Aan mijn ouders

Voor Lia en Lisette

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THE REACTION OF THE IMMUNE SYSTEM OF FISH TO VACCINATION

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THE REACTION OF THE IMMUNE SYSTEM OF FISH TO VACCINATION

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op woensdag 17 april 1985
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

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STELLINGEN

1 Ziektepreventie door middel van gerichte vaccinatieprogramma's kan een stuk van de huidige onzekerheid uit de visteelt wegnemen.

2 Melano-macrofagen centra in de lymfoïde organen van vissen kunnen beschouwd worden als de vroege fylogenetische voorlopers van de kiemcentra van vogels en zoogdieren.

3 Beenvissen bezitten een lokaal of mucosaal immuunsysteem.

4 Ondanks de (in vergelijking met zoogdieren) ogenschijnlijk eenvoudige bouw van het spijsverteringskanaal van vissen doet het entero-endocrine systeem in complexiteit weinig onder voor dat van zoogdieren.

Rombout, J.H.W.M. & Reinecke, M. (1984). Cell & Tissue Res. 237, 57-65.
El-Salhy, M. (1984). Histochemistry 80, 193-205.

5 De veronderstelling van Little et al., dat de amplificatie van proto-oncogenen in weefselkweek relatie heeft met de maligne activering van dezelfde genen in tumoren, is vooralsnog gebaseerd op te weinig informatie.

Little, C.D., Nau, M.M., Carney, D.N., Gazdar, A.F. & Minna, J.D. (1983). Nature 306, 194-197.

6 De aanwezigheid van een tweede Ig-klasse in een rog *(Raja kenojei)* rechtvaardigt niet de door Kobayashi et al. geuitte veronderstelling, dat het immuunsysteem van kraakbeenvissen verder ontwikkeld is dan dat van beenvissen.

Kobayashi, K., Tomonaga, S. & Kajii, T. (1984). Mol. Immunol. 21, 397-404.

7 De experimenten van Kaastrup & Koch verschaffen relevante gegevens over de alternatieve complement activatie in karper-achtigen, maar geven, in tegenstelling tot de suggestie van de auteurs, geen aanleiding het bestaan van de klassieke complement activering bij deze dieren in twijfel te trekken.

Kaastrup, P. & Koch, C. (1983). Dev. Comp. Immunol. 7, 781-782.

8 De binnenlaag van het ectoderm, of "inner nervous layer", die zich vooral bij vissen en amfibiën manifesteert, en waarin neurale buis, neurale lijst en placoden hun oorsprong vinden, kan beschouwd worden als een vierde kiemblad.

Pearse, A.G.E. (1973). Digestion 8, 372-385.
Rollhäser - ter Horst, J. (1980). Anat. Embryol. 160, 203-211.

9 Karperachtigen zijn minstens zo geschikte laboratorium proefdieren als knaagdieren.

10 Gezien het feit, dat in bepaalde landen waar mensenrechten met voeten worden getreden, regelmatig het doodvonnis wordt gewezen, is het uitermate ongepast bij dergelijke executies te spreken van terechtstellen.

11 De functie van een promotor is drieledig; hij treedt namelijk op als start-, stuur- en remmotor.

C.H.J. Lamers
The reaction of the immune system of fish to vaccination.
Wageningen, 17 april 1985.

CONTENTS

VOORWOORD

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ABBREVIATIONS

A-layer	additional layer
a.f.	after fertilization
B-cell	bursa (equivalent) derived lymphocyte
BSA	bovine serum albumin
C_1-C_9	components of the complement system
Con A	concanavalin A
CMI	cell mediated immunity
CRP	C-reactive protein
Cy	cyclophosphamide
DI	direct immersion
DMSO	dimethylsulfoxide
DNP	dinitrophenol
DTH	delayed type hypersensitivity
ECP	extra cellular products
EM	electron microscope
ERM	enteric redmouth (disease)
ETE	*Exteinascidua turbina* extract
Fc	crystallizable fragment of immunoglobulin
FCA	Freund's complete adjuvant
H-chain	heavy chain
HGG	human gamma globulin
HI	hyperosmotic infiltration
HMW	high molecular weight
HSA	human serum albumin
Ig	immunoglobulin
IFN	interferon
i.m.	intramuscular
i.p.	intraperitoneal
IPN	infectious pancreatic necrosis
i.v.	intravenous
J-chain	joining chain
Kd	kilo dalton
KLH	keyhole limpet haemocyanine
L-chain	light chain
LD90	lethal dose 90%
LMW	low molecular weight
LPS	lipopolysaccharide
MI	migration inhibition
MGG	May-Grünwald Giemsa
MMC	melano-macrophage centre
MoAb	monoclonal antibody
MW	molecular weight
NCC	non-specific cytotoxic cells
PAS-GL	periodic acid-Schiff positive granular leucocyte
p.h.	post hatching
PHA	phytohaemagglutinin
PBL	peripheral blood leucocytes
RER	rough endoplasmic reticulum
sBSA	soluble bovine serum albumin
sIg	surface immunoglobulin
SRBC	sheep red blood cells
T-cell	thymus derived lymphocyte
VHS	viral haemorrhage septicemia

ALPHABETICAL LIST OF FISH SPECIES

Common name	Scientific name
albacora tuna	*Thunus alalunga*
Atlantic salmon	*Salmo salar*
Australian catfish	*Tachysurus australis*
ayu	*Plecoglossus altivelis*
bluegill	*Lepomis macrochirus*
bowfin	*Amia calva*
bream	*Abramis brama*
brook trout	*Salvelinus fontinalis*
brown trout	*Salmo trutta*
carp	*Cyprinus carpio*
catfish	*Ictalurus melas* (Garavini)
channel catfish	*Ictalurus punctatus*
chinook salmon	*Oncorhynchus tshawytsha*
coho salmon	*Oncochynchus kisutch*
cutthroat trout	*Salmo clarkii*
dace	*Leuciscus leuciscus*
dogfish	*Scyliorhinus canicula*
eel (American)	*Anguilla rostrata*
eel (European)	*Anguilla anguilla*
eel (Japanese)	*Anguilla japonica*
giant grouper	*Epinephelus itaria*
goldfish	*Carassius auratus*
golden orfe	*Leuciscus idus*
largemouth bass	*Micropterus salmoides*
margate	*Haemalon album*
Mozambique mouthbrooder	*Tilapia mossambica*
nurse shark	*Ginglymostoma cirratum*
paddlefish	*Polyodon spathula*
perch	*Perca fluviatilis*
pike	*Esose lucius*
plaice	*Pleuronectes platessa*
rainbow trout	*Salmo gairdneri*
roach	*Rutilus rutilus*
rosy barb	*Barbus conchonius*
sea lamprey	*Petromyzon marinus*
sheepshead	*Archosargus probatocephalus*
snapper	*Lutjanus griseus*
sockeye salmon	*Oncorhynchus nerka*
sunfish	*Lepomis* sp.
tench	*Tinca tinca*

1 *GENERAL INTRODUCTION*

According to old Chinese writings and Egyptian bas-reliefs, the knowledge of fish farming dates back for several thousand years (Brown, 1977). At present fish farming is practised world wide and about 100 species of finfish are cultured in fresh, brackish or marine water. During the last decades the importance of small and large scale fish farming has grown enormously. The total world fish catch and production has more or less stabilized around 70 million tons (MT) after 1970. However, the total yield of inland fisheries has grown considerably during this period, predominantly due to aquaculture. The production of fish farming has increased from 2,6 MT in 1970 to 4,5 MT in 1975 (Pillay, 1976, cited by Brown, 1977), and from 1976 to 1982 a general increase of inland fisheries of about 25% was observed. However, this increase was mostly due to a few fish families that are popular for farming (Cichlidae, Salmonidae and Cyprinidae; see Table 1). Although inland fisheries still cover only a small part of the world animal protein production, its local importance must not be underestimated. It is an important protein source in Asia, a fast growing source in Africa and covers a high percentage of the total fish catch in these areas. For North America and Europe the contribution of inland fisheries is relatively small (Anonymous, 1983, 1984).

One of the obstacles for fish farming activities is the occurrence of infectious diseases. Certain diseases are related to unfavourable environmental conditions, whereas others are just induced by contact of fish with a critical level of the pathogen. The combat of these infectious diseases requires a multidiciplinary approach, and should include: hygiene, sanitary prophylaxis, immunoprophylaxis chemotherapeutics and. These aspects are discussed by Ghittino et al. (1984).

TABLE 1. The world fish catch and production in 1976 and 1982.

	1976	1982	1982 as % of 1976
Total world finfish catch	62,300*	67,600	109
- marine	55,700	59,600	107
- fresh water	6,600	8,200	124
Fresh water fish species			
- Cyprinidae	568	762	134
- Cichlidae	305	498	164
- others	4,847	5,723	118
Diadromous fish species			
- Eels	67	85	127
- Salmonidae	561	798	142
- Milkyfish/Shads	769	947	123
- others	126	153	121

* in thousands of tons

Immunoprophylaxis in fish was first applied by Duff (1942) and Schäperclaus (1954), but it is only since the last decade that there is straight forward research on fish vaccination (Anderson & Hennessen, 1981; Van Muiswinkel & Cooper, 1982; Anderson et al., 1983; De Kinkelin & Michel, 1984). Promising results have been obtained with vaccines against vibriosis and yersiniosis. This success was augmented by the development of new vaccination methods for large fish numbers (bath and spray vaccination). To date vaccination against these diseases has been realized on commercial scale, and the vaccines are also commercially available*. These results have been achieved largely on empiric grounds, but similar attempts to develop vaccines against other diseases had much less success. Recently Ellis (1985) clearly stated that a scientific analysis of the underlying factors affecting the efficacy of a vaccine and the constitution of protective immunity are required to make progress in this field. According to his view future research should concentrate on both host and pathogen. The different elements that should be included in these studies are summarized in Table 2. These studies should lead to the development of effective vaccines by allowing the appropriate choice

TABLE 2. Aspects related to effectivness of vaccination.

Studies on the host:

- pathology of the disease e.g. mode of entry
- optimal conditions for immunization
- determination of what constitutes protective immunity
- standardization of challenge protocols for potency testing of vaccine
- problems of carrier-status induced by vaccination
- tolerance by early exposure and vertically transmitted disease
- potentials of passive immunization
- improving immune responsiveness of fish populations by selective breeding

Studies on the pathogen:

- mechanisms of infection, e.g. attachment
- *in vivo* studies to determine the important antigens
- mechanism of disease e.g. toxin production
- mechanisms of avoiding host defences
- development of attenuated forms
- potential of non-virulent antigenically-related organisms
- culture requirements for antigen production

* Wildlife Vaccines Inc., 11475 West 48th Ave, Wheat Ridge, Colorado 80033, U.S.A.: Enteric redmouth bacterin (ERB); *Vibrio anguillarum* bacterin (VAB-2); *Aeromonas salmonicida* bacterin (ASB); moreover, combination vaccines are available: ASB/VAB-2 and ASB/ERB. European agent: Aquaculture Vaccines Ltd., 37, Queens Street, London, EC4R-1BY, U.K.
Biomed Research Laboratories Inc., 115 East Pike Street, Seattle, WA 98122, U.S.A.: Enteric redmouth bacterin (*Yersinia ruckeri*); *Vibrio anguillarum* bacterin (BIOVAX).
IFFA Mérieux, 17, rue Bourgelat, 69002, Lyon, France: *Vibrio anguillarum* bacterin (VIBRIFFA); Enteric redmouth bacterin (*Yersinia ruckeri*; YERSIVAX).

of antigens for a vaccine and methods for vaccine administration. Furthermore they should provide data on testing the potency of vaccines and on the assessment of commercial requirements such as large scale production, cost effectiveness and vaccination strategies (Ellis, 1985). The present day methods for vaccinating fish in ascending order of effectiveness, with regard to level and duration of protection are as follows: oral, spray, immersion (direct and hyperosmotic), and injection.

The study presented in this thesis concentrates on several aspects of the host reaction on administration of bacterial antigens. The aim of these studies was to obtain insight in the immunological processes induced by bacterial antigen, administered by injection, immersion or orally. The investigations have concentrated on the humoral immune response and the formation of immunological memory, the handling and processing of antigen and the histophysiology of the immune response. These studies add more data to the knowledge of the immune system of teleost fish, and provide more insight in the processes that are involved in protective immunity and in the optimal conditions of fish immunization.

The experiments described in this thesis were carried out in common carp (*Cyprinus carpio* L.). This fish is an excellent subject for biological studies. It can be easily bred in the laboratory, which guarantees a continuous supply of experimental animals. Moreover, as previously stated carp is an important cultured fish. For immunization, bacterial preparations of *Yersinia ruckeri*, and *Aeromonas hydrophila* have been used. See for more details on the bacteria (Paragraph 4.1.1, 4.1.2, 4.2.2 and 4.2.4).

In the following chapters an overview of the present knowledge on defence mechanisms in teleost fish is presented. Moreover, some data are given on the factors affecting defence, the main fish pathogens and the results of vaccination. The original research reports are presented in the appendices 1-9.

2 *DEFENCE MECHANISMS OF TELEOST FISH*

2.1 INTRODUCTION

All vertebrates possess an extensive defence system, which enables the individual to survive and maintain its integrity in a hostile environment. The protective mechanisms are directed against foreign matter, including pathogens and malignant cells, and comprise a number of non-specific and specific reactions. The defence mechanisms can be arranged according to successive lines of defence that an invading substance will encounter (see Table 3).

TABLE 3. Defence mechanisms in teleost fish.

A. First line of defence: relatively stable physical or chemical barriers.
- epithelia and their secretions
- transferrin
- enzyme inhibitors
- lectins
- lytic enzyms
- complement*

B. Second line of defence: inducible and/or mobile system, which is non-lymphoid and non-specific.
- interferon
- C-reactive protein
- natural cytotoxicity
- granulocytes (inflammation)
- macrophages (phagocytosis, without lymphoid interaction)

C. Third line of defence: inducible and mobile system, which is lymphoid or can co-operate with lymphoid cells. The reactions are specific and memory does occur.
- cell populations
- systemic system
  - organs
  - response types: humoral
    cellular
- local system
- memory formation

* Complement activity is also involved in processes of the second and third line of defence (Complement will be discussed as a whole in paragraph 2.2.6).

2.2 FIRST LINE OF DEFENCE

The first line of defence comprises those defence mechanisms that form relatively stable physical or chemical barriers. They prevent penetration of foreign matter into the host or eliminate these substances immediately after penetration.

2.2.1 *Physical barrier*

The epithelial surfaces with their secretions form a physical barrier. It is of prime importance for fish to maintain the integrity of the epithelial layer, as it has also a role in osmoregulation. Wound healing is extremely rapid even at low temperatures (Bullock et al., 1978). Moreover, injury and non-specific irritation may result in a hyperplasia of the epidermal cells.

The epithelia of fish are covered by a mucus layer. Replacement of mucus by a continuous secretion by goblet cells in skin, gills and mucosa of the gastro-intestinal tract, may prevent colonization by bacteria, fungi or parasites. Upon infection or stress the mucus secretion might be increased (Pickering & Macey, 1977; Arillo et al., 1979). Pickering & Richards (1980) stated that the most important role of mucus is to prevent attachment of pathogens to epithelia. It is interesting to mention that in mucus also bacteriostatic and bacteriocidal substances have been detected. In skin mucus of plaice and channel catfish lysozyme has been demonstrated (Fletcher & White, 1973a; Ourth, 1980), furthermore Ramos & Smith (1978) detected low levels of C-reactive protein in skin mucus of the Mozambique mouthbrooder also the presence of complement in rainbow trout skin mucus has been reported (Harrell et al., 1976).

2.2.2 *Transferrin*

Transferrin is an iron binding glycoprotein, which is found in sera of most vertebrates. Throughout the Animal Kingdom transferrin exhibits a high degree of genetic polymorphism (cf. Ingram, 1980). As low levels of iron are bacteriostatic, transferrin may play an important role in resistance against many bacterial infections. In coho salmon the various genotypes of transferrin may be responsible for differences in individual resistance to bacterial kidney disease (Suzumoto et al., 1977). Certain genotypic variants were able to bind iron better than others and resistance correlated with higher avidities for iron.

Unfortunately some pathogens have developed mechanisms to get around this protective mechanism. Crosa (1980) indicated that virulent strains of *Vibrio anguillarum* all have a plasmid controlled, efficient iron-sequestering system, that permits them to grow under low iron conditions.

2.2.3 *Enzyme inhibitors*

These substances neutralize the activity of pathogen exo-enzymes, and thus function in the defence against bacterial penetration or local bacterial digestion. However, they probably act primarily against auto-digestion. Ellis et al. (1981) and Munro et al. (1980) identified a protease inhibitor in serum of rainbow trout; it neutralized the proteolytic and ichthyotoxic activity of the extracellular products (ECP) of *Aeromonas salmonicida*. A similar serum protein has been detected in plaice (Starkey et al., 1982) and it was suggested to be analogous to mammalian α_2-macroglobulin (Grisley et

al., 1984). Recently Ellis & Grisley (1985) reported the presence of an efficient anti-trypsin activity in normal trout serum.

2.2.4 *Lectins*

Lectins are involved in cross-linking molecules (predominantly carbohydrate residues) in solution (precipitins) or attached to foreign red blood cells or micro-organisms (agglutinins). By doing so they may act as opsonins. The literature concerning lectins or lectin-like substances, which are proteins or glycoproteins is rather bewildering (Ingram, 1980; Fletcher, 1982) and there is little information on their protective role. It is supposed that they might be involved in immobilization and aggregation of micro-organisms, neutralizing bacterial components, especially exotoxins with anti-host activities and thus rendering them more susceptible to phagocytosis. Davies & Lawson (1982) isolated and partially characterized a precipitin from atlantic salmon. It was not related to immunoglobulin (Ig), but resembled plant and invertebrate agglutinins (lectins). The serum level did not increase in infected fish (Davies & Lawson, 1985). Upon reaction with fungal extracts, it activated complement and appeared to be a mediator of the inflammatory reaction.

2.2.5 *Lytic enzymes*

Lytic activity may be attributed to single enzymes as lysozyme or chitinase or to enzyme complexes like the complement system (see 2.2.6).

Lysozyme is an enzyme with bacteriolytic properties and is ubiquitous in its distribution amongst living organisms. It attacks specifically structures containing muramic acid, and has also been reported to have anti viral and anti parasitic properties. Lysozyme has been detected in serum, mucus and in phagocytic cells of both fresh water and marine fish. The enzyme is produced by phagocytic cells (Lukyanenko, 1965; Fletcher & White, 1973a; Fänge et al., 1976; Fletcher & Grant, 1968; Ourth, 1980, Murray & Fletcher, 1976).

Chitinase hydrolyses special structures in chitine. It is able to attack cell walls of fungi, nematodes and arthropods. Chitinase is demonstrated in fish leucocytes (Fänge et al., 1976).

2.2.6 *Complement*

The complement system does not fit easily in our scheme of defence lines, as it is related to more than one defence line. The reactions that first appeared in phylogeny, the "alternative" pathway, acts as a first line of defence, whereas complement side products initiate second line defence mechanisms. Furthermore, the "classical" pathway is related to the specific reactions of the third defence line. Nevertheless, our overview on complement will be presented here as a whole for an easier understanding.

The complement system is formed by a series of protein and non-

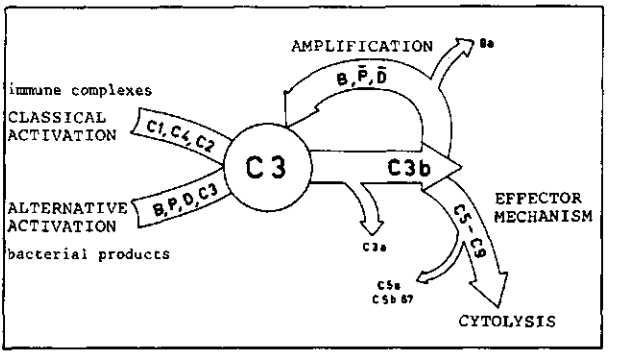

FIGURE 1. Schematic presentation of the complement system in mammals (Daha & Capel, 1979).

protein components, which upon activation act in a complex sequential, self-amplifying reaction that is responsible for foreign cell lysis. The two routes along wich complement can be activated are schematically presented in Figure 1. The "alternative" pathway can be activated by a variety of substances, usually polymers with repeating sequences, e.g. (lipo)polysaccharides (LPS) derived from bacterial or fungal cell walls. The "classical" pathway is activated by interaction of antibody with antigen. Both routes of complement activation also lead to a variety of other reactions, which are induced by complement side products. These reactions include chemotaxis of leucocytes, enhancement of adherance to, phagocytosis and killing by macrophages and hypersensitivity.

Complement activity has been demonstrated in serum of teleost fish. Its activity is thermolabile, requires Ca^{++} and Mg^{++}, and is mostly not exchangable between unrelated species (cf. Ingram, 1980; Fletcher, 1982; Rijkers, 1982a). Teleost complement is inactivated from 42 °C onwards (Sakai, 1981; Rijkers, 1982a). The optimal temperature for rainbow trout complement is 25 °C (Nonaka et al., 1981a); however, complement remains active over a wide temperature range. Perch complement retains its lytic activity even at 4 °C (Pontius & Ambrosius, 1972).

Only few data are present on the isolation and characterization of fish complement factors. Jensen & Festa (1981) identified, in nurse shark serum 6 components, three of which were equivalent with mammalian C1, C8 and C9 respectively. Nonaka et al., (1981b) isolated and characterized two complement factors from rainbow trout serum, which are thought to be the counterpart of mammalian C3 and C5. The serum concentration of rainbow trout complement factors is comparable to those in humans (C3 > 1 mg/ml, C5 about 210 µg/ml).

Griffin (1984) showed the generation of a leucocyte attracting activity after the interaction of antigen with antibody, in presence of whole complement. It is plausible that teleost complement, upon activation, also generates pharmacologically active products (proba-

bly C3a or C5a). These attractant factors might also be released in the alternative complement pathway, which is of prime importance for a fast inflammatory response.

2.2.6.1 *Alternative pathway*

Serum of the lowest vertebrates, Agnata or jawless fishes, contains both Ig and complement factors. However, their complement only functions via the alternative pathway (Fujii & Murakawa, 1981) and Ig was observed to act only as opsonin (Fujii, 1981). In teleosts both the alternative and classical pathway of complement activation are described by Nonaka et al. (1981a). Ourth & Wilson (1982a,b) indicated that the alternative pathway was important for the bactericidal activity of non-immune catfish serum against *Salmonella paratyphi* and other gram-negative bacteria. The alternative complement pathway in teleosts can easily be activated by substances that are known to activate this pathway in mammals too (e.g. inulin, zymozan) (Nonaka et al., 1981a; Kaastrup & Kock, 1983).

2.2.6.2 *Classical pathway*

The "classical" pathway of complement activation requires the presence of Ig and is clearly shown in teleost fish. For example, it is a prerequisite for the haemolytic plaque assay. This assay has been applied in many species (cf. Rijkers et al., 1980a). Both Nonaka et al. (1981a) and Giclas et al. (1981) reported that lysis of sheep erythrocytes required specific antibody and the presence of both Mg^{++} and Ca^{++} in rainbow trout, and in the albacora tuna respectively. Activation of the alternative pathway by inulin, zymozan or LPS, depleted the serum from antibody mediated lytic activity (Nonaka et al., 1981a).

2.3 SECOND LINE OF DEFENCE

The second line of defence can be defined as those processes that can be readily induced upon infection. The cells involved are non-lymphoid. Substances produced display only a relative low specificity and there is no memory formation.

2.3.1 *Interferon*

Interferon (IFN) is an anti-viral protein, that is produced by virus infected cells. Fish IFN is comparable with mammalian IFN, indicating that it is an phylogenetically conservative molecule. IFN acts against the intracellular phase of the viral growth cycle by damaging the RNA translation process (cf. Ingram, 1980).

IFN production has been demonstrated in several fish species following infection by various pathogenic viruses (De Kinkelin & Dorson, 1973; De Kinkelin & Le Berre, 1974). IFN has a broad antiviral activity, although it has a differential inhibitory effect on

various viral pathogens, e.g. IFN mediated resistance to infectious pancreatic necrosis (IPN) was less effective than to viral hemorrhagic septicaemia (VHS) (De Kinkelin et al., 1982). As in mammals, IFN in teleosts is species specific (De Kinkelin & Dorson, 1973). De Kinkelin et al. (1984) reported that the virulence of VHS virus strains was not (inversely) correlated with the quantity of IFN production, but with the differential sensitivity of the virus strains for IFN. The level of IFN produced was correlated with the number of infected cells. IFN levels in carp infected with VHS virus raised to high levels during the first two days of infection and declined from day 3 onwards; by day 14 IFN levels had disappeared (De Kinkelin et al., 1982). Transfer experiments with serum of VHS infected rainbow trout revealed that, provided that IFN was present at the moment of infection, protection against the virus was correlated with the IFN level.

It is known that outbreaks of various viral infections do not occur above certain temperatures, which is usually the optimal physiological temperature for the fish: e.g. clinical VHS in trout does not occur above 15 °C, and spring viraemia of carp does not occur above 20 °C (Amend, 1970; Dorson & De Kinkelin, 1974; Scherrer et al., 1974; Baudouy et al., 1980). This phenomenon might be correlated with the increasing rate of IFN production at higher temperatures, whereas viral growth is less affected by the temperature.

2.3.2 *C-reactive protein*

In teleost fish C-reactive protein (CRP) is a common serum component, that might significantly increase upon exposure to bacterial endotoxin (cf. Ingram, 1980). In presence of Ca^{++} CRP reacts with phosphorylcholine molecules, which are commonly present in the cell wall or surface structures of many invading micro-organisms. Specificity for such a common component makes CRP an important protective substance (Baldo & Fletcher, 1973). The CRP reaction results in agglutination of the pathogen and activation of complement and subsequently enhancement of phagocytosis. In mammals CRP is an acute-phase reactant in inflammatory reactions (Pepys & Baltz, 1983). In this respect, the results in fish are contradictory; upon administration of bacterial endotoxin or carrageenin, an extract of the marine alga *Chondrus cripus*, in plaice a significant increase of the CRP level was seen, but with other inflammation inducing substances, no change was observed (White et al., 1981). CRP serum levels in the Mozambique mouthbrooder strongly increased upon a physical tissue damage (Ramos & Smith, 1978).

Till the moment that an animal can mount a specific response, CRP is able to trigger several non-specific reactions by activating complement, serving as an opsonin or by agglutinating particles and promoting phagocytosis.

2.3.3 *Natural cytotoxicity*

Cells displaying non-induced and non-specific cytotoxicity have been described in a number of teleost fish, predominantly in fresh

water fish of the stenohaline type (Hinuma et al., 1980; Graves et al., 1984; Evans et al., 1984a,b). Non-specific cytotoxic cells (NCC) were detected in head and trunk kidney, spleen and peripheral blood, they lysed a variety of transformed target cells, were cytotoxic over a wide temperature range (16-37 °C) and displayed rapid killing kinetics (30 min.). Pettey and McKinney (1981) observed that in the nurse shark the natural cytotoxicity increased when fish were stressed by environmental conditions, as low water temperatures. Both Hinuma et al. (1980), for teleost fish, and Petty & McKinney (1981) in sharks, identified the effector cell as a glass adherent phagocytic cell. Evans et al. (1984a,b) suggested that NCC share some biophysical properties with mammalian natural killer cells. Moreover, NCC appeared to be under partial control of a radiation sensitive suppressor cell and also of a serum component, probably Ig.

2.3.4 *Inflammation*

The inflammatory reaction is a local reaction which confers some degree of protection by "walling off" an infected area from the rest of the body by an infiltration of granulocytes and macrophages. Histopathological studies in fish provide evidence for inflammatory responses in bacterial, viral, mycotic, protozoan and parasitic infections. Both acute and chronic inflammation occurs (cf. Finn, 1970; cf. Roberts, 1978; Van Muiswinkel & Jagt, 1984).

Acute inflammation responses in fish are comparable to those in mammals, but they are less intense and slower (Finn, 1970; Finn & Nielsen, 1971a). Upon i.m. or i.p. injection of *Staphylococcus aureus* or Freund's adjuvant in rainbow trout (at 15 °C), granulocyte infiltrations appeared after 12 to 24 hours; highest numbers of cells were detected after 2 to 4 days. Both granulocytes and macrophages appeared to be phagocytic, furthermore also many lymphocytes were present. The necrotic tissue was replaced by a fibrous granulation tissue between 8 and 16 days (Finn & Nielsen, 1971a). Lowering the temperature to 5 °C delayed the above described phenomena, and the whole process took twice the time as at 15 °C. Remarkable was the more prominent role of granulocytes at 5 °C (Finn & Nielsen, 1971b).

Chronic inflammation was evoked by injecting plaice with carrageenin (Timur et al., 1977a) or with *Mycobacterium piscium* (Timur et al., 1977b). In both cases the chronic inflammatory responses were analogous to those described in higher vertebrates and an encapsulated granuloma or typical focal lesions were formed.

2.3.5 *Phagocytosis*

Phagocytosis of foreign material is often called a non-specific defence mechanism. However, phagocytosis by macrophages is also recognized as the initial step of the specific immune response. Phagocytic blood leucocytes of fish have been identified as macrophages, monocytes and granulocytes (cf. Ellis, 1977a). However, the phagocytic capacity of granulocytes is still controversial.

2.3.5.1 *Phagocyte properties*

In vitro and *in vivo* studies showed that macrophages are highly phagocytic for inert and antigenic material (Ellis et al., 1976; McKinney et al., 1977). Macrophages from immune fish are more active in phagocytosis than normal phagocytes (Chronchorov, 1966, cited by Avtalion, 1981; Song & Kou, 1981).

The intracellular killing by teleost macrophages is slow compared to mammals (Finn & Nielsen, 1971a; Avtalion & Shahrabani, 1975). The following lysozomal enzymes have been demonstrated: alkaline and acid phosphatases and peroxidase (Ellis, 1977a; Garavini et al., 1981; Bielek, 1981; Braunnesje et al., 1982). Moreover, the production of oxygen metabolites (such as super oxide) have been demonstrated by chemiluminescence (Scott & Klesius, 1981; Stave et al., 1983). Some pathogens are able to escape from intracellular killing, e.g. *Edwardsiella tarda* cells, phagocytized by Japanese eel leucocytes were not killed, and even multiplied within the phagocyte (Miyazaki & Egusa, 1976).

At lower temperatures, the relative role of granulocytes in the inflammatory response of rainbow trout increased (Finn & Nielsen, 1971b). Moreover, Rijkers et al. (1981b) observed that lowering the temperature increased the number of granulocytes in the lymphoid organs of carp. Both studies suggested an enhanced non-specific cellular defence at lower temperatures. However, some data oppose this supposition: Tets (1969, cited by Avtalion, 1981) found in carp higher numbers of active phagocytes at 22 °C than at 7 °C. Moreover, the activity of brown trout phagocytes was significantly decreased below 10 °C (O'Neill, 1985), and Avtalion (1981) stressed that at low temperatures the intracellular killing by carp macrophages was abrogated.

2.3.5.2 *Antigen clearance*

Fish phagocytic cells are widespread in the lymphoid organs (head and trunk kidney and spleen), gills, peritoneum and atrium of the heart (Ellis et al., 1976; McKinney et al., 1977). These cells comprise the reticulo-endothelial cells lining blood sinuses and in addition to circulating blood phagocytes, this system is very efficient in clearing the bloodstream from foreign particles in a fashion similar to mammals.

Blood clearance has been studied in rainbow trout, using heat killed *Salmonella pullorum* (Ferguson et al., 1982, 1984), and in plaice using carbon and turbot erythrocytes (McArthur et al., 1983). The particle clearance is a biphasic process; after intravenous injection, up to 90% of the particles were removed from the circulation within 15-30 min. In the late phase the clearance rate slowed down considerably. Head and trunk kidney and spleen appeared to be the main phagocytic organs (McArthur et al., 1983). The first phase of the clearance was not affected by temperature (McArthur et al., 1983; Ferguson et al., 1984). However, different results were obtained for the total clearance of living virus particles (MS2 bacteriophage; O'Neill, 1980) or bacteria (*S. aureus*; Avtalion, 1981).

At optimal temperatures carp and brown trout completely cleared bacteriophages from the circulation in 4 to 7 days (O'Neill, 1980). With lowering of the temperature the rate of clearance decreased. Avtalion (1981) reported that carp still showed relatively high clearance rates at 10 °C; however, the phagocytes were unable to kill the bacteria. Therefore he supposed that by an infection at low temperatures there might be the risk that the fish become carriers.

2.3.5.3 *Membrane receptors on phagocytes*

A prerequisite for phagocytosis is the adherance of particles to the membrane of the phagocyte. Macrophage receptors are lectin-like structures that may react directly with components of bacterial surfaces (Weir & Ögmundsdótter, 1980), but there are also specific receptors for the Fc portion of Ig and for the complement factor C3b; these receptors facilitate phagocytosis of antigen complexed with antibody.

Information about the presence of specific receptors for Fc and C3 on the phagocytes of lower vertebrates is scant. Wrathmell & Parish (1980a,b) reported the lack of an opsonic activity of fish antibody and complement, and concluded to that Fc and C3 receptors were absent on teleost phagocytes. On the contrary, in the most primitive living vertebrates (lampreys), antibody acted as an opsonin in phagocytosis of SRBC (Fujii, 1981). Also in teleosts specific antibody may increase the rate of phagocytosis of bacteria (Post, 1966; Griffin, 1983) or enhance the intracellular killing (Avtalion & Shahrabani, 1975). Song & Kou (cited by Plumb, 1984) demonstrated that normal eel macrophages phagocytized 5,3 times as much *E. tarda* bacteria in presence of specific immune serum than in the presence of non-immune serum. Moreover, Sakai (1984), studying the phagocytic response of salmonid peritoneal exudate cells for viable *A. salmonicida* cells, demonstrated an accelerated phagocytosis of bacteria in presence of both specific antibody and whole complement. He suggested that salmonid macrophages may have receptors for both Fc and C3.

Macrophage action in mammals is mediated by humoral factors. In teleosts only little evidence is present for such substances; positive reactions in the migration inhibition test, with both mitogens (Manning et al., 1982b) and antigen (Jayaraman et al., 1979) suggest lymphokines exert influence on macrophage migration. However, there are no data on their role in enhancing phagocytic activity. Recently Griffin (1984) presented evidence for a leucocyte attracting factor in rainbow trout (see paragraph 2.2.6).

2.4 THIRD LINE OF DEFENCE

The third line of defence comprises reactions of lymphoid cells and the humoral and cellular components that are related to the response of these cells. This form of defence is characterized by specificity and memory formation. At first the cells and organs involved will be described, followed by a description of the immune response.

2.4.1 *Teleost Leucocytes*

The leucocytes of fish show distinct morphological similarities to those in birds and mammals. They comprise lymphocytes, plasma cells, mononuclear phagocytes and granulocytes (Lehmann & Stürenberg, 1975, 1981; Ellis, 1976; Davina et al., 1980; Boomker, 1981). Ellis (1977a) has extensively reviewed the morphology and function of fish leucocytes and noted an enormous variation among fishes.

Davina et al. (1980) described the blood cells of two cyprinid fish: rosy barb and common carp. In addition to erythrocytes and thrombocytes, blood leucocytes consisted mainly of lymphocytes, heterophylic granulocytes and rarely PAS-positive granulocytes and macrophages (Fig. 2). The cells are characterized as follows:

- Lymphocyte, a small round cell (diameter 3-5 µm), with a relatively large nucleus surrounded by a thin layer of basophilic cytoplasm.
- Heterophilic granulocyte, a cell (diameter 6-7 µm) with fine granulated, slightly acidophilic cytoplasm and a lobed or curved nucleus. The granules did not stain with a May-Grünwald/Giemsa (MGG) stain, nor with Romanovsky (cf. mammalian neutrophils). Moreover, the granules were of irregular shape and contained cristalloid inclusions (cf. mammalian eosinophils).
- PAS-positive granulocyte (PAS-GL), a round cell (diameter 8-9 µm) with an eccentric, disc-shaped nucleus and basophilic cytoplasm containing large granules staining Periodic Acid Schiff (PAS), but not with MGG or toluidin blue.
- Macrophage, a large amoeboid cell (diameter 10-12 µm), with vacuolated, basophilic cytoplasm and round nucleus.

Some confusion exists concerning monocytes in teleost fish. Davina et al. (1980) did not describe this cell type in cyprinid fish, although no enzyme histochemistry was applied. Ellis (1977a) stated that about 0,1% of the blood leucocytes were monocytes, and that these cells, in plaice, were the only phagocytic cells in the circulation. In addition to small lymphocytes also large lymphocytes, or lymphoblasts have been observed in the circulation of the plaice (Ellis, 1976; Ferguson, 1976b). These active cells have an extended cytoplasm and are up to 12 µm in diameter.

Two other cell types have to be mentioned, usually located outside the circulation (Fig. 2):

- Melano-macrophage, large irregular cells with large pigment containing vacuoles. They might originate from macrophages storing non-digestible material (see also appendix paper 8). These cells may be present as solitary cells, e.g. in the intestinal epithelium (Davina et al., 1982; appendix paper 9), or in large clusters (see section 2.4.3).
- Plasma cell, a cell of variable size, with a round to lobed nucleus (Weinberg, 1975) and a relatively extended pyroninophilic cytoplasm containing Ig. These cells do not show the typical mammalian plasma cell characteristics (Ellis, 1977a), although they have a well developed rough endoplasmatic reticulum (RER) (Weinberg, 1975), which sometimes shows dilatations (Zapata, 1982).

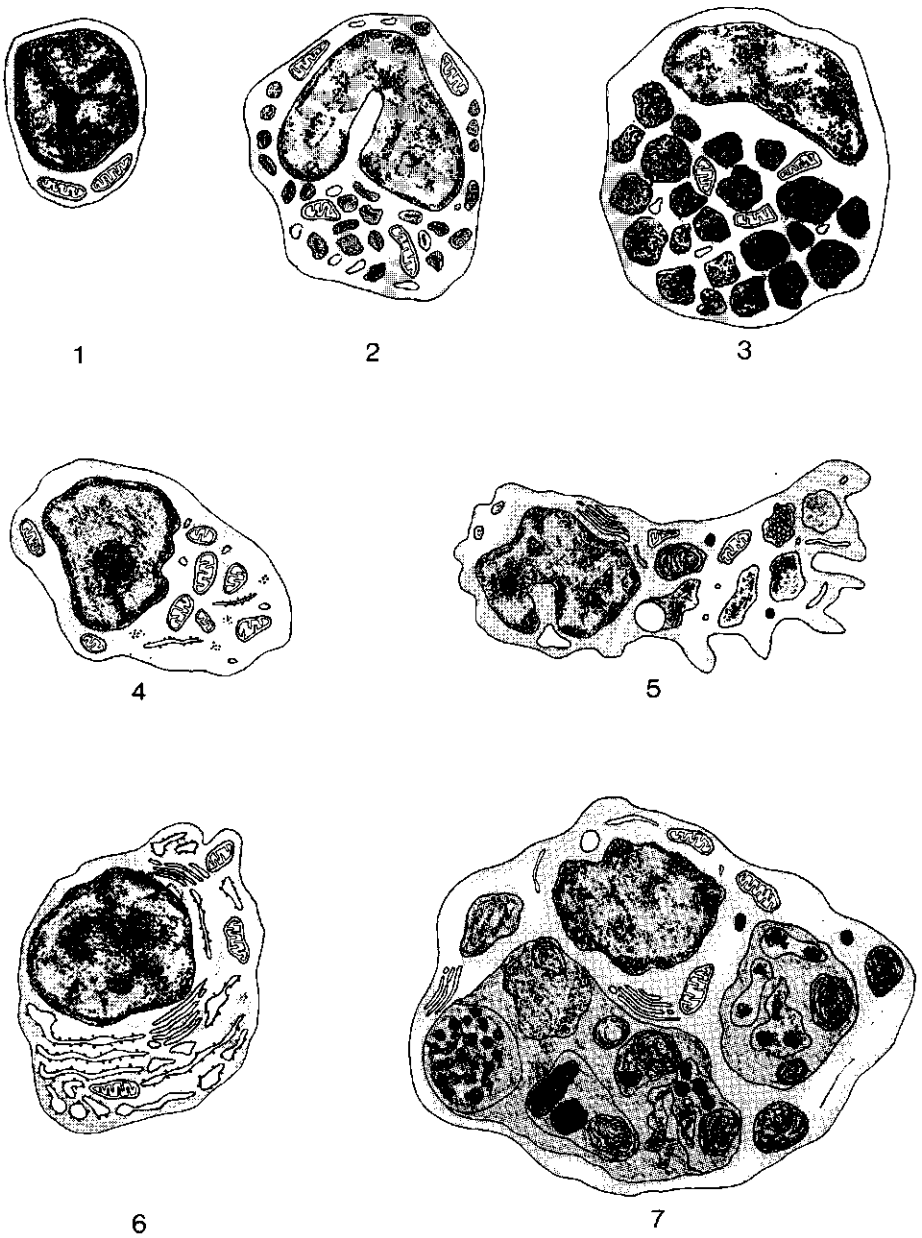

FIGURE 2. The leucocytes and related cells of carp. These drawings were made according to EM-pictures; 1) small lymphocyte; 2) heterophilic granulocyte; 3) PAS-positive granulocyte; 4) medium to large size lymphocyte; 5) macrophage; 6) plasma cell; 7) melano-macrophage.

Some properties of the granulocytes and macrophages have been indicated in the paragraphs on inflammation (2.3.4) and phagocytosis (2.3.5). The PAS-GL is considered as the teleost counterpart of the mammalian mast cell (Barber & Westermann, 1978). Recently Ellis (1982) indicated that in certain teleost species this cell type, although it probably does not contain histamine, may be involved in immediate hypersensitivity reactions. In plaice and in salmonids the eosinophilic granulocyte might be involved in these reactions. In these fish the PAS-GL has not been observed (Ellis, 1977a; Ezaesor & Stokoe, 1980).

2.4.2 *Lymphocyte subpopulations in teleosts*

The lymphoid cells mediating the specific immune responses in mammals can be divided into two populations: B- and T-lymphocytes. This distinction is based on structural and functional differences. B-cells display Ig on their cell surface (sIg), whereas T-cells are characterized by the absence of sIg, but they share non-Ig determinants. The search for lymphocyte subpopulations in teleost fish has concentrated on demonstrating functional heterogeneity of cells and on the determination of lymphocyte surface markers.

2.4.2.1 *Functional heterogeneity*

Functional heterogeneity of teleost lymphocytes has been investigated by means of hapten-carrier responses, mitogen responsiveness, and differential temperature sensitivity.

Hapten-carrier. The hapten-carrier system demonstrates co-operation between different lymphocyte populations during an immune response. Haptens are small molecules that can not induce antibody formation by themselves. In fish, the anti-hapten response was only obtained when the hapten was administered conjugated with a larger carrier molecule, provided that the fish was exposed previously to the same carrier (Yocum et al., 1975; Stolen & Mäkelä, 1975; Avtalion et al., 1975). In fish there is a need for co-operation between carrier-specific helper cells and hapten-specific antibody forming cells to achieve the anti-hapten antibody formation, analogous to the T- and B-cell co-operation in mammals. Ruben et al. (1977) separated hapten-reactive and carrier-reactive cells, using nylon wool column adherance. They observed that the hapten-reactive cells were present in the pronephros, but not in the thymus. This observation is suggestive for the idea that hapten-reactive cells are "B-like" cells.

Responses to mitogens. It is possible to induce proliferation of fish lymphocytes *in vitro* by mammalian T-cell (PHA, Con A) and B-cell (LPS) mitogens. Etlinger et al. (1976) showed an organ compartmentalization of the mitogenic responsiveness of rainbow trout lymphocytes, analogous to that in mice. However, Warr & Simon (1983) could not reproduce these results and their observations in rainbow trout were like those observed in the bluegill by Cuchens & Clem (1977), who reported that the mitogen reactive cell populations

showed no clear-cut organ distribution. Caspi et al. (1984) studied sequential or simultaneous stimulation with one or two mitogens and concluded that PHA and Con A responsive cells belonged to the same lymphocyte population, whereas LPS responsive cells form another distinct population. Moreover, they showed that these two cell populations differed in cell morphology. Cells in long-term culture with PHA had a smooth surface with wide pseudopods; in the cytoplasm many mitochondria were present. Cells in culture with LPS were round and had many small microvilli; in the cytoplasm many strands of RER were seen, whereas these cells had fewer mitochondria than the PHA cells.

Temperature-sensitivity of lymphocytes. Cuchens and Clem (1977) reported that mitogenic responses of bluegill pronephros lymphocytes showed differential temperature sensitivity. Stimulation by PHA and Con A was optimal at 32 °C, whereas the response to LPS was maximal at 22 °C. Moreover, they showed that the *in vitro* antibody response to the "T-dependent" antigen SRBC was good at 32 °C, but inhibited at 22 °C. Recently, Clem et al. (1984) reported similar results for mitogen stimulation of channel catfish peripheral blood lymphocytes. However, they stressed the importance of the preceding *in vivo* temperature. Their results indicate that T-like cells need higher temperatures than the B-like cell population which might be caused by a different membrane homeoviscosity (Abruzzini et al., 1982). The foregoing *in vitro* observations correlated well with *in vivo* experiments by Avtalion et al. (1980) and Whiskovsky & Avtalion (1982), who showed in carp that the development of the T-helper function for the humoral response was temperature dependent. After initial T- and B-cell co-operation at a relatively high temperature, the humoral response could develop at lower temperature.

2.4.2.2 *Lymphocyte surface markers*

All fish lymphocytes, including thymocytes, are positive for surface Ig (sIg^+) when analyzed with conventional antisera to fish serum Ig (Ellis & Parkhouse, 1975; Emmrich et al., 1975; Warr et al., 1976). The supposition that teleost thymocytes bear sIg seems to be supported by the observation that most monoclonal antibodies (MoAbs) made against carp thymocytes recognize serum Ig (Secombes et al., 1983a). However, in the same experiments only a small percentage of the MoAbs made against serum Ig react with thymocytes. One possible explanation for these data is the presence of only part of the antigenic determinants of serum Ig on the surface of teleost thymocytes. This is in agreement with Ambrosius et al. (1982), who provided evidence that the antigen-specific receptor of carp thymocytes is based on a dimer of slightly modified serum Ig heavy chains, homologous to the mammalian μ chain, whereas light chains are lacking. An alternative explanation is based on the observation that some of the anti-carp thymocyte MoAbs also stain certain cell populations in the brain, suggesting the presence of a teleost thymocyte surface marker, partially homologous to serum Ig, with properties similar to those of mammalian Thy-1. The cytolytic activity of an antiserum, raised against bluegill brain cells, for PHA-responsive ("T-like") lymphocytes (Cuchens & Clem, 1977) corresponds with this hypothesis.

Based on the observation that in teleosts some anti-serum Ig MoAbs do not react with lymphocytes from the thymus, several research groups (Secombes et al., 1983a; Warr et al., 1983; Lobb & Clem, 1982) have recently tried to identify lymphocyte subpopulations. Their results indicate that 40% or less of the lymphocytes from spleen and pronophros display a positive reactivity with anti-Ig MoAbs and might thus be considered equivalent to B-lymphocytes from higher vertebrates, carrying Ig as the antigen-specific receptor on their surface (sIg^+). Whatever the remaining lymphocytes carry on their surface cannot be a complete Ig molecule, and these cells should, therefore, be characterized as sIg^-. When fish lymphocytes are depleted of such B-cell equivalents, e.g., by "panning" procedures, the response of the remaining lymphocyte subpopulation to LPS, but not to Con A, is significantly reduced (Warr et al., 1983; Clem et 1984). Also, only sIg^+ catfish lymphocytes are required for an antibody-forming cell response *in vitro* to T-independent antigens, whereas for the response to T-dependent antigens both sIg^+ and sIg^- lymphocytes must be present. These results have led Clem and co-workers to conclude that catfish sIg^+ and sIg^- lymphocytes are functional equivalents of B- and T-lymphocytes from higher vertebrates.

2.4.3 *Melano-macrophages*

Melano-macrophages, solitary or in clusters, are conspicuous elements in the lymphoid organs of most teleost fish. They are not restricted to lymphoid organs and may also appear at other sites of extreme phagocytosis of foreign substances or autologous cellular material. The phylogeny, ontogeny and functional significance of the melano-macrophages has been extensively investigated by Roberts (1975); Agius (1979, 1980, 1981a,b, 1983); Agius & Roberts (1981) and Agius & Agbede (1984). Their conclusions may be summarized as follows:

- During evolution, from Agnatha to Teleosts, there is a progressive increase in number of melano-macrophages, their organization in centres and their preference for location in the main lymphoid organs.
- During ontogeny the first melano-macrophages appear in haemopoietic tissues at the first feeding. Other processes coincide also with the time of first feeding, like the onset of immunological maturation. The number of melano-macrophages in the lymphoid organs increases with age and during starvation.
- The identified pigments are melanin, lipofuscin and haemosiderin which are probably resulting from melanosome ingestion, peroxidation of unsaturated lipids and haemoglobin breakdown respectively. The function of melanin might be related to protection against free radicals, produced by phagocytic cells during extracellular killing.
- Melano-macrophages may be multifunctional, from acting as scavengers, involvement in bacterial killing, depot for iron, to a possible involvement in the immuno-regulation.
- Groups of melano-macrophages are proposed to represent primitive germinal centres. This aspect will be discussed in more detail in appendix paper 8.

2.4.4 *The systemic immune system I (Lymphoid organs)*

The immune system can be divided in: a) systemic system, involving the lymphoid organs, and the immune processes taking place in the inner part of the animal, and b) local system, involving the surface, including the intestine, of the animal and its immune reactions.

The lymphoid organs of teleosts are: thymus, spleen, head kidney and trunk kidney (Corbel, 1975, Fänge, 1982). Moreover high numbers of lymphoid cells are present in the intestinal mucosa (Bullock, 1963; Zapata, 1979a; Davina et al., 1980). Also in the gills leucocytes are present (personal observations). However, no data are available on their numbers and functioning. Fish lack bone marrow and lymph nodes, whereas the presence of lymph vessels described for plaice (Wardle, 1971), is questioned for other species like rainbow trout and tench (Vogel & Clavier, 1980; Vogel, 1981). The location of carp lymphoid organs are visualized in Fig. 3.

2.4.4.1 *Thymus*

The thymus of teleosts is a paired organ located near the brancheal cavity. It is covered by the pharyngeal epithelium. The thymus is composed of lymphocytes and lymphoblasts, arranged within a network of reticular epithelial cells. The morphology varies between species as far as a clear cortex/medulla distinction is concerned. Epithelial cysts and myoid cells have been observed, whereas Hassal's corpuscles are lacking (Sailendri & Muthukkaruppan, 1975; Smith et al. 1970; Grace & Manning, 1980; Zapata, 1981a; Chilmonczyk, 1983). The thymus in fish can be regarded as a central lymphoid organ as in mammals. This is demonstrated by the fact that it is the first lymphoid organ to appear during ontogeny (Ellis, 1977b; Grace & Manning, 1980; Van Loon et al., 1982). No phagocytosis and antigen processing occurs in the thymus (Ellis et al., 1976; Ellis, 1980). Moreover, Ellis & De Sousa (1974) found that radiolabelled auto-

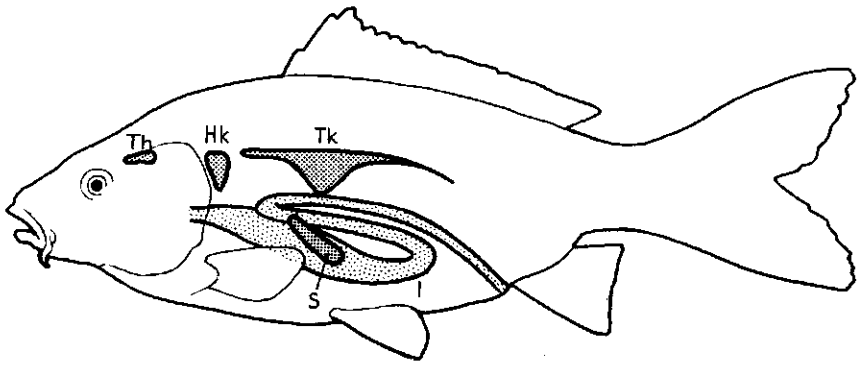

FIGURE 3. The lymphoid organs of carp. HK = head kidney; I = intestine; S = spleen; Th = thymus; TK = trunk kidney.

logous lymphoid cells of plaice did not migrate into the thymus after intravenous injection.

2.4.4.2 *Spleen*

The spleen is a haemopoietic organ including lymphoid tissue. Contrary to mammals, the spleen of teleosts does not contain distinct white pulp compartments or germinal centres. However, there are considerable differences in morphology between various teleost species (Haider, 1966). The splenic structure almost entirely consists of red pulp and has a distinct function in erythro-, granulo- and thrombopoiesis (cf. Haider, 1966). Zwillenberg (1964) and Haider (1966) mentioned that after removing the red blood cells from the spleen by washing a clear compartmentation of the splenic pulp could be observed. Each compartment was surrounded by bundeled reticular fibres and contained a sheathed arterial capillary or ellipsoid. These ellipsoids possess a thick wall, composed of endothelial cells, macrophages and reticular fibres and are mostly encircled by a blood sinus. Ellipsoids are conspicuous elements of the spleen of all teleosts (cf. Haider, 1966; cf. Pitchappan, 1980). In carp and sunfish the sheath macrophages have many cytoplasmic processes, forming an extensive network, the meshes of which are filled with lymphocytes (Graf & Schlüns, 1979; Fullop & McMillan, 1984). In most teleosts the white pulp is rather limited and diffusively distributed (e.g. in rainbow trout, Zwillenberg, 1964; plaice, Ellis et al., 1976; carp, Graf & Schlüns, 1979). In some species distinct lymphoid accumulations occur around small blood vessels and ellipsoids (e.g. in goldorfe, Haider, 1966; mozambique mouthbrooder, Sailendri & Muthukkaruppan, 1975) and around melano-macrophage centres (MMC) (Ferguson, 1976a; Sailendri & Muthukkaruppan, 1975; Zapata, 1982). These MMC are often seen in association with blood vessels or ellipsoids and may be surrounded by a fibrous reticular network.

The role of the spleen in immune reactivity has been questioned. The teleost spleen becomes lymphoid rather late in ontogeny (Ellis, 1977b; Grace & Manning, 1980). Moreover, Ferren (1967) reported that splenectomy had no effect on the antibody response in the snapper. Splenectomy in rainbow trout may slightly reduce antibody levels in serum (Van Muiswinkel & Anderson, personal communication). On the other hand considerable numbers of antibody-producing and antigen-binding cells have been detected in the spleen of various teleost species (cf. Rijkers, 1980). Secombes et al. (1982a) reported that after antigenic stimulation clusters of pyroninophilic cells appeared in the ellipsoid walls. They stressed the importance of ellipsoidal reticular fibres in binding immune complexes (Secombes et al., 1982b).

2.4.4.3 *Kidney*

The kidney is an important lympho-myeloid organ in teleosts: it is divided into head kidney and trunk kidney (pronephros and opisthonephros). The head kidney has lost its secretory function because renal tubules are absent in adult animals. The lympho-myeloid

tissue of both head and trunk kidney has a similar organization. In the trunk kidney it is located in the intertubular spaces (Ellis et al., 1976; Grace & Manning, 1980; Zapata, 1979b, 1981b). All tissues are supported by a reticular framework, interspersed with many thin-walled blood vessels. The lymphoid tissue is diffusely distributed (Ellis et al., 1976) or may show some clustering around blood vessels and sinuses (Smith et al., 1970; Sailendri & Muthukkaruppan, 1975).

The kidney has a high capacity for lympho- and plasmacytopoiesis (Smith et al., 1970; Zapata, 1979b, 1981b). The overall picture of the kidney has lead to the idea that the teleost kidney is analogous to mammalian bone marrow (Zapata, 1979b; Rijkers, 1980). Both in head kidney and trunk kidney high numbers of antibody-producing cells have been demonstrated (cf. Rijkers et al., 1980c). In species like bluegill and carp the head kidney was even more important in antibody production than the spleen (Smith et al., 1967; Rijkers et al., 1980c). It is concluded that the kidney functions as primary lymphoid organ (stem cells) and secondary lymphoid organ (antibody production).

The role of fish lymphoid organs in antigen clearing and antigen processing are discussed in detail in appendix papers 6, 7 and 8.

2.4.5 *The systemic immune system II (Humoral immunity)*

Since the beginning of this century it has been known that fish are capable of producing antibodies (cf. Corbel, 1975). Most early studies were performed in order to obtain protective immunity. Later on a more fundamental interest in the immune system of fish has developed. Both primary and secondary antibody responses have been registered in different teleost species and to a variety of antigens, e.g. heterogeneous erythrocytes, bacteria and bacterial extracts, virus particles, proteins, lipopolysaccharides and haptens (cf. Rijkers, 1980).

2.4.5.1 *Immunoglobulin*

The structure of fish immunoglobulin (Ig) has been the research object of several laboratories and this did result in a number of reviews (Carton, 1973; Corbel, 1975; Marchalonis, 1977; Rijkers, 1980; Dorson, 1981). In this paragraph a short summing-up of the present data will be given.

Ig of teleost fish has one heavy chain isotype, which corresponds with mammalian μ chain. In most teleosts, serum Ig is found exclusively in a tetrameric form (Shelton & Smith, 1970; Acton et al., 1971). In mammals a joining polypeptide (J chain) plays an important role in the assembly of IgM polymers. However, in teleost fish this polypeptide could only be demonstrated in some species (e.g. channel catfish) whereas in others it is apparently absent (e.g. carp). The physicochemical properties of teleost Ig slightly varies between species. Data for carp Ig are given in table 4.

TABLE 4. Physicochemical properties of carp Immunoglobulin*.

Sedimentation coefficient (S20)	14 - 15	S
MW native molecule	608 - 720	Kd
H-chain	71 - 77	Kd
L-chain	24	Kd
Carbohydrate content	6.8	%
Formule	(L2μ2)4	

* Data from Ambrosius et al. (1967); Shelton & Smith (1970); Marchalonis (1971); Richter et al. (1973); Andreas et al. (1975); Kd = kilo dalton

Ig has also been demonstrated in secretions (skin mucus, intestinal mucus, bile). In bile and skin mucus also a dimeric form is present in addition to tetrameric IgM (Lobb & Clem, 1981b,c). Ig levels in serum and secretions of carp, are given in table 5.

Ambrosius et al. (1982) tested the antigenic relationship of carp Ig and HMW Ig from other vertebrate species. They confirmed the assumption that IgM is phylogenetically the most early Ig. It is the only Ig type which is present in all vertebrate classes. In most teleosts this MHW IgM is the only Ig type. Following immunization no shift to LMW Ig takes place. However, in Chondrichthyes and some marine teleosts (e.g. grouper, margate and sheepshead) a LMW

TABLE 5. Immunoglobulin levels in carp[1)].

Source	Concentration	
	μgIg/ml	μgIg/mg protein
Serum	2000	67
Bile	2	1
Skin mucus	nt	1 - 2
Intestinal mucus 1[2)]	nt	≦ 0,5
Intestinal mucus 2[3)]	nt	1 - 4
Serum[4)]	1700	60

1) Ig levels in non-immune carp, measured by ELISA. The fish were kept at 20 ± 1 °C, and daily fed with pelleted dry food at about 2% of the body weight (Lamers & Den Bieman, unpublished); nt = not tested
2) First gut segment
3) Second gut segment
4) Data from Richter et al. (1973)

IgM has been detected, which was antigenically identical with HMW IgM (Clem, 1971; Lobb & Clem, 1981a).

Immunoglobulin subclasses. In recent years Lobb & Clem (1981a, b,c,d, 1983; Lobb et al., 1984) have published a series of studies providing evidence for structural diversity in fish Ig. The predominant HMW Ig in serum of channel catfish, sheepshead and salmon appear to exhibit structural variation in the covalent (disulfide bridges) architecture of the tetramer (Lobb & Clem, 1981a, 1983; Kobayashi et al., 1982). In other studies, on the structure of secretory Ig in fish, also indications were found for heterogeneity among H-chains. Sheepshead bile contained a dimeric Ig, which was antigenically similar to serum Ig. However, the MW of the H-chain (MW about 55 Kd) differed from that of serum Ig (i.e. HMW H-chain ± 70 Kd, LMW H-chain ± 45 Kd) (Lobb & Clem, 1981c). Cutaneous mucus of sheepshead contained both tetra- and dimeric Ig. However, their H-chains were in every respect identical to H-chains of serum HMW Ig (Lobb & Clem, 1981b). Lobb & Clem (1981d) showed in transfer experiments with radiolabelled homologous Ig that the bilary and cutaneous mucus antibody were not derived from serum. Foregoing data are suggestive for distinct H-chains in fish Ig. Moreover, they indicate that a local synthesis of secretory Ig may exist in fish.

Lobb et al. (1984) presented evidence for heterogeneous L-chains in channel catfish: 1) they showed that L-chains had different MW, 22, 24 and 26 Kd respectively; furthermore, DNP elicited preferential Ig with 22 and 24 Kd L-chains, which corresponds to the observation in mammals that haptens preferentially induce Ig containing a distinctive class of L-chain (λ or κ) (Nussenzweig & Benacerraf, 1967); 2) two antigenically different L-chains could be demonstrated by means of MoAbs, 22/24 Kd versus 26 Kd; 3) peptide mapping showed distinctive structural differences between the 2 L-chain types. The relative ratio of the two different L-chain types in an individual was constant for the whole fish population studied: 60% of 22/24 Kd and 40% of 26 Kd, which is consistent with the constant ratio of L-chain types in mammals. Lobb et al. (1984) concluded that these groups of L-chains can be designated as classes or subclasses.

Diversity and affinity. In teleost fish the antibody response against a particular antigen is polyclonal. Even a small antigen as DNP induces antibody directed to 4 different determinants on the molecule. However, as in other lower vertebrates, in fish only a limited antibody diversity has been reported (Machulla et al., 1980; Du Pasquier, 1982; Vilain et al., 1984).
In mammals antibody affinity increases during the response, which is accompanied by a change in Ig class. Although no change in Ig class occurs in teleosts, an increase in functional affinity has been demonstrated during a response against a T-dependent antigen in carp (DNP-HSA, Fiebig et al., 1979). Secombes & Resink (1984) speculated on production of higher affinity carp IgM in the secondary response against HGG, based upon the enhanced precipitating capacity.

2.4.5.2 *Antibody response*

It takes some time (lag period) before the first antibody appears in the circulation after injection of antigen. During that time a complex sequence of events has taken place. It is known from mammals that these processes comprise: antigen uptake from the circulation, antigen processing and presentation, T and B cell activation, T-B co-operation, resulting in cell proliferation and differentiation. There are indications in fish for the requirement of macrophages during the onset of the response (Smith & Braun-Nesje, 1982); the exposure of antigen, whether or not complexed with antibody in splenic ellipsoids (Secombes et al., 1982b) or on melanomacrophages (Ellis, 1980); "T" and "B" cell co-operation, in hapten-carrier experiments (Avtalion et al., 1975; Stolen & Mäkelä, 1975); induction of helper (Avtalion et al., 1980; Wishkovsky & Avtalion, 1982) and suppressor activity (Serero & Avtalion, 1978); production of interleukins (Caspi & Avtalion, 1984; Grondel & Harmsen, 1984) and occurrence of lymphocyte proliferation in the lymphoid organs (Manning et al., 1982a). These data suggest similar mechanisms for immune response induction as observed in mammals.

The cell proliferation and differentiation process results in formation of plasma cells secreting specific antibody. After appearance of the first antibody producing cells in the lymphoid organs, their numbers increase exponentially. A rather fast decay of the response usually follows after a sharp peak. Serum antibody titres are first observed during the exponential phase or at the peak day of the antibody forming cell numbers. Serum antibody titres increase exponentially and reach a plateau; depending on the type of antigen and fish species the subsequent decay may be fast or very slow (cf. Rijkers, 1980). The phases of the humoral response are visualized in Figure 4.

After a second delivery of the same antigen, the latent period is shorter, the response is accelerated and higher numbers of antibody producing cells and higher serum antibody levels are reached (see Fig. 4). A real anamnestic response is only observed when animals were able to form immunological memory after the first contact with the antigen (see paragraph 2.4.8).

2.4.6 *The systemic immune system III (Cellular immunity)*

Cell mediated immunity (CMI) is attributed to the activity of T-lymphocytes. These cells can act as regulatory (T-helper and -suppressor) or effector cells (T-killer). *In vivo* manifestations of CMI are: delayed type of hypersensitivity (DTH), transplantation and tumor immunity; whereas *in vitro* tests are: direct contact cytotoxicity, mixed lymphocyte reaction, macrophage migration inhibition and antigen induced blastogenesis of lymphocytes.

CMI in fish is less understood. Most studies are dealing with transplantation immunity. The phenomenon of graft rejection has been reviewed by Hildemann (1970), Botham et al. (1980) and Rijkers (1982b). On one hand it is apparent that lymphocytes and mononuclear phagocytic cells are involved in this process, but on the other hand

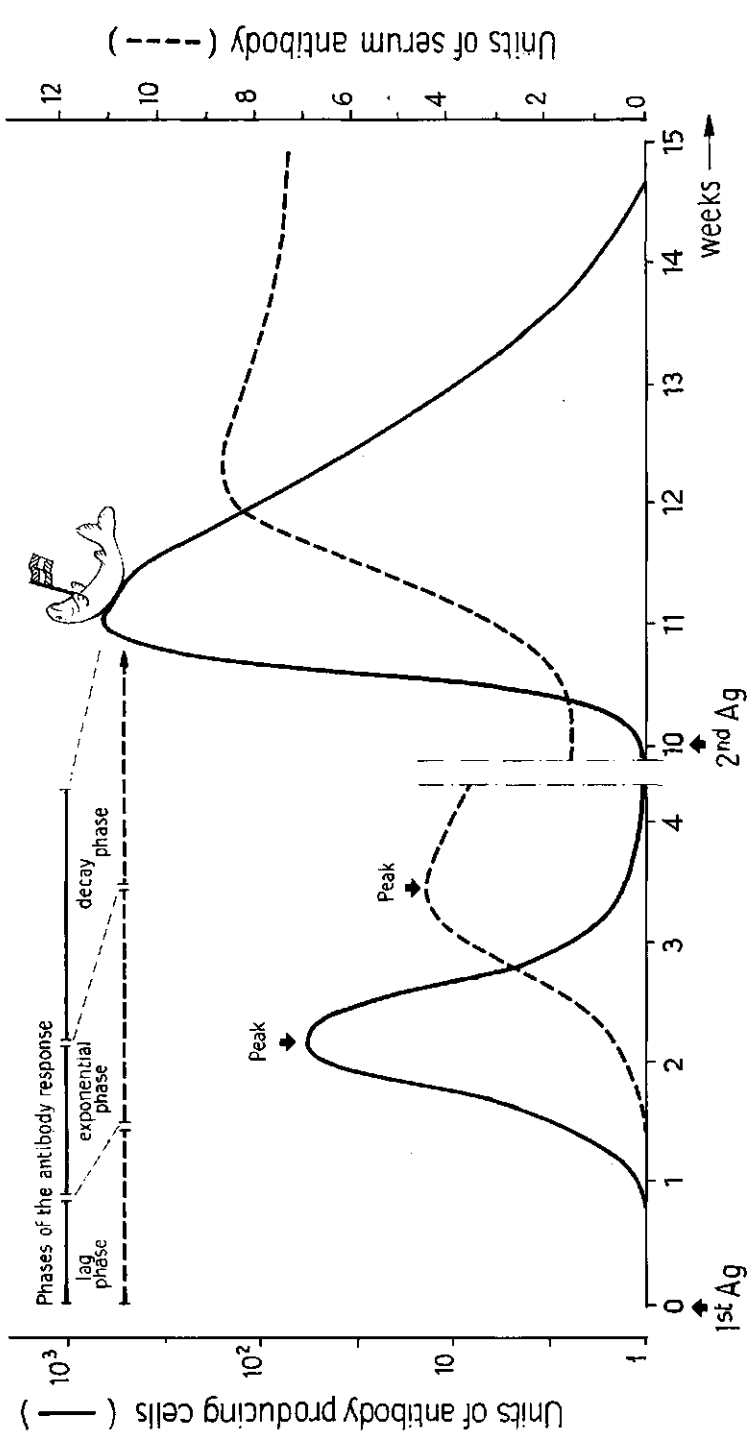

FIGURE 4. A schematic representation of a primary and secondary immune response in fish.

the mechanism is still not well documented. For example no specific cytotoxic lymphocytes could be demonstrated in fish up to now. The rejection of grafts reflects the ability of fish to act effectively against antigens on foreign cells including virus infected cells and tumor cells. However, little is known about CMI to pathogens like bacteria and parasites.

Tests for DTH gave variable results. A positive DTH was detected to soluble antigens of *Ascaris* in the bowfin (Good & Papermaster, 1964), to tuberculin derivatives in sea lampreys, paddlefish and rainbow trout (Finstad & Good, 1966; Ridgeway et al. 1966; Bartos & Sommer, 1981). Negative results are reported in studies on protein, viral and bacterial antigens in dogfish, eel, plaice and channel catfish (Parish, 1981; Pauwley & Heartwell, 1983). Using the macrophage migration inhibition (MI) test, Smith et al. (1980) showed that lymphocytes of rainbow trout immunized against *A. salmonicida*, the pathogen causing furunculosis, inhibited macrophage mobility in the presence of the antigen. About 75% of the vaccinated fish showed a positive reaction. 40% were positive in a MI test with a heterologous *A. salmonicida* strain, whereas 3-10% of control fish showed positive MI with both strains. The percentage of fish found resistant to furunculosis in a field trial corresponded closely to the percentage with positive MI against the strain causing the infection.

The reactivity of fish lymphocytes to antigens has also been tested *in vitro*. Sigel et al. (1978) demonstrated substantial blastogenic responses of peripheral blood lymphocytes (PBL) from nurse shark and snapper to protein and viral antigens. Chilmonzcyk (1978) reported a study with the viral fish pathogen causing VHS; inactivated virus induced stimulation of PBL in 82% of trout, which had survived a natural VHS infection, and in 58% of trout which were protected to VHS by previous vaccination. This response was specific and controls never responded to viral stimulation. Liewes et al. (1982) showed in carp the presence of specific sensitized leucocytes in thymus and spleen after bath vaccination against *Fexibacter columnaris*. None of these fish had developed antibody titres. However, a similar vaccinated group of carp proved to be protected in a natural challenge (Liewes et al., 1982).

Smith & Braun-Nesje (1981, personal communication) studied the CMI of *Salmo salar* to a *Vibrio* vaccine. Lymphocytes of vaccinated fish were highly stimulated by preparations of *V. anguillarum*, but control fish were stimulated as well. It is not clear whether the stimulation induced by *Vibrio* is due to mitogenic properties of the bacterin or that it is a real manifestation of a previous contact with *Vibrio* bacteria. Similar results were found by Lamers & Ideler (unpublished) in carp vaccinated with *A. hydrophila* bacterin.

From these few studies it is clear that antigenic stimulation of lymphocytes can take place in fish. It is a promising method to asses the acquired immunity to vaccination, especially after vaccination procedures which do not always generate antibody titres, e.g. the bath and spray method (Gould et al., 1978; Liewes et al., 1982).

2.4.7 *The local immune system*

The local or mucosal immune system, involving surface structur-

es and their immune reactions, has not yet been studied extensively in fish. Mucosal immune processes are very important in fish, as these animals have such an extensive mucosal barrier (skin, gills and intestine). The gills will not be taken into account due to lack of data.

2.4.7.1 *Gut associated immunity*

In teleosts leucocytes are present in the epithelium as well as in the lamina propria of the gut (Bullock, 1963; Pontius & Ambrosius, 1972; Weinberg, 1975; Zapata, 1979a). In carp and rosy barb mainly lymphocytes and heterophilic granulocytes and occasionally some macrophages, PAS-positive granulocytes and melano-macrophages were observed (Davina et al., 1980). The total number of cells is considerable, but they do not show concentrations, as have been reported in roach (Zapata, 1979b), and perch (Pontius & Ambrosius, 1972). In rosy barb the number of leucocytes per 100 epithelial cells varied from 20 to 50, depending on the region of the gut (Davina et al., 1982); in 20 weeks old carp it varied from 40 to 70 (Davina et al., 1980), whereas in 1 year old carp the number varied from 90 to 130 (Lamers, unpublished results). At a rough estimate 100-300 $x10^6$ leucocytes will be present in the intestinal epithelium of 1 year old carp.

Reports on intestinal immunity in fish are scarce, notwithstanding the numbers of lymphoid cells in the intestine. These are certainly high and might surpass the numbers in other lymphoid organs. The presence of both macrophages and lymphocytes indicates that in the intestinal mucosa specific immunological processes might occur. Plasma cells have been detected both in the epithelium (Weinberg, 1975) and in the lamina propria (Zapata, 1979b; Lamers, unpublished results). Pontius & Ambrosius (1972) demonstrated specific antibody producing cells in the pyloric region of the perch after SRBC injection in the heart region. Moreover, the presence of Ig has been demonstrated in the intestinal mucus (Fletcher & Grant, 1969; Bradshaw et al., 1971), and specific Ig was observed after oral vaccination (Fletcher & White, 1973b). Davina et al. (1982) observed an increase of intra-epithelial leucocytes in rosy barb, within 30 minutes after oral administration of a *Vibrio* bacterin. The increase was most pronounced in the bulbus and in the posterior gut. However, the short time interval in which the reaction occured suggests that it might be a non-specific reaction (inflammation). Worth mentioning is that feeding rainbow trout with *A. salmonicida* suppressed the humoral immune response to injected antigen (Udey & Fryer (1978); this resembles the phenomenon observed in mammals, that antigen processed by the intestine may result in a local immune response, while simultaneously inducing the formation of suppressor cells for the systemic humoral response (Mattingly & Waksman, 1978). Moreover, remarkable observations are reported by Kawai et al. (1981). They orally vaccinated ayu against vibriosis and although fish developed considerable protection, no agglutinating antibody activity was detected in serum and intestinal mucus; however, a distinct increase in anti-*Vibrio* agglutinin occurred in the skin mucus. This anti-*Vibrio* activity prevented colonization of viable *V. anguillarum* cells on the

skin. This is the first study providing data on the possible existence of a common mucosal immune system in fish.

2.4.7.2 *Skin associated immunity*

The defensive role of skin mucus is suggested by both non-specific and specific defence substances. Ig has been demonstrated in skin mucus in addition to lysozyme, CRP and complement (Fletcher & Grant, 1968; Bradshaw et al., 1971; Harris, 1972). Thus pathogens may be inactivated in mucus by both antibody mediated complement activation, and via the alternative pathway by activation through bacterial substances, or by means of CRP. The protective action of the skin may be enhanced; vaccination effectively inhibited bacterial or parasital colonization on the skin (Kawai et al., 1981; Hines & Spira, 1974; Goven et al., 1980).

Few data are available on the cellular aspects of local immunity in fish. DiConza & Halliday (1971) observed a diffuse pattern of lymphoid cells in fish skin. Some other studies report the presence of granulocytes and macrophages (Mittal & Munshi, 1971; Phromsuthirar, 1977; Ferri & Macha, 1982). St-Louis Cormier et al. (1984) demonstrated the presence of plasma cells in the cutaneous dermis and mucus of rainbow trout.

2.4.7.3 *Secretory immunoglobulin*

Ig has been demonstrated in intestinal mucus (Fletcher & White, 1973b; DiConza & Halliday, 1971) and bile (Lobb & Clem, 1981c,d) in addition to the presence in cutaneous mucus.

The finding that these IgM-like mucus antibodies are similar to serum antibodies (Ourth, 1980; Bradshaw et al., 1971; Fletcher & Grant, 1969; Lobb & Clem, 1981b,c), has focussed interest on the origin of antibodies of mucosal surfaces. Goven et al. (1980) observed that i.p. injection of parasite antigen (*Ichthyophtirius multifiliis*) in catfish resulted in both serum and mucus antibodies. This phenomenon is also reported by St.Louis-Cormier et al. (1984), after injection of SRBC in rainbow trout. Goven et al. (1980) supposed that the induction of the systemic antibody production was followed by a concentration of antibody in mucus. Also Ourth (1980) postulated that mucus antibodies of catfish originated from serum.

On the contrary several authors proposed a local production of Ig. DiConza & Halliday (1971) immunized the Australian catfish by injection with BSA, and detected IgM-like molecules in both skin and intestinal mucus. However, in contrast to serum, no specific antibody activity was present in these secretions. Furthermore they postulated that the diffuse lymphoid cells in skin and intestine might be involved in local Ig synthesis. Harrell et al. (1976) passively immunized rainbow trout with anti-*V. anguillarum* antibodies, but were unable to detect specific Ig in mucus and suggested that mucus antibody did not originate from serum. Recently Lobb & Clem (1981d) injected a marine teleost intravenously with purified radiolabelled Ig and subsequently compared amounts of label in serum, mucus and bile.

The low amount of labelled Ig which appeared in mucus and bile supported the hypothesis that a secretory immune system similar to that in mammals (Bienenstock & Befus, 1980), also exists in fish. The same authors also identified in cutanous mucus Ig the presence of an extra protein (95 Kd), which might be equivalent to mammaliam secretory piece (Lobb & Clem, 1981b).

Foregoing data on secretory Ig and induced mucus immunity are suggestive for the existence of a (common) local or secretory antibody system in fish.

2.4.8 *Memory*

An important feature of the immune system is the capacity to develop memory. After a first contact with antigen effector cells are usually induced which mediate the immune functions. These cells are mostly short-lived. Among the progeny of the original lymphocytes are also some long-lived cells, which may retain the capacity of being (re)-stimulated by the original antigen. In case of "positive memory" they cause a much faster and more vigorous immune response at a second exposure to the same antigen. When challenge produces a depressed response or non at all, a negative-type of memory (tolerance) has developed. Some properties are summarized in table 6.

The presence of memory lymphocytes is usually examined by monitoring the secondary response. This response will show anamnestic characters when memory is present; it is faster and more vigorous (Fig. 4). Moreover, in tetrapods there is also a shift to antibody with a higher affinity, which is correlated with the shift from HMW Ig to LMW Ig (e.g. in mammals IgM → IgG).

TABLE 6. Properties of positive Memory and Tolerance in Mammals*

	Positive Memory	Tolerance
Examples	Specific resistance to reinfection Higher and faster developing antibody titers Changes in class of antibody Hypersensitivity states Homograft immunity	Self-tolerance Acquired tolerance to foreign antigens; easier to induce neonatally, and to relatively small antigens; tolerance to both high and low antigen dose may develop
General Properties	Specific Temporary or long-lasting (depending on antigen presence)	Specific Temporary or long-lasting (depending on antigen presence)
Probable causes	Increased numbers of specific T or B cells or both Changes in nature of reactive cells (e.g. receptor affinity)	Specific deletion of reactive cells Reversible inactivation of T and/or B cells Presence of active suppressor T cells

* slightly modified after Cunningham (1978)

Positive memory has also been demonstrated in fish for cellular and humoral immune reactions (Botham et al., 1980; Rijkers et al., 1980b; Rijkers, 1982b). Several authors have questioned the existence of a classical anamnestic humoral response in fish (e.g. for goldfish, Desvaux & Charlemagne, 1981; rainbow trout, Dunier, 1985). In fish the ratio between secondary and primary antibody responses never reached the high levels obtained in mammals (about 10 in fish, up to 100 in mammals). However, Rijkers et al. (1980c) demonstrated that the height of the secondary antibody response in teleost fish was dependent on temperature. Carp kept at 18 °C elicited a secondary response which showed no enhancement above the primary response. Nevertheless, it differed from the primary response by an earlier appearance and by being more extended. At 20 °C the secondary response exceeded the primary about 10 times, and at 24 °C the ratio was even about 50.

The data on secondary responses in Salmonids have not been convincing and Dunier (1985) expressed doubts on the existence of memory in these fish. She did not find any difference between primary and secondary antibody responses against both "T-dependent" and "T-independent" antigens in rainbow trout. On the contrary Botham et al. (1980) reported an accelerated second skin graft rejection in rainbow trout, indicating the existence of memory for the cellular responses in trout.

Immunological tolerance has also been demonstrated in fish. Serero & Avtalion (1978) completely suppressed the anti-BSA response in adult carp by i.v. injection of soluble BSA. The induction of tolerance was not dependent on antigen dose, but on the route of injection and the nature of the antigen. Wishkovsky & Avtalion (1982) showed that at relatively low temperatures only a high dose of soluble BSA induced tolerance, which was specific and long-lasting (> 16 months). It was suggested that this long-term negative memory was stored in the body by specific suppressor cells.

There are also indications, that immunization, before a certain stage of immunological maturation may lead to a state of unresponsiveness (Van Loon et al., 1981; Manning et al., 1982b; Van Muiswinkel et al., 1985). (See also paragraph 3.2.4.2 on ontogeny of immune reactivity).

3 *IMMUNOMODULATION*

The immune response of teleost fish is influenced by several external and internal factors.

3.1 EXTERNAL FACTORS AFFECTING THE IMMUNE SYSTEM

It is important to realize that the kinetics of the immune response are influenced by a number of factors: temperature, the antigen and several other substances.

3.1.1 *Temperature*

In poikilothermic animals metabolic processes are directly influenced by the ambient temperature. The effect of temperature upon antibody synthesis has been known for a long time (Bisset, 1948).

In a series of studies Avtalion and his group investigated the effect of temperature upon antibody production in carp and tilapia against BSA (cf. Avtalion, 1981). They also used temperature as a means to separate helper, suppressor and antibody producing functions during the immune response. Bisset (1948) was the first to put forth the idea that dissection of certain stages in the immune response of lower vertebrates could be obtained using the temperature effect. He suggested that antibody synthesis and its release were affected by temperature. However, Avtalion (1969) showed that both synthesis and release of antibody could take place at low temperatures (12 °C) if fish are kept at the high temperature (25 °C) during the latent period. Using a hapten-carrier system Weiss & Avtalion (1977) showed that the generation of T-helper cells in fish was a temperature sensitive event. Moreover, the processing of antigen by macrophages seemed to be affected by temperature and Avtalion (1981) suggested that the temperature sensitive events in the humoral immune response probably were related to antigen processing by macrophages, or to cell interactions.

Avtalion et al. (1973) showed the presence of a temperature sensitive period between day 0 and 4 after immunization. They observed that specific antibodies were produced in carp only if kept at a high temperatures for more than 3 days, before transfer to low temperatures. Avtalion (1981) tested whether the whole period (day 0-4) or only the final events, leading to differentiation of helper activity was temperature sensitive. It turned out that the temperature sensitive period in the humoral response was located between day 3 and 4 after priming at 25°C. Avtalion (1981) proposed a scheme showing the different stages of the antibody formation process (Fig. 5).

Rijkers et al. (1980c) observed that primary anti-SRBC responses in carp were delayed at low temperatures, but the height of the response was unaltered. However, the capacity to mount a clear anamnestic response was impaired at lower temperatures (18 °C or lower). The relationship between temperature and peak day in the primary hu-

moral response closely matched the relationship between temperature and the survival time of allografts in goldfish (Hildemann & Cooper, 1963; Rijkers et al., 1980c). Rijkers (1982b) stated that the humoral and cellular immunity in fish are affected by temperature in a similar way.

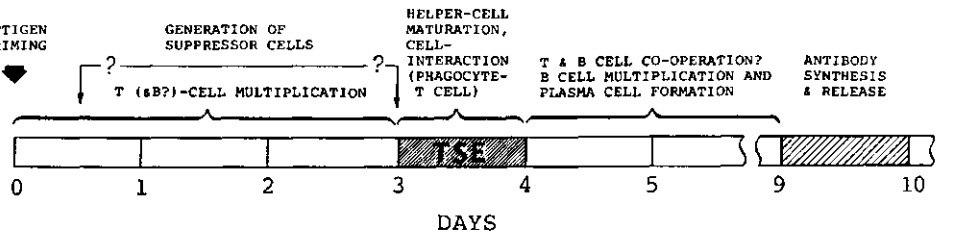

FIGURE 5. Chronological localization of the temperature-sensitive event (TSE) and dissection of the immune processes (Avtalion, 1981).

3.1.2 *Dose, nature and administration route of the antigen*

The dose of antigen may affect the latent period of the primary antibody response (Ambrosius & Schäker, 1964), and the height of the primary (Jayaraman et al., 1979) and secondary response (Rijkers et al., 1980b). Moreover, the dose of antigen is important for whether humoral or cellular immunity will predominate (Jayaraman et al., 1979), or whether or not a response or tolerance is induced (Avtalion et al., 1973). The effect of antigen dose is discussed in more detail in Appendix paper 3 and 4.

The nature of antigen. There are good and poor immunogens for a particular fish species, with respect to serum antibody production. For example SRBC stimulate a high antibody production in carp (Rijkers, 1980), whereas in rainbow trout only a moderate production occurs (Dorson et al., 1979; Anderson, personal communication). Moreover, Hodgins et al. (1967) reported that the soluble protein antigen BSA elicited no response in rainbow trout, whereas keyhole limpet haemocyanine (KLH) was highly immunogenic in this species.

In paragraph 3.2.2 the effect of a slight chemical modification of the BSA molecule on the ability to induce serum antibody will be mentioned (Avtalion, 1981). Jayaraman et al. (1979) immunized the mozambique mouthbrooder with fresh, or with formalin fixed SRBC. Fresh SRBC induced a strong humoral response, whereas with formalin fixed cells only a weak humoral response occured. Interestingly enough in the last case a strong cellular immune reaction was found.

Antigen immunogenicity is important for vaccination. As most fish pathogens can be immunogenic in fish, it is of utmost importance to detect the antigenic determinants which will elicit the synthesis of protective antibodies or induce other protective properties. This item will be discussed in more detail in paragraph 4.2 "antigens of fish pathogens".

Route of administration. The route of antigen administration has a clear effect on the outcome of immunization. Several authors report the efficacy of i.m. above i.p. injections (Harris, 1973; Ingram & Alexander, 1976). Rijkers et al. (1980b) found that priming carp with SRBC by the i.m. route induced better memory than i.v. priming. Soluble BSA might be tolerogenic when injected i.v., whereas it was not after i.m. injection (Serero & Avtalion, 1978). Following vaccination by immersion circulating antibody is often absent, although high protection levels can be achieved (Croy & Amend, 1977). Oral immunization may elicit antibody synthesis in the intestinal mucosa (Fletcher & White, 1973b).

3.1.3 *Antigenic competition*

Simultaneous vaccination against two or more pathogens includes the risk for antigenic competition. Antigenic competition is defined as inhibition of the immune response to one antigen or antigenic determinant caused by administration of another antigen. In mammals antigenic competition between mixed antigens is dependent on the relative proportions of the antigens (Taussing, 1977), and might be a feature of T-cells (macrophages and B-cells might also be involved in the competition between intra molecular determinants).

The phenomenon of antigenic competition has also been demonstrated in teleost fish. Richter & Ambrosius (1972) found that bovine and human gamma globulin competed in immunogenicity when they were conjointly injected in carp. O'Neill (1981) injected brown trout and carp with two bacteriophages MS_2 and X174. The clearance of the two bacteriophages was mutually suppressed in conjoint administration. Desvaux & Charlemagne (1981) recorded an inhibition of the anti-SRBC antibody synthesis in goldfish simultaneously injected with Xenopus red blood cells.

On the other hand Amend & Johnson (1984) provided evidence for the lack of interference with protective immunity, when various combinations of *V. anguillarum, Y. ruckeri, A. salmonicida* and *Rennibacterium salmoninarum* were administered to salmonid fish. The level of protection obtained with the cocktail vaccine was comparable with that obtained with the separate monovalent vaccines. Also Schäperclaus (1970) stated that *A. hydrophila* bacterins composed of several strains were more effective in raising protection than a monovalent vaccine.

It can be concluded from these data that the phenomenon of antigenic competition occurs in fish. However, to date there are no indications for interference with protective immunity. Thus potential polyvalent vaccines* may be used for protection of fish.

3.1.4 *Response enhancing substances*

In fish immunology several compounds are used which enhance antibody production (adjuvants). Several authors reported the enhancing

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* At present commercial combination vaccines are available. See footnote at page 16.

effects of Freund's Complete Adjuvant (FCA), (Krantz et al., 1963; O'Neill, 1979; Dunier, 1985). Both a higher serum antibody response and a longer persistence of antibody titres was obtained. However, FCA induces necrotic lesions, which is an obstacle for commercial use.

Considerable attention is devoted to the development of adjuvants for practical use. Anderson et al. (1984) immersed rainbow trout in dimethylsulfoxide (DMSO) followed by immersion in antigen solution and observed an elevated humoral response. It is known that DMSO facilitates attachment to receptor sites and internalization of antigen (Bartfield & Goldstein, 1975). Anderson et al. (1984) suggested that DMSO might stimulate the activity of antigen-presenting cells, as well as gill macrophages. Another substance that has been described to enhance phagocytosis in fish is ETE (extract, derived from the tunicate *Exteinascidua turbina*), known as an immuno stimulant in mice. According to McCumber et al. (1981), ETE could abrogate the suppressive effect of low temperature, and these authors suggested that it is a promising adjuvant.

3.1.5 *Response suppressing factors*

The effects of pollutants on fish health have been recognized (Reichenbach-Klinke & Ahne, 1981). The immune response is clearly affected by heavy methals, organic solvents and pesticides; these effects have been reviewed by Zeeman & Brindley (1981) and Anderson et al. (1984).

The outbreaks of several diseases in fish have been correlated with stress inducing events, such as crowding and handling (Snieszko, 1974). The influence of stress on the immune response of fish has been recently reviewed by Ellis (1981). Stress is visualized by an increase of plasma cortisol. It is expected that stress will impede an immunization, because corticosteroids can be immunosuppressive in fish.

Some antibiotics (e.g. Oxytetracycline) have been found to have a suppressive effect on *in vivo* (Rijkers et al., 1980d) and *in vitro* (Grondel & Boesten, 1982) immune reactions. Recently Grondel (in prep.) observed that the *in vivo* effect of the antibiotic was more a delay than a suppression of the humoral response.

3.2 INTERNAL FACTORS AFFECTING THE IMMUNE RESPONSE

Some immunoregulatory mechanisms, already described in mammals, have been found also in fish.

3.2.1 *Immunoregulatory mechanisms*

Antibody feed-back inhibition. Rijkers et al. (1981a) published an indication for this immunoregulatory mechanism in fish. They found that a high dose of passively transferred specific antibody inhibited the subsequent anti-SRBC response. Moreover, they reported

that fish i.m. primed with a high dose of SRBC gave only a moderate secondary antibody forming cell response upon a booster injection (Rijkers et al., 1980b). This observation might have been due to antibody feed-back regulation (Grantham & Fitch, 1975).

Immune complexes. The elimination of antigen is facilitated by the formation of complexes of antigen with antibody; these immune complexes may also have an immunoregulatory function. Rijkers et al. (1981a) observed that a low dose of passively transferred specific antibody had a stimulatory effect on antibody production, probably by *in vivo* formation of immune complexes after antigen injection. Secombes et al. (1982b) reported that injection of immune complexes or injection of antigen in immune animals resulted in an increased rate of antigen processing. Furthermore, priming carp with immune complexes induces a better memory than priming with antigen alone. It also induced the production of antibody with higher affinity (Secombes & Resink, 1984).

In mammals T-helper and suppressor cells play an important role in the humoral response.

3.2.2 *Helper activity*

The existence of helper activity in fish has been mentioned in a previous paragraph (3.1.1). Carrier specific helper cells co-operate with hapten specific antibody producing precursor cells (Stolen & Mäkelä, 1975; Avtalion et al., 1975). Pre-immunization with the carrier followed by a booster with the hapten-carrier complex resulted in an enhancement of the anti-hapten antibody response in carp (Avtalion et al., 1980), which suggests formation of helper-memory cells after priming. Furthermore, Weiss & Avtalion (1977) and Avtalion et al. (1980) showed that acetylated BSA molecules lost their ability to induce antibody formation in carp and tilapia but still stimulated helper-memory formation. This helper-memory was long-lived and it was still detectable at day 250 after priming. The formation of helper activity is abrogated at a low temperature (12 °C), but, once helper activity has developed at a high temperature (25 °C), it still functions when the fish is transferred to lower temperatures (Avtalion, 1981).

3.2.3 *Suppressor activity*

Serero & Avtalion (1978) found that intracardiac priming of carp with soluble BSA (sBSA) induced a state of specific unresponsiveness, which was of long duration (longer than 16 months). In contrary to the helper function, it could be induced at high or low temperatures. At a low temperature (12 °C) complete suppression was only obtained with a high antigen dose (10-50 mg sBSA). Wishkovsky & Avtalion (1982) demonstrated this phenomenon also for several other protein antigens.

Manning et al. (1982a) provided tentative evidence for a regu-

latory role of thymus derived cells in antibody formation. Thymectomy in adult carp and rainbow trout resulted in higher antibody responses than in normal fish.

In mammals the alkylating agent cyclophosphamide (Cy) predominantly blocks T-suppressor cells. Observations by Anderson et al. (1982) suggest similar effects in fish, for they found that Cy slightly reduced splenic lymphocyte numbers in rainbow trout, whereas antibody production was enhanced.

Udey & Freyer (1978) vaccinated rainbow trout against *A. salmonicida* and showed that fish which were fed antigen and subsequently injected showed suppressed serum antibody levels compared to the fish injected with antigen alone. These data suggest that after oral administration of antigen suppressor cells are formed for the systemic response.

3.2.4 *Ontogeny*

Research on the ontogeny of immunity in fish is of high basic and applied importance. Some diseases threaten young fish (IPN in particular). Most losses occur during the first half of the fish commercial life. It is important to know at what age a fish can be vaccinated in order to minimize these losses.

3.2.4.1 *Ontogeny of immune organs*

At 22 °C the lymphoid development in carp starts with the appearance of the thymus at 4 days after fertilization (a.f.) which is 2 days post-hatching (p.h.) (Botham & Manning, 1981) and in rainbow trout (at 14°C) at 5 days pre-hatching (Grace & Manning, 1980). Haemopoietic stem cells are first present in the kidney (Ellis, 1977b; Grace & Manning, 1980). Secombes et al. (1983b) studied the appearance of antigenic determinants on carp lymphoid cells using MoAb directed against thymocytes and Ig. As soon as the thymus appeared a thymocyte surface determinant could be observed. Cells carrying this determinant were not detected in head kidney until day 7 a.f. MoAb, that recognize both Ig and adult thymocytes stained thymocytes from day 7 onwards, and head kidney cells from day 12-16 a.f. onwards. Ig positive cells were first detected in head kidney from day 16 onwards. By day 16 a.f. the staining pattern of thymus resembled that of adult fish. These results showed that the appearance of cells with certain determinants in the thymus preceeds their appearance in head kidney, suggesting the thymus to be the primary lymphoid organ in carp. The spleen is probably not vital for immunological maturity as it is still present as a rudiment, when thymus and kidney lymphocytes already carry surface Ig and display mixed lymphocyte reactions (Ellis, 1977b).

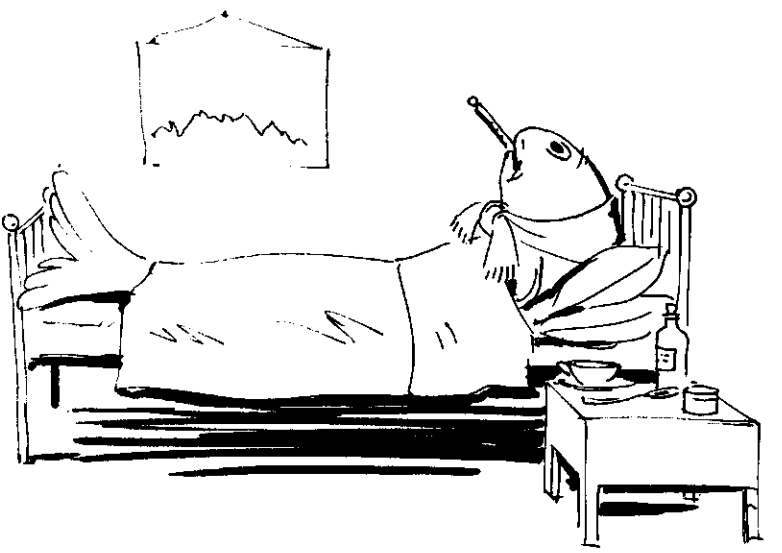
3.2.4.2 *Ontogeny of immune reactivity*

It has been shown that functional macrophages are present during the period of lymphocyte immaturity (Grace & Manning, 1980). At

4 days p.h. rainbow trout fry had already an efficient system of phagocytes in skin, gills and kidney. In the later phase cellular immunity developed rapidly: carp aged 16 days (p.h., 22 °C) were capable of mounting an allograft response (Botham et al., 1980). In rainbow trout the ability to respond to skin grafts was also correlated with maturation of thymus and kidney (Manning et al., 1982a,b).

The first Ig synthesis in pike started at day 18 (p.h), but Ig levels were still low at 1 month of age (Clerx, 1978). The actual antibody synthesis upon antigenic stimulation showed a different picture. Van Loon et al. (1981) injected 4 weeks old carp with SRBC, but could not elicit an anti-SRBC response. At reimmunization 3 month later the fish failed to respond, whereas animals receiving the first immunization at 4 months showed a normal anti-SRBC response. So too early injection of an antigen may cause tolerance. Manning et al. (1982a) reported that young trout were not able to elicit an antibody response against HGG 21 days p.h., whereas they responded at the same time to the bacterial antigen *A. salmonicida*. The trout developed a state of tolerance to HGG, for they did not respond to a second administration 8 weeks later. Mughal (1984) observed the same phenomenon in carp, using HGG in a soluble form or attached to latex beads. The tolerogenic effect persisted up to the end of the observation period (23 weeks). Using formalin-killed *A. salmonicida*, Mughal (1984) obtained different results: four weeks old carp responded to this antigen and upon a second exposure an enhanced antibody response was monitored.

It can be concluded from these results that thymic and nephric tissues develop rapidly, and at that early stage the phagocytic defence is soon supplemented with cellular immunity. Ab response and memory can be induced in young (2 month) fish using certain antigens. The ability to respond to some "T-dependent" antigens like SRBC did develop much later. These results indicate an asynchronous development of lymphoid functions in fish. This will probably have implications for vaccination schedules.



4 *DISEASES AND VACCINATION*

The number of known fish diseases has grown simultaneous with the expansion of fish culture . Extensive reviews on fish diseases have been published (Snieszko & Axelrod, 1971; Roberts, 1978; Reichenbach-Klinke, 1979; Amlacher, 1981). During the last decades more knowledge has been gathered on the causative agents of diseases, their etiology, pathogenesis, diagnosis, treatment and prevention (cf. Anderson et al., 1983).
A number of economically important diseases is included in the zoosanitory code of the Office International des Epizooties (De Kinkelin & Michel, 1984). At present immunoprophylaxis is applied in the case of 13 diseases (5 viroses, 7 bacterioses and 1 parasitosis). In most cases this was achieved at a small experimental, but sometimes even at large and practical scale. Most efforts have been invested in vaccination against bacterioses. The information in this chapter will be restricted to this subject.

4.1 BACTERIAL DISEASES

The bacterial diseases which cause serious problems in fish farming are usually septicaemic infections. The onset of these diseases is strongly linked to environmental conditions. Most bacterioses occur above 15 °C and are often linked to stress conditions. General information on pathogenic bacteria is summarized in Table 7.

Most of these organisms behave as typical pathogens, but *A. hydrophila* and also *F. columnaris* can act as opportunistic pathogens. They are normally present in or around the fish, and encounter those fish whose resistance is lowered. For specific data on pathology, etiology, diagnosis and treatment reference can be made to fish disease handbooks (Roberts, 1978; Reichenbach-Klinke, 1979; Amlacher, 1981; Anderson et al., 1983).

In this thesis bacterial preparations have been used from *A. hydrophila* and *Y. ruckeri*. Some supplementary data will be given on these bacteria.

4.1.1 *Aeromonas hydrophila*

A. hydrophila strains are ubiquitous habitants of surface waters, where they are often present in high numbers. Their density is directly related to water temperature and eutrophication (Hazen et al., 1978). Bacteria, referred to as *A. hydrophila* are very heterogeneous both biochemically, genetically, serologically and phenotypically. Therefore they have been described by several authors under many synonyms (e.g. *A. liquefaciens, A. punctata, Bacterium punctatum*; Newman, 1983).

The bacteria belong to the natural bacterial flora of all fresh water fish and play a significant role as potential pathogen (Heuschmann-Brunner, 1978). The opportunistic pathogen causes outbreaks of bacterial hemorrhagic septicaemia in situations in which fish suffer

TABLE 7. Gross characteristics of the causative agents of the major bacterial infections in fish (after De Kinkelin et al., 1985).

Bacterial taxonomy	Host range	Geographical range	Gross pathology
AEROBIC			
CYTOPHAGACEAE			
Flexibacter columnaris	all tested fish species	cosmopolitan	necrosis of integument and gill
FACULTATIVE ANAEROBIC			
ENTEROBACTERIACEAE			
Edwardsiella tarda	*Anguilla* sp. *Ictalurus punctatus* *Carassius auratus* *Micropterus salmoides* *Evynnis japonicus*	North America Asia	haemorrhagic septicaemia, "red pests", sometimes systemic granulomatosis
Edwardsiella ictaluri	*Ictaluridae*	North America	haemorrhagic septicaemia
Yersinia ruckeri	*Salmonidae* *Anguilla* sp. *Psetta maxima* *Notemigonus atherinoides*	North America Europe Australia	haemorrhagic septicaemia plus enteritis
VIBRIONACEAE			
Vibrio anguillarum	most species in sea and brackish water	cosmopolitan	haemorrhagic septicaemia
Vibrio ordalii	*Salmonidae*	North America	*id.*
Aeromonas hydrophila	most species in fresh and brackish water		*id.* "red pests"
Aeromonas salmonicida	*Salmonidae* and many fresh water species	cosmopolitan	haemorrhagic and ulcerative septicaemia; furunculosis
A.s. subsp. achromogenes	*Salmonidae*	cosmopolitan	*id.*
A.s. subsp. nova	*Cyprinus carpio*	cosmopolitan	erythrodermatitis

from physiological discomfort, injury or stress, as can occur after wintering under bad conditions, or following handling procedures (i.e. vaccination, transport), or as a second invader during infections of viral, bacterial or parasite nature (Newmann, 1983). *A. hydrophila* has been recognized as the major bacterial component in diseases of multiple ethiology, as "infectious dropsy" or "septicaemic-dermovisceral syndrome" (Amlacher, 1981).

Esch & Hazen (1980) observed a correlation of the prevalence of *A. hydrophila* induced "red sore disease" in large mouth bass, with body condition and stress. The bad condition was reflected by reduced red and white cell counts and elevated cortisol levels. Hazen et al. (1981) reported that the seasonal fluctuation in prevalence of diseased fish was positively correlated with the environmental temperature, but negatively correlated with the percentage of fish showing serum agglutinins to *A. hydrophila*.

The histopathology of *A. hydrophila* infected fish is variable (Newman, 1983). In large mouth bass (Huizinga et al., 1979), the external lesions range from pinpoint lesions affecting a few scales, to extensive chronic ulcerations. Many hemorrhagies occur in all organs, but liver and kidney are most affected. Bach et al. (1978) reported that the spleen was extremely affected in channel catfish. Toxic products of *A. hydrophila* may cause complete destruction of the integrity of this organ.

4.1.2 *Yersinia ruckeri*

The disease caused by *Y. ruckeri* is known as enteric redmouth disease (ERM). It is an acute to chronic systemic infection in Salmonid fish. The disease takes its name from the characteristic reddening of the mouth and opercula, which is caused by subcutaneous hemorrhages. Other clinical signs include inflammation and erosion of jaws and palate, darkening of the skin, exophtalmiasis, and sluggish behavior. Moreover the distal portion of the intestine is heavily affected (Bush, 1983). *Y. ruckeri* has a significant impact on the commercial trout industry. Mortality caused by ERM occurs in fish of 30 g and larger and losses reach a 10-20% level. Outbreaks of the disease generally follow stress producing procedures and probably result from shedding of bacteria by non-symptomatic carriers.

ERM disease has been effectively controlled by means of antibiotics (oxytetracycline and sulfamerazine), but repeated use of these drugs has led to serious resistance problems. The first vaccination trials revealed that both oral delivery, injection, and later also immersion provided protective immunity. *Y. ruckeri* vaccine was the first licenced vaccine for fish in the U.S.A. (Bush, 1983; Bullock & Anderson, 1984).

Successful vaccination against fish diseases requires knowledge of the major pathological mechanisms, development of vaccines containing the essential antigens and appropriate vaccination methods. In the following paragraphs attention will be paid to the "antigens of bacterial fish pathogens" and "vaccination methods".

4.2 ANTIGENS OF BACTERIAL FISH PATHOGENS

The isolation of fish disease antigens is of prime importance, firstly for the development of tests to detect and identify disease agents, and secondly for the development of effective vaccines. The first objective requires isolation of antigens that are strain or species specific. These antigens are usually related to surface structures such as lipopolysaccharides (LPS). LPS is an important immunogenic structure to which antisera can be raised for serodiagnosis.

To date serodiagnosis is widely applied, but reagents still need to be extended and perfected. The potential of monoclonal antibodies can be predicted. Furthermore the ELISA offers good prospectives for diagnostic tests. The Elisa has already been introduced at several laboratories for diagnosis of viruses (Ahne, 1981), *A. salmonicida* (Griffin, 1979; Smith, 1981), edwardsiellosis (Rogers, 1983). The second purpose is identification of those antigens that can evoke the immune response to give protective immunity. For both purposes the antigens in view do not need to be identical. Antigens "essential" to induce protection might be related to pathological features, or be hidden or only present in minority. In order to achieve successful vaccination it is important to identify these antigens and to compose vaccines in which these antigens will be optimally presented. Protective immunity for vibriosis and yersiniosis has been obtained by vaccination with plain bacterial preparations. However, for *A. salmonicida* and *A. hydrophila* this approach gave inconsistent results. In the following paragraphs the antigens of these four bacteria will be discussed.

4.2.1 *Vibrio anquillarum*

Successful vaccination against vibriosis has been achieved with bacterial cell preparations (Evelyn, 1984). The "protective antigens" of *V. anguillarum* are apparently LPS structures. These cell wall antigens also count for the serotypic specificities (Aoki et al., 1981). The evidence that LPS is the protective antigen is based on several observations: the immunogen that induced protection was heat stable (Harrell et al., 1975), had a large size (MW over 100 Kd, Evelyn & Ketcheson, 1980), and could be extracted by phenol and perchloric acid (Aoki et al., 1981; Agius et al., 1983). Protection is clearly related to cell associated antigens, but culture supernatant may also contain protective substances. However, these antigens appeared to be identical to cell wall LPS (Evelyn & Ketcheson, 1980), and are probably shed into the culture medium during growth. Some controversial data have been presented on the existence of non-LPS protective antigens (cf. Evelyn, 1984).

4.2.2 *Yersinia ruckeri*

Successful yersiniosis bacterins were killed bacterial cell preparations, delivered in the diet (Ross & Klontz, 1965; Anderson & Ross, 1972), by injection or immersion (Bullock & Anderson, 1984).

To date it is not exactly known which are the protective antigens of *Y. ruckeri*, but in comparison with *V. anguillarum* it is believed to be the LPS structures (Amend & Johnson, 1981).

The immunogenicity of *Y. ruckeri* has been studied extensively by Bush (1978), testing the humoral agglutinating antibody responses in rainbow trout to a variety of *Y. ruckeri* preparations. The water soluble protein antigens were more immunogenic than the carbohydrate antigens which were soluble in organic solvents. Formalin or phenol killed cells gave antibody titres as high as the water soluble antigen titres. However, the most immunogenic was an ethanol extracted cell wall preparation, designated as O-antigen. With a haemagglutination test, using SRBC coated with the water soluble antigen, the prevalence of the disease was monitored and carrier state could be detected (Bush, 1978). Anderson et al. (1979 a, b, c) demonstrated that the bacterial cell wall preparation of *Y. ruckeri* O-antigen was very immunogenic in rainbow trout upon injection and exposure (direct immersion, flush exposure). The preparation, used for testing antibody production, contained mainly a relatively short LPS without many repeating polysacharide chains (Evenberg, personal communication). No correlation has been made with protection. Amend et al. (1983) reported that lysis of the *Y. ruckeri* bacteria at pH 9,8 (for 60-120 min), followed by formalin inactivation, significantly increased its protective efficacy. They suggested that the O-antigen might be released by this treatment.

4.2.3 *Aeromonas salmonicida*

The LPS or endotoxin of *A. salmonicida* strains is antigenically homogeneous (Hahnel et al., 1983). Injection of coho salmon with killed *A. salmonicida* cells resulted in serum antibody which was largely directed against LPS (Patterson & Fryer, 1974a,b). However, the LPS is not related to pathogenesis and anti-LPS antibody did not reflect protection (Michel, 1979). So the importance of this antigen for conferring protection must be questioned. On the contrary Duff (1942) and Krantz et al. (1964) induced protection against furunculosis using cellular preparations of *A. salmonicida*. These inconsistent results have stimulated extensive research on virulence factors of *A. salmonicida* and their suitability for protection induction. The virulence factors can be divided in cell associated and extracellular substances.

Cell associated virulence factors. Udey & Fryer (1978) and Bootsma & Blommaert (1978) were the first to demonstrate that virulent strains of *A. salmonicida* possessed an additional protein layer (A-layer), external to the cell wall. The A-layer is strongly hydrophobic (Trust et al., 1982), it causes auto-agglutination and is thought to be responsible for bacterial adhesion to fish tissue (Udey & Fryer, 1978). Furthermore, the A-layer forms a barrier and protects the underlaying cell wall components for chemical modification (Kay et al., 1981), and against the bactericidal activity of complement, both in the presence or absence of specific antibody (Munn et al., 1982).

Evenberg et al. (1982) analysed the A-layer protein of a vari-

ety of *A. salmonicida* isolates and concluded that this protein was similar for all isolates, regarding molecular weight (about 50 Kd) and antigenicity. Furthermore, its amino acid composition resembled the virulence related outer layer protein of enteropathogenic strains of *Escherichia coli* (K88 fimbrial protein) (Evenberg & Lugtenberg, 1982). Trust et al. (1982) also purified the A-layer protein and observed that it was immunogenic in rabbits. The antiserum reacted with virulent cells, but not with their avirulent forms. The immunogenicity in fish has not yet been documented.

McCarthy et al. (1983) provided evidence for the protective role of a cellular protein antigen, probably the external A-layer protein. Furthermore, in their experiments cell free culture supernatant gave no protection at all.

Extracellular virulence factors. The pathological effects of *A. salmonicida* can be induced by cell free culture supernatants (Pol et al., 1980). Upon injection in trout, these substances, designated as extra cellular products (ECP), are strongly ichthyotoxic and lytic for both red and white blood cells (Munro et al., 1980). Their toxic activity is attributed to enzymes such as proteases and phospholipases and to haemo- and leukocytolytic substances (cf. Munro, 1984).

Culture supernatants may contain, in addition to the exotoxins, shed cellwall components. In table 8 the presently known extracellular substances of *A. salmonicida* are listed supplemented with their role in pathology, their immunogenicity and possible suitability for vaccination.

The studies by Cipriano are worth mentioning. Fractionation of ECP resulted in 4 fractions; fraction 1,2,3 had toxic properties; fraction 2 was proteolytic and fraction 4 contained a glycoprotein with cytolytic activity (Cipriano et al., 1981; Cipriano, 1982b). Vaccination experiments revealed that protection to furunculosis could be obtained in trout by inoculation with crude ECP, but also with ECP fraction 4, and to a lesser extent with fraction 1 (probably LPS) (Cipriano, 1982a,b; Cipriano, 1983a). An immersion vaccine, rich in ECP fraction 1 and 4, conferred significant protection in a field trial (Cipriano & Starliper, 1982).

The contradictory results obtained by McCarthy et al. (1983) and Cipriano (1983a), might be explained by different culture periods of the bacteria before composing the vaccine (1 and 5 days respectively). The cells in 1 day cultures contained high amounts of A-layer related protective antigen (McCarthy et al., 1983), which in long-term cultures might be shed into the medium. In 5 day cultures also high concentrations of exotoxins have accumulated. Their supernatants may be a rich source of antigens which evoke protection.

Austin & Rodgers (1981) reported on a test using *A. salmonicida* vaccines, composed of inactivated toxoid, whole cells, or toxoid enriched whole cell preparations. Toxoid containing vaccines were successful in laboratory, but failed in the field trial; a heavy mortality occurred due to the opportune pathogen *A. hydrophila*, whereas this was not the case in the other experimental group. It was suggested that the toxoid products might have induced immune suppres-

TABLE 8. Virulence factors of *Aeromonas salmonicida*

Factors	Chemical nature	Molecular weight	Type of strain	Role in virulence	Immuno-genicity	Suitability for vaccination	Reference
CELLULAR							
Endotoxin	lipopoly-saccharide		all strains	slightly toxic	+	±	1,2
A-layer	protein	± 50,000	virulent	attachment, protection pathogen, haemagglutination?	±	+	3
EXTRACELLULAR							
Complex			all strains	cytolytic proteolytic lipolytic toxic	+	+	4,5,6
Fractionated							
Fraction 1	lipopoly-saccharide		all strains	toxic	+	±	7,8,9
2	?			toxic, leucocytic, proteolytic	?	-	
3	?			toxic	?	-	
4	glycoprotein	67,000		cytolytic	+	+	
Purified							
Free endotoxin	lipopoly-saccharide		all strains	slightly toxic	+	±	10
Protease	protein	43,000	non-virulent	proteolytic	?	?	11
		11,000	?	proteolytic			12
		?	virulent	toxic (serine-protease)			13,14
		?	virulent	non-toxic (non-serine-protease)			
		87,000	virulent	proteolytic			15
		70,000	non-virulent	toxic			16
Phospholipase	protein	24,000	?	?	?	?	17
Haemolysin	?	?	virulent	proteolytic, slightly haemolytic	?	?	18,19
			non-virulent	haemolytic, slightly proteolytic			
Leucocyto-lytic factor	glycoprotein	100-300,000	virulent	leucocytolytic	+	±	9,10,20

1) Patterson & Fryer, 1974a; 2) Patterson & Fryer, 1974b; 3) McCarthy et al., 1983; 4) Munro et al., 1980; 5) Pol et al., 1980; 6) Ellis et al., 1981; 7) Cipriano, 1982a; 8) Cipriano, 1982b; 9) Cipriano, 1982c; 10) Cipriano, 1983b; 11) Dahle, 1971; 12) Shieh & McLean, 1977; 13) Sheeran & Smith, 1981; 14) Sheeran et al., 1984; 15) Mellegaard, 1983; 16) Findley, 1983; 17) McIntyre et al., 1980; 18) Tittball & Munn, 1985; 19) Tittball et al., 1984; 20) Fuller et al., 1977.

sion. Or possibly a toxicity reversion had taken place, as also has been observed in formalin inactivated cholera toxin (Holmgren et al., 1977).

The foregoing studies indicate good prospects for future vaccination against furunculosis. Although there are still few data on protective potency of *A. salmonicida* virulent factors. A theoretical potent vaccine might be composed of formalin killed whole broth of a 1 day culture (McCarthy et al., 1983), enriched with ECP fraction (1 and) 4 of a 5 day culture (Cipriano, 1983a). To avoid problems as mentioned by Austin & Rodgers (1981), ECP fractions of avirulent strains should be used, as these strains also produce high levels of the ECP fraction 4 glycoprotein (Cipriano, 1982b).

4.2.4 *Aeromonas hydrophila*

As for furunculosis, vaccination against *A. hydrophila* has been only a little successful thus far. However, the problems with *A. hydrophila* are related to the enormous heterogeneity among the different strains (cf. Newman, 1983). This feature is responsible for the fact that bacterial cell preparations may induce considerable protection against the homologous pathogen, but not to heterologous strains (Post, 1966; Schäperclaus, 1970). Therefore, successful vaccination will depend on antigens other than LPS, but "common" for many strains.

Although the study on *A. hydrophila* virulence factors has not been as extensive as for *A. salmonicida*, several promising observations have been reported. There is some evidence for a common cellular antigen, shared by the virulent *A. hydrophila* strains. Mittal et al. (1980b) and Lallier et al. (1981) examined 25-30 different *A. hydrophila* strains on surface characteristics and virulence. The virulent strains shared a common "O"-antigen and were resistant to the bactericidal action of mammalian serum. Moreover, autoclaving of *A. hydrophila* cells resulted in two types of antigens, a soluble, strain specific antigen and a particulate component, which was polyvalent of nature (Lewis, 1978). Both components were highly immunogenic and especially this polyvalent antigen should be examined on its protective nature.

A. hydrophila strains vary enormously in virulence, and De Figueiredo & Plumb (1977) showed that strains isolated from fish were more virulent than those isolated from water. Also in the pathogenesis of diseases induced by *A. hydrophila* the role of ECP has been confirmed. It has already been recognized by Schäperclaus & Mann (1939) that bacterial toxins were involved in infectious dropsy in carp. *A. hydrophila* strains produce many extra cellular enzymes (cf. Newman, 1983), which might be virulence factors or virulence determinants. Stevenson & Allen (1981) showed that ECP of *A. hydrophila* possess both proteolytic and haemolytic activities, and that injection of salmonid fish with this ECP induced pathological effects; this activity was heat sensitive. The ECP of a protease deficient mutant was significantly more toxic to trout than the parenteral strain, which implied that the ECP lethal factor was not the protease, but probably the haemolysin. Wakabayashi et al. (1980)

and Hsu et al. (1981) studied many *A. hydrophila* strains, and correlated virulence with presence of gelatinase and elastase activity in ECP. Especially the quantity of these products was very important in virulence. Thune et al. (1982a) demonstrated that *A. hydrophila* LPS alone was not lethal to channel catfish, further they isolated two lethal factors from ECP: a haemolysin, labile at 56 °C for 10 min., and a relatively heat stable protease activity. This protease activity appeared to have a heat labile and a heat stable component (Thune et al., 1982b). The lethal activity of i.p. injected ECP in channel catfish was related to the haemolysin, and to the heat stable protease.

Some studies indicate a possible serologic relatedness of *A. hydrophila* extracellular antigens. Liu (1961) reported that extra cellular toxins of various strains were neutralized by antiserum against the toxins of a single strain. Also Newman (1983) reported a personal communication by Thune, who had found that antiserum against the haemolysin of an *A. hydrophila* strain neutralized haemolysins of all other strains examined. Thus the potential usefulness of extracellular antigens in immunoprophylaxis is an area that deserves further research.

To achieve successful vaccination to *A. hydrophila*, toxoid or cell preparations have to be developed, containing antigens that are common for a variety of *A. hydrophila* strains.

4.3 VACCINATION METHODS

To date successful vaccination can be obtained against *Vibriosis, Yersiniosis,* and to a lesser extent against furunculosis and some other infectious diseases. At present several methods are available for vaccine administration in fish. These methods are: injection, oral administration, immersion (hyperosmotic and direct) and spray or shower. The various methods and their application in aquaculture have been reviewed also by Harrell (1979).

4.3.1 *Injection*

In most studies on the immune response the antigen is administered by injection. This is an effective method to induce immune responses. The differences between injection via the i.m., i.p. and i.v. route have been considered already in paragraph 3.1.2. It can be mentioned that Schäperclaus (1970) confirmed the idea that injection of *A. hydrophila* along the i.m. route resulted in higher serum antibody levels than i.p. injection. However, he also observed that i.p. injected carp developed a higher level of protective immunity. Repeater syringes are often used to give the i.p. injection in large scale vaccination. This method was shown to reduce mortalities due to infectious diseases in the laboratory. For vibriosis, yersiniosis and to a lesser extent for furunculosis and *A. hydrophila* field trials also successfully reduced losses.

The injection method usually results in considerable serum antibody levels. Injection permits the use of certain adjuvants. An-

tigen emulsified in FCA not only resulted in higher antibody levels (e.g. to *V. anguillarum*, Harrell et al., 1975; Evelyn, 1971a; to *A. salmonicida*, Krantz et al., 1964; Weber & Zwicker, 1979; to *R. salmoninarum*, Evelyn, 1971b), but also in a significant improvement and duration of protection (e.g. to *A. hydrophila*, Post, 1966; furunculosis, Krantz et al., 1964a; *R. salmoninarum*, Evelyn, 1971b). In general the use of FCA is avoided, with respect to the marketing of fish. However, there may be other means to enhance protective immunity e.g. the use of liposomes. Entrapment of antigen in liposomes has been proven to act as a powerful adjuvant in mammals (Allison & Gregoriadis, 1974; Van Rooyen & Van Nieuwmegen, 1983). Moreover, Secombes & Resink (1984) have reported the potential of immune complexes to enhance the immune responses in carp.

Apparently, injection is the best vaccination method, in terms of acquired protective immunity, provided that the vaccine contains the appropriate antigens. However, it also has some disadvantages: it imposes stress on fish, due to handling; it cannot be used with very young fish; and finally it is not a convenient method for mass-vaccination, for it is very laborious. Nevertheless, vaccination by injection is recommended for relatively small groups of valuable fish, e.g. broodstock.

4.3.2 *Oral vaccination*

Duff (1942) was the first to attempt to control infectious fish diseases by immunization. He reported that prolonged feeding of cutthroat trout with a diet containing chlorophorm killed *A. salmonicida* cells resulted in a good protection. Upon a water borne challenge, the vaccinated fish showed 25% mortality, compared to 75% in the non-vaccinated fish. Upon i.m. challenge with a LD90 the mortality was 68%. Furthermore, the vaccinated fish showed slightly enhanced serum agglutinin titres. After publication of this classic study on oral vaccination, considerable attention has been paid to this route of administering vaccines, despite several unsuccessful oral vaccination trials (Snieszko & Friddle, 1949) and the fact that injection consistently gave higher levels of protection. An advantage of oral vaccination is the fact that it is an appropriate method for mass-vaccination in young fish. It does not impose stress on the fish because handling is absent. However, it may result in an uneven distribution of the immunization, as fish consume different amounts of food. Moreover, high concentrations of vaccine are needed, and in most cases only moderate levels of protection have been achieved.

Oral administration of vaccine has been applied in trials against *A. hydrophila*, *A. salmonicida*, *F. columnaris*, *V. anguillarum* and *Y. ruckeri*. The main results are presented in Table 9. When protection obtained after oral vaccination is tested by injecting virulent cells, the bacterial dose is very important. Too high dosages may overwhelm a moderate protection or surpass local defense lines. A much better protection could be recorded when a subtle artificial challenge or a natural field challenge was applied. So it seems that the challenge method after oral vaccination should follow the normal route of pathogen entry. However, Udey & Fryer (1978) did

TABLE 9. Review of oral vaccination reports

Pathogen and Reference		Fish species	Challenge route	% Mortality vaccinated (control)	Protection
Aeromonas hydrophila					
Post	(1966)	*S. gairdneri*	i.p.	60-90 (80-100)	±
Aeromonas salmonicida					
Duff	(1942)	*S. clarkii*	bath	25 (75)	+
			i.p.	68 (90)	±
Snieszko & Friddle	(1949)	*S. fontinalis*	i.p.		-
Spence et al.	(1965)	*O. kisutch*	scrape/bath		-
Overholsen	(1968)	*O. kisutch*	field	3 (22)	+
Klontz & Anderson	(1970)	*S. fontinalis*	field	0 (58)	+
Udey & Fryer	(1978)	*O. kisutch*	field		-
Michel	(1979)	*S. gairdneri*	i.m.		-
Smith et al.	(1980)	*S. trutta*	field	35-62 (86)	+
Flexibacter columnaris					
Fujihara & Nakatani	(1971)	*S. gairdneri*	field	19 (27)	±
		O. kisutch	field	8 (48)	+
Vibrio anguillarum					
Hayashi et al.	(1964)	*S. gairdneri*	field		±
Schreckenbach	(1974)	*Anguillidae, Salmonidae*	field		+
Gunnels et al.	(1976)	*Salmonidae*	field		-
Braaten & Hodgins	(1976)	*S. gairdneri*	i.p.	7 (100)	+
Hastein et al.	(1977)	*S. gairdneri*	field		+
Prescott	(1977)	*various marine tropical fish*	sub cutaneous	9 (62)	+
Sawyer & Strout	(1977)	*S. kisutch*	field	3 (24)	+
Fryer et al.	(1978)	*O. tshawytsha*	field	7 (80)	+
Kusuda et al.	(1978)	*P. altivelis*	cohabitation	10 (90)	+
Gould et al.	(1978)	*O. nerka*		23 (58)	+
Nakajima & Chikahata	(1979)	*P. altivelis*	cohabitation	22 (50)	+
Baudin-Laurencin & Tangtronpiros	(1980)	*S. gairdneri*	i.p.	32 (34)	-
Evelyn & Ketcheson	(1980)	*O. nerka*	field	18 (42)	+
Amend & Johnson	(1981)	*O. kisutch*	bath	27 (52)	+
Kawai et al.	(1981)	*P. altivelis*	cohabitation	2 (100)	+
Horne et al.	(1982)	*S. gairdneri*	i.p.	94 (100)	-
Agius et al.	(1983)	*S. gairdneri*	i.p.	40-100 (100)	±
Johnson & Amend	(1983b)	*O. nerka*	bath	15-45 (55)[a]	±
				0 (55)[b]	+
Yersinia ruckeri					
Ross & Klontz	(1965)	*S. gairdneri*	i.p.	10 (90)	+
Anderson & Ross	(1972)	*S. gairdneri*	sub cutaneous	10 (100)[c]	+
				90 (100)[d]	±
				100 (100)[e]	-
Anderson & Nelson	(1974)	*S. gairdneri*	i.p.		+
Johnson & Amend	(1983b)	*S. gairdneri*	bath	24 (65) [a]	+
				3 (65) [b]	+

a = oral intubation
b = anal intubation
c = fish were fed with chloroform killed cells
d = " " " " phenol killed cells
e = " " " " sonicated formalin killed cells

not get protection in a natural challenge of orally vaccinated fish. They suggested that vaccine feeding might have induced immune suppression. However, Smith et al. (1980) showed that protection developed to a certain *A. salmonicida* strain in the vaccine did not protect against the heterologous strain encountered in nature. It might be possible that the results of Udey & Fryer (1978) can be explained this way.

Antigen dose. Oral vaccination requires relatively high antigen doses in order to induce a moderate level of protection. Protection of the bacterin from proteolytic activity or low pH might enhance the efficacy of the vaccine. Anders (1978) reported that *Vibrio* bacterin, mixed with a substance protecting against gastric acid, was about 10 times more effective in inducing protection in eel and rainbow trout. Furthermore, Johnson & Amend (1983b) demonstrated that a single *anal* administration, to by-pass antigen destruction in the upper intestine, of *V. anguillarum* to sockeye salmon, resulted in 100% survival upon a water borne challenge after 59 days, compared to 55-85% and 45% for oral administration and controls respectively. These observations open new prospectives for oral vaccination. It shows that high levels of protection are obtained when antigen can reach the posterior part of the intestine, relatively unaltered. Micro-encapsulation might be an effective mode to protect vaccine from destruction. This will also allow a more efficient use of the vaccine.

The observations by Johnson & Amend (1983b) confirm the speculations by Stroband et al. (1979), Stroband & Van der Veen (1981) and Davina et al. (1982) on the function of the second gut segment in uptake and processing of antigenic substances for immunological purposes. The mode of antigen entry through the intestine and the possible immunological significance of the second gut segment is extensively discussed in Appendix paper 9.

4.3.3 *Immersion vaccination*

The lack of an appropriate vaccination method for use on large scale, has initiated the development of new vaccination methods, based on exposure of the external surface of fish to the vaccine: hyperosmotic infiltration (HI), direct immersion (DI) and spray or shower. The immersion methods were initiated by Amend & Fender (1976). They showed that juvenile trout, immersed in a hypertonic salt solution containing BSA, resulted in an uptake of a considerable amount of BSA in the blood within 3 minutes. Similar results were obtained when the hyperosmotic treatment directly preceeded the immersion in the BSA solution. Subsequently it was demonstrated that HI was an effective vaccination method (Antipa & Amend, 1977; Croy & Amend, 1977).

Extensive laboratory and field testing has revealed that HI vaccination is very promising in conferring protection to vibriosis in salmonids (Antipa & Amend, 1977; Lannan, 1978; Antipa et al., 1980; Evelyn & Ketcheson, 1980; Giorgietti et al., 1981), and in ayu (Itami & Kusuda, 1978; Aoki & Kitao, 1978; Nakajima & Chikahata, 1979; Aoki et al., 1984), to yersiniosis (Tebbit et al., 1981) and

to furunculosis (Smith et al., 1980; Palmer & Smith, 1980). The advantage of HI vaccination is the induction of a high protection level. It can be applied on a large scale and it is not limited by fish size. One of the drawbacks is that a high level of stress is attended with it, induced by handling and the osmotic shock. This might initiate subclinical or latent infections by opportunistic pathogens or even cause post vaccination mortalities (Harrell, 1979; Hunter et al., 1980).

Omission of the hyperosmotic step did not influence the level of protection. However, in DI the bacterin concentration and exposure time needed slight adjustment (Croy & Amend, 1977; Antipa et al., 1980). This procedure reduced stress caused by osmotic shock, but still induces handling stress. Egidius & Andersen (1979) and Anderson et al. (1979c) introduced flush exposure, which eliminated handling of the fish, by "just pouring the vaccine into the aquarium". The efficacy of DI and flush exposure has been demonstrated in laboratory and field trials for vaccination against vibriosis in salmonids (Egidius & Andersen, 1979; Egidius et al., 1982; Antipa et al., 1980; Amend & Johnson, 1981; Johnson et al., 1982a, b; Horne et al., 1982), in eel (Song et al., 1980) and in ayu (Itami & Kusuda, 1980), against yersiniosis (Johnson et al., 1982a, b; Newman & Majnarich, 1982; Johnson & Amend, 1983a; Amend et al., 1983), against furunculosis (Johnson & Amend, 1984), against edwardsiellosis (Song et al., 1982) and against *A. hydrophila* (cf. Plumb, 1984). For protection against both furunculosis and edwardsiellosis multiple immersion was required.

The spray or shower method has been developed simultaneously with immersion vaccination. This method involves vaccine spraying onto fish skin. Initially high pressure spraying was performed (Gould et al., 1978; Itami & Kusuda, 1978). However, low pressure spraying appeared to be as effective (Gould et al., 1978; Rosenkvist -Jensen, 1982). In these experiments this procedure induced protection against vibriosis in coho salmon, ayu and rainbow trout.

The HI, DI and spray vaccination gave comparable degrees of protection (Itami & Kusuda, 1978; Gould et al., 1978). It is important for this comparison to consider the challenge route. Amend & Johnson (1981) obtained 1 and 3% mortality in DI and spray vaccinated coho salmon against vibriosis after bath challenge. Much higher mortality levels were found in DI vaccinated fish after an i.p. challenge (Tebbit & Goodrich, 1983; Newman & Majnarich, 1982).

The advantage of the immersion methods is that they can be applied with a minimum of handling to high numbers of fish, especially small animals. Moreover, these methods are very rapid: only a few seconds exposure to the vaccine is required to get maximum protection (Johnson et al., 1982a). This has been confirmed by Tatner & Horne (1983a), testing the uptake of radiolabelled *V. anquillarum* in rainbow trout. The maximum uptake was reached in 10 seconds.

The route of antigen entry. The amount of antigen uptake by a fish during immersion vaccination is small, and ranges from 0,01 to 0,2% of the initial bath vaccine concentration (Tatner & Horne, 1983a). The entry route of antigens administered by immersion or spray is still under research. Amend & Fender (1976) suggested that

antigen was taken up principally through the lymphatic system, associated with the lateral line, and also through the gills. In a later publication they expressed doubt on the role of the gills (Fender & Amend, 1978). However, Bowers & Alexander (1980) and Alexander et al. (1981) showed that *E. coli* bacteria had a rather quick access to the bloodstream of brown trout, when only head and gill region were immersed. Moreover, Smith (1982) showed that small amounts of radiolabelled soluble and particulate antigen were taken up by the skin and gut of rainbow trout in direct immersion, but the majority of the radiolabel was found in the gills. Antigen appeared in the lymphoid organs within 24 h. Particulate antigen was taken up more effectively than soluble antigen. The appearance of cellular antigen in gills and skin suggested an active uptake by specialized cells. It is interesting to mention that Mittal et al. (1980) have described the presence of Langerhans-like cells in teleost skin. Also Anderson et al. (1984) published a hypothesis on active antigen uptake through epithelia.

In an elaborate study Hockney (1985) investigated the trunk region of rainbow trout for antigen uptake through the skin following spraying with HGG and killed *V. anguillarum*. No antigen could be traced in the blood even after long-term spraying of the posterior part of the fish. Using immunofluorescence Hockney (1985) observed antigen binding to skin. No cells were involved and in a later phase antigen was lost by shedding from the surface. The author concluded that the posterior part of the fish was not important for antigen uptake in spray vaccination. In contrast to adults the skin in young fish is rather thin (e.g. 3 cell layers in 2 weeks old carp), which offers better prospectives for antigen entrance. We immersed 2 weeks old carp fry in a HRP solution (Lamers & Helfrich, unpublished results). It was seen with the electronmicroscope that HRP was bound to the outer surface. Subsequently, it was pinocytosed by surface cells and later on it became localized in macrophages present under the skin.

Although a role of the skin in antigen uptake cannot be excluded, the importance of gills is obvious. At present no detailed electronmicroscopical studies have been reported and no clear data are available on the mechanisms of uptake through skin.

4.3.4 *Comparison of vaccination methods*

Some benefits or drawbacks of the different vaccination methods have been mentioned in paragraph 4.3.1 to 4.3.3. A comparison of their efficacy is shown in Table 10. Vaccine injection confers the best protection, followed by immersion, spray and finally oral administration. In most cases the challenge route determines the success. Especially for immersion and oral vaccination the efficacy was demonstrated most clearly in a "mild" challenge, e.g. bath or field. Immersion vaccination is very effective, and protection figures are almost equal to those obtained for injection, in cases when a bath or field challenge was used. It is clear that oral vaccination is the least effective, although moderate protection may be obtained. Enhancing its efficacy seems possible in view of the results obtained by Johnson & Amend (1983b). However, at present oral vac-

TABLE 10. Comparison of the efficacy of various vaccination methods (in % mortality)*

Pathogen and Reference		Delivery method					Fish species
		None	Injection	Immersion	Spray	Feeding	
Vibrio anguillarum							
Hastein et al.	(1977)	46	19	42	nt	76	*S. gairdneri*
Itami & Kusuda	(1978)	80	34	nt	27	nt	*P. altivelis*
Gould et al.	(1978)	58	nt	nt	0	23	*O. nerka*
Nakajima & Chikahata	(1979)	50	nt	0	nt	22	*P. altivelis*
Rosenkvist-Jensen	(1980)	66	0	19	11	nt	*S. gairdneri*
Baudin-Laurencin & Tangtrongpiros	(1980)	34	1,5	2	nt	32	*S. gairdneri*
Evelyn & Ketcheson	(1980)	42	0,7	2	nt	18	*O. nerka*
Amend & Johnson	(1981)	52	0	4	1	27	*O. kisutch*
Horne et al.	(1982)	100	7	53	nt	94	*S. gairdneri*
		100	56	-	-	78	
Johnson & Amend	(1983b)	55				15-45[a] 0[b]	*O. nerka*
Yersinia ruckeri							
Ross & Klontz	(1965)	80	nt	nt	nt	10	*S. gairdneri*
Newman & Majnarich	(1982)	70-83	nt	3-30	nt	nt	*O. kisutch*
Johnson & Amend	(1983a)	79	2	23	nt	nt	*S. gairdneri* (2 g fry)
		58	nt	2	6-14	nt	*S. gairdneri* (4 g fry)
Johnson & Amend	(1983b)	65	nt	18	nt	24[a] 3[b]	*S. gairdneri*

* = after challenge with the virulent pathogen
nt= not tested
a = oral intubation
b = anal intubation

cination is not widely used, because of the bad predictability of the outcome.

It is obvious that the preferred vaccination method is along the natural entrance route of the pathogen. Enhancing protection at those entry sites probably will prevent the pathogen from becoming harmfull. In this respect it is interesting to mention that Tatner et al. (1984) observed that live *A. salmonicida* bacteria, after direct immersion, were present for 2 days on the gills of rainbow trout and up to 5 days on the skin. In the same period no bacteria were recovered from the gut, suggesting that immersion is the best vaccination route for this pathogen. However, it has to be mentioned that oral administration may also lead to a higher protection level in the skin (Kawai et al., 1981).

4.4 AGE AND SIZE EFFECT ON VACCINATION

Johnson et al. (1982a) studied the ontogeny of immunity in Salmonid fry of 6 different species, vaccinated by direct immersion in vibriosis and yersiniosis vaccine. The minimal fish size at which protection occurred was between 1,0 and 2,5 g. Here immunity appeared to be a function of size and not of age. The duration of protective immunity varied with bacterin concentration, fish size and species. Generally, it lasted for about 120 d in 1 g fish, about 180 d in 2 g fish and longer than a year in 4 g fish (Johnson et al., 1982b). Dorson (1977) showed that much smaller/younger fish may be successfully vaccinated by i.p. injections. He vaccinated 15 days old (p.h.) rainbow trout (< 0,15 g) against IPN. Moreover, Khalifa & Post (1976) immunized rainbow trout at 3 weeks after the onset of feeding (± 0,3 g) against *A. liquefaciens* by i.p. injection, and obtained both precipitating antibody and protection.

Tatner & Horne (1983b) found that rainbow trout fry were not susceptible for *V. anguillarum* administered by i.p. injection or by bath until they reached the age of 7 or 8 weeks p.h. respectively. Vaccination by direct immersion of 2, 4, 6 and 10 weeks old fry resulted in increasing protective immunity upon an i.p. challenge. I.p. vaccination of 10 weeks old fry resulted in 100% protection. Tatner & Horne (1983b) suggested a correlation between the attainment of immunological maturity and the sudden increase in the relative weight and cell numbers in the thymus and kidney of rainbow trout at about 8-12 weeks of age (Tatner & Manning, 1983). They suggested that immunological competence is achieved at a critical number of immune active cells, rather than depending on the appearance of a certain cell type.

4.5 PROTECTIVE IMMUNITY

All immunological phenomena should be considered (paragraph 2.1-2.4.8), in the search for immunological features that might reflect protection. It is possible that protection is the result of different immunological and non-immunological defence mechanisms. No generalizations can be made because a particular reaction may be specific for just one fish species. Till now, most attention was

paid to specific mechanisms, and to the role of antibody in particular.

There are two distinct phases in active humoral immunity: the production of the protective mechanism (antibody), for a relatively short period, followed by the long-lasting state of positive memory. Immunization usually refers to the establishing of this memory state. Protection depends - at least in part - on the quality and quantity of memory lymphocytes. However, to date only few studies have been published on this important immunological feature in fish, especially with respect to bacterial antigens (see also Appendix paper II, IV and V).

To date little is known about CMI to pathogens. Only few studies have shown an *in vitro* antigenic stimulation of lymphocytes. This is a promising method to asses the aquired immunity to vaccination, especially after vaccination procedures which do not always reveal antibody titres, e.g. bath, spray (Gould et al., 1978; Liewes et al., 1982). This method has some drawbacks; it is no simple test and requires sophisticated laboratory equipment. Moreover, we have to be aware of the fact, shown by Chilmonzcyk (1978), that not all fish protected against VHS were positive with this test, like not all protected fish had detectable serum antibody. Much more data are required to decide whether or not, or in which cases, CMI will be the appropriate approach to asses the acquired immunity to vaccination.

In the case of vibriosis a protective role of antibody has been clearly demonstrated. Harrell et al. (1975) passively immunized rainbow trout with immune serum and observed protection upon challenge. The protective effect could be abrogated by preincubation of the immune serum with *Vibrio* bacterin. Moreover, the decay of the injected immunoglobulin did correspond with the decrease in protection (protection decreased by 60% in about 5 weeks time). Comparable results have been obtained by Viele et al. (1980). They observed that the protective effect of serum increased up to 100% in the first 4 weeks after vaccination. Transfer of lymphoid cells from head kidney conferred also protection. However cells from spleen and thymus did not. These experiments clearly demonstrate that the humoral response provides a good protection against vibriosis. Immersion vaccination can be followed by serum antibody production (Garrison & Gould, 1976; Antipa & Amend, 1977). However, there are cases in which protection was observed in the absence of serum antibodies (Croy & Amend, 1977; Gould et al., 1978) (see also paragraph 2.4.6 and 2.4.7). In this respect the role of local and/or cellular immunity has to be mentioned.

In most studies on oral vaccination against vibriosis no serum antibody was observed although a certain protection level was obtained (Kusuda et al., 1978; Fryer et al., 1978; Gould et al., 1978; Kawai et al., 1981). Fletcher & White (1973b) demonstrated specific antibody in intestinal mucus after feeding of *Vibrio* bacterin. As serum levels were quite low, these data suggested a local production of antibody. Kawai et al. (1981) found that orally vaccinated ayu developed an anti-*Vibrio* agglutinin in skin mucus, whereas no antibody was found in serum or intestinal mucus. Moreover, they showed that the lack of agglutinating serum antibodies did not deprive the

animal of protective immunity. Serum from orally vaccinated ayu, which did not contain anti-*Vibrio* agglutinins, was transferred to non-immunized fish, and conferred protection after challenge. Moreover, in immunized fish the colonization of the skin by viable bacterial cells was significantly reduced, especially in the mouth region and on the gills. Also in the stomach and intestine no bacterial colonization occurred, despite the fact that in intestinal mucus no anti-*Vibrio* activity could be detected (Kawai et al., 1981).

In the case of yersiniosis, successful protection has been achieved with whole cell bacterins. *Y. ruckeri* cell wall preparations are very immunogenic, and will be mainly directed to LPS. In this case, the protective role of antibody seems obvious, although it has not yet been proven by serum transfer (cf. Bullock & Anderson, 1984; Bush, 1983). In accordance with the efficacy of immersion vaccination against *Y. ruckeri*, Anderson et al. (1979a,b,c) demonstrated that O-antigen of *Y. ruckeri* induced antibody production in rainbow trout, when it was given by immersion or by flush exposure. As little as 5 µg/ml O-antigen elicited an immune response. High levels of serum antibody and antibody forming cells in the spleen were detected. An attempt to demonstrate the formation of immunological memory, induced by immersion failed (Anderson & Dixon, 1980). This observation can be explained by the thymus-independency of the O-antigen used.

It is unclear whether antibody plays a role in protection against furunculosis (*A. salmonicida*). Spence et al. (1965) protected young coho salmon against furunculosis, by passive immunization with serum from immunized trout. Upon vaccination with cellular components, the antibody is mainly directed against LPS structures (Paterson & Freyer, 1974a,b), but the presence of these antibodies was not correlated with protection (Michel, 1979). McCarthy et al. (1983) suggested that antibody against the A-layer protein was protective. However, the immunogenicity of A-layer protein for fish has still to be established.

Cipriano (1983b) observed that natural resistance of rainbow trout to an experimental pathogenic challenge was correlated with the ability to neutralize *A. salmonicida* cytotoxic activity, and not with the level of natural serum agglutinins. Neutralizing serum components might be the α-migrating enzyme inhibitors (Ellis et al., 1981). Whether the protective effect of the ECP fraction 4 glycoprotein is related with induction of neutralizing antibodies is still to be proven. Smith et al. (1980) studied the cell mediated immunity in brown trout vaccinated against *A. salmonicida*, using the macrophage inhibition test. They observed that the percentage of fish reacting positively in the test correlated with the percentage that was protected in a homologous challenge. Also the percentage of fish reacting upon a heterologous bacterial strain closely corresponded with the percentage protected against that strain. In case of *A. salmonicida* probably T-cell/macrophage co-operation is involved in bacterial killing, whereas humoral responses might be important for neutralizing toxins. This implies that both parameters have to be monitored to test vaccination efficacy against furunculosis.

4.5 VACCINATION AGAINST *AEROMONAS HYDROPHILA*

The first vaccination trials against bacterial hemorrhagic septicaemia were performed by Schäperclaus (1954), using killed and living *A. hydrophila* cells. He obtained a decrease in the average loss in immunized yearling carp. Moreover, carp surviving a natural epizootic were highly resistant to experimental challenge. Post (1966) obtained protection in rainbow trout immunized with heat killed *A. hydrophila* cells, against the homologous strain, up to 8 months after vaccination. Acuigrup (1980) tested monovalent *A. hydrophila* vaccine of an endemic strain in a field trial with rainbow trout. They only obtained low protection and a slightly better food conversion. Schäperclaus (1970) reported that vaccination with an *A. hydrophila* strain, results in protection against the homologous strain, but not against heterologous strains.

Thune (1980) used a polyvalent sonicated *A. hydrophila* bacterin in channel catfish. Direct immersion in the bacterin induced protection against the *A. hydrophila* strains incorporated in the vaccine, whereas poor protection against heterologous strains was recorded. This author also tested the potential of young channel catfish to develop protective immunity. Bath vaccinated sac fry and swim-up fry showed protection upon a water borne challenge with 7,6% and 13% mortality respectively, against 45% for non vaccinated fish.

Up to now *A. hydrophila* vaccines have been composed of inactivated whole or lysed bacterial cell cultures, composed of a single or multiple strains. Most of the work has been done with monovalent vaccines. Polyvalent vaccines preferably contain strains that share antigenic determinants with a number of known isolates from that particular area. Toxoid products or the common cellular components have not yet been used in vaccines.

Post (1966) reported that rainbow trout produced significant antibody against *A. hydrophila*. Heat killed bacterin, mixed with adjuvant, gave higher antibody levels and better protection than bacterin in saline. Moreover, antibody titres were present for a longer period in the adjuvant group. Feeding rainbow trout with 1 mg dry weight of bacteria each day, for 9 months, resulted in distinct serum antibody titres, which declined when feeding was stopped. Upon homologous i.p. challenge with a LD90, the orally vaccinated fish showed about 20% and the injected fish about 70% reduction in mortality. Post (1966) also reported that phagocytes from immunized fish showed a 3,6 times higher phagocytic activity than cells from control fish. Injection of *A. hydrophila* in eel resulted in serum antibody levels, which were highest with a live attenuated strain, followed by formalin or heat killed bacteria and soluble bacterial extracts (Song & Kou, 1976). Thune & Plumb (1982) reported that injection of a sonicated preparation of *A. hydrophila* in channel catfish resulted in significantly higher antibody titres than either freeze-thaw or whole cell preparations.

No conclusive evidence is available on the protection to *A. hydrophila*. However, the present data might permit some speculation. Khalifa & Post (1976) vaccinated rainbow trout by i.p. injection with heat killed *A. liquefaciens* cells. Serum antibodies were higher

and persisted for a longer time when bacteria had been suspended in FCA. Upon homologous challenge, the fish that showed an antibody titre of 1:32, were 100% protected, and fish with an antibody titre of 1:8 showed mortalities. It appeared from several vaccination trials that vaccination induced protection against the homologous strain, however, no protection developed against heterologous strains (Schäperclaus, 1970). Probably antibody directed against LPS confers protection, but the heterogeneity in *A. hydrophila* LPS might account for the poor cross protection. Therefore, future research on vaccination to *A. hydrophila* will have to concentrate on common antigens, whether associated with the cell wall or in the ECP.

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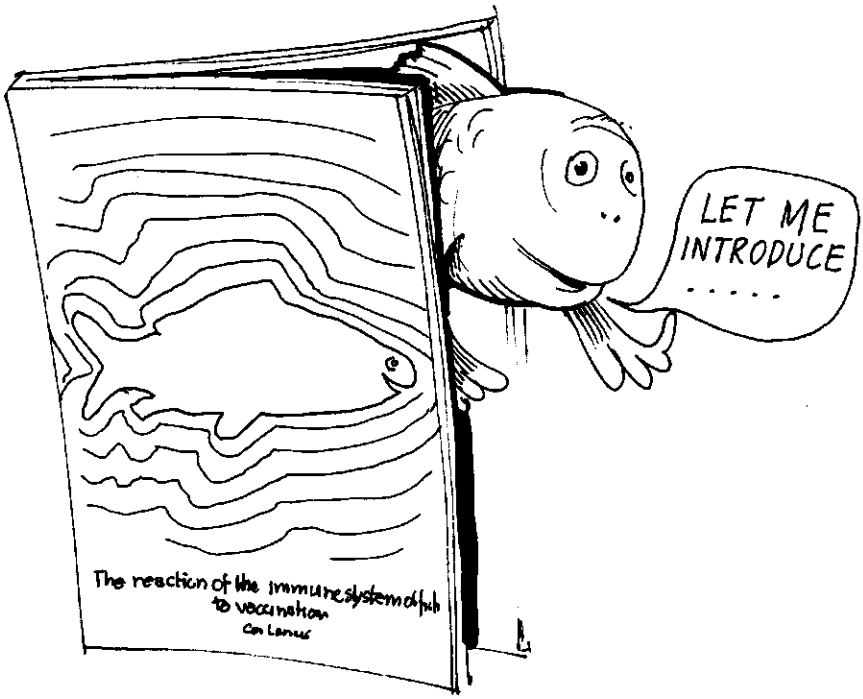

LET ME INTRODUCE .....
The reaction of the immune system of fish to vaccination
Con Lanus

INTRODUCTION TO THE PAPERS

At present the nature of antigens inducing protective immunity, the mode of antigen entry into the fish after bath, spray and oral immunization, and the nature of immunological features that reflect protective immunity are still poorly understood. A very limited number of studies have been carried out to determine the immunological processes occurring after vaccine administration by bath or feeding. The same holds true for data on the histophysiology of the immune response in fish. Although the knowledge on factors affecting the immuno-responsiveness of fish is continuously extending, there is still a need for more investigations of these factors, especially with regard to vaccination. The ultimate goal is to define the optimal conditions for vaccination.

The aim of the studies presented in this thesis was to determine the effect of administration of a bacterial preparation on the immune system of fish, using immunological and histological parameters. In this project, besides injection also vaccination methods, which are more useful for large scale use, have been taken into account. Attention was paid to the way antigen enters the fish, the processes of antigen handling and processing in the lymphoid organs, the influence of antigen nature and dose on the kinetics and duration of the humoral immune response and the development of immunological memory.

In *appendix 1* and *2* the humoral immune response was studied against a bacterial cell wall preparation (O-antigen) of *Y. ruckeri*, which is regarded as a T-independent antigen in mice (Anderson & Dixon, 1980). Attention was paid to the antigen dose, the route of administration (intramuscular, intraperitoneal, direct immersion), and the formation and duration of immunological memory. Furthermore, the processing of antigen in the lymphoid organs was studied after injection of antigen.

In the studies presented in *appendix 3, 4, 5, 6* and *8*, bacterial preparations have been used of *A. hydrohila*, an ubiquitous bacterium in the aquatic environment, which is an opportunisitc pathogen for many fish species.

The immunogenicity of two injected *A.hydrophila* preparations (whole formalin killed cells or heat killed and disrupted cells) and the influence of the antigen dose on the humoral immune response were studied in *appendix 3*. Furthermore, the occurrence of a natural serum agglutinin was considered, and the nature of the antigens, playing a role in the induction of the immune response was discussed.

In *appendix 4* the development and duration of immunological memory was investigated in carp injected with formalin killed *A. hydrophila* cells. Special attention was paid to the effect of the antigen dose on the induction of memory and the evocation of the secondary response.

In *appendix 5* the humoral response and development of immunological memory was studied following direct immersion in bacterin. Based on the results it was suggested that the route of entry during the first and the second administration of the antigen has to be the same, in order to show the presence of immunological memory. The existence of local or mucosal immunity in fish was suggested.

In *appendix 6* a brief morphological description of carp lymphiod organs is given, followed by detailed data on the localization and processing of bacterial antigen in these organs. Both the initial and later stages (up to 12 months after antigen administration) were taken into account.

In order to determine which of the processes observed in the study on antigen handling are of immunological interest, and which are just a non-specific reaction, in *appendix 7* the fate of injected inert material (carbon) has been studied for comparison.

In *appendix 8* simultaneous histological and immunological tests were carried out after injection of antigen, in order to get a better view on the functioning of the immune system. Besides the antibody response and antigen handling, also the distribution of tissue bound immunoglobulin and the pyroninophilia in the lymphoid organs were studied. The effect of emulsifying the antigen in adjuvant was also taken into account. Studies presented in the *appendices 6, 7* and *8* raised the question as to whether melano-macrophage centres (MMC) play a role in the long-term retainment of antigen. It is supposed that MMCs might be early phylogenetic analogues of mammalian germinal centres.

Up to now oral vaccination has not been applied frequently, as it is relatively little effective. However, in a practical sense it is the most preferable vaccination method. To achieve this goal much more basic information has to be obtained on the possiblities of inducing mucosal immunity and on the properties of antigens which may be succesfull in affecting the immune system by the intestinal route. In *appendix 9* the uptake and transport of intact macromolecules, ferritin and horse radish peroxidase, in the intestinal epithelium has been studied by electron microscopy. The transport of antigens from the intestinal lumen through the enterocyte to the intra-epithelial leucocytes might have immunological implications e.g. induction of local immunity.

SUMMARY

The studies presented in this thesis deal with the effect of bacterial antigens of *Yersinia ruckeri* and *Aeromonas hydrophila* on the immune system of carp. The antigens were administered by injection or by bath treatment. The effect on the immune system was studied by measuring the numbers of antibody forming cells (AFCs), the level of serum antibody and the processing of antigen in the lymphoid organs. The antigen dose and the formation of immunological memory were taken into account. Moreover, in view of oral vaccination, a study was carried out on the uptake and transport of macromolecules through the intestinal epithelial cells.

Y. ruckeri O-antigen was very immunogenic in carp. Both i.m. and i.p. injection of antigen and direct immersion in antigen solution evoked distinct levels of AFCs and serum antibody (Appendix paper *I*). The height of the response was dependent on entry route; e.g. it was clearly lower after direct immersion. Contrary to the numbers of AFCs, which showed a fast decrease after a peak at day 10, serum antibody persisted for a long time, especially at the highest antigen dose. It was suggested that a long-term presence of antigen in the lymphoid organs, as observed by immunofluorescence, might account for this feature.

Secondary antibody responses in carp injected or bathed in *Y. ruckeri* O-antigen gave indications for the formation of immunological memory on the first contact with the antigen for both priming routes. Highest secondary responses were obtained in animals in which antibody levels resulting from the priming had decreased to background levels (Appendix paper *II*).

Upon i.m. injection of two *A. hydrophila* bacterins, formalin killed cells (F-*Ah*) and heat killed and disrupted cells (H-*Ah*), considerable serum antibody titers were found in carp. H-*Ah* induced higher levels of antibody than F-*Ah*, which in both cases persisted for more than 8 months (Appendix paper *III*). Whereas antigen dose clearly affected the height of the response, the peak day depended on the type of bacterin (day 14 and day 20 for all doses of H-*Ah* and F-*Ah* respectively). The effect of mixing the antigen with adjuvant found expression in a second increase of AFCs after a "normal" peak in the primary response, and a prolonged increase of serum antibody levels (Appendix paper *VIII*).

Antisera raised against the two *A. hydrophila* bacterins showed different agglutinating properties (Appendix paper *III*). Antibody induced by F-*Ah* was mainly directed to lipopolysaccharide (LPS), whereas with H-*Ah* this was not the case. It was concluded that LPS is an important antigen of *A. hydrophila*. However, there is at least one other immunogenic component.

Fractionated immune (anti F-*Ah*) and non-immune control sera were tested for immunoglobulin (Ig) by an Elisa and for agglutinating properties. It was shown that Ig was restricted to the first high molecular weight protein peak. In addition to the antibody in this peak also a non-Ig agglutinin was present in lower molecular weight fractions, probably a natural agglutinin.

Memory formation was monitored by studying secondary responses. Upon a primary i.m. injection, carp developed memory to *A. hydrophila* bacterin (Appendix paper *VI*). The height of the secondary responses was directly correlated with the priming dose. Moreover, the dose of the booster injection determined the outcome of the formed memory; corresponding priming and boosting doses expressed the highest memory levels. The development of maximum memory took some months, and its presence was still demonstrable at 12 months. The low dose priming induced only a weak memory, which was limited in time.

A single bath in *A. hydrophila* bacterin was not followed by the appearance of serum antibody. However, it did induce formation of memory, for a second bath resulted in clear secondary responses. This memory was maximum at 3 months after priming and was limited in time, for it was gone at 12 months. When a booster was given by i.m. injection instead of immersion, the induced memory was not expressed as a clear secondary response. This feature suggests the importance of local processes during the immune response after bath vaccination (Appendix paper *V*).

In Appendix paper *VI* a brief morphological description is given of the spleen, head kidney and trunk kidney. An antigen localization study was carried out after i.m. injection of *A. hydrophila* bacterin, by means of immunofluorescence. The antigen first appeared in the splenic ellipsoids and solitary phagocytic cells of the head and trunk kidney. Later on it gradually concentrated in or near melanocentres (MMCs) in all organs studied. In the later phases antigen was only located in and around MMCs, both intracellularly and bound to cell membranes, and it remained so for a full year. No antigen could be traced in the lymphoid organs following bath immunization. The question arose if the observed processes were of immunological interest, and which were just a non-specific reaction. Therefore the fate of injected inert material (carbon) was studied for comparison (Appendix paper *VII*). The processing of carbon was comparable to that described for *A. hydrophila* antigen. However, at two points there was a difference: 1) in the spleen carbon was transported to MMCs by cells leaving the ellipsoids, whereas this was not clear for *A. hydrophila* antigen, which appeared near and in MMCs bound to the surface of cells; 2) carbon was only seen intracellularly. Based on these observations it was assumed that the membrane bound antigen in the MMCs had immunological relevance. To test this assumption simultaneous histological and immunological tests were carried out after injection of *A. hydrophila* antigen (Appendix paper *VIII*). In those tests it was found that extracellular bound antigen in MMCs was not observed until circulating antibody was present, which is suggestive for the antigen to be complexed by antibody. A further support for this assumption was given by the simultaneous presence of tissue bound Ig and *A. hydrophila* antigen in MMCs.

The possible immunological importance of MMCs is discussed in Appendix papers *VI-VIII*. There are several indications for MMCs to be early phylogenetic analogues of the mammalian germinal centres.

In Appendix paper *IX* a study is presented on uptake and transport of intact macromolecules in the intestinal epithelium of carp.

This study was started as oral administration is an interesting vaccination method. Electronmicroscopical observations showed that two protein tracer-molecules, horse radish peroxidase (HRP) and ferritin were absorbed more extensively in the second than in the first gut segment. Moreover, HRP and ferritin were processed by the enterocytes in a different way. HRP seemed to be taken up by membrane binding and was subsequently transported to the intercellular space, where it contacted the abundant intra-epithelial leucocytes. There was no notable intracellular digestion of HRP. However, ferritin was taken up by pinocytosis and ended up in lysosome-like bodies or supranuclear vacuoles (2nd segment), where it was digested slowly. Although no ferritin was found in the intercellular space, large macrophages, with phagosomes containing ferritin, were present between the epithelial cells. Also some smaller macrophages containing ferritin, have been observed in the lamina propria and even in the spleen. These data were discussed in terms of both selective and non-selective absorption of macromolecules and the possible immunological implications. It was concluded that orally administered antigens reach intra-epithelial leucocytes, and might induce a (local) immune response. As most antigens are transmitted in the second segment, this part of the gut probably has an important immunological function.

FINAL CONCLUSIONS

- The humoral immune response in carp can be evoked by soluble and particulate bacterial antigens from *Yersinia ruckeri* and *Aeromonas hydrophila*.

- The height of the humoral response and the antigen dose are directly correlated.

- The height of the humoral response is dependent of the route of antigen administration. Injection gives higher serum antibody levels than bath vaccination.

- The peak day of the response is not antigen dose dependent, but is influenced by the bacterin type.

- Circulating antibody in these animals can be very persistent (e.g. 1 year). This phenomenon is due to continuous stimulation by antigen in or on macrophages (especially melano-macrophages).

- Local processes in the skin and gills play a regulatory role in the response after bath vaccination.

- Memory develops after both antigen injection and bathing in antigen solution.

- The development of memory in fish takes much more time than the antibody response itself. Maximum levels are usually not reached before 3 months after the initial contact with the antigen.

- The height of the secondary responses can be used as an indirect

method for the quantification of memory. The data obtained can be used for calculating a memory factor.

- The height of memory levels achieved and the priming antigen dose are directly correlated.

- The expression of memory is affected by several factors, such as residual "priming" antibody at the moment of boosting, the ratio between the first and second antigen dose, and whether or not the priming and boosting routes correspond.

- In the early phase of the response mononuclear phagocytes in the kidney and spleen are responsible for antigen handling and stimulation of immuno-competent cells. In the later phase melano-macrophage centres (MMCs) become more important for the stimulation of the response and for memory induction.

- MMCs probably are the early phylogenetic analogous of mammalian germinal centres.

- Epithelial cells of the second segment of the intestine are important for the initial antigen uptake. Certain antigens are not digested in the cells, but released in the intercellular space (circulation) and taken up by intestinal macrophages. It is unclear if the intestinal lymphocytes react upon these antigens.

- Successful application of bacterial vaccines to fish is possible. Injection methods can be used for small numbers of fish. Bath methods are possible for large scale application or young animals. Oral administration is not as effective as injection or immersion, but recent data provide good prospectives for future use.

- The best results in terms of protection can be expected when the vaccination route (e.g. bath) corresponds with the route of pathogen entry (e.g. gills and skin).

- In tests for the determination of vaccination efficacy the same challenge route as the vaccination route should be preferred.

- It will be an advantage when fish, after a vaccination, can develop immunological memory during a period of 2-3 months at optimal temperatures in a relatively clean environment (e.g. specific pathogen free hatcheries).

SAMENVATTING

De snelle groei die de visteelt de laatste jaren heeft doorgemaakt, heeft mede geleid tot een toenemende kennis betreffende een aantal visziekten. De bestrijding van deze infectieuze ziekten vraagt een multidisciplinaire aanpak. Het belang van immunoprofylaxis voor een gezonde bedrijfsvoering wordt steeds meer erkent. Tot nu toe zijn er goede resultaten behaald met vaccinatie tegen de bacteriële ziekten vibriosis (*Vibrio anguillarum*) en yersiniosis (*Yersinia ruckeri*), terwijl vaccinatie tegen andere ziekten, o.a. veroorzaakt door *Aeromonas hydrophila* en *A. salmonicida* minder succes opleverde.

A. hydrophila is een opportuun pathogeen en veroorzaakt veelal problemen bij, reeds door andere ziekten, verzwakte vissen, waardoor deze bacterie een ernstige bedreiging voor de visteelt vormt. De enorme heterogeniteit tussen de vele *A. hydrophila* stammen geeft problemen bij vaccinatie. Zoals ook het geval is voor *A. salmonicida* richt het onderzoek aan *A. hydrophila* zich momenteel op het identificeren van de virulentiefactoren, met name op gemeenschappelijke antigenen bij virulente stammen. Effectieve vaccinatie tegen *A. hydrophila* zal afhankelijk zijn van deze "algemene virulentie factoren" en de vaststelling van de optimale condities voor het toedienen van vaccins. Hierbij is het van belang goed geïnformeerd te zijn omtrent de reactie van het immuunsysteem op toediening van deze vaccins.

Op dit ogenblik is er weinig bekend over de aard van de antigenen die bescherming kunnen induceren, over het mechanisme van antigeenopname na een bad vaccinatie of na toediening met het voer, en over de immunologische reacties die direct verantwoordelijk zijn voor bescherming. Tot nu toe is er weinig aandacht besteed aan de immuunreacties na badvaccinatie of na antigeen toediening met het voer. Hetzelfde geldt voor de histofysiologie van de immuunrespons van vissen. Ondanks de steeds toenemende kennis omtrent de factoren, die de afweerreacties van vissen beïnvloeden, is er toch behoefte aan meer informatie, met name over het effect van vaccinatie. Uiteindelijk zullen dan optimale condities voor het uitvoeren van vaccinaties kunnen worden vastgesteld.

Dit proefschrift beschrijft het effect van toediening van bacteriepreparaten van *Yersinia ruckeri* en *A. hydrophila* op het afweerapparaat van de karper. De bacteriepreparaten werden via injectie of badbehandeling toegediend. Het effect op het immuunsysteem werd bestudeerd aan de hand van het aantal antilichaam vormende cellen (AVC) in de lymfatische organen, de hoogte van de antilichaamspiegel in het bloed en de wijze waarop antigeen in de lymfatische organen wordt verwerkt. Verder werd, met het oog op orale vaccinatie, een studie verricht naar de opname en transport van eiwitmoleculen door darm epitheelcellen.

Het O-antigeen preparaat van *Y. ruckeri* was sterk immunogeen in de karper (Appendix *I*). Zowel na injectie van het preparaat in de rugspieren als in de buikholte of na een bad in de antigeenoplossing werden aanzienlijke aantallen AVC en hoge antilichaamtiters gemeten. De hoogte van de reactie was afhankelijk van de toedieningsroute b.v. de reactie was duidelijk geringer na badbehandeling.

In tegenstelling tot het aantal AVC dat na een piek op dag 10 snel afnam, bleef het niveau van de antilichamen in het serum lange tijd hoog, vooral na injectie van een hoge dosis antigeen. Het ligt voor de hand dat een langdurige aanwezigheid van antigeen in de lymfatische organen, zoals m.b.v. immunofluorescentie kon worden vastgesteld, verantwoordelijk is voor dit fenomeen.

De secundaire immuunresponsen in karpers na een tweede toediening van antigeen via injectie of bad, geven indicaties voor de vorming van een immunologisch geheugen na een eerste contact met het antigeen. De hoogste secundaire responsen werden verkregen in vissen, waarvan de antilichaam niveaus, die nog resteerden na de eerste vaccinatie, waren gedaald tot achtergrondswaarden (Appendix *II*).

Van *A. hydrophila* zijn twee bacterie preparaten gebruikt nl. intacte formaline gedode cellen (F-*Ah*) en hitte geïnactiveerde en stuk gevroren cellen (H-*Ah*). Injectie van beide preparaten leidde tot de productie van aanzienlijke hoeveelheden serum antilichamen. H-*Ah* induceerde meer antilichamen dan F-*Ah*, maar in beide gevallen bleven de antilichamen lange tijd aanwezig, minimaal gedurende 8 maanden (Appendix *III*). Terwijl de hoogte van de reactie duidelijk gecorreleerd was met de antigeendosis, was de dag waarop de piek werd bereikt afhankelijk van het type bacteriepreparaat (dag 14 voor H-*Ah* en dag 20 voor F-*Ah*). De invloed van het mengen van antigeen met adjuvant kwam tot uiting in een tweede toename van het aantal AVC, na de normale piek, en een verlengde productie van antilichamen (Appendix *VIII*).

De antisera, opgewekt in de karper, tegen de twee *A. hydrophila* preparaten vertoonden verschillende agglutinerende eigenschappen (Appendix *II*). Antilichamen opgewekt tegen F-*Ah* waren hoofdzakelijk gericht tegen lipopolysaccharide (LPS), terwijl dit met H-*Ah* niet het geval was. LPS is een belangrijk antigeen van *A. hydrophila*, maar er is tenminste nog één andere immunogene component. Immuun serum (anti-F-*Ah*) en niet-immuun controle serum zijn gefractioneerd en de fracties werden vervolgens onderzocht op aanwezigheid van immunoglobuline (Ig) m.b.v. een ELISA en op agglutinerende activiteit t.o.v. de bacteriepreparaten. Het bleek, dat de aanwezigheid van Ig beperkt was tot de hoog moleculaire eiwit fracties. In het immuun serum correleerde Ig met aanwezigheid van agglutinerende activiteit. In zowel immuun als niet-immuun serum was er een agglutinerende activiteit in laag moleculaire eiwit fracties, hetgeen waarschijnlijk een spontaan voorkomende agglutinine is (Appendix *III*).

De vorming van immunologisch geheugen is bestudeerd aan de secundaire responsen. Er kan geconcludeerd worden, dat na injectie van *A. hydrophila* een geheugen gevormd wordt (Appendix *IV*). De hoogte van de secundaire respons was direct gecorreleerd met de gebruikte antigeendosis bij de eerste (priming) injectie. Bovendien bepaalde de antigeendosis van de tweede (booster) injectie de mate waarin het gevormde geheugen tot expressie kwam. Het beste geheugen werd gemeten bij overeenkomende priming en booster doses. Het duurde enige maanden voordat het geheugen z'n maximale waarde had bereikt, en het was in de meeste gevallen na 12 maanden nog aanwezig. Een lage

antigeendosis induceerde een zwak geheugen, dat bovendien van korte duur was.

Een enkel bad in een *A. hydrophila* preparaat, werd niet gevolgd door het verschijnen van circulerende antilichamen, maar het induceerde wel de vorming van een geheugen, want een herhaald bad gaf wèl een duidelijke antilichaamrespons (Appendix *V*). Dit geheugen was maximaal na 3 maanden. Het was tijdelijk van aard, want het was weer verdwenen na 12 maanden. Indien de booster werd gegeven via injectie, kwam het gevormde geheugen niet tot expressie als een duidelijk verhoogde secundaire respons. Bovenstaande gegevens onderstrepen het belang van locale processen in de kieuwen en de huid voor de immuun respons na badvaccinatie.

In Appendix *VI* is een korte morfologische beschrijving gegeven van de milt, kopnier en rompnier. Na injectie van een *A. hydrophila* preparaat is er m.b.v. de immunofluorescentietechniek een studie verricht naar de verwerking van het antigeen in genoemde lymfatische organen. Kort na injectie verscheen het antigeen in de hulscapillairen van de milt en in verspreid voorkomende fagocyten in de kop- en rompnier. Vervolgens concentreerde het zich rond centra van melanomacrofagen in al de bestudeerde organen en in latere fasen was het antigeen uitsluitend in en rondom de melano-macrofagencentra (MMC) aanwezig, zowel intracellulair als gebonden aan celmembranen. Deze situatie bleef meer dan een jaar bestaan. Na een badvaccinatie kon geen antigeen worden aangetoond in de lymfatische organen.
De vraag rees, welke van de waargenomen processen immunologische betekenis hadden en welke eventueel een niet specifieke reactie waren. Daarom werd de verwerking van inert materiaal (koolstof) bestudeerd ter vergelijking (Appendix *VII*). Op twee punten waren er verschillen met de verwerking van *A. hydrophila* antigeen: 1) koolstof werd uitsluitend intracellulair waargenomen, 2) in de milt werd koolstof naar de MMC getransporteerd door migrerende cellen, die de hulscapillairen verlieten, terwijl dit met *A. hydrophila* niet duidelijk het geval was. Het antigeen verscheen in de MMC gebonden aan het oppervlak van cellen.

Mede gebaseerd op deze gegevens werd verondersteld, dat membraan gebonden antigeen in de MMC van immunologische betekenis zou zijn. Om deze veronderstelling te testen werden na injectie met *A. hydrophila* antigeen tegelijkertijd histologische en immunologische tests uitgevoerd (Appendix *VIII*). Het bleek, dat extracellulair, membraan gebonden antigeen in de MMC niet kon worden waargenomen voordat circulerende antilichamen detecteerbaar waren. Dit suggereert, dat er sprake was van antigeen-antilichaamcomplexen. Een verdere aanwijzing hiervoor is de gelijktijdige aanwezigheid van Ig en antigeen in de MMC.

De mogelijke immunologische betekenis van MMC is besproken (Appendix *VI - VIII*). Er zijn verschillende aanwijzingen die suggereren, dat MMC vroege fylogenetische analogons zijn van kiemcentra, zoals beschreven voor zoogdieren.

Appendix *IX* beschrijft een electronenmicroscopische studie naar de opname en transport van 2 "tracer" eiwit-moleculen, nl. horse radish peroxidase (HRP) en ferritine, in het darmepitheel van

de karper. In het tweede darmsegment werd méér intact materiaal opgenomen dan in het eerste segment; bovendien was de verwerking van beide moleculen door het darmepitheel verschillend. HRP werd waarschijnlijk opgenomen via membraanbinding en pinocytose en vervolgens getransporteerd naar de intercellulaire ruimten, waar het in contact kwam met de intra-epitheliale leucocyten. Er vond geen waarneembare intracellulaire vertering van HRP plaats. Daarentegen werd ferritine opgenomen via pinocytose en kwam uiteindelijk terecht in lysosoomachtige structuren of supranucleaire vacuolen (in het 2e segment), waar het langzaam werd verteerd. Hoewel er geen ferritine in de intercellulaire ruimte kon worden aangetoond, waren macrofagen met grote ferritine bevattende vacuolen aanwezig tussen de epitheel cellen. Kleinere macrofagen, die ook ferritine bevatten, zijn in de *lamina propria* en zelfs in de milt gedetecteerd. Deze gegevens kunnen geïnterpreteerd worden in termen van selectieve en niet selectieve absorptie van macromoleculen en de mogelijke immunologische betekenis daarvan.

Er kan geconcludeerd worden, dat oraal toegediende antigenen de intra-epitheliale leucocyten bereiken en dat ze mogelijk een locale immuunrespons kunnen induceren. Aangezien het meeste antigeen in het 2e segment wordt opgenomen, zou dit deel van de vissedarm een belangrijke immunologische functie kunnen hebben.

ALGEMENE CONCLUSIES

- In de karper kan een humorale immuunrespons worden opgewekt met oplosbare en deeltjes antigenen van de bacteriële vispathogenen *Yersinia ruckeri* en *Aeromonas hydrophila*.

- De hoogte van de humorale respons hangt af van de route waarlangs het antigeen wordt toegediend. Injectie leidt tot een hoger niveau aan antilichamen in het bloed dan een badvaccinatie.

- De dag waarop de piek van de respons wordt bereikt, wordt niet bepaald door de antigeen dosis, maar door het type antigeen.

- In vissen kunnen circulerende antilichamen erg lang aanwezig zijn (b.v. 1 jaar). Dit fenomeen wordt veroorzaakt door een voortdurende stimulatie van het immuunsysteem door antigeen, dat zich in of op macrofagen bevindt (speciaal melano-macrofagen).

- Na badvaccinatie spelen locale processen in de huid en kieuwen een regulerende rol in de immuun respons.

- De hoogte van de secundaire responsen kan worden gebruikt als een indirecte methode om geheugen te quantificeren, d.m.v. het berekenen van een geheugenfactor.

- Zowel na injectie van antigeen als na een bad in een antigeenoplossing wordt er een immunologisch geheugen gevormd.

- De vorming van een geheugen in een vis kost veel meer tijd dan het tot stand komen van een antilichaamrespons. Na een eerste

contact met antigeen duurde het gewoonlijk 3 maanden of langer voordat het gevormde geheugen z'n maximale waarde had bereikt.

- Er bestaat een directe correlatie tussen de antigeendosis, gebruikt voor priming, en de maximale waarde die het geheugen bereikt.

- De mate waarin het geheugen tot expressie komt, wordt beïnvloed door verschillende factoren, zoals het restant aan primaire antilichamen op het moment van de booster, de verhouding tussen eerste en tweede antigeendosis en het al of niet overeenkomen van de routes waarlangs priming en booster hebben plaatsgevonden.

- Kort na antigeentoediening zijn mononucleaire fagocyten in de milt en nier verantwoordelijk voor de verwerking van antigeen en voor de stimulatie van immunocompetente cellen. In een latere fase spelen macrofagen in melano-macrofagen centra (MMC) een rol bij de continue stimulatie van de respons en de vorming van een geheugen.

- MMC kunnen worden beschouwd als de vroege fylogenetische analogons van de kiemcentra bij vogels en zoogdieren.

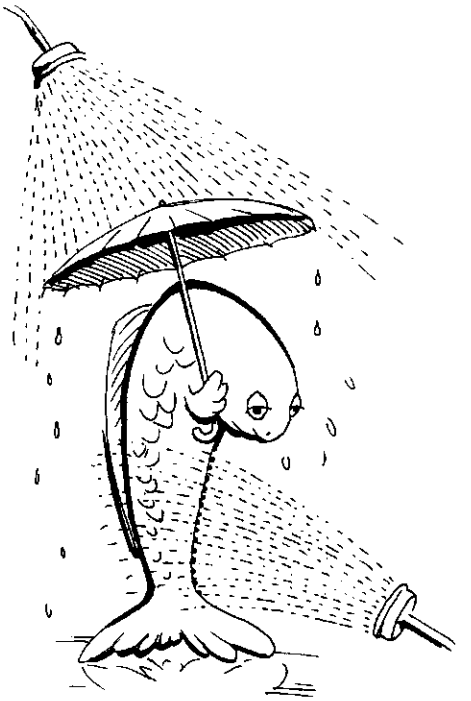
- Epitheelcellen van het 2^{e} darmsegment van de karper zijn belangrijk voor opname van antigeen. Bepaalde antigenen worden niet in de cellen verteerd, maar doorgesluisd naar de intercellulaire ruimten (circulatie!) en opgenomen door "darm"-macrofagen. Het is niet duidelijk of "darm"-lymfocyten op deze antigenen reageren.

- Het is mogelijk om vissen met succes te vaccineren. Injectiemethoden kunnen worden aangewend voor kleine aantallen vis. Badmethoden zijn mogelijk voor toepassing op grote schaal of voor jonge dieren. Orale toediening is niet zo effectief als injectie of bad, maar recente ontwikkelingen bieden goede vooruitzichten voor de toekomst.

- De beste resultaten in termen van bescherming kunnen worden verwacht als de vaccinatieroute (b.v. bad) overeenkomt met de route waarlangs de ziekteverwekker onder normale omstandigheden binnen dringt (b.v. kieuwen en huid).

- In tests ter bepaling van de effectiviteit van een vaccinatie zou de route waarlangs de "challenge" (blootstelling aan pathogeen) wordt gegeven dezelfde behoren te zijn als de weg waarlangs het vaccin is aangeboden.

- Het verdient aanbeveling om vissen na een vaccinatie, gedurende 2-3 maanden, bij optimale temperatuur en in een relatief schone omgeving (pathogeen vrije kwekerijen) een immunologisch geheugen te laten ontwikkelen.



SPRAY OR SHOWER VACCINATION

CURRICULUM VITAE

Op 17 april 1954 ben ik als Cornelis Henricus Johannes Lamers geboren te Driel (gem. Heteren).
Reeds op de kleuterschool oefende de natuur op mij een grote aantrekkingskracht uit, getuige de vele pogingen tot het "transplanteren" van crocusjes.
Na de St. Stanislaus lagere school te Driel heb ik, volgens traditie in ons gezin, de RK Ulo-school "St. Aloysius" te Elst (Gld) bezocht, waar mij op 12 juni 1970 het diploma Mulo A en B met aantekening handelskennis werd overhandigd.
Met de Mammoet op de hielen ben ik overgestapt naar het St. Canisius College te Nijmegen en behaalde op 20 juni 1972 het HBS-B diploma.

In datzelfde jaar heb ik een aanvang gemaakt met de Biologie studie, waarvan de plaatsingscommissie bepaalde, dat dit aan de Landbouwhogeschool te Wageningen zou zijn. Misschien door mijn agrarische achtergrond heb ik me er prima thuisgevoeld en op 8 juni 1976 kon mij het kandidaatsdiploma "met lof" worden uitgereikt.

In de doctoraalstudie heb ik leeronderzoeken verricht in het kader van:
- een verzwaard hoofdvak Celbiologie (Ontwikkelingsbiologie) bij prof.dr. L.P.M. Timmermans, met als onderwerpen:
  a) De herkomst van entero-endocriene cellen in larven van *Barbus conchonius*
  b) EM studie naar de differentiatie van het darmepitheel van *Barbus conchonius* larven (beide uitgevoerd o.l.v. dr. J.H.W.M. Rombout)
  c) Migratie en differentiatie van neurale lijstcellen in interspecifieke neurale buis transplantaten tussen kip en kwartel (uitgevoerd o.l.v. prof.dr. N.M. LeDouarin, Lab. d'Embryologie, Nogent-sur-Marne, Frankrijk).
- een hoofdvak Dierkunde (Parasitologie) bij dr. W. Dorsman, met als onderwerp: Produktie en weefsellocalisatie van IgE in de rat tijdens een infectie met *Trichinella spiralis* (uitgevoerd o.l.v. prof.dr. F.J. Ruitenberg, RIVM, Bilthoven).

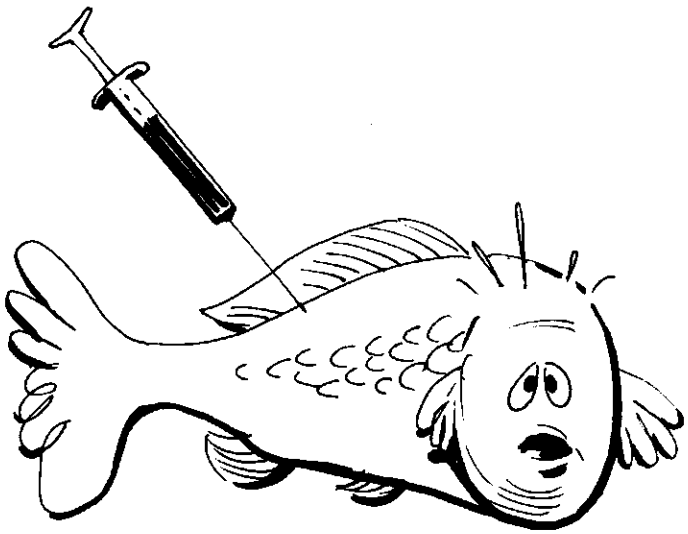
Als afsluiting van deze boeiende en leerzame periode mocht ik op 26 januari 1979 het ingenieursdiploma Biologie "met lof" in ontvangst nemen.

Ik ben in de gelukkige omstandigheid geweest mijn wetenschappelijke scholing nog enige jaren te hebben kunnen voortzetten. Van 1 maart 1979 tot 15 oktober 1980 heb ik bij de vakgroep Experimentele Diermorfologie en Celbiologie (EDC) van de Landbouwhogeschool, in het kader van een vooronderzoek naar "De herkomst, functie en regulatie van entero-endocriene cellen in karperachtigen" i.s.m. dr. J.H.W.M. Rombout een studie verricht naar de migratie en differentiatie van neurale lijstcellen bij vissen. In deze periode, waarin het oorsponkelijke tijdelijke dienstverband van 9 maanden zeven maal werd verlengd, heb ik ook de afsluitende hospiteerstages voor mijn onderwijsbevoegdheid Biologie verricht.

Op 15 oktober 1980 heb ik een begin gemaakt met een promotie-onderzoek bij de vakgroep EDC onder verantwoording van prof.dr. L.P.M. Timmermans (sectie Histologie/Ontwikkelingsbiologie) en dr. W.B. van Muiswinkel (sectie Celbiologie).
Het betrof een driejarig promotie-assistentschap van de Landbouwhogeschool met als titel: "De reactie van het afweerapparaat op toediening van vaccin bij vissen".

Op 1 januari 1984 werd dit onderzoek officieel afgesloten en 383 uitkeringsdagen later kon het manuscript van dit proefschrift aan de promotie-commissie worden voorgelegd.

A P P E N D I C E S



INTRAMUSCULAR VACCINATION

APPENDIX PAPER I

IMMUNE RESPONSE AND ANTIGEN LOCALIZATION IN CARP (*CYPRINUS CARPIO*) AFTER ADMINISTRATION OF *YERSINIA RUCKERI* O-ANTIGEN[a)]

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SUMMARY

In this paper a study in carp, kept at 20°C or 22°C, is described with *Yersinia ruckeri* O-Antigen (O-Ag). The O-Ag of *Y. ruckeri* was administred by intramuscular (i.m.), intraperitoneal (i.p.) injection or by bath. After injection a clear plaque forming cell response in the pronephros was observed. At 20°C serum antibody titres increased from day 12 onwards in all groups. After bath the titre was relatively low. Antibody (Ab) titres stayed at a high level up to day 56; even after 4 month these levels were still relative high. Antigen localization was studied in the spleen, pro- and mesonephros using indirect fluorescence. After i.m. and i.p. injection, the O-Ag was first seen in the spleen and in the free macrophages of all organs (day 2-8). From day 16 onwards the O-Ag was localized in clusters of macrophages (mc's).

INTRODUCTION

During the last decades interest has grown to develop vaccination schedules evoking protective immunity in fish (1,2,3). The methods most commonly used for vaccine administration are: oral,

a) Developmental and Comparative Immunology, Supplement 2, 107-114, 1982.

hyperosmotic immersion, direct immersion and spray or shower. Injection is only used on small scale. In most cases the efficacy of vaccination was determined by virulent challenge (2,3,4,5). Only few studies were made on immunological parameters after vaccination (6,7,8) and on the relation between these immunological parameters and protective immunity (9,10). More knowledge is required on the way antigen (Ag) enters after different routes of administration and memory formation.

Teleost fish are capable to respond upon antigenic stimulation by humoral and cellular immune responses (11,12). Primary and secondary antibody (Ab) responses and allograft rejection have been observed in a wide range of species. However, it is difficult to draw general conclusions from results obtained in different fish species. Within one species the lag period, the kinetics of the Ab response, memory formation and clearance of the Ag from the circulation are dependent on temperature, type, nature, dose and route of administration of the Ag (13,14,15,16,17,18). For each species conditions have to be tested when new Ag is used.

Yersinia ruckeri is the causative agent of enteric redmouth (ERM) disease in salmonids. In this model study in carp the humoral immune response and Ag localization are studied after i.p. or i.m. injection and direct immersion with the O-Ag of *Y. ruckeri*.

MATERIALS AND METHODS

Animals

Common carp, ranging from 7 to 20 month of age and 50 to 280 g in weight were either bred in our laboratory or obtained from the Organization for Improvement of Inland Fisheries (O.V.B., Nieuwegein). They were kept in aquaria with running tap water at 20°C or 22°C and daily fed with pelleted dry food (K30, Trouw and Co., Putten).

Antigen

The O-Antigen is an isolate of *Yersinia ruckeri* (no. 11.29, National Health Research Laboratory, Leetown, W.V., U.S.A.). The lyophilized powder was boiled for 1 h at 1 mg/ml in phosphate buffered saline (PBS) and cooled before administration or coating sheep red blood cells (SRBC).

Immunization

Animals were injected i.p. or i.m. in the dorsal region with Ag in 0.1 ml PBS or were bathed for 1 h with aeration.

- In the first experiment, animals kept at 22°C were injected i.p. or i.m. with 10 µg O-Ag or were incubated in a solution of 15 mg/l aquarium water. The control group was not treated.

- In a second experiment the serum antibody level and Ag localization after different routes of Ag administration was determined. Fish kept at 20°C were injected i.p. with 100 µg, i.m. with 10 µg or 100 µg or bathed as described above.

The haemolytic plaque assay

The haemolytic plaque assay was carried out according to Rijkers et al. (19). As indicator system SRBC were used, coated with the O-Ag, according to Anderson (8). Bream (*Abramis brama*) serum was used as complement source. Plaques were counted after incubation at 25°C for 4 h. Viability of cells in the suspension was determined by the dye exclusion assay (0.2% Trypan Blue in PBS). Results are expressed in plaque forming cells per 10^6 white cells (PFC/10^6WC).

Passive haemaglutination test

Specific antibody titres to *Y. ruckeri* O-Ag were determined using the standard method of passive haemagglutination in microtitre plates. Coated SRBC were used. As control for SRBC specific agglutinating antibodies, sera were also tested with non-coated SRBC.

Immunofluorescence

To detect the O-Ag in the tissue, an indirect immunofluorescent test was carried out on 5 µm cryostat sections using rabbit anti O-Ag antiserum and fluorescein labelled goat anti rabbit immunoglobulin antiserum (Nordic, Tilburg). The anti O-Ag serum (titre 1:1024) was made in our laboratory.

RESULTS

The pronephros showed a clear primary PFC response between day 4 and 17 after O-Ag administration (Fig. 1). The maximum PFC number was found on day 10 (22°C) in all groups. However, the response after bath administration was significantly lower (20 PFC/10^6WC) than after i.m. or i.p. injection (300 and 210 PFC/10^6WC respectively). Agglutinating Ab titres were determined in the period of 4 months after Ag administration. It is clear from Fig. 2 that in all groups the level of anti O-Ag antibodies increased from day 12 onwards and reached high levels between day 17 and 34 (20°C). In the 10 µg group Ab remained at this high level for a long period and decreased slow-

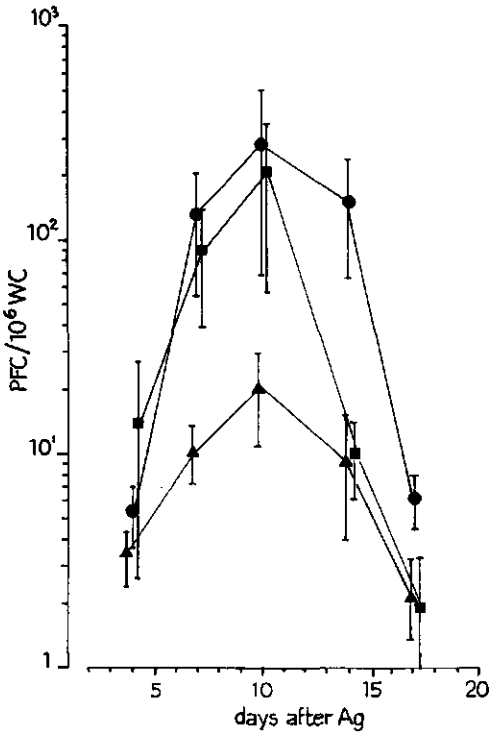

FIGURE 1.
Kinetics of the primary PFC response in the pronephros of carp kept at 22°C. Fish were injected i.m. 10 µg (●); i.p. 10 µg (■) or were incubated in a O-Ag solution (▲).
PFC/10^6WC = Plaque Forming Cells per 10^6 White Cells. Each point represents the arithmetic mean ± 1 SE (n=4).

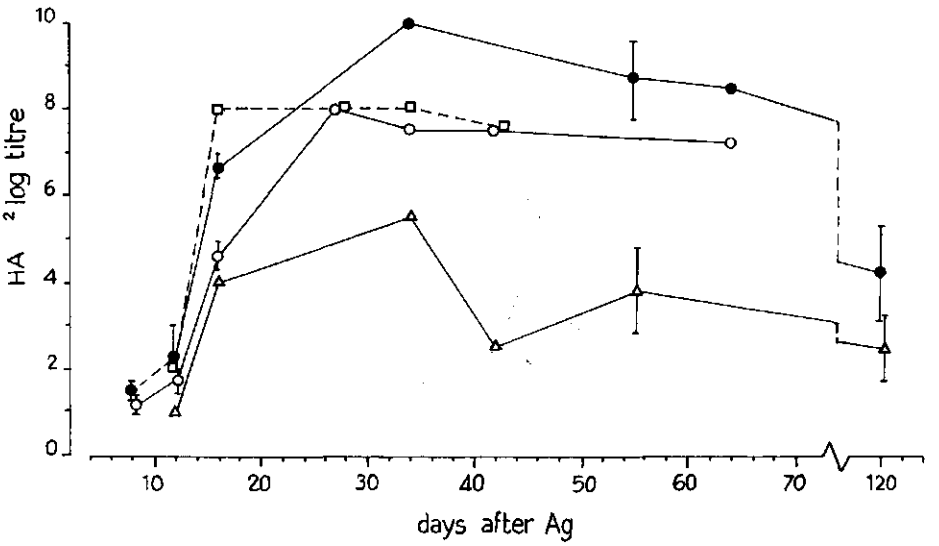

FIGURE 2. Serum levels of agglutinating antibodies in carp after primary administration of O-Ag (20°C). Animals were injected i.m. 10 µg (●) or 100 µg (○); i.p. 100 µg (□) or incubated in antigen solution 15 mg/l (△). Antibody levels are given as ^{2}log dilution. Per point 2-4 animals were bled.

ly after day 55. The 100 µg injected group stayed on the high level up to 4 months. There is a difference between the two dosages of O-Ag used. After i.p. or i.m. injection of 100 µg Ag the antibody titres were lower than after i.m. injection of 10 µg. the titres after bath were considerably lower than those after injection and were still present after day 55. At 4 months after Ag administration the titres in all experimental groups were still enhanced compared with the controls. Whereas in the bath and the 10 µg injection group Ab levels were decreasing from day 55 onwards, in the 100 µg group the Ab level stayed at the high level up to 4 month.

Antigen localization has been studied in the spleen, pro- and mesonephros at day 2,5,8,12,16,22,27,34,42 and 120 after Ag administration. The results obtained with the immunofluorescent method, are summarized in Table 1. There were fluctuations in Ag content between animals at the same day. Therefore these results must be regarded as preliminary. Throughout the whole experiment Ag was localized intracellularly. After injection of 10 µg Ag almost no fluorescing material was found. Much better pictures were obtained with 100 µg Ag. At day 2 after i.m. injection the first fluorescence was seen in the walls of the ellipsoids. At day 5 fluorescent cells could be detected in the splenic pulp and from day 16 onwards Ag was detectable in macrophage clusters (mc's). In carp, cells of the macrophage clusters contain large amounts of ceroid substances, however, generally no melanin is found as described in plaice (22) and turbot (23). The fluorescence was still present in these clusters at day 120. Up to day 42 there was some Ag in the cells of the ellipsoid walls. In the pro- and mesonephros solitary cells in the pulp contained Ag in the first 2-8 days. Later on an Ag shift to the mc's took place. At day 120 a lot of fluorescence was still present in the mc's and also a single fluorescent cell was observed.
After i.m. injection usually less fluorescence was present in the spleen than in the pro- and mesonephros. After i.p. injection the spleen contained relatively more fluorescing cells than the pro- and mesonephros, whereas the localization in time resembled the i.m. injection.

TABLE 1. Antigen localization in spleen, pronephros and mesonephros of carp after administration of *Y. ruckeri* O-Antigen, visualized by indirect immunofluorescence.

Organ		Days after i.m. injection 2-8	16-22	28-42	120	Days after i.p. injection 2-8	34-42
Spleen	ellipsoids	++	+	++	-	+++	±
	pulp	±	+	+	-	+	+
	mc	-	±	++	+	±	+
Pronephros	pulp	±	+++	+	±	+	±
	mc	-	+	++	+++	-	+
Mesonephros	pulp	±	+++	+	-	+	±
	mc	-	++	+++	++	-	+

mc = macrophage cluster
+++, much fluorescence; ++, moderate fluorescence; +, little fluorescence; ±, very little fluorescence; ±, single fluorescing cell; -, no fluorescence

DISCUSSION

The enteric redmouth disease, caused by *Yersinia ruckeri*, is thus far only described in salmonid fish; vaccination schedules developed to prevent considerable losses are satisfying and are applicable to large scale trout culture (3). More knowledge on the mechanism of vaccination will not improve these results, except when an increase in vaccine quality can be achieved. However, vaccination schedules of other bacterial diseases do not work as well. Therefore we are convinced that model studies on the influence of the application route and the nature and dose of Ag on the immune system are important. These experiments combined with studies on protection to natural or artificial challenge can provide better insight in the way protective immunity can be achieved.

In trout PFC responses to the O-Ag were demonstrated after i.p. injection, direct immersion and flush exposure (8,20,21). In carp, both i.p. injection and direct immersion gave a clear response. However, lower figures than in trout were found. Different routes of administration of Ag gave differences in latent period and antibody response (17). All groups in our first experiment showed a PFC

peak on day 10, but comparing the kinetics after i.m. and i.p. injection the PFC maximum of the i.p. group can be expected on day 9. This can be correlated with the earlier increase of antibody titre in the i.p. group than in the i.m. group. The 10 µg i.m. group gave a higher response than the 100 µg i.p. and i.m. group, whereas antibody levels with the high dose stayed constant for a longer period. Bath also induced an Ab response: a relatively low level was reached, which decreased slowly after day 55. At day 120 this level was still enhanced when compared with the controls. This was comparable with the 10 µg i.m. injection group. The different levels of Ab may be explained by different amounts of Ag present in the fish, with an optimum for the Ag concentration. There seem to be relative high Ab levels for a longer period with increasing Ag concentration. It is remarkable that when the PFC response in the pronephros decreased after day 10 (22°C), circulating Ab titres just started to rise and went on increasing when no more PFC could be detected. Comparable results are obtained in trout using O-Ag of *Y. ruckeri* (20). The most important organs in PFC response in carp are pronephros, mesonephros and spleen, in which this response takes place simultaneously (13). It is unlikely, that large number of PFC will be formed in other organs, but even small numbers of such cells and a low catabolism of Ab, might account for the rather persistent Ab levels. On the other hand the two tests used, demonstrated different types of Ab, namely lytic and agglutinating Ab. It may be that in course of time a shift takes place in the nature of the produced antibody.

The process of O-Ag localization, both the initial and the later stages, are rather comparable to the localization of non-antigenic particulate material (22) and bears various resemblances to what occurs to antigenic particulate material (24). No resemblances are observed with the antigen trapping process or the way soluble antigen is lozalized (24,25). All fluorescence was localized intracellularly and no extracellular fluorescence was detected. Similar to *A. salmonicida* (24) O-Ag stayed present for a longer time in the splenic ellipsoids and macrophages in spleen, pro- and mesonephros when compared with carbon (22). At the time Ab levels rise in the serum, the strongest fluorescence was seen in free macrophages and in splenic ellipsoids. In the 100 µg group, in which a large amount

of fluorescing material was present in mc's at day 120, a high Ab level could still be detected. In the other groups which received a lower dose of O-Ag the Ab level was significantly decreased at that time. With the method used no O-Ag was detected in the tissue of these groups during the whole experiment, so only speculations can be made on the relation of the long persistance of Ab and the long presence of O-Ag in the tissue. The high Ab level might be caused by a low continuous Ab production induced by continuous Ag exposure. Some authors (24,25) speculate on: Ag exposed in mc's to be involved in memory formation, comparable to what occurs in germinal centres in mammals and Ag bound in the form of immunecomplexes at metalophil reticular fibres, present in the ellipsoid walls and peri-ellipsoidal areas might be involved in regulation of Ab response. It is suggested in mice (26), that Ag retained in follicles could play a role in maintaining and regulating serum Ab levels for a long time after immunization. In our experiments no Ag trapping was observed and therefore there might be limited possibilities for Ab response regulation (termination). Induction of Ab production might have been caused by exposure of Ag on macrophages, which contained Ag, and it may be maintained by the Ag exposure on the rare free macrophages or by exposure in the mc's. If mc's are involved in memory formation, differences in amount of Ag and time of Ag present in mc's might influence this process. At present a study on memory formation is been carried out using O-Ag of *Y. ruckeri*, which might provide data on Ag dose and rate of memory formation. The illucidation of the evens occurring in mc's may give an insight into the longterm effectiveness of vaccination.

ACKNOWLEDGMENTS

The authors wish to thank Dr. D.P. Anderson (Leetown, U.S.A.) for supplying the O-Antigen of *Yersinia ruckeri*, Drs. W.B. Van Muiswinkel and C.J. Secombes for helpful advice during performance of this study and preparation of the manucript and Mr. W.J.A. Valen for drawing the figures. This work was supported by a scholarship of the Dutch Ministry of Education and Sciences for A. Pilarczyk.

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APPENDIX PAPER II

PRIMARY AND SECONDARY IMMUNE RESPONSE IN CARP (*CYPRINUS CARPIO*) AFTER ADMINISTRATION OF *YERSINIA RUCKERI* O-ANTIGEN[a)]

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INTRODUCTION

In salmonid fish the gram negative bacterium *Yersinia ruckeri* causes a chronic to acute disease called "Enteric Redmouth" (ERM). Outbreaks mostly follow stress-inducing procedures such as handling and crowding. Losses from ERM frequently reach 10-20% of the fish population. In the past control of ERM was performed by incorporation of antibiotics (i.e. oxytetracycline, sulfamerazine) into the food. However, repeated use of these antibiotics have led to serious problems with drug resistant bacteria. Recently bacterins of *Y. ruckeri* were developed for vaccination. Rainbow trout were succesfully immunized by oral administration or by injection (Ross & Klontz, 1965; Anderson, 1974). In a two year field evaluation by Wildlife Vaccines Inc. & Clear Spring Trout Co. (Wheat Ridge), in which 23×10^6 (4-5 g) rainbow trout were vaccinated by direct immersion, significant reduction in mortality attributable to ERM was demonstrated, when compared with non-vaccinated control groups (Tebbit et al., 1981). In most cases vaccination efficiency is determined by virulent challenge. This is an expensive and time and material consuming method. Although it has been shown that protec-

a) Published in: Fish Diseases, Fourth COPRAQ Session (Ed. by Acuigrup). ATP, Madrid, p. 119-127, 1984, (slightly revised).

tive immunity can be achieved in fish (see review by Corbel, 1975) still more knowledge is needed on the mechanism of immunity produced by vaccination, especially on antigen (Ag) localization after different routes of antigen administration, immune response and memory formation. Comparison of these basic studies with protection experiments might lead to tests by which protection can be measured on immunological features. The formation of memory after the first contact with Ag plays an important role in protective immunity. Immunological memory is characterized by specificity, earlier antibody production and the attainment of higher Ab levels after a second contact with the same Ag (Nossal, Austin & Ada, 1965).

In our laboratory carp (*Cyprinus carpio*) is used in studies on the immune system. This study was performed in order to obtain more information about the effect of different vaccination routes, using a bacterial antigen.

MATERIALS AND METHODS

In our experiments we studied the primary and secondary immune response in carp (kept at 20°C and weighing 60-300 g) after administration of O-Antigen (O-Ag) of *Yersinia ruckeri* following the scheme presented in Fig. 1. The *Y. ruckeri* O-Ag is a gift of Dr. D.P. Anderson of the National Fish Health Research Laboratory, Leetown, U.S.A. Just before and at successive intervals after O-Ag administration 0.3 ml blood was taken by caudal vein puncture. Both Ag injection and blood sampling were performed under MS 222 anaesthesia. Serum Ab levels were determined by the passive haemagglutination test. Sheep red blood cells (SRBC) were coated with O-Ag according to Anderson et al. (1979b).

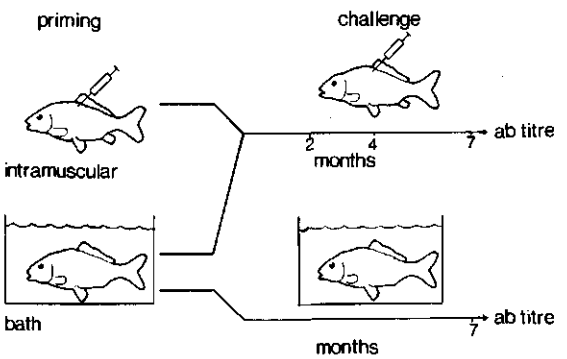

FIGURE 1. Immunization schedule for secondary immune responses in carp against *Y. ruckeri* O-antigen.

RESULTS

Primary immune response

The primary immune response was studied in fish after i.m. injection of 10 or 100 µg O-Ag per fish in 0.1 ml phosphate buffered saline (PBS) or after direct immersion in 15 mg Ag/l aerated aquarium water for 1 h. The results are shown in Fig. 2. It is interesting that at day 0 some carp already had an anti O-Ag titre. This is probably due to cross-reacting antigens in the environment. From day 10 onwards in all groups Ab levels were increasing and highest levels were reached at day 28. From this day onwards Ab titres in the 10 µg group decreased slowly. At day 220 still an enhanced titre could be observed when compared with non-immunized animals. The 100 µg group reached a higher Ab level than the 10 µg group and had still a relatively high level on day 190, when the last sample was taken. After direct immersion (bath) a clear Ab response was seen, but the levels reached were lower and titres decreased more rapidly than after injection. After 4 months the control level was reached.

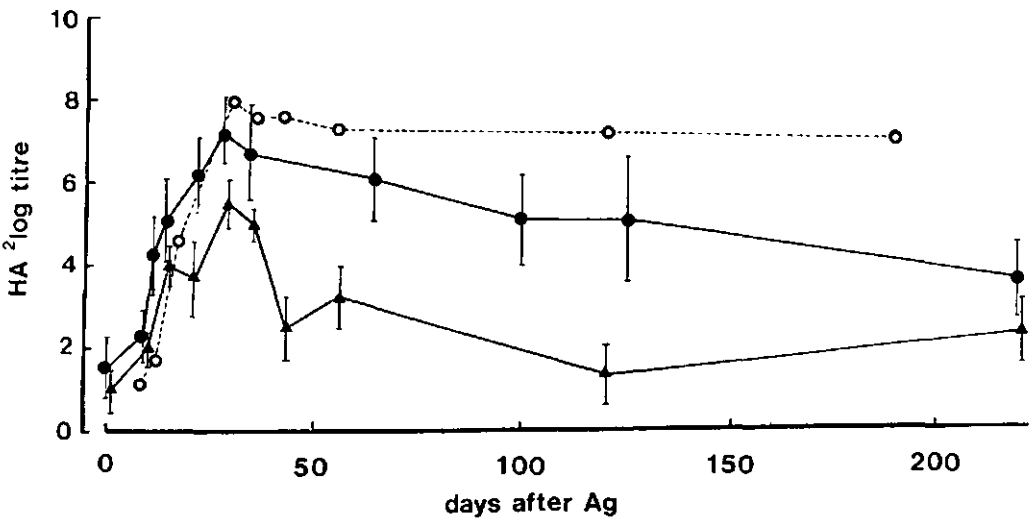

FIGURE 2. Serum levels of agglutinating antibodies after primary administration of O-antigen in carp (20°C). Animals were injected i.m. 10 µg (●); 100 µg (O) or incubated in 15 mg Ag/l aquarium water (▲). Titres are given as geometric mean of ^{2}log dilution (± 1 S.E., when 3 or more animals per point were used).

Secondary immune response

In order to evoke a secondary immune response a booster was given 2, 4 or 7 months after the first administration of Ag (10 µg i.m. injection or bath 15 mg/l). From the kinetics of the primary response (Fig. 2) it can be seen that at 2, 4 and 7 months Ab levels are still present, especially in the injection groups.

In Fig. 3 the shaded area represent the range of the primary response after injection of 10 µg Ag. When the same treatment is given 2 months after the first the animals start with a high Ab level at day 0, which does not alter during the 2 months after secondary injection. This contrasts with animals not given a secondary injection in which a decrease in Ab level was observed during the same period.

When a second injection was given 4 or 7 months after the first a clear rise in the agglutinating titres was observed during the first 15-20 days (Fig. 3). A second injection 4 months after starting the primary response did not give rise to titres which were significantly higher than during a primary response. However, a real secondary response with significantly higher titres after day 20 was observed when animals were injected with an interval of 7 months.

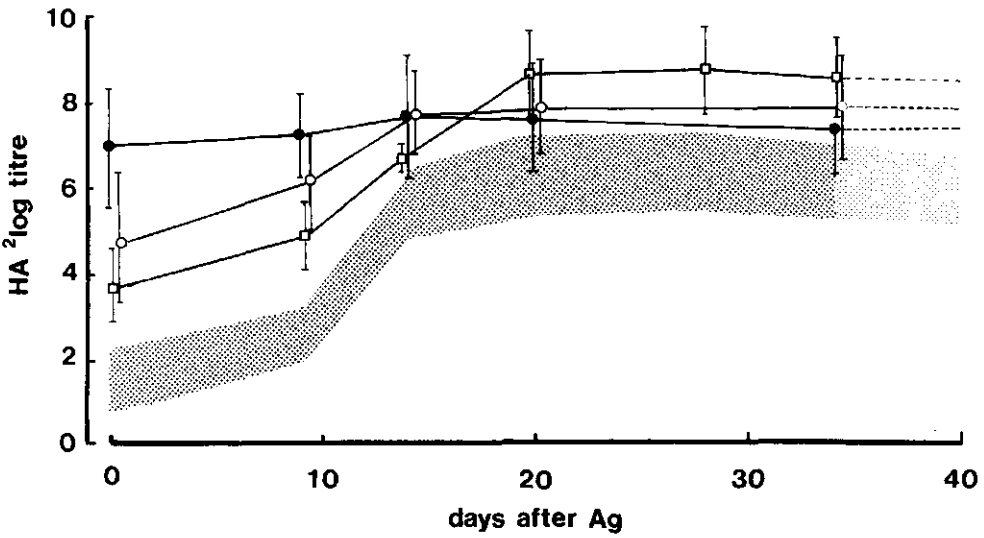

FIGURE 3. Serum levels of agglutinating antibodies after second administration of 10 µg O-antigen by i.m. injection in carp. The shaded area represents the primary response after i.m. injection of 10 µg O-antigen. The second administration was given 2 (●); 4 (O) or 7 (□) months after the first.

It is obvious that the development of memory takes time.

Another group of animals was i.m. injected with 10 µg Ag/fish at 2,4 or 7 months after direct immersion (Fig. 4). The grey area in this figure represents the range of the primary response after injection of 10 µg Ag. At day 0 in all animals, except the 2 months group, the titre was not higher than in non-immunized animals. During the subsequent 20 days Ab titres increased as during a primary response. No significantly elevated level was reached in animals immersed 2 months previously. However, from day 10 onwards a significantly higher level was reached which stayed up to day 70 in animals immersed 4 months earlier. Amazingly the response of the 7 months group did not differ much from a primary response.

In the last group of animals, pretreated by direct immersion, the second administration of Ag was also performed by bath. The interval was 7 months. The secondary response, as shown in Fig. 5, has to be compared with the shaded area which represents the primary response after direct immersion. A clear Ab increase in titre is seen and relatively high titres are reached when compared with the primary response after bath. The response was significantly higher than after the first bath.

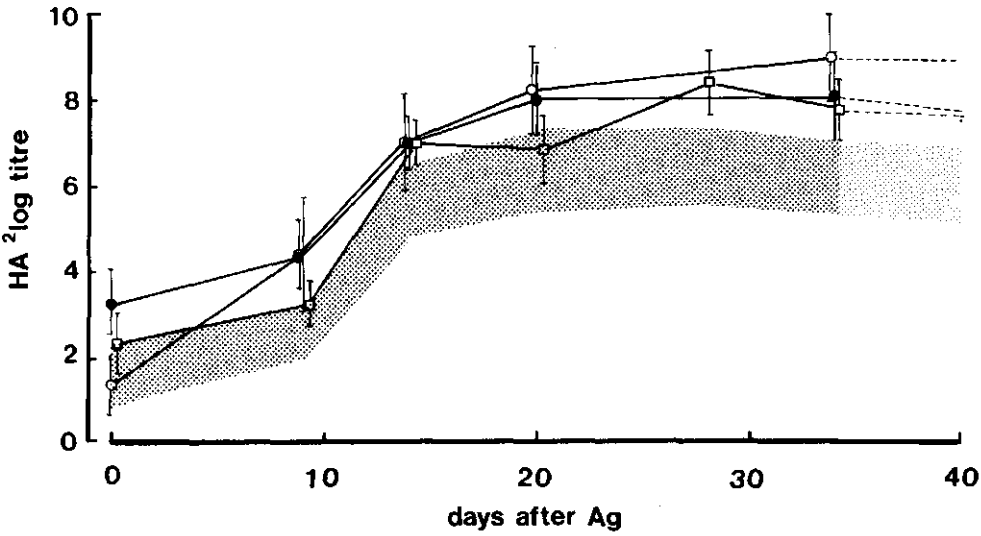

FIGURE 4. Serum levels of agglutinating antibodies in carp. The shaded area represents the primary response after i.m. injection (10 µg). The lines give the responses in animals which were primed by bath (15 mg/l) and which received a second administration by i.m. injection (10 µg) at 2 (●); 4 (○) or 7 months (□) after the bath.

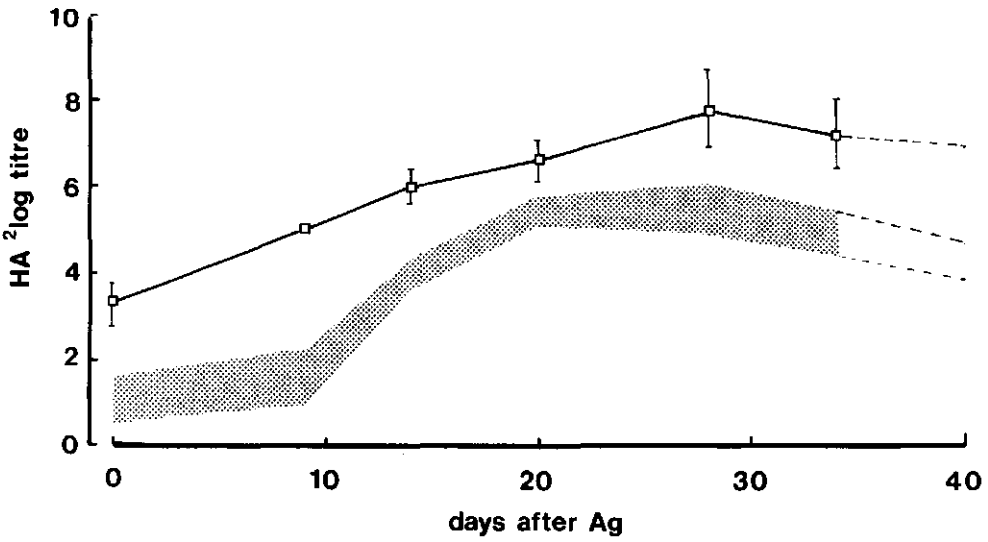

FIGURE 5. Serum levels of agglutinating antibodies in carp after a second bath in O-antigen (15 mg/l). The shaded area represents the primary response after bath. A second bath (□) was given 7 months after the first.

DISCUSSION AND CONCLUSIONS

Both injection and direct immersion gave a clear primary humoral immune response to *Y. ruckeri* O-Ag in carp. This response was studied by determing serum Ab levels, but it can also be done by determing plaque forming cells (PFC) (Lamers & Pilarczyk, 1982). In trout PFC responses to *Y. ruckeri* O-Ag were studied after i.p. injection, direct immersion and flush exposior (Anderson et al., 1979a,b,c). In carp responses were comparable with those in trout, however, in carp lower figures were found. Comparing the 10 and 100 µg injection doses, it can be said that a 10 fold higher Ag dose did not influence significantly the rate of Ab production or the peak level reached. However, the continuation of the Ab production seemed to be related with the Ag dose. In other experiments it was shown using immunofluorescence techniques, that Ag was still present in the kidney and the spleen at day 120 after 100 µg injection. This may be responsible for the persisting Ab level in the serum (Lamers & Pilarczyk, 1982).

From the experiments on the secondary immune response it can be concluded that after both injection and bath immunological memory to *Y. ruckeri* O-Ag is developed. However, the secondary responses are low compared with other antigens like SRBC. In the groups challenged by injection there is a tendency to higher Ab levels after a second injection if the remaining level at the moment of the second injection is less enhanced.

In a study on anti SRBC memory formation in carp Rijkers et al. (1980) have shown that priming with a low antigen dose (10^5 SRBC) evoked only a low primary response, but a high secondary response after boosting with 10^9 SRBC. However, an optimal memory formation needed about 6 months to develop. In general, high dose priming gave excellent primary, but poor secondary response. Our results seem to be in accordance with these data on anti-SRBC plaque forming cells, but as different parameters (PFC vs. titres) were used, we have to be careful in drawing conclusions. Anderson & Dixon (1980) found an absence of immunological memory after flush exposure using the same Ag in rainbow trout. Using athymic nude mice they also proved O-Ag to be T-independent in mammals. In the response to such Ag's no T-cell help is needed. In B-memory cell formation T-cells are involved and immune responses to T-independent Ag's lack a negative feedback (Lafrenz & Feldbush, 1981; White & Nielsen, 1975). Both the long persistent Ab levels and the limited memory formation in our experiments may be explained by T-independency of this O-Ag.

Protective immunity

Until now only few studies have been made to answer the question: which immunological features reflect protection. Studies by Hayashi et al. (1964), Harrell et al. (1975) and Viele et al. (1980) showed that in rainbow trout the level of *Vibrio anguillarum* Ab was correlated with protection against natural challenge. Also injection of non-immune fish with head kidney cells of vaccinated fish gave protection. However, after oral administration of *Vibrio* bacterin, protection could be achieved, although no serum titre was seen. In intestinal mucus of the same animals relatively high levels of Ab were demonstrated (Fletcher & White, 1973). The presence of secretory Ig combined with complement factors in the (skin) mucus (Harrell

et al., 1976) may protect rather effectively, by preventing penetration of pathogens. The initiation of this kind of protection may be an case of "local activation" which may lead to the suggestion to apply bath vaccination for pathogens which usually penetrate through skin and gills and oral vaccination for pathogens of the gastro-intestinal tract. More research has to be focussed on these routes of vaccination regarding the properties of the pathogen. The cell mediated immune response should also be taken in account. Only few authors have published data on cellular immunity in fish evoked by bacterins/vaccines (Chilmonczyk, 1978; Liewes & van Dam, 1982; Smith et al., 1980).

Bath vaccination

For obvious economic reasons most vaccination research has been carried out on salmonids. Recently other fish i.e. plaice, carp and catfish have received more attention. Very little is known about direct immersion in carp. We have shown in this study that using this route a humoral immune response to a bacterial Ag can be induced. Maas & Bootsma (1982) provided interesting data on protection of carp against a natural outbreak of Columnaris disease after immersion in *Flexibacter* bacterin (disrupted cells). In their study bacterial Ag was detected in the spleen after immersion, which indicates that using this method Ag really enters the fish. Other authors already described the same phenomenon in salmonid fish (Amend & Fender, 1976; Bowers & Alexander, 1980; Smith, 1982). More data (e.g. other antigens) are needed on vaccination routes in carp and other fish. At this moment it is clear that direct immersion is a promising vaccination method for fish farming.

Final conclusions

- An immune response and immunological memory for *Y. ruckeri* O-antigen can be evoked in carp both by injection and by direct immersion.
- After primary injection, Ab peak levels are not related with Ag dose in the range of 10-100 µg Ag/fish, but persistence of Ab is dose dependent.
- Indications for immunological memory were only obtained in those animals in which Ab titres were at background levels at the moment of the challenge.

- There seems to be an inverse relationship between the Ab level at the moment of the challenge and the peak response after challenge.
- The combined results of primary and secondary responses indicate that *Y. ruckeri* O-Ag might be a T-independent Ag in carp.
- Despite the poor memory formation, the O-Ag might be protective due to the long persistence of Ab induced by relatively low Ag dose.

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APPENDIX PAPER III

THE IMMUNE RESPONSE IN CARP (*CYPRINUS CARPIO* L.). ACQUIRED AND NATURAL AGGLUTININS TO *AEROMONAS HYDROPHILA*[a)]

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SUMMARY

Formalin killed *A. hydrophila* cells (F-*Ah*) and heat inactivated and fragmented *A. hydrophila* cells (H-*Ah*) both evoked agglutinin titres in carp at 21 °C, which persisted for at least 8 months. The height of the agglutinin response depended on antigen dose and bacterin. H-*Ah* injection induced the highest agglutinin production. The peak day of the reponse was dependent not on antigen dose, but on bacterin type (day 14 and 20 for H-*Ah* and F-*Ah* respectively). Antisera raised against the two bacterins differed in agglutinating properties. F-*Ah* induced agglutinins which were mainly directed to *A. hydrophila* lipopolysaccharide (LPS), whereas H-*Ah* induced activity against other bacterial antigens. Sephadex G200 fractionation of immune (anti F-*Ah*) and non-immune control sera resulted in four and three protein peaks respectively. Immunoglobulin (Ig) was restricted to the first, high molecular weight protein peak. The first peak fractions of immune serum agglutinated both F-*Ah* and H-*Ah*; the third peak fractions also contained activity, however, they agglutinated H-*Ah* only. In non-immune serum only fractions of the second/third peak showed agglutination, and it was mainly directed against H-*Ah*.

INTRODUCTION

Bacteria belonging to the heterogeneous species *A. hydrophila* are widely distributed throughout the aquatic environment. Certain

a) Submitted for publication.

strains have produced massive mortalities of cultured and feral fish. This opportunistic pathogen can cause outbreaks of bacterial hemorrhagic septicemia following handling during vaccination procedures (Austin & Rodgers, 1981; Munn & Trust, 1983), or simultaneous with other diseases (cf. Newman, 1983).

Few studies are performed on the immune responsiveness of fish to *A. hydrophila*. In *A. hydrophia* infected wild largemouth bass (*Micropterus salmoides*) Hazen et al. (1981) observed fluctuation in serum agglutinin levels, due to season and prevalence of the disease. In channel catfish (*Ictalurus punctatus*) the highest serum Ab response was traced after injection of fragmented bacterin (Thune & Plumb, 1982). In the case of *Vibrio*-disease distinct serum Ab titres might reflect protection (Harrell et al., 1975; Viele et al., 1980). However, for *A. hydrophila* this correlation is unclear. Whereas Post (1966) reported that serum Ab titres in rainbow trout reflected protection against the homologous strain, Song & Kou (1981,1982, cited by Plumb, 1984) and Schäperclaus (1970) found that protection against heterologous strains did not correlate with serum Ab levels in eel (*Anguilla japonica*) and carp respectively. Up to now vaccination against *A. hydrophila* has not been efficacious, for protection usually is limited to the *A. hydrophila* strains that were included in the vaccine (Schäperclaus, 1970; Acuigrup, 1980). Therefore it will be necessary to identify the antigens responsible for inducing protective immunity, to search for "common protective antigens", and to trace the immunological events evoked by these antigens.

In general, the humoral immune response in fish is influenced by several factors: antigen nature, dose and route of administration and environmental factors as pollution and temperature (cf. Dorson, 1984). In fish serum several non-inducible proteins have been demonstrated e.g. natural agglutinins, which might have protective properties (Ingram, 1980; Rijkers, 1982).

In this study we compared the immunogenicity of two *A. hydrophila* bacterins, and traced the influence of antigen dose on the humoral immune response. Subsequently the immune carp sera were tested for cross-reactivity, in order to get a better view on the different antigens involved in induction of the immune response. Sephadex G200 fractionation of immune and non-immune sera, followed

by testing for the presence of agglutinating activity and Ig were carried out to characterize the nature of a natural occurring agglutinating activity against *A. hydrophila*.

MATERIALS AND METHODS

Animals

Common carp (*Cyprinus carpio*) were reared in our laboratory. At the start of the experiments the carp were about 9 months old and weighed 50-100 g. Carp were kept in aquaria with recirculated, filtered and U.V. sterilized water. Water quality was checked weekly, and during the experiments the following conditions were registered: temperature 21 ± 1 °C; pH = 7.5 ± 0.3; NO_2^- ± 0.05 mg/ml; NH_4^+ ± 0.02 mg/ml; O_2 > 7-9 mg/ml. The fish were fed daily with pelleted dry food (K30, Trouw and Co., Putten, The Netherlands).

Antigen

Bacterins of *A. hydrophila* (*Ah*, freshwater isolate V81/149) were prepared and kindly donated by Dr. R. Bootsma (Dept. of Pathology, Veterinary Faculty, University of Utrecht, The Netherlands). Bacterins were prepared by culturing *A. hydrophila* in tryptose phosphate broth (pH 7.3, Difco), at 25 °C. The medium was inoculated with *A. hydrophila* cells from a pre-culture in exponential phase. The culture was incubated for 24h under constant agitation until bacteria attained stationary growth. The cell yield (approximately 10^9 cells/ml) was estimated by determing the number of colony forming units on tryptic soy agar plates. For preparation of F-*Ah*, the culture was inactivated by adding formalin to a final concentration of 0.3% (v/v), and stored sterile at 4 °C. H-*Ah* bacterin was prepared by heating the culture for 30 min at 60 °C, followed by three freeze/thaw cycles to disrupt the cells. The bacterin was stored at -20 °C. Bacterins were centrifuged 10 min at 10,000 x g and washed three times in phosphate buffered saline (PBS, pH 7.2). F-*Ah* preparation was considered complete formalin fixed cells, whereas H-*Ah* consists of relative large bacterial cell envelopes.

The *A. hydrophila* LPS fraction was isolated from a cell wall extract, prepared according to the method of Edwards & Ewing (1972). This extract was boiled for 1h in PBS, and then centrifuged for 10 min at 6,000 x g. The water soluble fraction was used as LPS. Analysis of this fraction was carried out by sodium dodecylsulphate polyacrylamide gel electrophoresis (Lugtenberg et al., 1975) on 11.5% (w/v) gels, followed by LPS specific staining (Hitchcock & Brown, 1983). This analysis, carried out by Dr. D. Evenberg (Dept. of Molecular Cell Biology, University of Utrecht, The Netherlands), showed that the preparation contained almost exclusively low molecular weight LPS. Sheep red blood cells (SRBC) were coated with this LPS preparation by the method described by Anderson et al. (1979).

Immunization procedure and antibody titration

Carp were immunized by intramuscularly (i.m.) injection of 10^5, 10^6, 10^7 or 10^9 F-*Ah* or H-*Ah*/100g body weight, in 0.1 ml PBS in the

dorsal region. Concentrations were determined spectrophotometrically. At regular intervals up to day 240 the fish were bled by caudal vein puncture and the sera stored at -20 °C. The 10^6 group were monitored up to day 60.

Serum agglutinin titres were determined with a standard microtechnique for bacterial agglutination or passive haemagglutination. In the bacterial agglutination, washed bacteria were used at a final concentration of 2.5 x 10^8 bacterial cells/ml. For the passive haem-

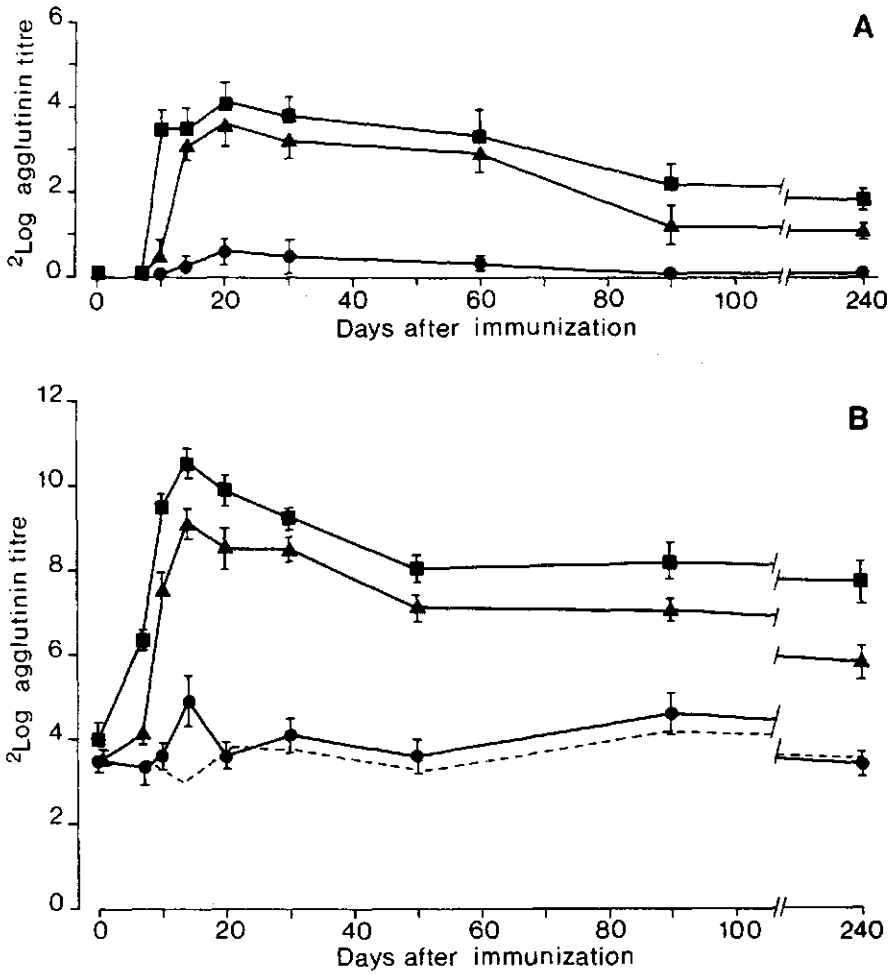

FIGURE 1. Dose dependency and kinetics of the primary agglutinating antibody response against formalin killed *A. hydrophila* cells (Figure 1A) and heat inactivated and disrupted *A. hydrophila* cells (Figure 1B). The animals were (i.m.) injected with 10^5 (●), 10^7 (▲) or 10^9 (■) bacterial cells. Agglutinin titres are expressed as ^{2}log of the highest serum dilution still giving clear agglutination. Each point is the mean ± 1 SE (n = 10). ----- = agglutinin activity in non-immune control sera.

agglutination test coated SRBC were used at a final concentration of 10^8 cells/ml, stabelized with 0.5% non-immune carp serum. The serum agglutinin titres are expressed as ^{2}log of the highest serum dilution, still giving a clear agglutination.

Serum fractionation

Pooled immune (5ml) and non-immune (15ml) sera were used. Lipoprotein was removed and the non-immune serum was 3 times concentrated in an Amicon concentrator (Ym-10). Subsequently all sera were dialyzed against tris-buffer pH 8.0 (150mM NaCl, 50mM Tris, 0.01% NaN_3). Samples were fractionated at 4 °C on a Sephadex G200 gel. All fractions containing protein (absorbance at 280nm) were tested for the presence of immunoglobulin (Ig) and for agglutination with F-*Ah* and H-*Ah*.

ELISA for detection of Ig

Monoclonal Ab directed to carp Ig was used to perform a "sandwich" ELISA. Mouse anti carp Ig (MAC/Ig) was produced and characterized at our laboratory by Dr. E. Egberts as WCI 16 (Egberts et al., 1982; Secombes et al., 1983). Flat bottom microtitre plates (96 wells, Linbro) were coated with 5 µg/well MAC/Ig in 0.1M carbonate buffer (pH 9.6) for 4 h at 37 °C. After 3 washings with tapwater the wells were incubated with PBS + 1% bovine serum albumin (BSA) and 0.05% Tween 80 for 1h at room temperature. Subsequently test samples (diluted 1:2 in PBS + 0.2% Tween) were incubated overnight at 4 °C. After 3 washings (tapwater) the wells were incubated for 3h at 37 °C with MAC/Ig conjugated to Horse-radish peroxidase (MAC/Ig-PO). The MAC/Ig-PO was diluted 1:1000 in PBS containing 0.5 M NaCl, 0.05% Tween and 0.5% BSA. After 3 more washings (tapwater) the plates were incubated with substrate (0.1% 5-aminosalicylic acid with 0.005% H_2O_2 adjusted to pH 6.0 with 1N NaOH). After 60 min the absorbance at 450nm was read with a titretek Multiskan apparatus (Flow Laboratories).

Statistics

For the analysis of the agglutinating properties of the anti F-*Ah* antisera, regression analysis was performed. The test for linearity was performed by analysis of variance (Piazza, 1979). The linear regression line: $y = a + bx$ is determined by the reduced major axis method (Millar & Kahn, 1962); a is the y intercept and b is the slope or regression coefficient. In addition the standard error (SE) of the slope b, and the correlation coefficient r were determined.

RESULTS

Agglutinin response to A. hydrophila

Following injection of various doses of the two *A. hydrophila* bacterins the serum agglutinin responses were monitored up to 8 months (Fig. 1). Sera of non-immune carp showed "background" agglu-

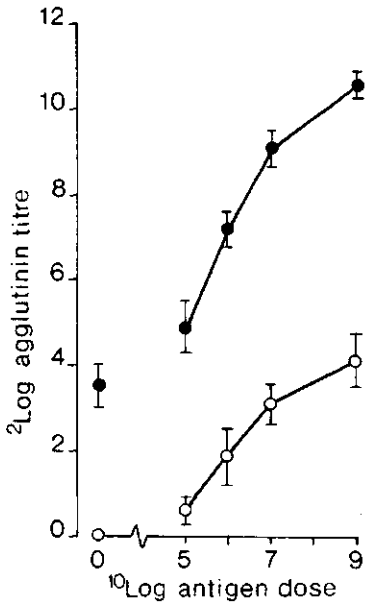

FIGURE 2.
Dose dependency of the primary anti-*A. hydrophila* serum agglutinin response. Animals were i.m. injected with 10^5, 10^6, 10^7, or 10^9 F-Ah (O), H-Ah (●). Serum agglutinin titres at the peak day of the response are shown. For further ex-planation see Figure 1.

tination with H-*Ah* (Fig. 1B; mean titre 3.5 ± 0.43), whereas they did not with F-*Ah* (Fig. 1A). After injection of bacteria a lag period of 7 days was registered with F-*Ah*, but H-*Ah* induced an increase of the agglutinin level within the first week. Maximum titres were observed at day 14 and 20 for H-*Ah* and F-*Ah* respectively. Animals injected with 10^5 F-*Ah* or H-*Ah* responded poorly, and significant increases in agglutinin titres were only seen at the peak day. Injection of 10^6, 10^7 or 10^9 bacteria resulted in clear agglutinin titres. With increasing antigen dose a marked increase in agglutinin titre was observed, i.e. the peak responses are 0.6 ± 0.3; 1.85 ± 0.67; 3.1 ± 0.45 and 4.1 ± 0.6.4 for 10^5, 10^6, 10^7 and 10^9 F-*Ah* respectively and 4.9 ± 0.6; 7.2 ± 0.35; 9.1 ± 0.35 and 10.6 ± 0.25 for 10^5, 10^6, 10^7 and 10^9 H-*Ah* respectively (Fig. 2). Agglutinin titres decreased slowly untill the end of the experiment at day 240. Even at that time clear agglutinin titres were still present in fish injected with 10^7 or 10^9 bacteria.

A number of immune carp sera, with various agglutination titres, and resulting from immunization with F-*Ah* (n = 72) or H-*Ah* (n = 10), and non-immune control sera (n = 15) were tested for their

TABLE 1. Agglutination properties of carp sera against *Aeromonas hydrophila*

Serum	Antigen used in test		
	F-Ah	H-Ah	LPS
anti F-Ah	+++[a)]	++++	+++
anti H-Ah	±	++++	-
non-immune control	-	++	-

a) + = agglutination; - = no agglutination
Abbraviations used in the Table: F-Ah = formalin killed *A. hydrophila*; H-Ah = heat killed and disrupted *A. hydrophila*; LPS = lipopolysaccharide extracted from *A. hydrophila*; anti F-Ah = antiserum against F-Ah; anti H-Ah = antiserum aganist H-Ah.

agglutinating properties with F-*Ah*, H-*Ah* and SRBC coated with *A. hydrophila* LPS. Non-immune control sera were not able to agglutinate F-*Ah* or coated SRBC, however, with H-*Ah* distinct agglutination occurred (Fig. 1B, Table 1). The sera obtained after immunization with H-*Ah* were only reacting with H-*Ah*, but not with F-*Ah* or coated SRBC. The antiserum raised against F-*Ah* agglutinated F-*Ah*, H-*Ah* and coated SRBC; however, the titre of this antiserum against the three bacterial preparations was not identical (Table 1). Agglutinin titres of individual anti F-*Ah* antisera as occurred with F-*Ah*, H-*Ah* or coated SRBC were mutually correlated. Analysis of variance demonstrated a linear relation for all combinations. Regression analysis was performed to characterize the relation between the H-*Ah*, F-*Ah* and LPS titres (Fig. 3, Table 2; relation H-*Ah*/LPS titres is not shown in Fig. 3).

TABLE 2. Linear regression on F-Ah, H-Ah and LPS agglutinin titres, obtained with anti F-Ah antisera.

Correlated agglutinin activity	Number of sera	Regression line	SE of slope	Correlation coefficient
H-Ah/F-Ah	85	H = 3.38 + 0.62F	0.05	0.75
LPS/F-Ah	42	L = -0.71 + 0.78F	0.07	0.79
H-Ah/LPS	42	H = 4.12 + 0.87L	0.09	0.76

H, F, L : agglutinin titres obtained with H-Ah, F-Ah and LPS respectively

The nature of serum agglutinin

The question raised whether the agglutinating activity of F-*Ah* antisera and non-immune sera could be attributed to Ig only, or that the sera also contained other agglutinating factors (of non-Ig nature). Therefore, Sephadex G200 chromatography of both pooled F-*Ah*

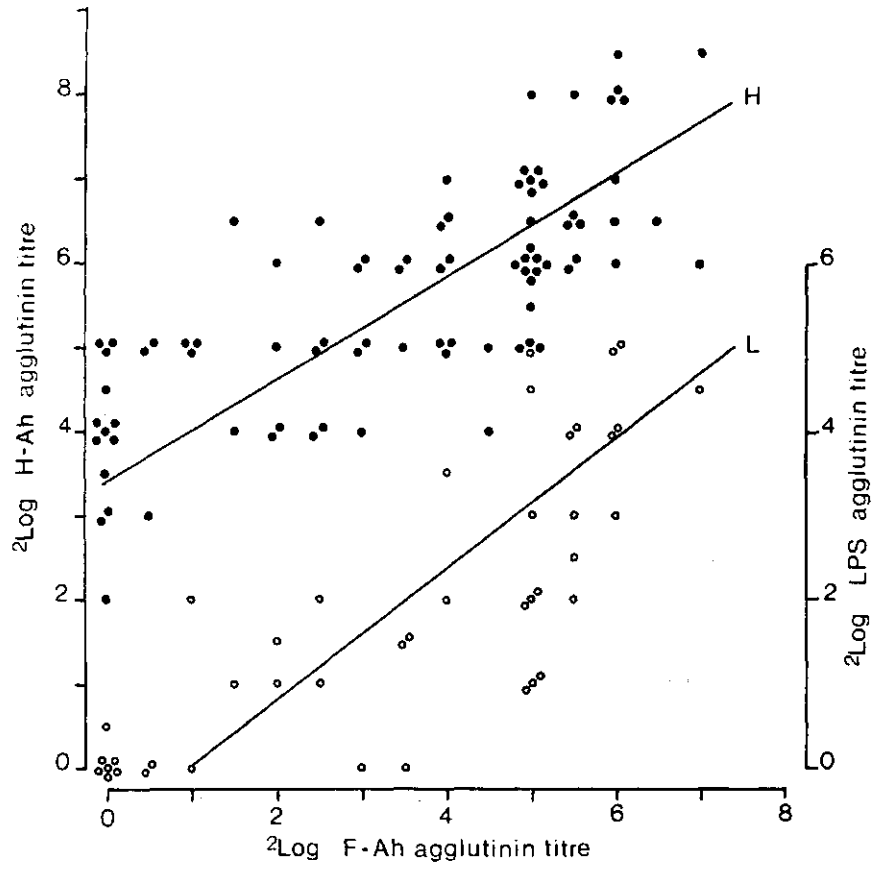

FIGURE 3. Agglutinating properties of antisera raised against formalin killed *A. hydrophila* (F-Ah) and tested with F-Ah, heat killed and disrupted *A. hydrophila* (H-Ah) and SRBC coated with *A. hydrophila* lipopolysaccharide (LPS). Agglutinin titres are expressed as ^{2}log of the highest serum dilution still giving clear agglutination. The relation of the anti H-Ah/anti F-Ah (●) and the anti LPS/anti F-Ah (O) ^{2}log titres of individual sera are shown. The relation of anti H-Ah/anti F-Ah titres is given by the regression line H: H = 3.38 + 0.62F (r = 0.73). The relation of anti LPS/anti F-Ah titres is given by the regression line L: L = -0.71 + 0.78F (r = 0.79).

antisera and pooled non-immune control sera were performed, and fractions were tested for agglutinating activity and the presence of Ig (Fig. 4). Non-immune control serum was concentrated three times, in order to amplify the agglutinating activity in the fractions. The relative high protein loading of the column might be responsible for the different separation patterns of non-immune serum (3 protein peaks) and the F-*Ah* antiserum (4 peaks).

Using an ELISA it was established that Ig was mainly associated with the first, high molecular weight (HMW) protein peak (Fig. 4A,B; fraction 33-40, immune serum; fraction 38-48, non-immune serum), but traces of Ig were detected up to fraction 49 and 66 in the immune and non-immune serum respectively.

The ability to agglutinate F-*Ah* or H-*Ah* was determined for all fractions containing protein. Two distinct peaks of agglutinating activity were present in immune serum (Fig. 4C); one peak eluted at fractions 33-40, and this peak also contained Ig. However, the second peak eluted in the later, low molecular weight protein fractions (fraction 57-64), in which no Ig was present. Fractions from non-immune serum showed very weak agglutination in the first peak (fraction 38-48; Fig. 4D). It has to be mentioned that concentrating the sera will have resulted in the high Ig content of these fraction. Distinct agglutinating activity was present in the later fractions (fraction 55-75).

For both immune and non-immune sera the agglutinating activity in the first HMW protein peak was directed towards both F-*Ah* and H-*Ah*, whereas the later fractions, which did not contain Ig, only agglutinated H-*Ah*. This agglutinating substance was not increased by vaccination with F-*Ah* antigen.

DISCUSSION

Antigen dose and the immune response

The magnitude of the serum agglutinin response to *A. hydrophila* bacterin is dependent on the amount of injected antigen, in the dose range used in this study (10^5 - 10^9 bacteria/100g body weight). Although several reports provide data on humoral responses against

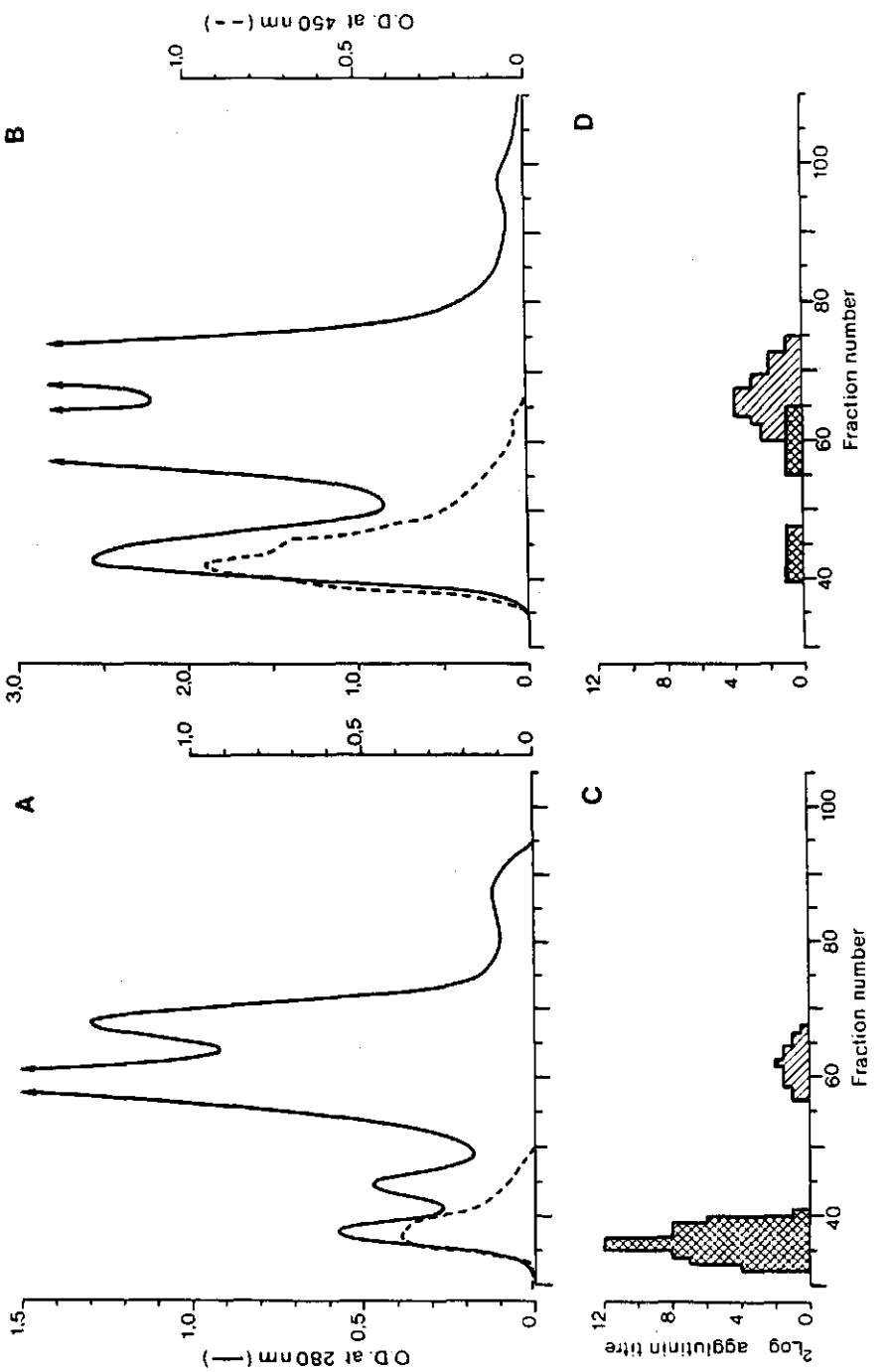

FIGURE 4. (Legend at page 139).

bacteria in fish (Post, 1966; Antipa & Amend, 1977; Thune & Plumb, 1982), up to now studies on the relation of the dose of bacterial Ag and the height of the induced immune response were lacking. In our experiment the minimal Ag dose to evoke a detectable response was about 10^5 F-*Ah* or H-*Ah*. In the dose range 10^5 - 10^6 - 10^7 bacteria, the agglutinin level increased exponentially (linear on log scale). A relative high Ag dose of 10^9 bacteria resulted in high agglutinin levels, although the rate of the increase between 10^7 - 10^9 was less than that observed between 10^5 - 10^7. Therefore it is expected that a further increase in Ag dose will only induce a limited enhancement of the response. Our observations on the dose dependency of the humoral response in carp are in agreement with data obtained with a non-bacterial antigen as SRBC (Rijkers et al., 1980).

The obtained serum agglutinin levels in our experiment decreased slowly. Notably the bacterial Ag was detectable in carp lymphoid organs for a long time (Lamers & De Haas, 1985). This long-term presence of Ag might reflect its low degradability, and might account for a continuous stimulation of cells involved in Ab production.

From our study it is obvious, that if serum Ab levels correlate with protection as e.g. with *Vibrio anguillarum* (Harrell et al., 1975; Viele et al., 1980), and protection is required shortly after immunization, it seems advisable to vaccinate fish with rather high Ag doses. However, when vaccination is supposed to give a long-term protection, the Ag dose should be adapted to the optimal dose for induction of immunological memory. High Ag doses, which are optimal for primary responses, might be suboptimal for induction of immunological memory (Rijkers et al., 1980). However, data on the formation of immunological memory to *A. hydrophila* indicate that development of considerable levels of immunological memory and the production of high serum Ab levels not necessarily need to be conflicting in-

FIGURE 4. Sephadex G200 fractionation of carp serum from fish immunized with formalin killed *A. hydrophila* (Fig. 4A and 4C) and from non-immune control animals (Fig. 4B and 4D). ——— = relative protein level (Absorbance at 280nm); ------ = relative immunoglobulin (Ig) level (Absorbance at 450nm in ELISA). Agglutinating properties of serum fractions are expressed as ^{2}log of the highest serum dilution still giving clear agglutination: ▒▒▒ = agglutination with both F-Ah and H-Ah, ▨ = agglutination with H-Ah only.

terests (Lamers et al., 1985).

Few reports are available on cellular immunity in fish, and more data are required on its role in protection against pathogens, especially in view of its suggested significance after bath immunization (Croy & Amend, 1977). By bath vaccination a low dose of Ag enters the fish (Tatner & Horne, 1983), and in this respect it is notably that optimal cellular immunity is obtained with relative low Ag doses which only induce a minor humoral response (Jayaraman et al., 1979).

Properties of anti A. hydrophila agglutinins

In this paper we showed that H-*Ah* evoked higher agglutinin titres than F-*Ah*. Harrell et al. (1975) reported similar results in a study with heat and formalin killed *V. anguillarum*. These observations might be explained as follows: A) formalin treatment probably induces structural or chemical alterations in the antigen, resulting in a modified antigen processing and subsequent Ab response (Dennert & Tucker, 1972). This is illustrated by the observation that in *T. mossambica* formalin treated SRBC induced cellular immunity, whereas Ab formation was absent. On the other hand, the application of the same dose of fresh SRBC gave inverse results (Jayaraman et al., 1979); B) Heat killing followed by freezing and thawing as done in our experiments may also induce important changes. Preparation methods by which bacterial cells are disrupted, like freezing/thawing, sonication (Schiewe & Hodgins, 1977; Thune & Plumb, 1982), trypsin digestion (Egidius & Andersen, 1979) or autoclaving (Gould et al., 1978), release intracellular factors. These preparations are usually more immunogenic than whole cell bacterins.

It is suggested by our cross-agglutinating experiments (table 1, Fig. 3) that antibodies in anti H-*Ah* antiserum were directed against antigenic determinants which are hidden, or altered in F-*Ah*. These antibodies were not raised against LPS structures, which is illustrated by the absence of agglutination with LPS coated SRBC. Disruption of cells will have exposed (or released) internal constituents such as lipoprotein (Overbeeke et al., 1980), which subsequently might have competed in immunogenicity (Pross & Eidinger, 1974) with cell surface structures as LPS.

Anti F-*Ah* antisera agglutinated F-*Ah*, H-*Ah* and LPS coated SRBC. From the regression analysis on the agglutinating properties of the individual sera it might be concluded, that not all anti F-*Ah* agglutitins recognized H-*Ah* determinants, for the slope is only 0.62. A better correlation was found between titres obtained with LPS coated SRBC and F-*Ah* or H-*Ah* (b is 0.78 and 0.87 respectively). So, a substantial part of the agglutinin in anti F-*Ah* was directed against LPS. And this LPS was detectable on both F-*Ah* and H-*Ah*. Furthermore, the F-*Ah* apparently does contain at least one more immunogenic component in addition to the predominant LPS.

From these results, and especially from the observation that anti H-*Ah* antisera contained a high level of agglutinin that did not react against LPS, we can conclude that it is essential to use the same Ag preparation for immunization and Ab titration. When this is not done, great care is required with the interpretation of the results, e.g. testing anti H-*Ah* antisera with LPS-coated SRBC may give the false impression that an immune response is absent. Further more, vaccine preparation should be directed to the exposure of the essential antigens for raising protection, and to avoid immunological competence with non-essential antigens. Therefore, in first instance, it will be very important to define the essential antigens of fish pathogens.

The nature of serum agglutinins

After Sephadex G200 fractionation of fish sera the "antibody activity" is usually associated with the first, HMW protein peak (Hodgins et al., 1967; Bush, 1978; O'Neill, 1979; Clerx et al., 1980), but also, although to a lesser extent, with the second or third protein peak. This last activity was not expected to be antibody, but a natural occurring substance (Hodgins et al., 1967). With respect to foregoing, we just proved what formerly was expected, by adding the discrimination between "antibody activity" and immunoglobulin. In our experiments, using an ELISA, high levels of Ig could be demonstrated in all fractions of the first protein peak, whereas some Ig was also detected in the second peak. The agglutinating activity of immune serum, to both F-*Ah* and H-*Ah* cells, was restricted to the first peak, whereas some agglutination with H-*Ah* cells was

also seen in the third peak. The agglutinating activity in the third peak was due to a non-Ig natural agglutinin, because Ig was absent in these fractions. In non-immune serum almost all agglutinating activity can be attributed to non-Ig natural agglutinins.

Bacterial agglutinins, haemagglutinins and haemolysins are present in non-immune sera of many fish ranging from elasmobranchs (Sigel et al., 1970) to teleosts (Sindermann and Krantz, 1968; Evelyn, 1971). The "natural" agglutinins have to be devided into "induced" agglutinins of Ig nature, caused by contact with environmental micro-organisms (Hazen et al., 1981), and a LMW non-Ig natural agglutinin. The induced agglutinins of Ig nature might account for the seasonal and temperature dependent fluctuations in serum agglutinin titres as reported by Fujihara & Hungate (1972) and Hazen et al. (1981). The nature of the LMW natural agglutinin in bony fish is not well documented. Most authors agree about the broad specificity of these proteins. The biological function of this natural agglutinin is probably a base line defence against common pathogens in immune and non-immune animals.

ACKNOWLEDGMENTS

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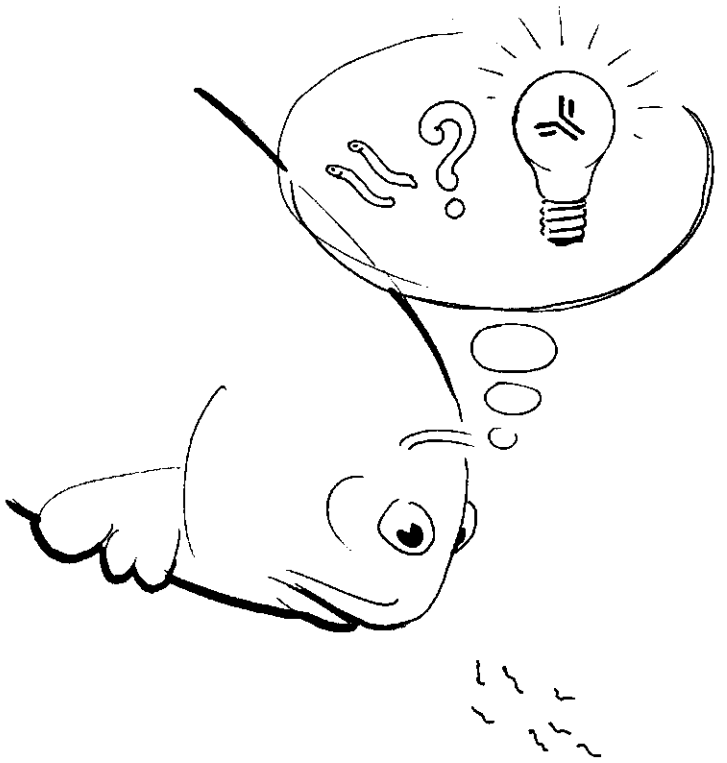

IMMUNOLOGICAL MEMORY

APPENDIX PAPER IV

HUMORAL RESPONSE AND MEMORY FORMATION IN CARP AFTER INJECTION OF *AEROMONAS HYDROPHILA* BACTERIN[a)]

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SUMMARY

The humoral immune response of carp (*Cyprinus carpio*) upon a bacterial fish pathogen *Aeromonas hydrophila* was studied in relation to memory formation. After a single intramuscular (i.m.) injection of formalin killed *A. hydrophila* cells (F-*Ah*), maximum serum antibody (Ab) titres were observed at day 20. Distinct titres were still seen at day 360 in the groups injected with a medium or high antigen (Ag) dose (10^7 respectively 10^9 F-*Ah*). The effect of a second immunization with a high Ag dose was studied in fish primed 1, 3, 8 or 12 months earlier with 10^5, 10^7 or 10^9 F-*Ah*. The height of the secondary Ab response was positively correlated with the height of the priming Ag dose. Challenge with a low Ag dose (10^6 F-*Ah*) gave the best results with 10^7 F-*Ah* primed animals. The highest secondary responses were obtained with combinations of corresponding priming and challenge dosages. It is concluded that fish are able to form immunological memory to this bacterial Ag. However, optimal memory levels are reached after a relative long period (3-8 months).

INTRODUCTION

Immunocompetence combined with the formation of memory is present in vertebrates and some invertebrates (1). In vertebrates immunological memory is a feature of cells involved in cellular and

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a) Developmental and Comparative Immunology 9, 1985 (in press).

humoral immune responses. One type of memory is illustrated by an accelerated rejection of a second set allograft. Several data on this type of cellular immunity are available for bony fish (2,3). The secondary humoral response is characterized by an earlier rise in Ab production and by attainment of higher Ab levels than in the primary response. In tetrapods there is also a shift from high molecular weight immunoglobulin (HMW Ig) to low molecular weight (LMW) Ig (e.g. IgM → IgG in mammals; IgM → IgG-like in birds, reptiles and anurans). In bony fish the secondary Ab titre is not always very impressive and no switch in Ig class occurs. Most fish species possess only HWM Ig, a tetramer IgM-like molecule (4,5).
In some cases immunological memory in fish is absent or very weak (6-8). In other cases clear secondary responses have been registered to a variety of antigens, including erythrocytes (9,10), bacteria and bacterial extracts (11,12), virus particles (13), proteins (14, 15), lipopolysaccharides (16) and haptens (17,18).

Secondary responses in fish do not always show both anamnestic characters (earlier rise and higher levels). This phenomenon was observed when the effect of temperature upon the response was studied. Rijkers et al. (10) showed in carp clear anamnestic plaque forming cell (PFC) responses to sheep red blood cells (SRBC) at 20-24°C, but at 18°C no enhanced PFC numbers were seen. However, at lower temperatures PFC's were observed during a longer period than in the primary response.

The expression of memory to haptens is dependent upon the presence of carrier induced helper function (17,18). The existence of the carrier effect in fish indicates the presence of a long term memory function, maintained by helper cells and sensitized precursor cells for Ab production.

Most studies on immunological memory in fish are relative short-term experiments (1-2 months). Long term memory (8-10 months) was demonstrated only for bovine serum albumin (BSA) (19) and SRBC (9). It is important to gather more information on the development and continuation of immunological memory to bacterial antigens, as this is needed for succesful protection against pathogens. In this study the effect of Ag dosage on the induction of immunological memory and the evocation of the secondary response was investigated.

MATERIALS AND METHODS

Animals

Common carp (*Cyprinus carpio* L.) were bred in our own laboratory. At the start of the experiment the animals were about 8 months old and weighing 50-150 g. They were kept at 21 ± 1°C in aquaria with running tap water or with water recirculating along biological filters and U.V. sterilizers. Water quality was monitored weekly. The observed values were: pH=7.5 ± 0.3; NH_4^+ < 0.02 mg/ml; NO_2^- < 0.05 mg/ml; O_2 > 7-9 mg/ml. The fish were fed daily with pelleted dry food (K30, Trouw & Co., Putten, The Netherlands).

Antigen

Bacterin of *Aeromonas hydrophila* (*Ah*) was used as antigen. *Ah* is the causative agent of a complex of diseases in a variety of fish species, called bacterial hemorrhagic septicemia. Bacterin of *A. hydrophila* (isolate V81/149) was kindly donated by Dr. R. Bootsma (Dept. of Pathology, Faculty of Veterinary Medicine, University of Utrecht, The Netherlands). In short: *Ah* was grown in a liquid culture (tryptose phosphate broth, Difco) for 24 h at 25°C, resulting in cell yield of approximately 10^9 cells/ml. The liquid culture was killed by formalin (0.3% final concentration) and kept sterile at 4°C. Before use the formalin killed *Ah* cells (F-*Ah*) were centrifuged 10 min at 10,000 x g and washed three times in phosphate buffered saline (PBS).

Immunization procedure and antibody titration

The primary response was started by an i.m. injection in the dorsal region with 10^5, 10^7 or 10^9 F-*Ah*. At regular intervals up to day 360 the fish were bled by caudal vein puncture and sera were stored at -20°C. The secondary response was started at 1, 3, 8 or 12 months after priming with 10^5, 10^7 or 10^9 F-*Ah*. Most fish received a high dose challenge by i.m. injection of 10^9 F-*Ah*/100 g body weight. Moreover, in one experiment a low dose challenge of 10^6 F-*Ah*/100 g body weight was given.

Ab titres were determined with a standard microtechnique for bacterial agglutination (2.5 x 10^8 F-*Ah*/ml final concentration). Results were expressed in ^{2}log of the highest dilution still showing a clear agglutination.

Memory factor and statistics

The memory factor (MF), as proposed by Nossal, Austin & Ada (20), was used to quantify immunological memory. The MF is the net secondary titre, divided by the primary titre at the corresponding moment during the primary response. In both cases the same antigen dose and same route of application was used. MFs were calculated for peak values (d14-d20) and for day 60, using the reciprocal value of the highest serum dilution still showing clear agglutination.

$$MF = \frac{S(x) - S(O)}{P(x)}$$

S(x) = titre of fish sampled at day x after secondary injection. S(O) = the residual titre, resulting from priming, present at the day of secondary injection. P(x) = titre of fish of the same age, sampled at day x after primary injection.

The Student's t-test was used to compare geometric means of Ab titres. The p-value obtained, is an estimate of the probability that the observed differences in means is due to chance alone. If $p < 0.05$ the difference was considered significant.

RESULTS

Primary Ab response to F-Ah

The Ab levels of carp after injection with different dosages of F-*Ah* bacterin are shown in Fig. 1. The kinetics of the primary Ab response were studied over a period up to 12 months (details will be published elsewhere). Non-immune carp showed no background Ab titre to F-*Ah*. Animals injected with 10^5 F-*Ah* responded poorly. Hardly significant increases in Ab titre were seen only on the peak day. The peak response for all groups was observed on day 20. With increasing bacterial cell dose a marked increase in Ab titre was observed i.e. peak responses 0.6 ± 0.3; 3.1 ± 0.25 and 4.9 ± 0,64 for 10^5, 10^7 and 10^9 F-*Ah* respectively. Ab titres were very persistent and remained at relative high levels for a long period. All doses, 10^5 excepted, still showed significant enhanced Ab levels after 60 (data not shown here) or even 360 days (Fig. 1). Thus the height of the Ab response after the first injection of F-*Ah* was correlated with the Ag dose.

Kinetics of the secondary Ab response to F-Ah.

In an other experiment the effect of a second immunization with 10^9 F-*Ah* was studied in fish primed 1, 3, 8 and 12 months ear-

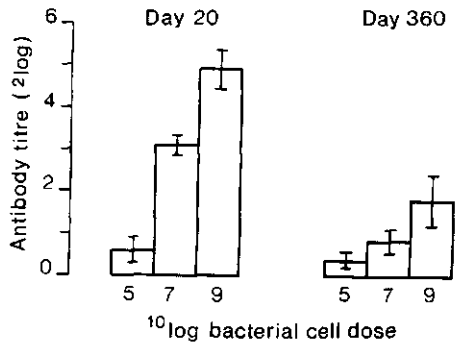

FIGURE 1.
Agglutinating antibodies in carp serum at day 20 (peak day) and day 360 after a single i.m. injection of 10^5, 10^7 or 10^9 formalin killed *A. hydrophila* cells. Ab levels are given as ^{2}log dilution. Each column represents the geometric mean ± 1 SE (n=10).

lier with 10^5, 10^7 or 10^9 F-*Ah*. The fish primed with 10^7 or 10^9 F-*Ah* still have an Ab titre at the time of the challenge, varying between 3.4-0.8 and 3.7-1.8 respectively. Ab responses following the second injection were compared with the corresponding primary response. The kinetics of the secondary responses are shown in Fig. 2A-2D.

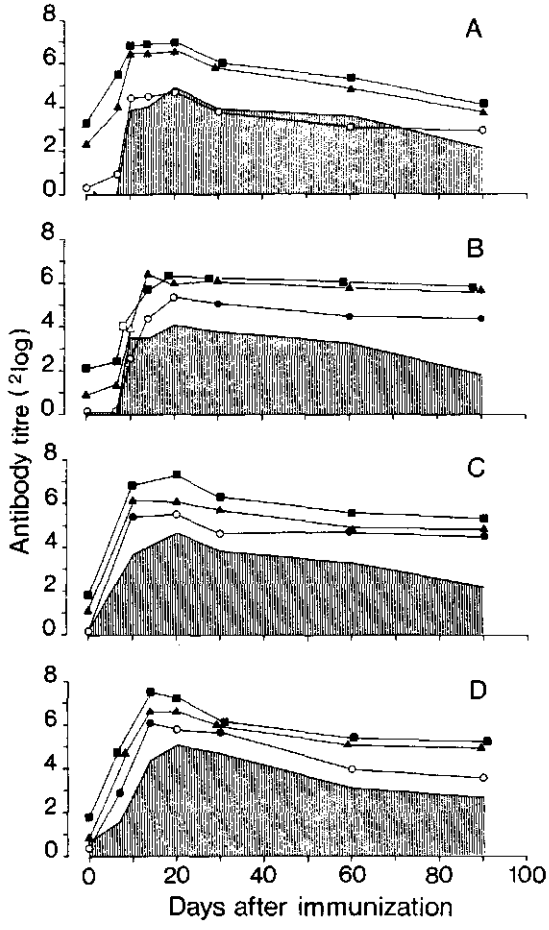

FIGURE 2.
Kinetics of primary and secondary responses in carp. Animals were primed by i.m. injection with 10^5 (○,●), 10^7 (△,▲) or 10^9 (□,■) formalin killed *A. hydrophila* cells (F-Ah). At 1, 3, 8, 12 months after priming (Fig. 2A, 2B, 2C and 2D respectively) 10 animals out of each group were challenged with 10^9 F-Ah (i.m.). Serum agglutinating Ab levels were monitored at regular intervals up to day 90. Each point represents the geometric mean (n=5-10). Closed symbols were used when the secondary response differed significantly from the primary response (Student's t-test $p < 0.05$). The shaded area represents the corresponding primary response after a single i.m. injection with 10^9 F-Ah.

Challenge at 1 month. It can be seen from Fig. 2A that a second injection given 1 month after priming with 10^5 F-*Ah* evoked an Ab response equal to the primary response. An earlier increase was seen at day 7 of the secondary response in the 10^7 or 10^9 primed groups. In these groups maximum Ab titres were reached in the period between day 10 and day 20. During the rest of the response sig-

nificantly enhanced Ab levels were observed, decreasing at the same rate as the control.

Challenge at 3 months (Fig. 2B). No earlier increase in Ab titres was seen when compared to the control. Maximum Ab titres were reached at day 14-20. Amazingly enough the high Ab levels were maintained up to the last day tested (day 90). Even the 10^5 primed group showed significant enhancement from day 30 onwards.

Challenge at 8 and 12 months (Fig. 2C+2D). The maximum Ab titres were reached between day 10 and day 20. Differences with the controls were significant for all days during the response of the 10^7 and 10^9 primed groups. At some days tested the low primed group showed significant secondary response too. It is interesting to see that the secondary responses in Fig. 2C and Fig. 2D show a gradual decrease after the peak day, which is different from the picture observed in Fig. 2B.

From Fig. 2A-2D we see that the peak day for primary responses is always at day 20. About half of the secondary responses showed also a peak on day 20, but the other half peaked already on day 10 or day 14. In agreement with this is the observation that some secondary responses showed an earlier rise in Ab production, compared with the primary responses (see Fig. 2A and Fig. 2D).

Quantification of memory

For a better comparability of the results MFs (see Materials and Methods) were calculated for Ab levels at peak days and at day 60 (Fig. 3). In our experiments MFs exceeding the value 2 appeared to be significant. Memory could be demonstrated during the whole experiment, using the Ab peak values from groups primed with 10^7 and 10^9 F-*Ah* (Fig. 3A). Priming with 10^9 bacterial cells gave consistently higher memory levels than priming with 10^7, except at 3 months. Priming with 10^5 cells induced only a weak memory (significant at 3 month for the peak day and day 60 and at 8 months for day 60). At 1 month after priming memory was still developing in all groups. Maximum MFs for Ab peak levels were reached after 3 months for priming with 10^5 (MF = 2.4) and 10^7 (MF = 4.8) and at 8 months for priming with 10^9 F-*Ah* (MF = 6.1). MFs for Ab levels at

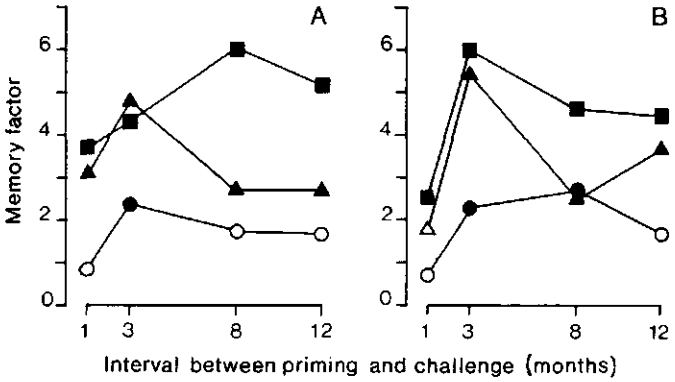

FIGURE 3. Development and quantification of immunological memory in carp. Memory factors (MFs) were calculated for peak days (Fig. 3A) and day 60 (Fig. 3B) after a second i.m. injection with 10^9 formalin killed *A. hydrophila* cells (F-Ah). The fish were primed with 10^5 (○,●), 10^7 (△,▲) or 10^9 (□,■) F-Ah and challenged 1, 3, 8, or 12 months later. Significant memory factors are represented by closed symbols.

day 60 after challenge peaked at 3 or 8 months and were 2.6, 5.5 and 6.0 for priming with 10^5, 10^7 and 10^9 F-*Ah* respectively (Fig. 3B). At 12 months a clear memory was still present in medium and high dose primed animals, but absent in the 10^5 animals.

Comparison of low and high dose challenge

Only at one moment after priming (8 months) a low dose challenge was given. Fish primed with 10^5, 10^7 or 10^9 F-*Ah* were boosted by i.m. injection of 10^6 F-*Ah*. A non-primed control group was also given 10^6 F-*Ah*. In the primed groups maximum Ab levels were reached at day 20 (10^5, 10^7) and day 30 (10^9) after challenge versus day 20 for the control group. After 10^5 priming the Ab titres were not significantly higher than in the non-primed group. In the medium and high primed animals Ab titres were consistently higher than in the control group (data not shown here).

Comparison of the level of immunological memory (MFs) evoked by high or low dose challenge is shown in Tabel 1. It is obvious that memory varies with the priming dose, but also with the challenge dose. It is interesting to see that as a result of high dose challenge the best memory (highest MF) was shown after priming with

TABLE 1. Memory factors after high and low dose challenge with formalin killed *A. hydrophila* cells in carp.

Priming dose	Challenge dose	
	low 10^6	high 10^9
10^5	1.11[a)]	1.73
10^7	5.30	2.73
10^9	3.63	6.05

a) Values for peak days of the secondary response started 8 months after priming

10^9 F-*Ah*, whereas the best results after challenge with a low dose were obtained with 10^7 primed animals.

DISCUSSION

Injection route and dose dependency of the response

In this study the i.m. injection route was used to immunize fish. Ingram & Alexander (6) and Harris (21) have shown that i.m. injection is more suitable for induction of primary responses than the intraperitoneal (i.p.) route. Moreover, it was demonstrated by Rijkers et al. (9) that the priming route is important for the magnitude of the secondary response. They showed that i.v. priming with SRBC in carp resulted in moderate secondary PFC responses. Higher levels in the secondary response were registered following i.m. priming.

In our experiments the magnitude of the primary response to F-*Ah* was dose dependent (Fig. 1). In other studies an increase of the Ag dose resulted in higher Ab titres and PFC numbers in carp and brown trout after application of particulate or soluble Ag (9,14,22). In contrast to these data no dose dependence was seen in rainbow trout after injection of FH_5 bacteriophage (23) or O-antigen of *Yersinia ruckeri* (24). In carp i.p. injection of BSA in Freund's complete adjuvant (FCA) was tolerogenic at low and high doses (0.04-0.2 mg/kg respectively 4-100 mg/kg). However, medium doses evoked Ab levels which were almost identical in the range

from 0.2-4 mg/kg (25). Similar results in carp were obtained by Secombes (26) using human gamma globulin (HGG) in FCA.

Persistence of antibody

Anti F-*Ah* Ab's are very persistent and were still demonstrated 1 year after injection. The lack of Ab titres in the control fish, suggests that a continuous antigenic stimulation by *A. hydrophila* bacteria in the laboratory tanks is very unlikely. It is more obvious that this phenomenon is due to a relative slow turn-over of Ig or a long-term persistence of antigen in the lymphoid organs of the fish.

Only few data are present on the half-life of fish Ig. The half-life of lemon shark Ig was determined at 4-5 days (27), corresponding with the half-life of 7S Ig in mammals (e.g. 5 days for IgG2a in the rat, (28)). From passive transfer experiments in rainbow trout (29) a biological half-life of approximately 7 days can be calculated. When this is valid for carp, it is suggested that enhanced levels of antibody are due to a continuous production of Ab, implying a long-term retention of Ag, in the form of Ag-Ab complexes (30). This is supported by Ag presence in the lymphoid organs for at least 12 months after injection of carp with 10^9 F-*Ah* cells (31). It was not fully proved that the retained Ag was present as immune-complexes. However, the phenomenon of Ag-trapping was observed in carp (32) supporting the idea that retained Ag might be in the form of immune-complexes (33).

In mice T-independent Ag (*E. coli* LPS) persists longer than T-dependent Ag (SRBC)(34). Moreover, in Ab responses to a T-independent antigen (*Salmonella adelaide* O-Ag) in chickens, for several weeks no switch from 19S to 7S Ig took place and consequently no feed back inhibition was initiated during that period (35). As carp has only one class of Ig (14-15S) no switch to 7S Ig occurs (36). However, production of Ig with higher affinity was observed in carp (37) which might have similar implications for regulation as 7S Ig in mammals and birds. It is not clear whether *Ah* bacterin is a T-dependent or T-independent antigen. In our opinion this bacterium contains both T-dependent and T-independent components, which are degraded at a different rate. In conclusion, Ab persistence might

be explained by continuous stimulation, caused by long-term Ag retention and T-independent nature of certain components of the Ag.

Secondary responses and quantification of memory

In our experiments the level of the secondary Ab response was positively correlated with the priming dose. The secondary responses usually reached a higher peak at an earlier day than after the first injection. The MF proved to be a useful tool to quantify memory and to compare secondary responses in different experiments. In the groups primed with 10^7 or 10^9 F-*Ah* cells MFs were between 2.7 and 6.1 from 1 to 12 months after priming. Injection with a low dose (10^5 F-*Ah*) was almost not immunogenic in the primary response and induced a very weak memory (maximum MF 2.4). Based on the calculated MFs some thoughts about the kinetics of the memory formation were developed. It is clear that an increase in memory was seen from 1 to 3 months after priming. A marked further increase (maximum at 8 months) was seen only for Ab peak levels in the group primed with 10^9 F-*Ah*. MFs for Ab levels at day 60 after challenge clearly demonstrate the maintenance of the anamnestic character of the secondary response. It is concluded that in most groups memory formation took place in the first 3 months after priming. Moreover, memory itself was a long lived phenomenon (>12 months).

Nossal et al. (20) injected rats with various dosages (10 pg-1 mg) *Salmonella* flagellar Ag and found that the secondary responses were evoked only when the second dose of Ag equaled or exceeded the first. Over a wide range of priming doses the amount of memory induced was dose dependent. Furthermore, a sub-immunogenic priming dose could induce memory. Our observations in carp agree with these findings. Our results with the low dose challenge (10^6 F-*Ah*) are more or less in agreement with Nossal et al. (20). In general, the best results were obtained with a succession of corresponding priming and challenge dosages. In other words: a low challenge dose fits better in low primed animals. Nossal et al. (20) explained the phenomenon that no secondary responses could be evoked with a challenge dose lower than the priming dose, by suggesting that a considerable amount of the priming Ag was still present in the lymphoid organs, depriving the low dose challenge of its effect. In this respect it

is interesting to mention that F-*Ah* was still present in spleen and kidney of carp 12 months after high dose priming (31).

Immunological memory has been demonstrated in several fish species and to various antigens. The peak level of the secondary responses usually increased 2-16 times, when compared with the primary response (38). Memory levels as determined by anti F-*Ah* Ab responses in this study are in agreement with these findings and are also confirmed by results from anti *A. hydrophila* PFC responses (Lamers, unpublished results). In short: Challenge with 10^9 bacterial cells at 4 months after priming with 10^7 or 10^9 *Ah* cells resulted in 7-18 times higher PFC numbers than in the primary response. The memory levels described for fish are distinctly lower than for mammals. Nossal et al. (20) found a MF of 100 in mice injected twice with 10 µg *Salmonella* flagellar Ag, whereas the maximum MF in our study was 6.1.

Rijkers et al. (10) observed in carp a temperature dependence of the secondary response. At 24°C the secondary PFC response to 10^9 SRBC was 50 times higher than the primary response, whereas this increase was only 10 times at 20°C and no increase at all was noticed at 18°C or lower. The moderate memory levels seen in some fish studies might be caused by the chosen temperature which was sub-optimal for the experimental animals. It is suggested by Wishkovsky & Avtalion (17) that the maturation of a helper effect in carp is a temperature sensitive event, which takes place at higher temperatures (25°C) but not at relative low temperatures (12°C).

From this study it is concluded that carp develop distinct memory to the bacterial antigen F-*Ah*. The maximum memory level was dependent on the priming dose. The best results were obtained with medium and high priming dosages. It is also important that priming and challenge dosages are corresponding. The development of maximal memory takes some months and considerable secondary responses were still observed after 12 months. Low dose priming induced only a weak memory which was limited in time.

At this moment the significance of secondary responses for disease protection in fish is still debatable. However, the fact that fish develop memory after contact with a killed pathogen, gives us hope for the future.

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APPENDIX PAPER V

THE REACTION OF THE IMMUNE SYSTEM OF FISH TO VACCINATION. DEVELOPMENT OF IMMUNOLOGICAL MEMORY IN CARP (*CYPRINUS CARPIO*) FOLLOWING DIRECT IMMERSION IN *AEROMONAS HYDROPHILA* BACTERIN[a)]

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SUMMARY

The development of immunological memory was investigated in carp following direct immersion in *Aeromonas hydrophila* bacterin. A single bath did not induce an increase in serum antibody (Ab) levels. A second bath at 1, 3 or 8 months resulted in clear secondary Ab responses. The highest Ab levels were reached in the group boosted after 3 months. However, at 12 months a second bath gave no further significant response. When a booster was given at the same intervals by intramuscular (i.m.) injection, instead of immersion, the induced memory was not expressed as a clear secondary response. It is concluded that direct immersion in a bacterial antigen did induce immunological memory. The duration of this slowly developing memory is limited in time (less than 12 months) and was only demonstrated following a second immersion. The last feature suggests the existence of a local or mucosal activity during the immune response after bath vaccination.

INTRODUCTION

During the last decade the interest in protection against fish diseases has grown enormously. Vaccination by injection can be used, but this method is time consuming. On the other hand, oral applica-

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tion of vaccines is not always effective. These problems have resulted in the development of new modes of vaccination for large scale use. At present hyperosmotic infiltration (Croy & Amend, 1977; Antipa, Gould & Amend, 1980) direct immersion (Egidius & Andersen, 1979; Antipa et al., 1980) and spray or shower (Gould, O'Leary, Garrison, Rohovec & Freyer, 1978) are in frequent use as methods for antigen exposure. The best results have been obtained by these methods for the protection of salmonids against vibriosis and yersiniosis. The level and duration of protection were variable, depending on bacterin concentration, animal size and fish species used (Johnson, Flynn & Amend, 1982a,b).

Immersion is effective in raising protection, but little is known about mechanisms behind this type of immunity. With this method low quantities of bacterin are taken up by fish (Tatner & Horne, 1983), predominantly by the gills (Alexander, Bowers, Ingram & Shamshoom, 1982; Smith, 1982). Immersion can result in serum Ab levels (Anderson, Roberson & Dixon, 1979; Lamers & Pilarczyk, 1982; Thune & Plumb, 1982), but it may also give protection without demonstrable serum Ab (Croy & Amend, 1977; Gould et al., 1978). It is interesting to mention that sensitized lymphocytes were demonstrated in peripheral blood of brown trout (*Salmo trutta*) using a migration inhibition test (Smith, McCarthy & Paterson, 1980) and in spleen and thymus of carp, using the leucocyte stimulation assay (Liewes, Van Dam, Vos-Maas & Bootsma, 1982), after immersion vaccination in bacterin of *Aeromonas salmonicida* or *Flexibacter columnaris* respectively. Studies on the development of immunological memory, one of the prerequisites for longterm protective immunity, are only scarce. An attempt has been reported by Anderson & Dixon (1980) and in our laboratory a moderate secondary response was observed after direct immersion of carp in *Yersinia ruckeri* O-antigen (Lamers & Van Muiswinkel, 1984).

To date only limited evidence has been presented for the existence and importance of a local defence system in fish. Fletcher & White (1973) demonstrated specific Ab in mucus of skin and intestine, independent from serum Ab level; and Lobb & Clem (1981a) isolated an extra protein (secretory component ?) from immunoglobulin of mucus and bile.

In this study we used a bacterin of *Aeromonas hydrophila*. This organism is an important aquatic pathogen causing bacterial hemorrhagic septicemia in a variety of fish species eg. carp, goldfish and channel catfish (Newman, 1983). The development and duration of immunological memory was investigated in carp following direct immersion in *A. hydrophila* bacterin. Special attention was also payed to the question as to whether the route of antigen entry during the first and second contact has to be the same, in order to show the presence of immunological memory; in other words, does it make any difference whether bath or injection are used for the booster after priming by bath.

MATERIALS AND METHODS

Animals

Common carp (*Cyprinus carpio* L.), bred in our laboratory, were maintained in aquaria with running tap water at 20 ± 1 °C. They were fed with pelleted dry food (K30, Trouw & Co., Putten, The Netherlands). At the start of the experiment the animals were 7-9 months old, weighing 50-100 g.

Antigen

Bacterin of *Aeromonas hydrophila* (isolate V81/149) was prepared by Dr. R. Bootsma (Dept. of Pathology, Veterinary Faculty, University of Utrecht, The Netherlands). *A. hydrophila* was grown in a liquid culture (tryptose phosphate broth, Difco) for 24 h at 25 °C, resulting in aproximately 10^9 cells/ml. The bacterin was constituted as a whole culture, which was heat inactivated (30 min at 60 °C), disrupted by at least 2 thawing/freezing cycles and stored at -20 °C. Prior to injection or the agglutination test, the cells were washed three times in phosphate buffered saline (PBS pH 7.2), by centrifugation for 10 min at 10.000 x g.

Experimental procedure

Bath vaccination was carried out by direct immersion in bacterin of *A. hydrophila* for 1 h with aeration. The bacterin was 1:10 diluted with aquarium water and had a concentration of aproximately 10^8 bacterial cells/ml. In the first experiment fish were given two immersions with an interval of 1, 3, 8 and 12 months between the first and the second treatment. In a second experiment fish were primed by direct immersion and boosted by intramuscular (i.m.) injection, at an interval of 1 or 3 months with 10^9 *A. hydrophila* cells, and at 8 and 12 months with 10^6 bacterial cells. The injections were given in the dorsal region with 0.1 ml bacterin suspended in PBS. In all experiments non-immune control and primary response groups were included for comparison. Up to 90 days after the last immunization blood samples (0.2 - 0.3 ml) were taken by caudal vein

puncture. Sera were stored at -20 °C and agglutinin titres were measured by bacterial agglutination in microtitre plates. Results are expressed as ^{2}log of the highest serum dilution giving a clear agglutination, as scored with dark ground illumination.

Memory factor and statistics

The memory factor (MF), as proposed by Nossal, Austin & Ada (1965), was used to quantify immunological memory. The MF is the net secondary titre, divided by the primary titre at the corresponding moment during the secondary, or primary response respectively. MFs were calculated for peak values and for day 60,using the reciprocal value of the serum dilution.

$$MF = \frac{S(x) - S(0)}{P(x) - P(0)}$$

S(x) = titre of fish sampled at day x after secondary immunisation. S(0) = the residual titre, resulting from priming, present at the day of secondary immunization. P(x) = titre of fish of the same age, sampled at day x after primary injection. P(0) = natural titre, present at the day of the primary immunization.

The Student's t-test was used to compare geometric means of Ab titres. If $p < 0.05$ the difference is considered significant.

RESULTS

Primary and secondary response following direct immersion

Sera of non-immune control groups and day 0 sera of experimental groups showed a background agglutination with heat killed *A. hydrophila* cells (mean titre 3.5 ± 0.43), due to the presence of a non-immunoglobulin bacterial agglutinin (Lamers & Van Muiswinkel, 1985). A single bath did not result in a significant increase of the agglutinin level (Fig. 1). However, a second bath given 1 month later induced an increase of the titre at day 14, and a maximum was reached at day 30 (titre 5.9 ± 0.35). Three months after priming a second bath even induced a higher agglutinin level (maximum titre 8.2 ± 0.45 at day 30). A bath at 8 months resulted in a response comparable with the situation at 1 month after priming (maximum titre 6.5 ± 0.5 at day 30). In all cases enhanced agglutinin levels after a second bath were maintained up to the end of the experiment (day 90). It is interesting that a second bath at 12 months after priming did not result in a response. Primary response groups tested simultaneously with the twice bathed fish never showed an increase

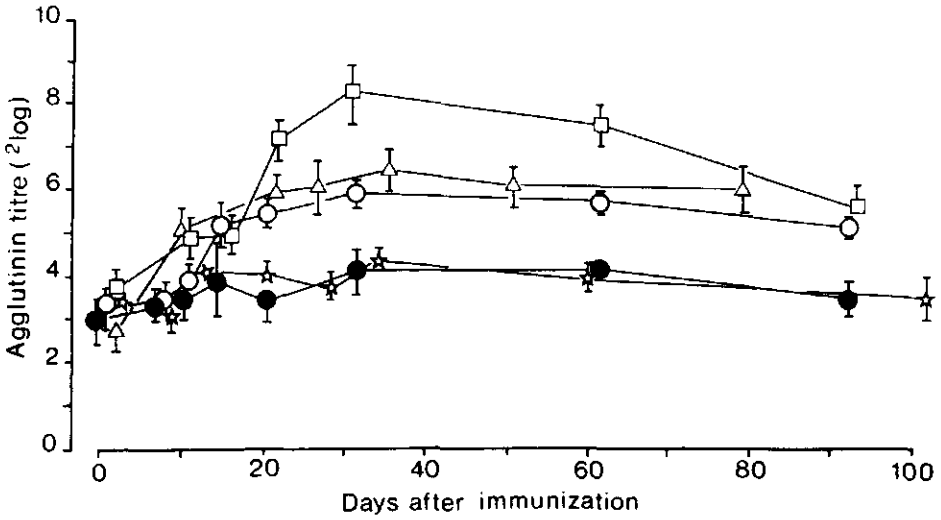

FIGURE 1. Kinetics of the primary (●) and secondary anti-*A. hydrophila* serum agglutinin response. Carp were vaccinated and boosted by direct immersion. The interval between the two treatments was 1 (○), 3 (□), 8 (△) or 12 (☆) months. Each point represents the geometric mean ± 1 S.E. of agglutinin titre (n=5-10).

in titre. Memory factors are calculated for the titres at the peak day (day 30) and at day 60 (Table 1A). Factors exceeding the value 2 were significant. It is clear from these factors that memory has developed at 1 month after priming, but the highest level was not reached before 3 months. After three months a gradual decrease was observed, resulting in the absence of memory at 12 months. During the whole observation period following the booster, essentially the same results were observed (eg. compare MFs calculated for the peak day and for day 60). So the magnitude of the secondary response is depending on the interval between the first and the second bath.

Booster by injection

In this case responses were monitored in fish which were primed by bath and boosted by i.m. injection. The response was compared with a primary response of non-primed fish which were injected at the same time (Fig. 2). At the moment of booster, both primed and non-primed fish showed a background agglutination; the mean titre

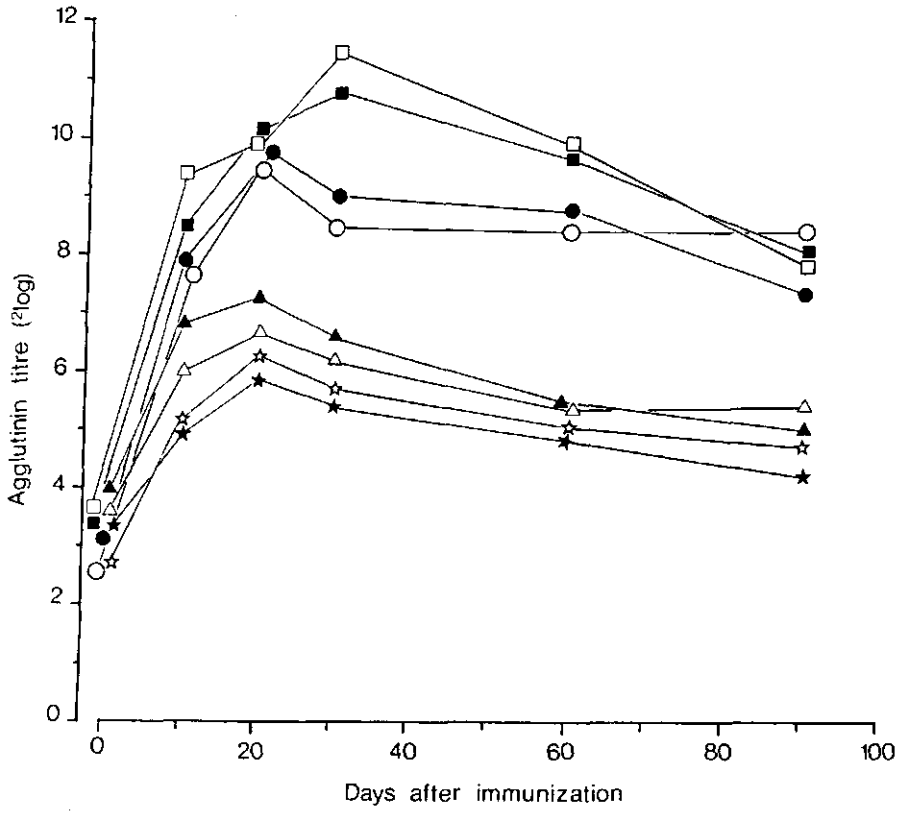

FIGURE 2. Kinetics of the primary and secondary anti-*A. hydrophila* serum agglutinin response. Carp were primed by direct immersion and boosted by i.m. injection. The interval between priming and booster was 1 (● ○), 3 (■ □), 8 (▲ △) or 12 (★ ☆) months. Open symbols are the responses of the boosted groups, whereas closed symbols are the corresponding primary responses, monitored in non-primed fish injected simultaneously. At 1 and 3 months a high dose (10^9 bacterial cells) was given, whereas at 8 and 12 months the fish received a low dose (10^6 bacterial cells). Each symbol represents the geometric mean of the agglutinin titre (n=5-10). The mean standard errors with their standard deviation (S.E. ± S.D.) for the different groups were for the non-primed and primed groups respectively: 1 months 0.34 (0.17) and 0.31 (0.10); 3 months 0.39 (0.05) and 0.98 (0.36; 8 months 0.23 (0.07) and 0.37 (0.08); 12 months 0.33 (0.08) and 0.57 (0.08).

of these groups varied between 2.6 to 4.0. The booster with a high dose (10^9 cells) resulted in a fast increase in serum agglutinin titres, which peaked at day 20 (titre 9.5 ± 0.29) and day 30 (titre 11.5 ± 1.3) at the 1 and 3 months interval respectively. The non-

primed groups injected simultaneously with the primed groups showed comparable responses (1 month, maximum titre 9.8 ± 0.75 at day 20; 3 months, maximum titre 10.8 ± 0.45 at day 30). The booster with a low dose of antigen (10^6 cells), given at 8 or 12 months after priming, resulted in agglutinin responses which peaked at day 20 (8 months, maximum titre 6.7 ± 0.36; 12 months, maximum titre 6.3 ± 0.52). The non-primed control groups gave similar primary results (8 months, maximum titre 7.3 ± 0.19; 12 months, maximum titre 5.9 ± 0.27). No significant differences were present between the responses after booster and the corresponding primary responses. Thus the introduction of antigen along the i.m. route never induced a classic secondary response in bath primed fish. The results of the boosted fish are also expressed as memory factors, calculated from titres at the peak day and at day 60. MFs for all groups were not significantly different and equaled the value 1 (Table 1B).

TABLE 1. Memory factors in carp, primed by direct immersion in *Aeromonas hydrophila* bacterin and calculated for the Ab peak day and day 60 after a booster.

Booster	Interval between priming and booster	Ab peak day	day 60
A. Direct immersion	1 months	4.9*	4.5
	3 months	28.8	16.4
	8 months	7.4	6.4
	12 months	0.7	0.5
B. I.m. injection			
10^9 Ah cells	1 months	0.8	0.7
	3 months	1.7	1.1
10^6 Ah cells	8 months	0.7	0.5
	12 months	1.3	1.0

* Memory factors exceeding the value 2 are significant ($p < 0.05$)

DISCUSSION

Development of immunological memory

In this study we showed that a repeated bath in *A. hydrophila* bacterin resulted in a distinct rise in serum antibodies. To our knowledge this is the first time that clear-cut immunological memo-

ry is shown following immunization by direct immersion. In addition, the magnitude of the memory was shown to be time dependent. Memory was already present at 1 month after priming, but the highest level was reached at 3 months. At 8 months it returned to the level at 1 month and at 12 months the immunological memory was no longer present. Therefore in our experiments memory was restricted in time and lasted for about 8 months. Former studies on the induction of memory following vaccination by immersion have been less succesful. Anderson & Dixon (1980) could not demonstrate an anamnestic response in rainbow trout after flush exposure to *Y. ruckeri* O-antigen 10 weeks after the first exposure, and in carp a second immersion in *Y. ruckeri* O-antigen 7 months after priming only resulted in a slightly higher Ab level (Lamers & Van Muiswinkel, 1984). The nature of the antigen might account for these observations: *Y. ruckeri* O-antigen is probably a T-independent antigen (Anderson & Dixon, 1980).

In our study, memory takes a long time (3 months) to reach the highest levels. This phenomenon might be a common feature in fish, for it has also been described after immunizing carp by i.m. injection with SRBC (Rijkers et al., 1980) or *A. hydrophila* cells (Lamers, De Haas & Van Muiswinkel, 1985). Whereas Rijkers et al. (1980) reported that only a low dose of antigen (10^5 SRBC) induced a clear memory, which was slow to develop (maximum at 6 months), Lamers et al. (1985) demonstrated this phenomenon for different doses of formalin killed *A. hydrophila* cells (10^5,10^7 and 10^9; maximum memory from 3-8 months). In contrary to 10^5 SRBC the low dose of formalin killed *A. hydrophila* induced only a very weak memory. The amount of antigen entering the fish by direct immersion is estimated to be 0.01 - 0.2% of the initial antigen concentration in the vaccine (Tatner & Horne, 1983). In our case this might have represented an uptake of 10^4- 2 x 10^5 bacterial cells per fish. An i.m. injection with a comparable antigen dose of the same bacterin (10^5 *heat* killed and disrupted *A. hydrophila* cells) induced a minimal primary Ab response and a booster injection after 8 months with the same antigen dose gave only slightly enhanced Ab levels, comparable to the secondary response following the repeated bath at 8 months (unpublished results).

Systemic and local immunity

By immersion, antigen enters the fish predominantly by the gills, but a smaller contribution of the skin and the gut also occurs (Smith, 1982; Alexander et al., 1982). The uptake is supposed to be an active process and it results at least in an activation of the "systemic" immune system. Following immersion antigen was detected in the lymphoid organs, spleen, head and trunk kidney (Alexander et al., 1982; Smith, 1982) and, as described above, it is clear that this procedure induces the production of serum Ab. Moreover, protection against vibriosis could be transfered to non-immune fish by both serum and cells from spleen and kidney from immersion vaccinated fish (Viele, Kerstetter & Sullivan, 1980).

The observation that in carp, immunized by bath, memory could only be demonstrated by a second bath and not by injection, argues for the importance of local processes in the induction of the humoral response. The uptake and processing of antigen by cells at the body surface is such a local process. However, up to now, data on this process in fish are scarce. Antigen presentation to the mucosa in mammals may induce homing of activated cells in the mucosa and the subsequent production of secretory immunoglobulin (sIgA; Bienenstock & Befus, 1980). It is tempting to speculate, in analogy to mammals, on homing of immunoreactive cells to the body surface or gills and intestine of fish vaccinated by immersion. In a way these cells might have contributed to the elevated serum titres by stimulating the sensitized "B"-cells in the lymphoid organs, by means of remigrating "T" helper cells from the skin or by long distance mediators (interleukins?). Another possiblity is the shedding of locally produced Ab into the circulation. The latter is unlikely because relative high numbers of Ab producing cells have been detected in spleen and kidney after direct immersion (Anderson et al., 1979; Lamers & Pilarczyk, 1982). On the other hand, clear indications are available for the presence of a local (secretory) immune system in fish. The presence of Ig has been demonstrated in skin mucus (Fletcher & Grant, 1969; Lobb & Clem, 1981a), gut mucus (Harris, 1972; Fletcher & White, 1973) and bile (Lobb & Clem, 1981c). In some studies, specific Ig activity could be detected in intestinal and cutaneous mucus following a parasite infection or vaccination

(Harris, 1972; Hines & Spira, 1974; Goven, Dave & Gratzek, 1980). As a high level of specific Ig activity was also present in the serum, these authors suggested a transfer of Ig from serum to mucus. Others have stated that the secreted Ig was produced locally e.g. passively transfered anti-BSA Ab (Di Conza & Halliday, 1971) or radioiodinated Ig (Lobb & Clem, 1981b) could not be detected in cutaneous mucus or bile; continuous oral administration of a bacterin preparation resulted in a higher level of specific Ab in mucus than in serum (Fletcher & White, 1973); moreover, Lobb & Clem (1981c) have isolated an extra protein from bile Ig, which might be analogous to the secretory component in mammals.

The inability to demonstrate the induced memory by injection, after priming by immersion, might also be explained in another way. Processing of antigen through gills and skin might induce alterations in the physico-chemical nature of the Ag. In mammals this has been shown for Bovine Serum Albumin (Strobel, McMowat, Drummond, Pickering & Ferguson, 1983). In our experiment, heat killed *A. hydrophila* cells, taken up from the water, might be processed in such a way that the following systemic immune reaction takes place to altered determinants. Later on the injected (unaltered) bacterin is not recognized by the memory cells and only a primary response is generated.

Bath vaccination and protection

After bath vaccination of salmonids against vibriosis and yersiniosis the level and duration of the protection was dependent on the bacterin concentration, fish size and fish species. Protection was better and lasted longer when higher bacterin concentrations and larger fish were used (Johnson et al., 1982a,b; Horne, Tatner, McDerment & Agius, 1982). Furthermore this protection lasted at most 3 to 12 months. These data are in agreement with our observations on the temporary character of memory following immersion. The observation that immunological memory induced by direct immersion found no expression by giving a challenge by injection, might have practical implications for challenge experiments to test protection after a bath or spray vaccination. When a bath challenge is used the pathogens meet both the activated local defence lines and

the activated systemic defence system. In case of the ectoparasite *Ichthyophthirius multifiliis* it is clear that mucus Ab, originating from vaccination or previous infection, prevents attachment of the parasite to the skin in a subsequent challenge (Hines & Spira, 1974; Goven et al., 1980). In several studies vaccination by immersion is almost as effective in raising protection as injection (Johnson et al., 1982a,b), whereas in other experiments immersion appeared less effective (Horne et al., 1982; Tebbit & Goodrich, 1983). In the first experiments the challenge was performed by bath, whereas in the latter injection was used, which may account for the observed differences.

From the present study it can be concluded that direct immersion induces immunological memory. This memory is limited in time and, under the conditions used, it could only be demonstrated after boosting by bath and not after boosting by injection. Therefore, it is obvious that the use of bath or environmental challenge should be preferred for monitoring protection after vaccination by immersion or spraying.

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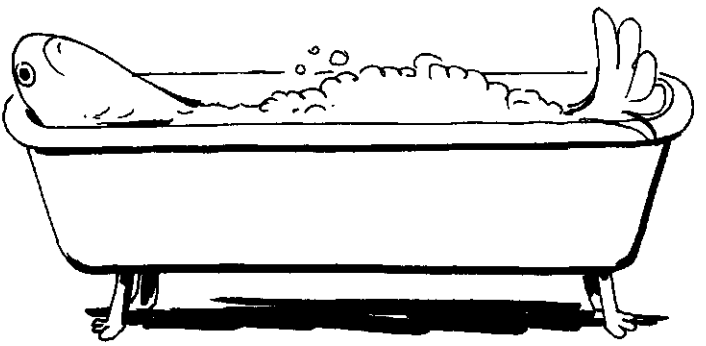

BATH VACCINATION

APPENDIX PAPER VI

ANTIGEN LOCALIZATION IN THE LYMPHOID ORGANS OF CARP (*CYPRINUS CARPIO*)[a)]

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SUMMARY

A brief morphological description is given of the spleen, head kidney and trunk kidney, the three peripheral lymphoid organs of carp. An antigen localization study was carried out using *Aeromonas hydrophila*, a cellular bacterial antigen and using an indirect immunofluorescence test. A study of the lymphoid organs at various times after injection (up to 12 months) showed that at first antigen was present in splenic ellipsoids and in solitary phagocytic cells in the spleen, head and trunk kidney. Later on the antigen was gradually concentrated in or near melano-macrophage centres in all organs studied. Simultaneously it disappeared from the splenic ellipsoids, and the number of solitary *A. hydrophila*-immunoreactive cells decreased too. In the later phase antigen was located extracellularly at the membrane of cells in and around the melano-macrophage centres and it remained so for a full year. No antigen was detected in the lymphoid organs following a bath immunization.

INTRODUCTION

The first phase of the immune response involves antigen (Ag) processing and recognition by lymphoid cells, resulting in cellular proliferation and differentiation into regulatory and effector cells. Both this phase and the subsequent effector phase of the response have been extensively studied in mammals. Injected antigenic

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a) Cell and Tissue Research (accepted for publication; slightly revised).

materials initially localize in the peri-follicular areas of spleen and lymph nodes (Nossal & Ada, 1971, Van Rooijen, 1972). It is associated with the onset of the immune response, which takes place at the periphery of the T-cell areas, by means of macrophages with surface bound Ag, which interact with both T- and B-cells (Langevoort, 1963; Van Ewijk et al., 1977; Unanue, 1972; 1981). Subsequently, Ag may be subjected to a longterm retention, complexed with immunoglobulin (Ig) and the complement factor C3, on the surface of dendritic cells in the follicle centres (Nossal & Ada, 1971; Tew & Mandel, 1979; Radoux et al., 1984). This probably is the only way by which Ag is held in the body for a considerable period of time (Nossal & Ada, 1971; Tew & Mandel, 1979). Its functional significance is related with immunological memory (Klaus et al., 1980) and feedback regulation of the immune response (Tew & Mandel, 1978; Tew et al., 1980).

Lymphoid organs of poikilothermic vertebrates lack follicles and germinal centres, and teleosts in particular have a poorly developed splenic white pulp (Good & Finstad, 1967; Borysenko, 1976; Pitchappan, 1980). It might be expected that this would impair the development of immunological memory, but in all vertebrate classes from teleost fish onwards distinct secondary humoral and cellular responses can be induced (Manning, 1980; Rijkers, 1982; Muthukkaruppan et al., 1982).

Studies on structure and function of fish lymphoid organs have been predominantly concentrated on the spleen. Several studies indicate the importance of special splenic structures, the terminal arterial capillaries or ellipsoids, in clearing a variety of substances from the circulation, such as vital dyes or carbon (Yoffey, 1929; cf. Ellis et al., 1976; Mori, 1980), particulate bacterial Ag (Secombes & Manning, 1980; Lamers & Pilarczyk, 1982; Maas & Bootsma, 1982) and soluble Ag (Ellis, 1980; Secombes & Manning, 1980). The ellipsoids in the spleen of carp closely resemble the sheathed capillaries of Schweigger-Seidel (SS-Sheaths) in the mammalian and avian spleen (Graf & Schlüns, 1979; Blue & Weiss, 1981). Little information is present on their function in mammals, but in the chicken spleen both carbon and antigenic material initially become localized in the periphery of the SS-Sheaths (White et al., 1970; Eikelenboom et al.,1983).

In fish, Ag can finally be localized in pigmented macrophages present in clusters in all lymphoid organs (Ellis, 1980; Lamers & Pilarczyk, 1982).

To date studies on Ag localization in fish are limited, with few data on longterm Ag retention. As this process is thought to play a role in the regulation of the immune response and memory formation, it is important to gather more information on this subject for vaccination purposes, using Ag of fish pathogens. In this study a bacterin of the fish pathogen *Aeromonas hydrophila* was used, and the localization of this bacterial Ag in lymphoid organs of carp was traced in detail, in both the initial and later phases after administration. As the structure of carp lymphoid organs is still poorly documented, a short description of the morphology of these organs is given, with special reference to their major lymphoid elements.

MATERIALS AND METHODS

Animals

In this study common carp (*Cyprinus carpio*) of approximately 8 months old and weighing about 70 g were used; they were reared at 20 ± 1 °C. The fish were kept in aquaria with recirculating, filtered and U.V. sterilized water and fed daily with pelleted dry food (K30, Trouw, Putten, the Netherlands).

Antigen

Aeromonas hydrophila bacterin was kindly provided by Dr. R. Bootsma (Dept. of Pathology, Veterinary Faculty, University of Utrecht, The Netherlands). The bacterin was heat inactivated (30 min at 60 °C) and disrupted by 3 freezing and thawing cycles. Before injection the broken cells were washed three times in phosphate buffered saline (PBS, pH 7.2) by centrifugation (10 min at 10,000 x g).

Experimental design

Ag localization was studied after: A) a single intramuscular (i.m.) injection in the dorsal region with 10^9 *A. hydrophila* cells in 0.1 ml PBS, or B) a single bath treatment in 1:10 diluted bacterin of *A. hydrophila* for 1h (concentration ± 10^8 bacterial cells/ml). At 1, 7, 14, 30, 60, 90, 120 and 360 days after the treatment 2 fish per group and a control fish were killed; spleen, head and trunk kidney were taken out and frozen in liquid nitrogen. Tissue samples were also taken from skin, gill, gut and heart (atrium). In addition some tissue samples were fixed in Bouin's fluid and processed for normal histology. Parraffin sections were stained with Heidenhain's iron haematoxilin and counterstained by light green, with Periodic Acid Schiff (PAS) and with silver according to the method of Gomori (1937) for reticulin fibres.

Immunocytochemistry

From the frozen tissues cryostat sections (7 µm) were cut and tested for the presence of *A. hydrophila* Ag using an indirect immunofluorescence test. Antiserum against *A. hydrophila* was raised in albino New Zealand rabbits. Animals were primed subcutaneously with 10^8 bacterial cells in PBS and boostered 5 weeks later intramuscularly with 10^{10} cells; they were bled 2 weeks after the last injection and sera were stored at -20 °C (bacterial agglutinin titre 1:4096). The antiserum was diluted 1:50 in PBS before use on sections. Fluorescein labelled goat anti rabbit Ig (Nordic; 1:40 diluted) was used as a second step. Control reactions were performed using the pre-immune rabbit serum (diluted 1:20). To prevent fading of the FITC dye sections were mounted in medium according to Johnson & Nogueira Araujo (1981).

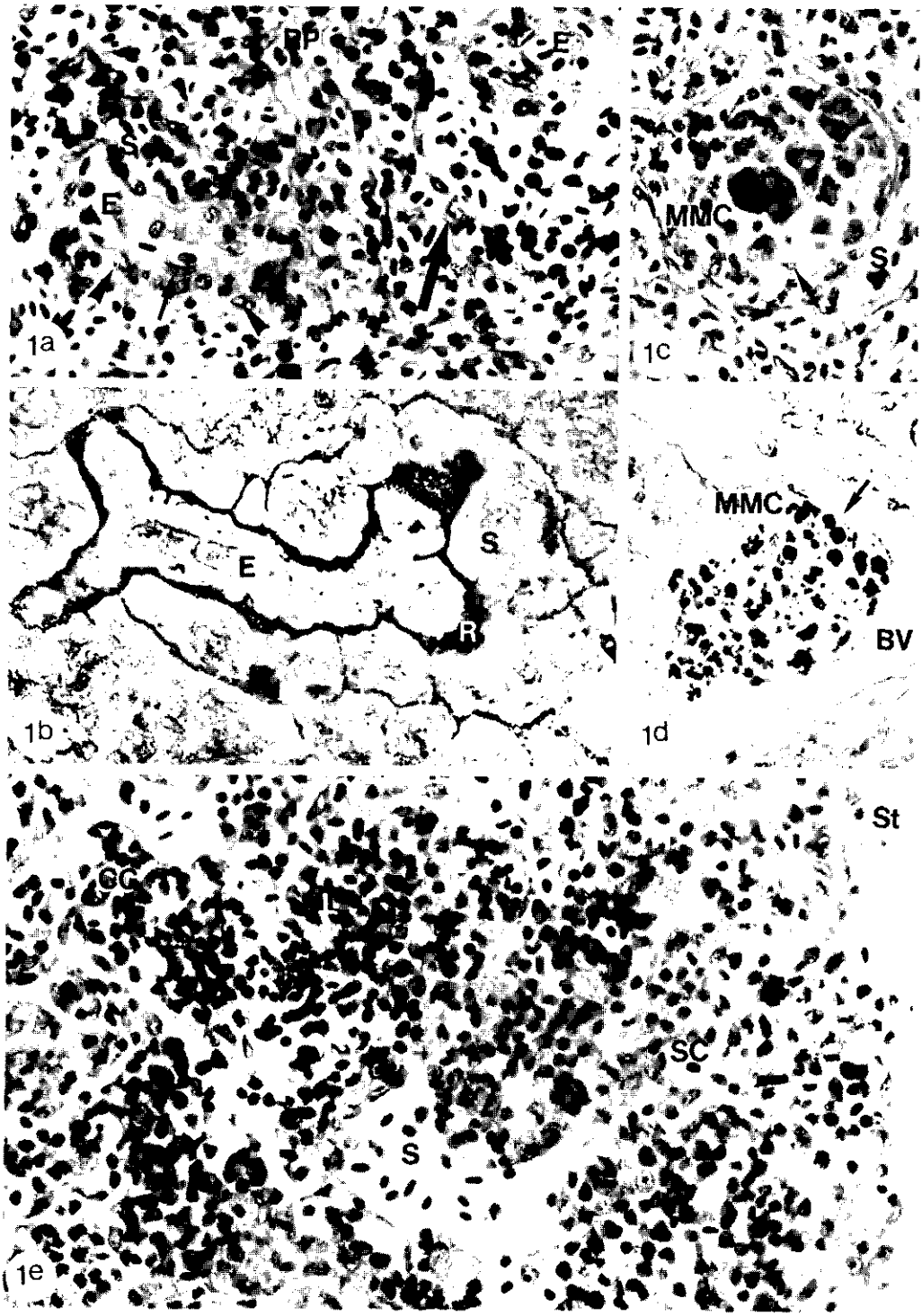
RESULTS

The structure of the peripheral lymphoid organs

The main lymphoid organs of cyprinid fish are the thymus, spleen and kidney. The thymus in fish, as in mammals, is a central lymphoid organ and was therefore not investigated in this study. The structure of the other lymphoid organs will be described briefly in relation to their major lymphoid elements.

The spleen. The spleen is delineated by a very thin layer of connective tissue. Almost the entire splenic tissue consists of red pulp, which is made up of a spongy cellular reticulum, intermingled with haemopoietic tissue and many blood sinuses (Fig. 1a). The white pulp is poorly developed and consists mainly of diffusely distributed lymphoid cells. However, especially after immunization, some con-

FIGURE 1a-e. Carp lymphoid organs. Sections of spleen (a,b,c,d) are stained by Heidenhain's iron haematoxylin and counterstained by light green (a, c) or with silver for reticulum (b,d); a) splenic ellipsoids (E) are small capillaries surrounded by a cuff of macrophages (arrow head) and lymphoid cells; the red pulp (RP) consists of many blood sinuses (S) and a cellular reticular network (thick arrow); small arrow points at an endothelial cell of ellipsoid. x 600; b) ellipsoids are enmeshed in a reticular sheath (R), and are surrounded by a blood sinus (S). x 525; c) melano-macrophage centre (MMC) in the red pulp, surrounded by the cellular reticulum (arrow) and associated with a blood sinus. x 600; d) large MMC at the bifurcation of a large blood vessel (BV), the MMC is surrounded by a thin reticular layer (arrow). x 350.
Section of head kidney (e) is stained with PAS. The parenchyma of head kidney consists of cords of cells(CC), which are separated by small capillaries (SC) and blood sinuses (S), the latter are surrounded by an accumulation of lymphoid cells (L). Larger sinuses often are bordered by a thick layer of steroidogenic cells (ST, lumen of the large sinus is not shown). x 525.


RP
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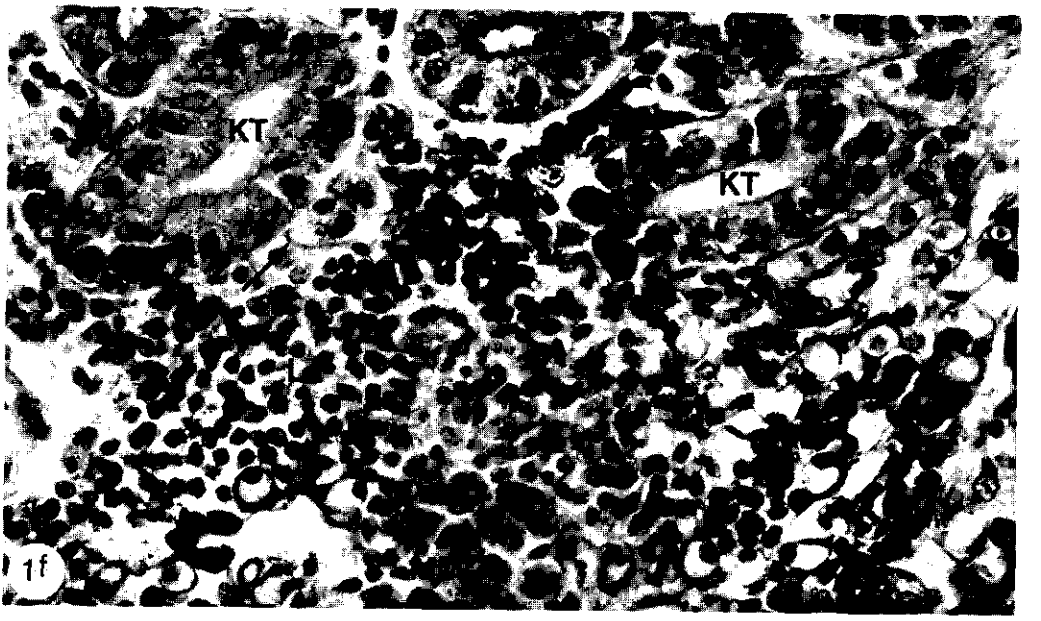


FIGURE 1f. Carp lymphoid organs. Section of trunk kidney stained with PAS. Lymphoid accumulations (L) are present between the kidney tubules (KT), the latter are surrounded by peritubular capillaries (arrow; MMC = melano-macrophage centre). x 690.

centrations of lymphoid cells are seen associated with the ellipsoids and sometimes with pigment containing cells. The ellipsoids are the terminal arterial capillaries, which are surrounded by a cuff of cells (mainly macrophages; Fig. 1a). This structure is enmeshed in a sheath of fibrous reticulum and is mostly encircled by a blood sinus (Fig 1b).

In the red pulp groups of pigment containing cells are present at the bifurcation of large blood vessels and also near the ellipsoids (Fig 1c); these are the so called "melano-macrophage centres" (MMCs). These cells are PAS-positive and show orange/yellow autofluorescence in U.V. light. The MMCs are surrounded by the cellular reticulum and sometimes a thin layer of connective tissue is present (Fig. 1c,d).

The kidney. The carp kidney is divided into a pro- and opisthonephros (head and trunk kidney).

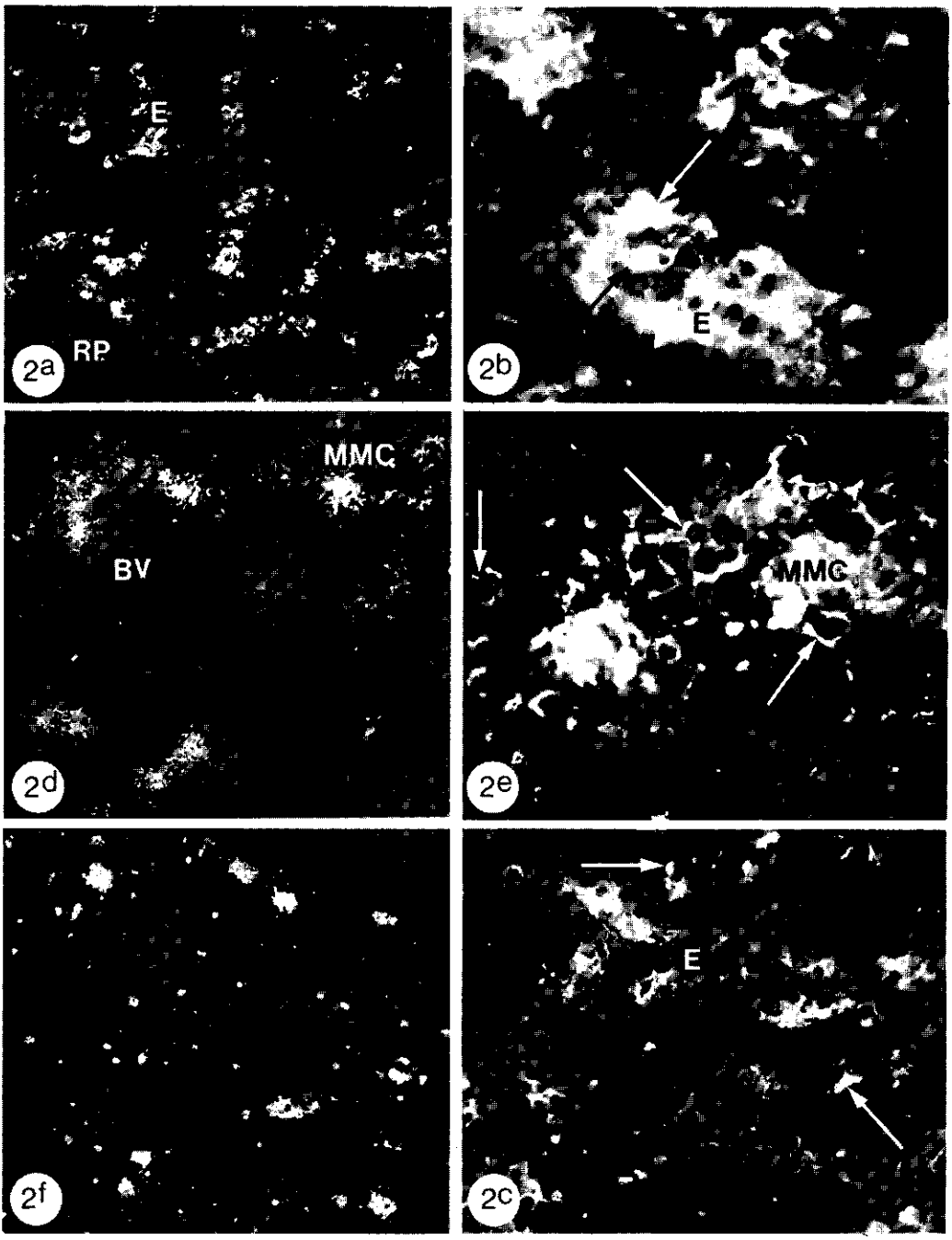
The head kidney has lost its excretory function and no pronephric tubules are seen (Fig. 1e). The organ is surrounded by a thin con-

nective tissue capsule and is supported by a cellular reticulum. The parenchyma is compact, densely cellular and interspersed with many blood vessels, consequently the tissue consists of strands of cells bordered by small blood sinuses. Large blood sinuses may have a thick mantle of steroidogenic cells distal to the endothelial lining (cf. Smith et al. 1970). The parenchyma of the head kidney is a lymphomyeloid tissue, with little patterned arrangement. Lymphoid cells usually cluster along veins or arteries in thick cords of cells between small sinuses ("white pulp"). The extensive non-lymphoid areas ("red pulp") are populated with a variety of cells undergoing erythro- and granulopoiesis. As in the spleen melano-macrophages are present throughout the organ, but in the head kidney they are usually less aggregated than in the spleen. If present, MMCs are located in the densely populated area around the larger sinuses and blood vessels.

The trunk or excretory kidney is also an important lympho-myeloid organ, because the intertubular spaces consist of a reticular network which is filled with haemopoietic and lymphoid parenchyma. The lymphoid cells are diffusely distributed in the parenchyma, but at certain areas some concentrations do occur (Fig. 1f). Reticular and endothelial cells with phagocytic properties line numerous sinuses, which are most clearly seen near the kidney tubules. Within the renal parenchyma melano-macrophages are also seen, but only few aggregate into MMCs (Fig. 1f).

Localization of A. hydrophila antigen

The spleen (Fig. 2). At *day 1* after injection (Fig. 2a,b) the ellipsoids showed general and intense immunofluorescence (IF) with some spots of more bright IF, indicating that the bacterial Ag was not only located intracellularly, but also extracellularly between the ellipsoidal phagocytic cells. In the splenic pulp many weakly fluorescing solitary cells were seen, indicating that they had ingested Ag. At *day 7* the ellipsoidal tissue showed less IF; the Ag appeared to be concentrated within cells of the ellipsoids. Moreover many solitary fluorescing cells were present throughout the splenic pulp. At *day 14* (Fig. 2c) IF of cells in the ellipsoids was weak and the surrounding tissue showed a diffuse reaction. The number of solitary cells containing Ag had increased and around MMCs cells ap-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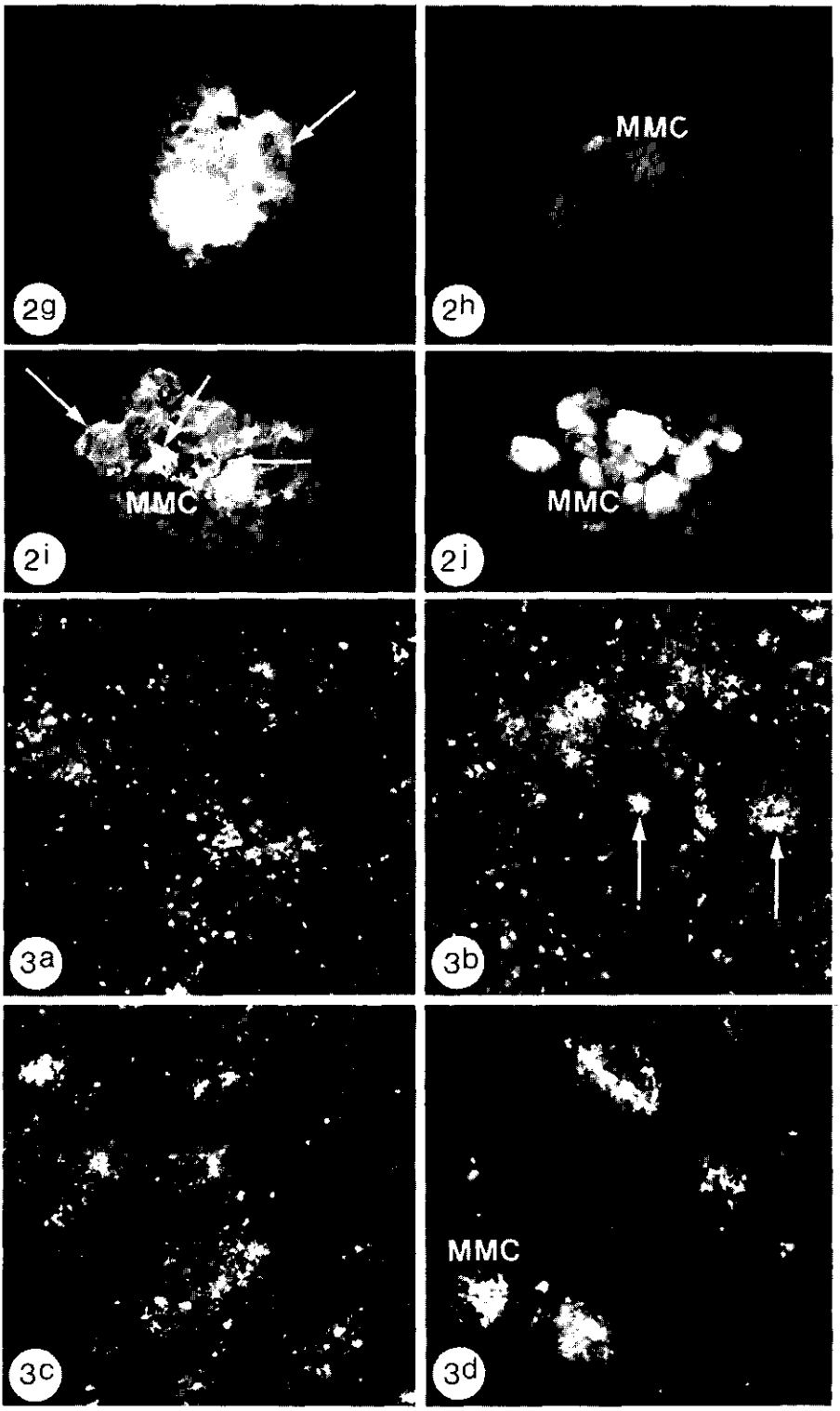
peared that showed IF at the cell surface. Using alternately FITC and TRITC filters, the FITC fluorescence (yellowish green) could be distinguished from the autofluorescence of the MMCs (orange; cf. Fig. 2g,h). At *day 30* (Fig 2d,e) Ag was no longer present in the ellipsoids and solitary IF cells in the splenic pulp were scarce. Most of the IF was aggregated near MMCs, mainly located on the outer surface of cells surrounding the centres, but also on the outer membrane of melano-macrophages. At *day 60* IF was only seen in a few large and small MMCs. Apparently, the number of MMCs had increased, especially the small ones. The Ag was mainly located at the surface of the cells in the centres and appeared as small rims of IF, but intracellular IF was also seen. At *day 90* and *day 120* observations were similar to those at day 60 (Fig. 2f,g,h). At *day 360* some MMCs still showed IF (Fig. 2i,j), but the number of positive MMCs had decreased.

Head kidney (Fig. 3a-f). At *day 1* several areas of the organ showed a diffuse IF and a number of solitary cells with distinct intracellular IF, scattered throughout the parenchyma (Fig. 3a). At *day 7* many solitary cells were seen throughout the tissue, whereas the diffuse tissue IF was no longer present. The surface of some melano-macrophages showed a weak reticular IF. At *day 14* besides in solitary cells, the Ag was concentrated in areas surrounding the MMCs, where it was located extracellularly on the surface of cells. From *day 30* to *day 120* the IF gradually increased within MMCs, showing both intracellular and membrane bound localization (Fig. 3b-f). At *day 360* only a few MMCs still showed specific IF.

Trunk kidney (Fig. 3g-i). At *day 1* a weak tissue IF was seen in certain areas and positive solitary cells were prominent. These

FIGURE 2a-f. *Aeromonas hydrophila* antigen in sections of the spleen after intramuscular injection of bacterin and stained by the indirect immunofluorescence (IF) technique; a) 1 day after injection: fluorescence is associated with ellipsoids (E) and solitary cells. (RP = red pulp) x 90; b) a larger magnification shows that the whole ellipsoidal structure is fluorescing, with a higher intensity in the cytoplasm of some cells (arrow). x 580; c) 14 days: fluorescence is moderate in ellipsoids and solitary cells (arrow). x 230; d) 30 days: fluorescence is concentrated in and around melano-macrophage centres (MMC); no ellipsoidal fluorescence is seen. (BV = blood vessel) x 90; e) A higher magnification shows that the fluorescence is located on the outer surface of cells in and around MMCs (arrow). x 580; f) 90 days: the fluorescence is concentrated in some large and several small MMCs x 90.


2g
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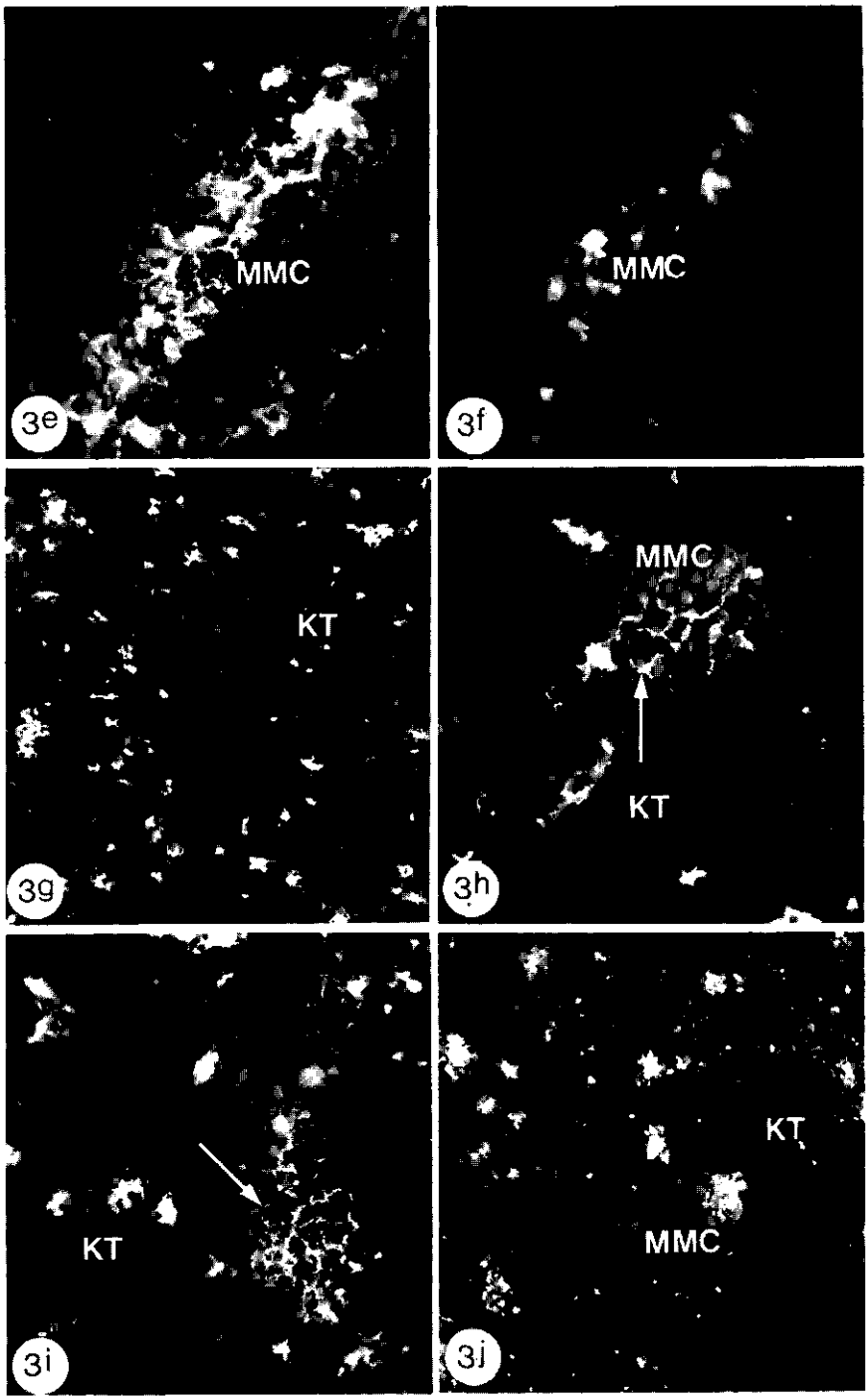
cells were closely associated with kidney tubules (endothelial cells of the peritubular capillaries) and only few cells were seen in the haemopoietic parenchyma. The *A. hydrophila*-immunoreactive cells were irregularly shaped and showed mainly intracellular IF. At *day 7* (Fig. 3g,h) no diffuse IF was seen. Many solitary fluorescing cells were present, still mainly located near kidney tubules. Some MMCs showed a delicate, but distinct rim of IF on the outer surface of the cells, giving the IF a lacework-like appearance (Fig. 3h,i). At *day 14* accumulation of Ag took place in MMCs, whereas positive solitary cells were still prominent. The cells of the MMCs and also the cells directly surrounding the MMCs showed intense IF on their outer surface. At *day 30* the Ag was mainly located in MMCs and the surrounding areas (Fig. 3j). As in the spleen and head kidney, from day 30 onwards the IF became more and more concentrated in MMCs. Although MMCs in trunk kidney were smaller and less compact than in spleen, they were more numerous.

In none of the other organs examined, e.g. gills and heart could Ag be traced after i.m. injection of *A. hydrophila* bacterin. Moreover all control animals were negative at all moments.

The localization of *A. hydrophila* Ag after i.m. injection is summarized in Table 1. After bath immunization Ag could not be demonstrated in the lymphoid organs, gill, skin and intestine up to 30 days after the treatment. Therefore localization after bath immunization was not studied further.

FIGURE 2g-j. *Aeromonas hydrophila* antigen in sections of the spleen after intramuscular injection of bacterin and stained by the indirect immunofluorescence technique; g) 90 days: the specific fluorescence is present in MMCs (arrow). x 580; h) the autofluorescence of the same MMC as shown in g, using TRITC filters. x580; i) 360 days: specific fluorescence present in and around a MMC (arrow). x 580; j) autofluorescence of the same MMC (i). x 580.

FIGURE 3a-d. *Aeromonas hydrophila* antigen in sections of the head kidney of carp, after intramuscular injection of bacterin and stained with the indirect immunofluorescence (IF) technique; a) head kidney at 1 day after injection: diffuse tissue fluorescence and distinct solitary fluorescing cells are present all over the tissue. x 90; b) head kidney at 30 days: IF is concentrated in groups of cells spread throughout the tissue (arrow). x 90; c) head kidney at 60 days: the aggregation of the IF is more pronounced than at 30 days. x 90; d) head kidney at 90 days: IF is only seen in large accumulations, melano-macrophages (MMC), in which the fluorescence is both intra- and extracellularly located. x 90.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DISCUSSION

In the present paper we have described the morphology of 3 lymphoid organs of carp (spleen, head kidney and trunk kidney) and the process of Ag localizaton after i.m. injection of *A. hydrophila* bacterin.

Lymphoid organs

There are considerable differences in morphology of the spleen among various teleosts (Haider,1966). In most teleosts the white pulp is very limited and diffusely distributed (e.g. in rainbow trout, *Salmo gairdneri*, Zwillenberg, 1964; plaice, *Pleuronectes platessa*, Ellis et al., 1976; carp, Graf & Schlüns, 1979), but in some species distinct lymphoid accumulations are present around arteries, veins or ellipsoids (e.g. golden orfe, *Idus idus*, Haider, 1966; mozambique mouthbrooder, *Tilapia mossambica*, Sailendri & Muthukkaruppan, 1975a). Especially in chondrostei and elasmobranchs the ellipsoids are surrounded by a distinct layer of lymphoid tissue (Good et al., 1966; Pitchappan, 1980). Ellipsoids are present in all teleosts; however, their structure is variable, which holds also for the extent to which MMCs are encapsulated and are in contact with ellipsoids or lymphoid cells. Our study is in agreement with the description of Haider (1966) and Graf & Schlüns (1979) with respect to carp ellipsoids and the limited amount of white pulp. Moreover, we noticed that in carp the MMCs have a limited connective tissue capsule and that they regularly contact ellipsoids and lymphoid cells.

The kidney is an important lympho-myeloid organ in teleosts. In the head kidney the presence of extensive lympho-, granulo- and erythrocytopoiesis is described; Smith et al. (1970) described the mor-

FIGURE 3e-j. *Aeromonas hydrophila* antigen in sections of the head kidney (e-f) and trunk kidney (g-j) of carp, after intramuscular injection of bacterin and stained with the indirect immunofluorescence (IF) technique; e) head kidney at 90 days: larger magnification of Fig. 2d, showing a melano-macrophage centre (MMC), in which the fluorescence is both intra and extracellularly located. x 420; f) autofluorescence of the same MMC as in e. x 420; g) trunk kidney 7 days: IF is associated with solitary cells surrounding the kidney tubules (KT). x 125; h,i) moreover, a fine reticular-like IF is present on the surface of cells in MMCs (arrow). x 230; j) trunk kidney at 30 days: most fluorescence is concentrated in MMCs, whereas still some solitary fluorescing cells are present. x 90.

TABLE 1. Antigen localization in carp lymphoid organs after i.m. injection of *Aeromonas hydrophila* bacterin, stained by indirect immunofluorescence

Organ	Days after injection with antigen							
	1	7	14	30	60	90	120	360
Spleen								
Ellipsoids	+++ $^{i/e}$	+ ^{i}	$\pm^{i}$	-	-	-	-	-
Cells in pulp	$\pm^{i/e}$	++ ^{i}	+ ^{i}	+ ^{i}	$\pm^{i}$	-	-	-
MMC	-	$\pm^{e}$	+ ^{e}	++ $^{e/i}$	++ $^{e/i}$	++ $^{e/i}$	++ $^{e/i}$	+ $^{e/i}$
Head kidney								
Cells in parenchyme	++ $^{t/i}$	++ ^{i}	+ ^{i}	+ ^{i}	+ ^{i}	-	-	-
MMC	-	-	+ ^{e}	+++ ^{e}	++ $^{e/i}$	++ $^{e/i}$	++ $^{e/i}$	+ $^{e/i}$
Trunk kidney								
Cells in parenchyme	+++ $^{t/i}$	++ ^{i}	+ ^{i}	$\pm^{i}$	$\pm^{i}$	-	-	-
MMC	-	+ ^{e}	++ ^{e}	++ ^{e}	++ $^{e/i}$	++ $^{e/i}$	++ $^{e/i}$	$\pm^{e/i}$

The quantity of fluorescence reflects the amount of A. hydrophila antigen present, and is indicated: +++ = much; ++ = moderate; + = little; ± = very little; - = no fluorescence; *i* = intracellular fluorescence; *e* = extracellular, cell surface or reticular bound fluorescence; *t* = diffuse tissue fluorescence; MMC = Melano-macrophage centre.

phology of carp head kidney, which in general is in agreement with our observations. However, in the present study more distinct lymphoid zones were described, especially located along veins or arteries. After immunization considerable numbers of plasma cells may be present in these areas (Lamers, 1985). Also in the mozambique mouthbrooder (Sailendri & Muthukkaruppan, 1975a), *Rutilus rutilus* and *Gobio gobio* (Zapata, 1979) distict lymphoid and non-lymphoid zones are present. Moreover, in several fish species antibody (Ab) forming cells have been demonstrated (e.g. in bluegill, *Lepomis macrochiris*, Smith et al., 1967; rainbow trout, Chiller et al., 1969; perch, *Perca fluviatilis*, Pontius & Ambrosius, 1972; mozambique mouthbrooder, Sailendri & Muthukkaruppan, 1975b). It has been established in carp that head kidney is even more important than spleen as far as Ab production is concerned (Rijkers et al., 1980). From both morphological and functional studies on teleost head kidney it is clear that it is an important lymphoid organ, however, the extent to which lymphoid tissue is organized or aggregated is variable among the different teleost species.

Between the tubules of the trunk kidney of teleost fish an extensive haemo- and lymphopoietic tissue is located (Ellis et al., 1976; Zapata, 1979). There is no clear organization or compartmentation of this tissue. In carp Rijkers et al. (1980) demonstrated that the trunk kidney is also an important Ab producing organ.

Melano-macrophages are prominent cells in the lymphoid organs of all teleosts. In many fishes the pigment melanin is present in these cells; however, in carp no melanin is observed; lipofuscin and haemosiderin are the main pigments (Agius, 1985). Lipofuscin is the component responsible for the autofluorescence and increases with the age of the fish. The presence, distribution, variation between species and several other properties of the pigment containing macrophages in teleost fish have been reviewed by Agius (1985).

Antigen localization

The tissue IF in the head and trunk kidney and the general IF of the splenic ellipsoids at the first day indicate that after injection a substantial part of the Ag was located extracellularly. Probably not all Ag was interiorized immediately. EM studies of ellipsoids (carp, Graf & Schlüns, 1979; sunfish, *Lepomis* spp, Fulop &

McMillan, 1984) showed an extended intercellular space containing a dense system of cytoplasmic processes between endothelial cells and between macrophages, allowing free communication between the capillary lumen and this intercellular space. At such places Ag might be kept before being interiorized by the macrophages (see also Lamers & Parmentier, 1985).

In carp, as in mammals, Ag localization appears to be a two phased process. In the first phase, untill Ab gains access to the circulation, the bacterial Ag is localized in a rather non specific manner, similar to carbon (Lamers & Parmentier,1985). In the second phase in mammals an extracellular localization of Ag takes place in the form of immunecomplexes, which coincides with the onset of the Ab production. In carp Ab production starts at day 7-10 after injection of *A. hydrophila* Ag (Lamers & Van Muiswinkel, 1985; Lamers, 1985), which coincides with the onset of the localization of Ag in the MMCs. The ellipsoidal reticular sheath and cells of the MMCs of carp are of importance in Ig binding during the immune response (Lamers, 1985), and it is tempting to speculate on immunecomplex binding by cells of the MMCs in fish.

Other studies in fish on localization of bacterial Ag, such as *A. salmonicida*, *Flexibacter columnaris* or *Yersinia ruckeri* O-antigen (Secombes & Manning, 1980; Maas & Bootsma, 1982; Lamers & Pilarczyk, 1982) are initially comparable with our observations. In the later phase this Ag also became localized in MMCs; however, no extracellular membrane associated localization was reported in these studies.

The fate of soluble Ag also has been studied in fish. In mammals both soluble and particulate Ag localize in a similar way and it only depends on the capacity of the Ag to bind to the membrane of macrophages. However, several soluble Ag's are not easily bound by macrophages, exept in a particulate form, aggregated or complexed with Ab (Unanue, 1972). Despite foregoing there seems to be a difference between localization of soluble and particulate Ag in fish, especially with respect to the involvement of MMCs. Injected soluble Ag, human gamma globulin (HGG) in carp (Secombes & Manning, 1980) and bovine serum albumin (BSA) in plaice (Ellis,1980), showed initially in spleen a diffuse distribution with a little binding to the endothelial lining of ellipsoids and blood vessels. In head and

trunk kidney HGG also showed a diffuse distribution, whereas BSA was only present intracellularly in macrophages and reticular cells. From the time Ab appeared in the circulation HGG localized in splenic ellipsoids only, mainly associated with the reticular sheath, and no MMCs were involved. In kidney HGG showed a fine cell associated IF, which appeared to correlate with cells that stained positive with pyronin (Secombes & Manning, 1980). BSA was seen in both ellipsoids and MMCs; it appeared in MMCs as a fluorescent lace-work and was associated also with small cells which clusterd adjacent to these MMCs (Ellis, 1980). The last observation corresponds with results in the present study on *A. hydrophila* Ag. Lamers (1985) reported that after immunization small pyroninophilic cells appeared around MMCs; they may be similar to the cells observed by Secombes & Manning (1980), Ellis (1980) and in the present study. The identity and significance of these small cells is not known.

The data obtained in the present study show that in the localization of particulate Ag in carp, the MMCs play an important role in a similar way to that described for BSA (Ellis, 1980), especially in the later localization. This might indicate that the MMCs are the place for longterm Ag retention, probably in the form of immune complexes and that MMCs might be primitive analogues of the avian and mammalian germinal centres. This supposition was first made by Ellis & De Sousa (1974), who observed that small lymphocytes percolate through the MMCs. However, the results of Secombes et al. (1982) on the localization of immune complexes are not in agreement with this hypothesis; HGG complexed with Ab localized faster than Ag alone, but never was associated with MMCs. Despite the controversy concerning the structures involved in Ag or immune complex retention, the functional significance of immune complexes in the immune response regulation in fish has been shown (Secombes & Resink, 1984). Priming of carp with immune complexes, induced a stronger immunological memory than priming with Ag alone and it possibly induced also the production of Ab with a higher affinity, two of the functions thought to be induced by immune complexes trapped in germinal centres of homoiotherms. It is clear that more detailed information is needed on immunecomplex trapping in fish lymphoid organs.

Antigen transport

In amphibia, birds and mammals carbon and Ag initially localize at a similar spot and from there Ag is "transported" to a place where long term retention occurs (White et al., 1975; Van Rooijen, 1981; Manning & Horton, 1982). A comparable situation is observed in cyprinid fish using carbon (Lamers & Parmentier, 1985) or using particulate *A. hydrophila* Ag (this study). On the contrary, localization of soluble Ag or immune complexes in fish seem to be held at the place of the first retention (Secombes & Manning, 1980; Secombes et al., 1982). Although carbon laden sheath macrophages seem to leave actively the ellipsoidal structure and migrate to MMCs (Lamers & Parmentier, 1985), we did not notice this phenomenon with *A. hydrophila* Ag, and the mechanism of Ag transport is open to speculation. Also in higher vertebrates a similar confusion exists concerning the transport of substances to the different lymphoid tissue compartments. In the bird spleen Ag localization takes place in the SS-sheaths and cellular transport of Ag and immune complexes from the SS-sheaths to germinal centres has been described (White et al., 1970; 1975). However, Eikelenboom et al. (1983) suggest this process to take place by diffusion, analogous to the movement of immune complexes to different compartments in the mammalian spleen (Groeneveld et al., 1983). Although Ag transport to different compartments of the lymphoid tissues is a common phenomenon in different vertebrate classes, there is a discrepancy in fish, depending on the type of antigen used. Furthermore it may be related to the more simple organization of the lymphoid tissue in lower vertebrates.

Bath vaccination

After bath vaccination no Ag could be detected in organs of carp. The amount of Ag taken up during immersion is 0.01-0.2% of the initial vaccin concentration (Tatner & Horne, 1983), and in our case this will have been 10^4 - $2x10^5$ bacterial cells/fish. Following injection of carp with a similar dose (10^5 *A. hydrophila* cells) no Ag could be traced in the lymphoid organs (data not published). It is worth mentioning that, although the dose of bacterin, used by bathing, was not sufficient for detection in lymphoid organs or for raising a serum Ab level, it was sufficient to induce the formation of immunological memory (Lamers, De Haas & Van Muiswinkel, 1985).

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APPENDIX PAPER VII

THE FATE OF INTRAPERITONEALLY INJECTED CARBON PARTICLES IN CYPRINID FISH[a)]

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SUMMARY

The lymphoid organs of rosy barb (*Barbus conchonius*) and carp (*Cyprinus carpio*) were investigated for their phagocytic properties on carbon, after its intraperitoneal injection. In general, carbon handling was similar in both species. Carbon was firstly detected in the lymphoid organs at 30 min after injection. During the first day carbon was phagocytized by macrophages situated in the spleen within the ellipsoids and in the red pulp. In head and trunk kidney carbon was localized in macrophages scattered through the haemopoietic parenchyma and in cells lining the blood sinuses. In the spleen, macrophages replete with carbon left the ellipsoidal structures and formed aggregates with pigment containing macrophages from day 6 onwards. In all lymphoid organs almost all carbon was ultimately concentrated in the melano-macrophage centres.

INTRODUCTION

In mammalian lymphoid organs antigenic or inert particles initially localize in a similar way; both substances are phagocytized by macrophages in the perifollicular areas. Subsequently however, they are processed in a different manner. Inert material, such as carbon, is accumulated in macrophages scattered through the red

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a) Cell and Tissue Research (accepted for publication; slightly revised).

pulp and marginal zone (Van Rooijen & Roeterink, 1980). However, antigenic material may be subjected to a long-term retention in the follicular centres (Nossal & Ada, 1971; Tew et al., 1980), which has functional significance for the regulation of the immune response (Klaus et al., 1980; Tew et al., 1980; Kunkl & Klaus, 1981).

In some species of teleost fish the major phagocytic sites of inert particles and the areas of eventual deposition are determined (Mackmull & Michels, 1932; Ellis et al.,1976; Mori, 1980). Also the fate of antigenic particulate material has been traced (Secombes & Manning, 1980; Lamers & Pilarczyk, 1982; Maas & Bootsma, 1982). Both inert material and antigenic particles seemed to be phagocytized and processed in a similar way: initially ellipsoids in the spleen, and macrophages and reticulo-endothelial cells in head and trunk kidney were involved, whereas finally material was deposited in clusters of pigmented macrophages, the so called "melano-macrophage centres" (MMCs; Roberts, 1975). Lamers & De Haas (1985) extensively described the processing of a bacterial antigen in the lymphoid organs of carp, and clearly demonstrated that the antigen eventually was located extracellularly at the membrane of cells in and around MMCs. This observation was of particular interest in relation to the possible role of MMCs in immune regulation. The discrepancy between the study of Lamers & De Haas (1985) and former reports on antigen processing in fish raised the question, which of the observed processes are of immunological interest and which are just a non-specific reaction. To elucidate this question a comparison has to be made between processing of antigenic and inert material. Therefore we investigated, in addition to the former study, the lymphoid organs of two cyprinid fish, carp and rosy barb, for their phagocytic properties on injected carbon.

MATERIALS AND METHODS

Animals

Animals used in this study were: 1) adult rosy barb (*Barbus conchonius*), weighing approximately 6 g (standard length about 7.5 cm), and reared at 23 ± 1 °C. 2) common carp (*Cyprinus carpio*), approximately 5 months old, weighing about 40g (standard lengh 10 - 12 cm), and reared at 20 ± 1 °C. The fish were kept in aquaria with recirculating, filtered and U.V. sterilized water and daily fed with pelleted dry food (K30, Trouw, Putten, The Netherlands).

Experimental design

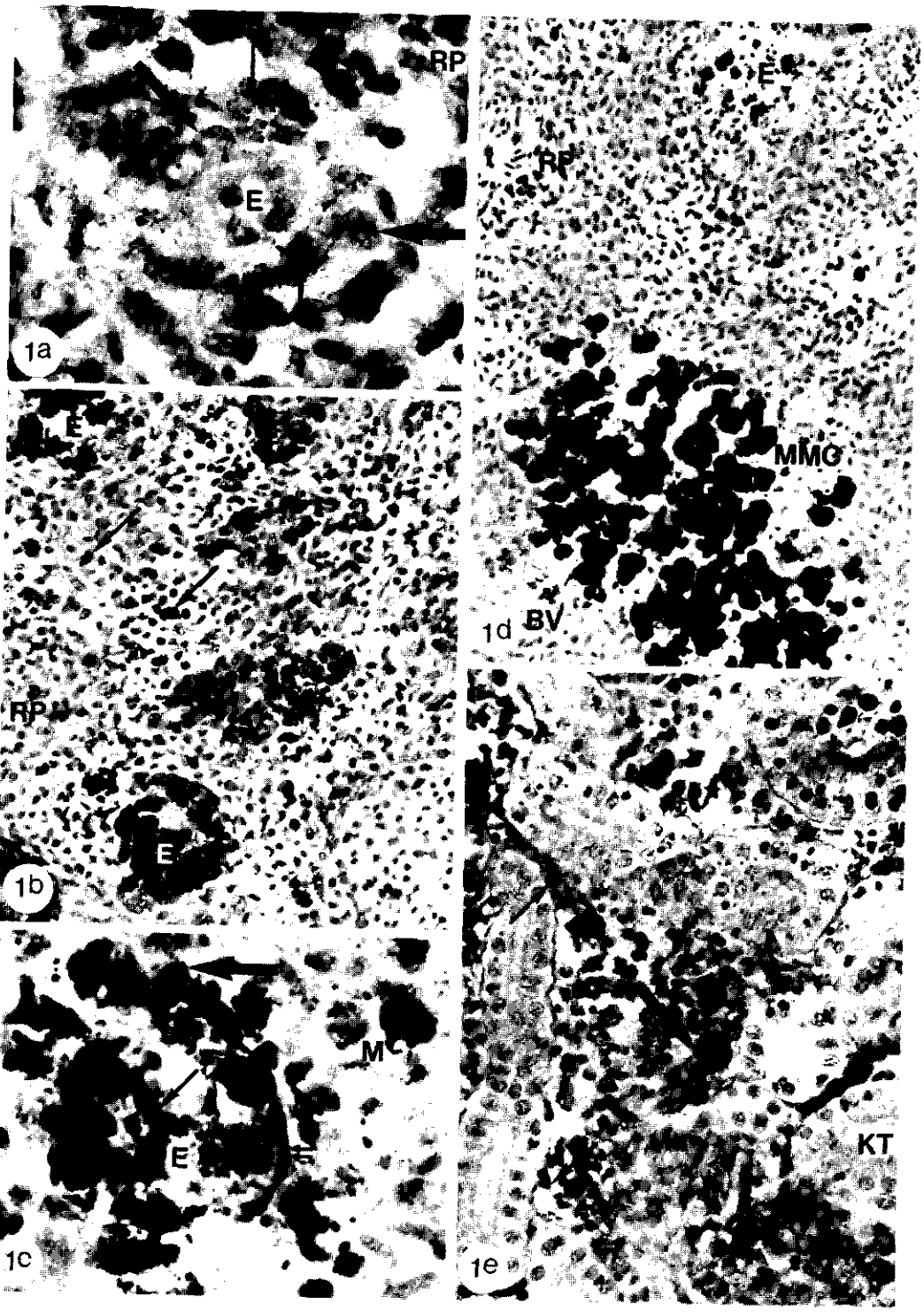
1) Rosy barb were injected intraperitoneally (i.p.) with 0.2 ml indian ink, diluted in PBS (pH = 7.2; 1:10). At 5 min, 30 min, 90 min, 5 h, 15 h, 2, 6, and, 19 days respectively 2 fish and a control fish were killed by decapitation and the organs of the abdomal cavity removed. 2) Carp were injected i.p. with 0.2 ml indian ink (1:10 diluted in PBS) and at 1 and 28 days respectively 2 fish and a control fish were killed and the spleen, head kidney and trunk kidney taken out. Tissues were fixed in Bouin's fluid, dehydrated, embedded in paraffin and sectioned at 5 µm. Sections were stained with Haemalum and Eosin. Prior to staining the sections were bleached in 15% hydrogen peroxide for 24h to facilitate discrimination between pigments and carbon.

RESULTS

Phagocytosis of carbon in rosy barb (Fig. 1a-f)

Extensive phagocytosis of carbon was observed in the mesenteries, spleen and trunk kidney. In the mesenteries macrophages were replete with carbon within a few hours and from 15h after injection onwards large accumulations of carbon containing macrophages were observed. Some phagocytic activity was seen in other sites in the abdomal cavity, notably the mucosa of the distal part of the intestine, biliary and pancreatic ducts and round blood vessels. At 6 and 19 days after injection some carbon containing macrophages even were located within the epithelium in the distal part of the intestine, some of these cells also contained ceroid.

From 30 min after i.p. injection onwards, carbon was traced in the lymphoid organs. In spleen carbon particles appeared in the ellipsoids, both in and around the endothelial cells and surrounding macrophages (Fig. 1a). During the next hours the ellipsoidal macrophages accumulated a considerable amount of carbon and also in the red pulp many macrophages contained carbon (Fig. 1b). Gradually the ellipsoidal macrophages became replete with carbon and several cells even bulged out, through the reticular structure, and seemed to leave the ellipsoid (Fig. 1c). At two days after injection the number of cells replete with carbon in the ellipsoids had decreased. Some ellipsoids seemed to be deprived of macrophages, whereas carbon particles were still associated with the endothelial and reticular structures. In the red pulp the number of carbon-laden cells had increased and a single cell was seen to join a MMC. Gradually more


RP
E
1a
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RP
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1b
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E
1c
E
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MMC
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BV
KT
1e

carbon-laden macrophages gathered near MMCs and at day, 19 after injection large accumulations of cells replete with carbon were present (Fig. 1d). In all of these accumulations melano-macrophages were present. Only a few macrophages were left in the red pulp and ellipsoids that contained some carbon.

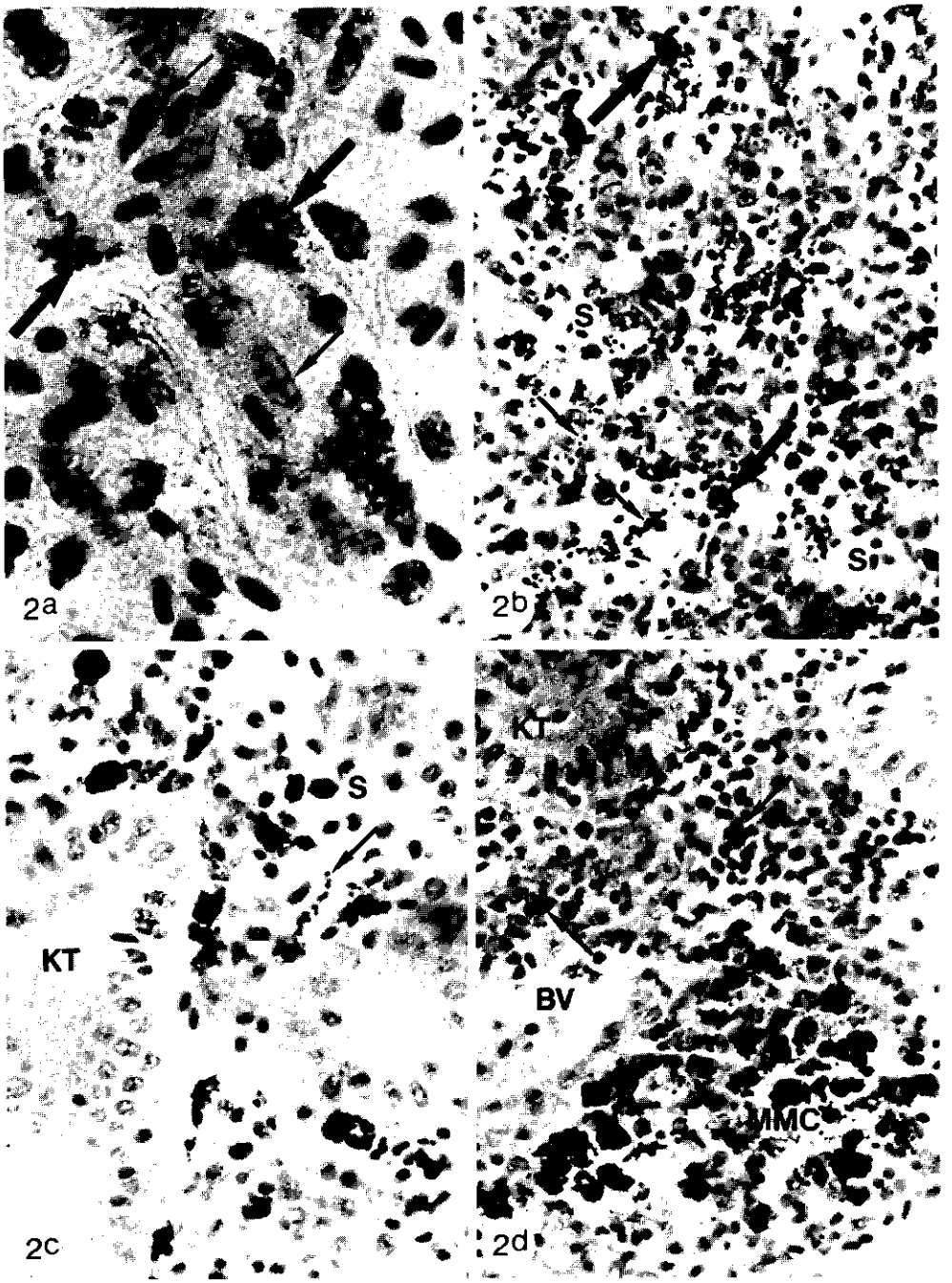
In the trunk kidney the first carbon particles were observed in association with reticulo-endothelial cells of thin walled blood vessels in the parenchyma and surrounding the kidney tubules. Subsequently, carbon was phagocytized by macrophages present in the haemopoietic and lymphoid parenchyma. In contrast to the reticulo-endothelial cells, which mostly phagocytized a low amount of carbon, macrophages became heavily laden (Fig. 1e). Finally, accumulations of carbon-laden cells appeared in association with melano-macrophages. However, when compared with the spleen, many relatively small aggregations were formed. By day 19, except in the aggregations, carbon was still present in some solitary macrophages, which were spread all over the tissue.

Head kidney was only looked at occasionally. The picture resembled that of the trunk kidney, although the amount of carbon was scanty when compared with spleen and trunk kidney. The tissue distribution of carbon in time is summarized in Table 1.

Phagocytosis of carbon in carp (Fig. 2a-d)

The distribution of carbon in carp was studied in the spleen, head kidney and trunk kidney at 1 and 28 days after injection. At 1 day carbon was present in cells of the ellipsoids and in some scat-

FIGURE 1a-e. Carbon in sections of spleen and trunk kidney of rosy barb after i.p. injection; a) spleen at 30 min after injection, showing transverse section of an ellipsoid (E), in which some carbon particles are present (small arrows); note the layer of macrophages (thick arrow, RP = red pulp). x 1500; b) 90 min: macrophages in ellipsoids contain carbon; macrophages throughout the red pulp also have ingested carbon (arrow). x 600; c) 5 hours: macrophages of ellipsoids are heavily laden with carbon and some seem to leave the ellipsoid (thick arrow); carbon is also present in endothelial cells (small arrow) and on the outside of the ellipsoid (double arrows) possibly associated with the reticular sheath; in the red pulp heavily laden macrophages (M) and free carbon particles are present. x 1500; d) 19 days: carbon is concentrated in a large melano-macrophage centre (MMC) bordering a blood vessel (BV); in ellipsoids still a little carbon is left. x 450; e) trunk kidney at 90 min after injection: carbon is taken up by macrophages in the haemopoietic tissue (thick arrow) and by cells lining the peritubular sinuses (small arrow, KT = kidney tubules). x450.


S
2a
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BV
MMC
2d

tered large macrophages in the red pulp of the spleen. The carbon was distinctly restricted to large vacuoles of the cuff macrophages, which surround the ellipsoids (Fig. 2a). In the head kidney many macrophages contained a considerable amount of carbon, but also the lining cells of the sinuses had taken up some particles (Fig. 2b). In the trunk kidney a similar picture was seen: many large macrophages replete with carbon were scattered through the tissue and cells in the lining of sinuses contained a few carbon particles (Fig. 2c). At 28 days some carbon was still present in the ellipsoids of the spleen, but it was also accumulated in small MMCs. In the head and trunk kidney many large concentrations of macrophages replete with carbon were present, which were mostly located near blood vessels (Fig. 2d). Moreover a few scattered heavily laden macrophages were seen. In carp, the head and trunk kidney played the major role in carbon entrapment, whereas in the spleen relatively little carbon was present. Furthermore, less carbon was observed in lymphoid organs of carp than in those of rosy barb, which is probably due to a relative lower dose of carbon given to carp.

DISCUSSION

The major phagocytic tissues of teleost fish, as summarized by Ellis et al. (1976), comprise spleen, head kidney, trunk kidney and mesenteries, whereas in some species phagocytic properties were reported also for cells in the atrium of the heart, liver and gill. Moreover some phagocytic activity is observed in the intestine (cf. Ferguson et al., 1982). The present paper on carbon phagocytosis and processing in the lymphoid organs of rosy barb and carp in gen-

FIGURE 2a-d. Carbon in sections of lymphoid organs of carp after i.p. injection; a) spleen at 1 day after injection, showing an ellipsoid (E) with macrophages containing vacuoles with carbon particles (thick arrow); note the endothelial cell (small arrow). x 1500; b) head kidney at 1 day after injection: macrophages have ingested much carbon (thick arrow), whereas cells lining the blood sinuses (S) contain few particles (small arrow). x 600; c) trunk kidney at 1 day after injection: macrophages have ingested much carbon, whereas cells lining the sinuses contain few particles (arrow, KT = kidney tubule). x 780; d) trunk kidney at 28 days after injection: large melano-macrophage centre (MMC) situated near a blood vessel (BV) and containing many carbon macrophages: a few carbon macrophages are still present in the haemopoietic parenchyma (arrow). x 600.

TABLE 1. Localization of carbon in the lymphoid organs of *Barbus conchonius* after i.p. injection*.

Organ	Time after injection						
	30 min	90 min	5 h	15 h	2 days	6 days	19 days
Spleen							
Ellipsoid	+[e]	++	+++	++++	+++	+++	±
Cells in pulp	±	+	++	++	+++	+++	+
MMC	-	-	-	-	±	+	+++
Trunk kidney							
Cells in parenchyme	±	++	+++	+++	+++	+++	++
MMC	-	-	-	-	-	-	-

* When not indicated, localization is within cells; e = carbon located extracellularly; -, ±, +, ++, +++, ++++: indication for the amount of carbon present; ± = a single cell with carbon; MMC = Melano-macrophage centre

eral agree with former studies, (e.g. in cunner, *Tautogolabrus adspersus*, Mackmull & Michels, 1932; plaice, *Pleuronectes platessa*, Ellis et al., 1976; and gold fish, *Carassius auratus*, Mori, 1980). However, in atrium, liver or gill no significant phagocytosis was observed. The differences between the two cyprinids, in the extent to which several organs participated in phagocytosis, might be related to the spot of injection and the distribution of carbon in the abdominal cavity after injection.

Within 30 min after i.p. injection in rosy barb the first carbon was seen in the lymphoid organs, mainy as free particles. Mackmull & Michels (1932) and Ellis et al. (1976) concluded that after i.p. injection carbon was absorbed by mesenteric blood vessels and was then transported to the phagocytic sites as free particles, for only few carbon containing leukocytes were detected in the blood or lymph. In rosy barb, carbon particles in first instance were generally distributed in the ellipsoids, which suggests an intra- and extracellular localization. Studies on particle clearance in plaice (McArthur et al., 1983) and in trout (*Salmo gairdneri*; Ferguson et al., 1982) revealed that up to 90% of intra veneously injected particles (carbon or bacteria) were removed from the circulation within the first 15 to 30 min. In this first phase of clearance a physical entrapment or passive uptake and sequestration of particles within the reticular meshwork and capillaries of all tissues will take

place. Most of the particles became immobilized in kidney, wheras also in spleen and gills a considerable amount of material was detected (Ferguson et al., 1982; McArthur et al., 1983). As there exists an extended extra cellular space with many cytoplasmic processes between endotholial cells and macrophages of the splenic ellipsoids (Graf & Schlüns, 1979), it is clear that ellipsoids are good sites for immobilizing particulate material, before it becomes internalized by cuff macrophages.

In our study massive phagocytosis was seen in both the spleen and the kidney at 90 min after injection, and within a few hours many macrophages in the splenic ellipsoids and kidney were replete with carbon; moreover these replete cells were observed to leave the ellipsoids. This phenomenon is also described by Ellis et al. (1976) and is probably induced by the fact that the cells replete with non-digestible material were not longer of use at that site. Carbon-laden macrophages formed large accumulations, which in rosy barb always were associated with pigment containing cells. In carp however, this was not always so, especially not in the head and trunk kidney. Mori (1980) stated that carbon-laden macrophages in goldfish always aggregated within pre-existing MMCs, whereas Ellis et al. (1976) suggested that in plaice the macrophages replete with carbon initially aggregated within MMCs and that later on they formed aggregates by them selves. In our view these observations are dependent on two factors: 1) the amount of phagocytized indigestible material and 2) the number of MMCs present. It has to be kept in mind that the number of MMCs present in fish lymphoid organs is correlated with the age of fish (Agius, 1981); the carp used in this study were juvenile animals (5 months), and had a relative low number of MMCs.

As to the origin of melano-macrophages and their centres only few data are available. In this respect Ferguson (1976) stressed the importance of ellipsoids, describing that in turbot (*Scophthalmus maximus*) infected with coccidial protozoa MMCs developed from the splenic ellipsoids. However, this phenomenon might rather be related to the strong local phagocytic activity due to the infection, than that it reflects the only mechanism of MMC formation. We think it very likely that MMCs are formed from macrophages at places of extensive phagocytosis of foreign or autologous cellular materi-

al, whereby the highly indigestible compounds are stored. This is illustrated by the observations that MMCs are involved in disease processes (Roberts, 1975), both in local infections, as seen in gills of *Ichtiobodo*-infected salmon (Ellis & Wootten, 1978) and in systemic infections observed in the liver and lymphoid organs of large mouth bass (*Micropterius salmoides*), infected with *Aeromonas hydrophila*, (Huizinga et al., 1979). Furthermore Secombes et al. (1982) reported the appearance of pigment containing macrophages in clusters of pyroninophilic cells in the lymphoid organs of carp, which might be due to a prominent cell death (the clusters disappeared within a few weeks), phagocytosis of cell debris and the accumulation of indigestible substances.

Initially particulate antigen (*A. hydrophila*, Lamers & De Haas, 1985) and inert particles (carbon, this study) are phagocytized and processed in a similar way, later on clear differences are observed. Whereas carbon transport towards the MMCs seems to be associated with cell movement, the mechanism of transport of antigen, which ultimately accumulates also within the MMCs, is unclear (Lamers & De Haas, 1985). In the MMCs carbon was always located intracellularly in large vacuoles. Secombes and Manning (1980) and Lamers & Pilarczyk (1982) observed that particulate antigenic material may be localized similar to carbon, however, Lamers & De Haas (1985) traced the fate of *A. hydrophila* bacterin in carp, and obtained convincing evidence that this antigen became mainly located extra cellularly, bound to the cell membrane of melano-macrophages and cells surrounding the centra. This phenomenon coincided with the onset of antibody production and the presence of immunoglobulin at the outer surface of melano-macrophages (Lamers, 1985). The possible significance for the regulation of the immune response of the extracellular binding of antigen in combination with the presence of immunoglobulin on the MMCs in lymphoid organs has been discussed elsewhere (Lamers & De Haas, 1985; Lamers, 1985). MMCs are prominent structures in the lymphoid organs of all teleosts; the identified pigments are melanin, lypofuscin and haemosiderin (Agius & Agbede, 1984). The cells execute a multiplicity of functions, from scavengers or depot for e.g. iron to a support in the immune regulation (Agius, 1985). It is clear that with inert material the cells of

the MMCs act as scavengers, whereas in processing of antigenic material they may have both scavenger and immuno-regulatory properties.

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APPENDIX PAPER VIII

HISTOPHYSIOLOGY OF A PRIMARY IMMUNE RESPONSE AGAINST *AEROMONAS HYDROPHILA* IN CARP (CYPRINUS CARPIO L.)[a)]

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SUMMARY

Histological and immunological parameters were monitored in carp, during the primary immune response against *Aeromonas hydrophila*. Localization of antigen in the spleen initially occurred in the ellipsoids, whereas in the head and trunk kidney it was diffusively distributed; when antibody titres raised (from day 9 onwards), antigen accumulated in melano-macrophage clusters in all the three organs, and it was mainly bound to cell membranes. Tissue bound immunoglobulin was present on several structures in the lymphoid organs; due to immunization, the number of cells with cytoplasmic immunoglobulin, and the amount of immunoglobulin associated with the reticular sheath of ellipsoids and membranes of melano-macrophages increased. Pyroninophilia was limited, and occurred in spleen associated with ellipsoids; also clusters of cells appeared displaying a small rim of pyroninophilic cytoplasm. In kidney the reaction was not conspicuous. The number of antibody forming cells in the head kidney peaked at day 9 and the serum antibody response followed with a peak at day 15. When the antigen was injected, emulsified in adjuvant, only the antigen processing and the antibody responses were affected. Antigen processing was retarded and a second burst of antibody production occurred from day 15 onwards. The relation of melano-macrophage centres with immunological processes is considered. It is suggested that in fish these centres might be primitive analogues of the mammalian germinal centres.

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a) Submitted for publication.

INTRODUCTION

Whereas teleost fish are fully immunocompetent and many studies on their immune response have been published (Dorson, 1984), the histophysiology of their immune response is poorly understood.

In mammals, antigenic stimulation results in histological changes in lymph nodes and spleen (Langevoort, 1963; Van Ewijk et al., 1977), which are characterized by two events: 1) the plasmacellular reaction: an increase in the number of pyroninophilic cells in the perifollicular area and subsequently the appearance of plasmacells, 2) the follicle centre reaction: the appearance of an area of large pale staining cells in the centre of follicles, the germinal centres. The latter reaction starts at the peak of the plasmacellular and coincides with the binding of antigen (Ag) complexed with immunoglobulin (Ig) to the surface of follicular dendritic cells. The functions of the germinal centres and their trapped immune complexes are related to processes involved in immune response regulation and memory formation (Klaus et al., 1980; Tew et al., 1980).

All poikilotherms lack germinal centres, and fish in particular have a poorly developed white pulp (Good et al., 1966; Pitchappan, 1980). Nevertheless, a pyroninophilic reaction takes place in the lymphoid organs of carp, following a strong antigenic stimulation (Secombes et al., 1982a). Ag localization in fish spleen in first instance occurs in the sheathed arterial capillaries (ellipsoids), whereas in head and trunk kidney it is dispersed all over the tissue (Ellis, 1980; Secombes & Manning, 1980; Lamers & De Haas, 1985). Later on, Ag concentrates in melano-macrophage clusters (MMCs), and may be localized at the outer membrane of cells. Ellis (1980) and Lamers & De Haas (1985) suggested that MMCs play a role in longterm Ag retention, probably involving immune complexes.

Immunological properties which in mammals are related to germinal centres are, to a greater or lesser extent, also present in fish: B-cell memory (Manning, 1980; Rijkers, 1982), immune complex trapping Secombes et al., 1982b), enhancing effect of immune complexes on both the primary and secondary humoral immune responses (Secombes & Resink, 1984) and the production of higher affinity antibody Ab during the course of the immune response (Fiebig et al.,

1979). It has been speculated that pyroninophilic cell clusters, as described by Secombes et al. (1982a) or MMCs (Ellis, 1980; Lamers & De Haas, 1985) might be analogous to mammalian germinal centres.

In order to get a better integrated view on the functioning of the immune system of fish, simultaneous histological and physiological tests were carried out after an intramuscular (i.m.) injection of carp with *A. hydrophila* bacterin. The following parameters were monitored: Ag localization, Ig distribution, and pyroninophilic cell reaction in the spleen, head kidney and trunk kidney. In addition, the number of Ab forming cells in the head kidney and the serum Ab titre were traced. The effect of emulsifying the antigen in adjuvant was also considered.

MATERIALS AND METHODS

Animals

In this study common carp (*Cyprinus carpio*) of approximately 8 months old and weighing about 70 g were used. They were reared at 20 ± 1 °C. The fish were kept in aquaria with recirculating, filtered and U.V. sterilized water and fed daily with pelleted dry food (K30, Trouw).

Antigen

Aeromonas hydrophila bacterin was kindly provided by Dr. R. Bootsma (Dept. of Pathology, Veterinary Faculty, University of Utrecht, The Netherlands). The bacterin was heat inactivated (30 min at 60 °C) and disrupted by three freezing/thawing cycles. Before injection the broken bacterial cells were washed three times in phosphate buffered saline (PBS) by centrifugation (10.000 x g for 10 min).

Experimental set up

Carp were i.m. injected in the dorsal region with 10^9 *A. hydrophila* cells in 0.1 ml PBS. In a second group the Ag was emulsified in an equal volume of Freunds complete adjuvant (FCA, Difco). At 1, 3, 7, 9, 12, 15, 24 and 45 days after injection 2 animals per group and 1 control animal were sampled. The animals were anaesthetized in MS222 (Sandoz, Basel, Switzerland), bled by caudal vein puncture, followed by removal and freezing in liquid nitrogen of spleen, head and trunk kidney. A part of the head kidney was processed to detect Ab forming cells by means of a passive haemolytic plaque assay. Sera were stored at -20 °C until testing for Ab by a bacterial agglutination test. Cryostat sections (7 µm) of the tissues were made for immunocytochemical use and for staining with methylgreen and pyronin.

Immunohistochemistry

Tissue sections were tested for the presence of *A. hydrophila* Ag and Ig, using an indirect immunofluorescenc test. Antiserum

against *A. hydrophila*, raised in rabbits as described previously (Lamers & De Haas, 1985), was diluted 1:50 in PBS, before use on sections. Mouse monoclonal Ab directed to carp Ig (MACIg) was produced at our laboratory by Dr. E. Egberts and characterized as WCI 16 (Secombes et al., 1983; Egberts et al., 1983); ascites fluid, 1:40 diluted in PBS, was used on sections. Fluorescein labelled goat anti rabbit Ig (GAR/FITC; Nordic) and rabbit anti mouse Ig (Fc piece; RAM(Fc)/FITC; Nordic) were used as second step antisera. Controls consisted of sections incubated with PBS, pre-immune rabbit serum, normal mouse serum in the first step, followed by the usual second step, and were negative in all tests. To prevent fading of the FITC dye, sections were mounted in PBS-glycerol (1/9, v/v), containing 1 mg p-phenyldiamine (Johnson & Nogueira Araujo, 1981).

Immunological tests

The haemolytic plaque essay, as described for carp by Rijkers et al. (1980a), was adapted in order to determine the anti-*A. hydrophila* Ab forming cells. As an indicator system sheep red blood cells (SRBC) coated with the O-antigen isolated from *A. hydrophila* were used (Lamers et al., 1985). Bream (*Abramis brama*) serum was used as complement source. Plaques were counted after incubation at 25 °C for 4h. Results are expressed in plaque forming cells (PFCs) per 10^6 viable white cells (PFC/10^6 WC). The number of viable white cells was determined with the dye exclusion assay (0.2% trypan blue in PBS). For control, tests were also performed using non-coated SRBC; the number of anti-*A. hydrophila* PFCs were corrected for the anti-SRBC PFCs (0.05 - 0.7 PFC/10^6 WC).

Antibody titration was carried out by bacterial agglutination on microtitre plates with washed *A. hydrophila* cells (5 x 10^8 cells final concentration). Results are expressed in ^{2}log of the highest serum dilution still giving clear agglutination.

RESULTS

Histological observations

Antigen localization (Table 1). The series of events with respect to the localization of *A. hydrophila* Ag in the lymphoid organs was comparable in both groups, and in general was similar to that described in a former study (Lamers & De Haas, 1985). For Ag in saline the process was as follows: in the spleen Ag appeared predominantly in the ellipsoidal structures and in some solitary cells. From day 15 onwards it also became localized in and around MMCs, mainly on the outer surface of cells in this area. By day 45 *A. hydrophila*-immunore activity had diminished in ellipsoids and solitary cells, but in MMCs it was increased. In head and trunk kidney the initial diffuse tissue reaction had disappeared at day 7 and solitary cells were scattered throughout the tissue up to day

TABLE 1. Antigen localization in the lymphoid organs of carp after i.m. injection of *Aeromonas hydrophila* bacterin, with or without adjuvant; staining with immunofluorescence.

Organ	Days after immunization (no FCA)				Days after immunization (with FCA)			
	1-3	7-9	12-15	45	1-3	7-9	12-15	45
Spleen								
Ellipsoid	+/+++	++	+	±	±/++	+/++	+++	±/++
Cells in pulp	±/+	+	+/++	+	±	±/+	+	±/+
MMC	-	-	±	++	-	-	±	+/++
Head kidney								
Cells in parenchyma	++	+	+	±	+	+	++	±/+
MMC	-	-	±	++	-	-	±/+	±/++
Trunk kidney								
Cells in parenchyma	+++	++	+	±	+	+	+++	+
MMC	-	-	+	++	-	-	+	+/++

The quantity of fluorescence reflects the amount of *A. hydrophila* antigen present, and is indicated: +++ = much; ++ = moderate; + = little; ± = very little; - = no fluorescence; +/++ = variation between animals; MMC melano-macrophage centre.

45. From day 15 onwards, Ag appeared in and around MMCs also predominantly located on the outer surface of cells in this area. The processing of the Ag injected in adjuvant was retarded when compared with the saline group (Table 1); the ellipsoidal and the diffuse general head kidney fluorescence was present from day 3 to day 45. However, the localization in MMCs was less retarded, although it was somewhat more variable than in the saline group.

Localization of Immunoglobulin (Table 2, Fig. 1,2). The specificity of the monoclonal Ab raised against carp Ig (WCI 16) was determined by Secombes et al. (1983) and Egberts et al. (1983). Our study showed several Ig-immunoreactive cells and structures in the spleen, head and trunk kidney of control animals: cytoplasmatic immunofluorescence (IF) of (plasma)cells, scattered troughout the tissue and associated with MMCs, in spleen also in or near ellipsoids; IF on the surface of a small amount of white cells, scattered troughout the tissue and present in splenic ellipsoids; IF on the surface of melano-macrophages, whether or not clustered; IF on the splenic ellipsoids (possibly the reticular sheath).

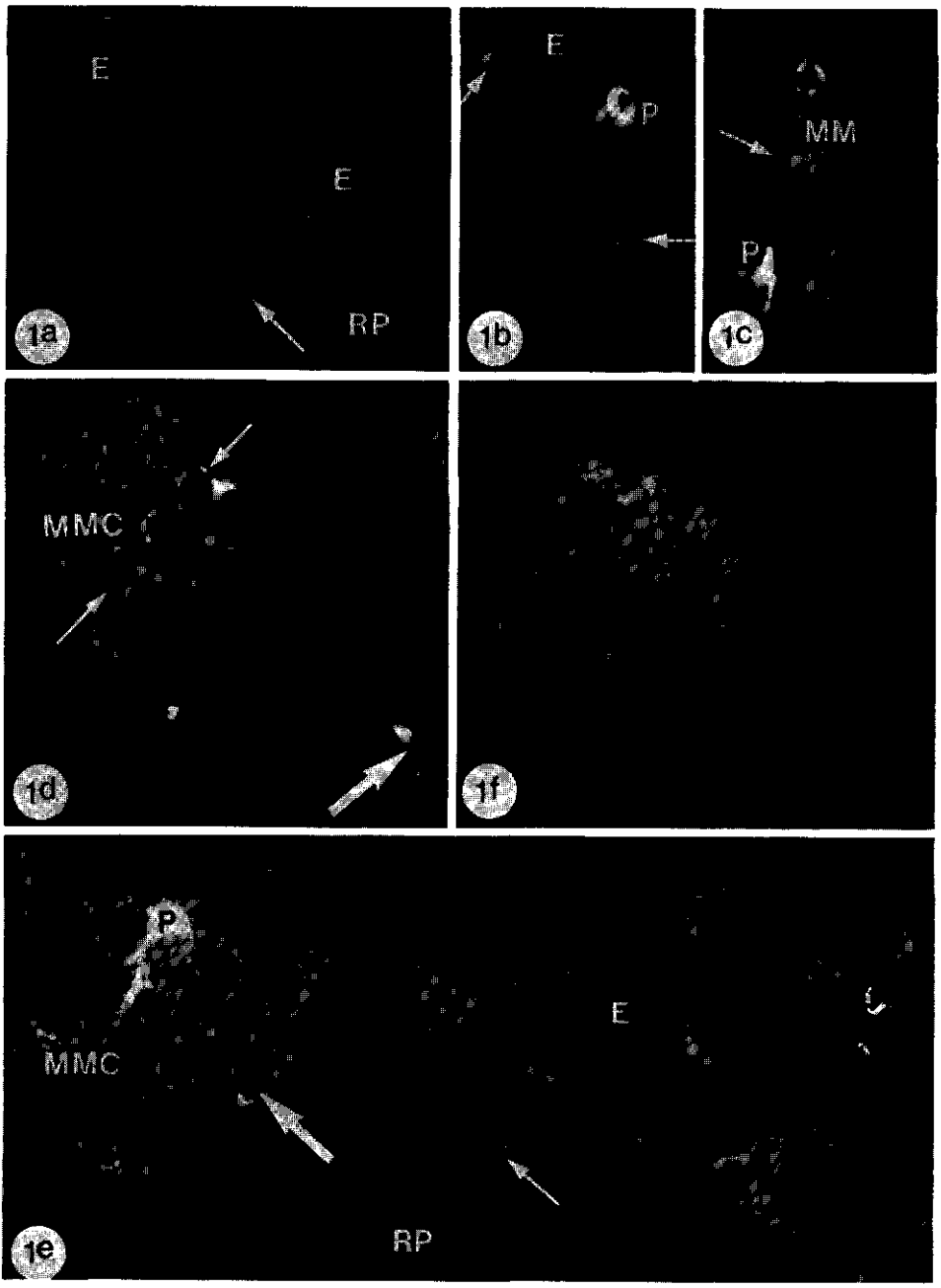

FIGURE 1. Immunoglobulin in sections of carp spleen after injection of *A. hydrophila* bacterin, and stained by indirect immunofluorescence; a) day 1: the ellipsoidal fluorescence appears to be associated with the reticular sheath;

The distribution of Ig and the changes as a result of immunization are given in Table 2. As no clear differences between the saline and adjuvant groups were seen, data were combined. In spleen a slight increase in IF of the ellipsoids and MMCs was found from day 7 onwards (Fig. 1). Moreover the number of plasma cells had slightly increased. In the head and trunk kidney the number of solitary cells with cytoplasmic IF increased (Fig. 2a,b,c), and they accumulated near large blood vessels. As in spleen the MMCs showed surface IF, which slightly increased in intensity at longer intervals after injection (Fig. 1c,d,f,2d).

Methylgreen Pyronin staining (Fig. 3). With pyronin not only plasma cells but also their precursors and cells of the haemolympho- and granulopoiesis will be stained. Especially in head and trunk kidney many pyronin positive cells were present. In spleen of control animals some pyroninophilic cells were associated with ellipsoids and MMCs, but in the red pulp they were only occasionally present. Emulsifying the Ag in adjuvant had no effect. After immunization a slight increase in number of positive cells was observed in and around the ellipsoids and MMCs (Fig. 3a). From day 15 onwards, groups of small cells were seen predominantly surrounding MMCs, and these cells had a small pyroninophilic rim of cytoplasm (Fig. 3b).

In the head and trunk kidney of control animals pyroninophilic cells were spread all over the organ, with a concentration near the larger blood vessels (Fig. 3c,d,e). An increase in number and a change in distribution of these cells following immunization was not conspicuous. In both organs small pyroninophilic cells appeared around MMCs (Fig. 3c,e).

FIGURE 1. (continued) note the solitary cells with membrane fluorescence (arrow, E = ellipsoid, RP = red pulp). x 580; b) day 9: a cell with cytoplasmic fluorescence, plasma cell (P), is associated with the ellipsoid; note the membrane fluorescence of some cells in the splenic pulp and in the ellipsoid (arrow). x 580; c) day 9: fluorescence is associated with the membrane of melano-macrophages (MM; arrow); note a plasma cell (P) associated with these cells. x 580; d) Spleen section at day 45 after injection, showing a cluster of melano-macrophages (MMC) not associated with an ellipsoid; note the fluorescence on the cell surface of the cells (small arrow) and the presence of a plasma cell (arrow). x 580; e) day 45: fluorescence is associated with the ellipsoidal sheath, the surface of melano-macrophages (thick arrow) and membrane of some solitary cells (small arrow, P = plasma cell). x 580; f) autofluorescence of the same MMC as in (e). x 580.

TABLE 2. Immunoglobulin localization in carp lymphoid organs after injection of *A. hydrophila* bacterin, stained by indirect immunofluorescence

Organ	Days after immunization				
	0	1-3	7-9	12-15	45
Spleen					
Ellipsoid[a)]	±	±	+	+	+
Cells in pulp[b)]	±	±	+/++	+	+
MMC	+	+	++	++	++
Head kidney					
Cells in parenchyma	+	+	+++	++	+
MMC	±	+	+/++	++	++
Trunk kidney					
Cells in parenchyma	+	+	++	++	+
MMC	+	+	++	+	++

a) ellipsoids and MMCs show extracellular fluorescence;
b) cells in splenic pulp and parenchyma of head and trunk kidney show cytoplasmic fluorescence;
-, ±, +, ++, +++: indication for the amount and intensity of fluorescence present, +/++ = variation between individuals; MMC melano-macrophage centre.

Immunological observations

The number of anti-*A. hydrophila* PFCs in the pronephros of carp was determined at successive days after immunization (Fig. 4a). At day 7 PFCs were already present and the response peaked at day 9 in both groups (Ag in saline: 141-231 PFC/10^6 WC; Ag in adjuvant: 63-137 PFC/10^6 WC; controls: 0.46-0.59 PFC/10^6 WC). No data were obtained at day 12. At day 15 in both groups the number of PFCs had decreased to approximately 10 PFC/10^6 WC. From day 15 onwards the responses of the two groups differed; whereas PFC numbers in the saline group showed a further decrease to little above control level (day 45: 0.7-3.9 PFC/10^6 WC), the PFC numbers in the adjuvant group gradually increased till the end of observation (day 45:, 19.2-40.1 PFC/10^6 WC).

Agglutinating serum Ab levels (Fig. 4b) were in accordance with the PFC numbers; Ab titres increased significantly from day 7 onwards and up to day 12 they were comparable in both groups. Day 15 was the peak day for the saline group (^{2}log titre 8.0), whereas in the adjuvant group the titres increased till the end of the experiment (day 45: titre 10.5). The titre in controls was 2.5 ± 1.4

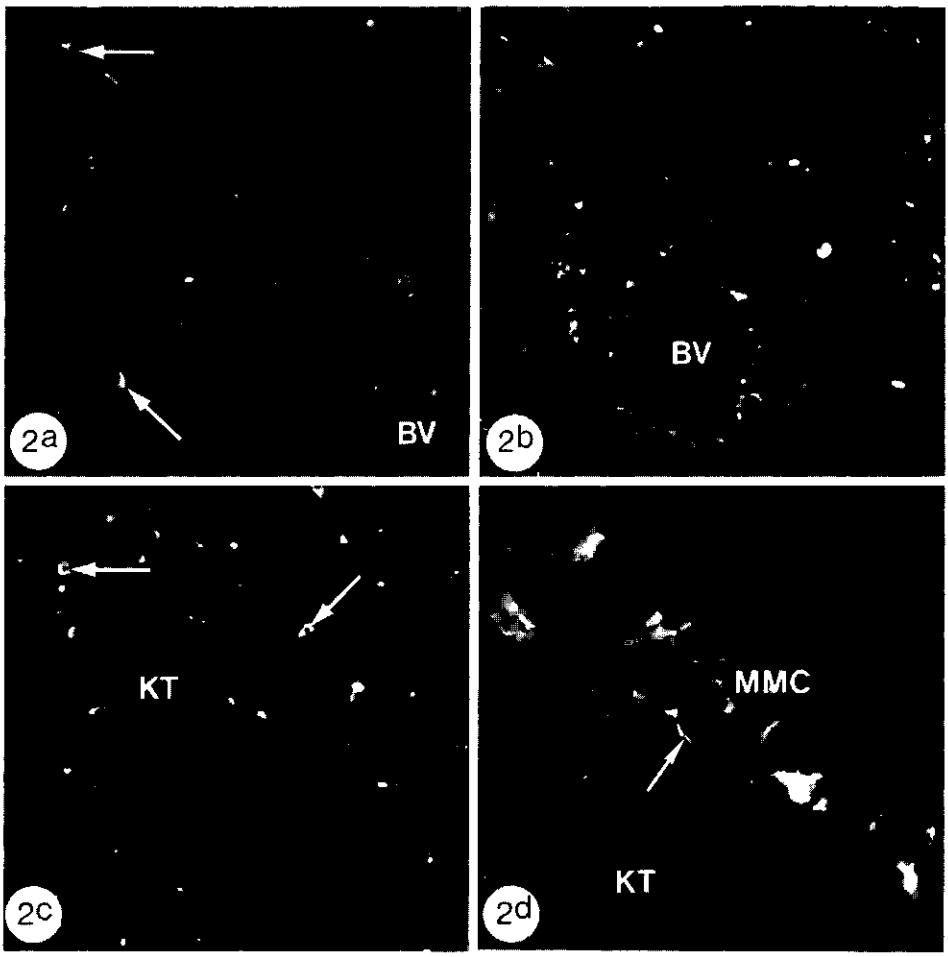

FIGURE 2. Immunoglobulin in sections of carp kidney, after injection of *A. hydrophila* bacterin, stained by indirect immunofluorescence. a) head kidney at day 1: some scattered cells show cytoplasmic fluorescence (arrow, BV = blood vessel). x 230; b) head kidney at day 9: several cells with cytoplasmic fluorescence are concentrated around a large blood vessel (BV). x 230; c) trunk kidney at day 9: several cells with cytoplasmic fluorescence are scattered in the haemopoietic parenchyme (arrow; KT = kidney tubule). x 230; d) trunk kidney at day 45, membrane fluorescence (arrow) of cells in the melano-macrophage centre (MMC). x 580.

(mean ± SD; n = 13). No statistical analyses were performed on data of the experimental groups, as only 2 animals per point were sampled.

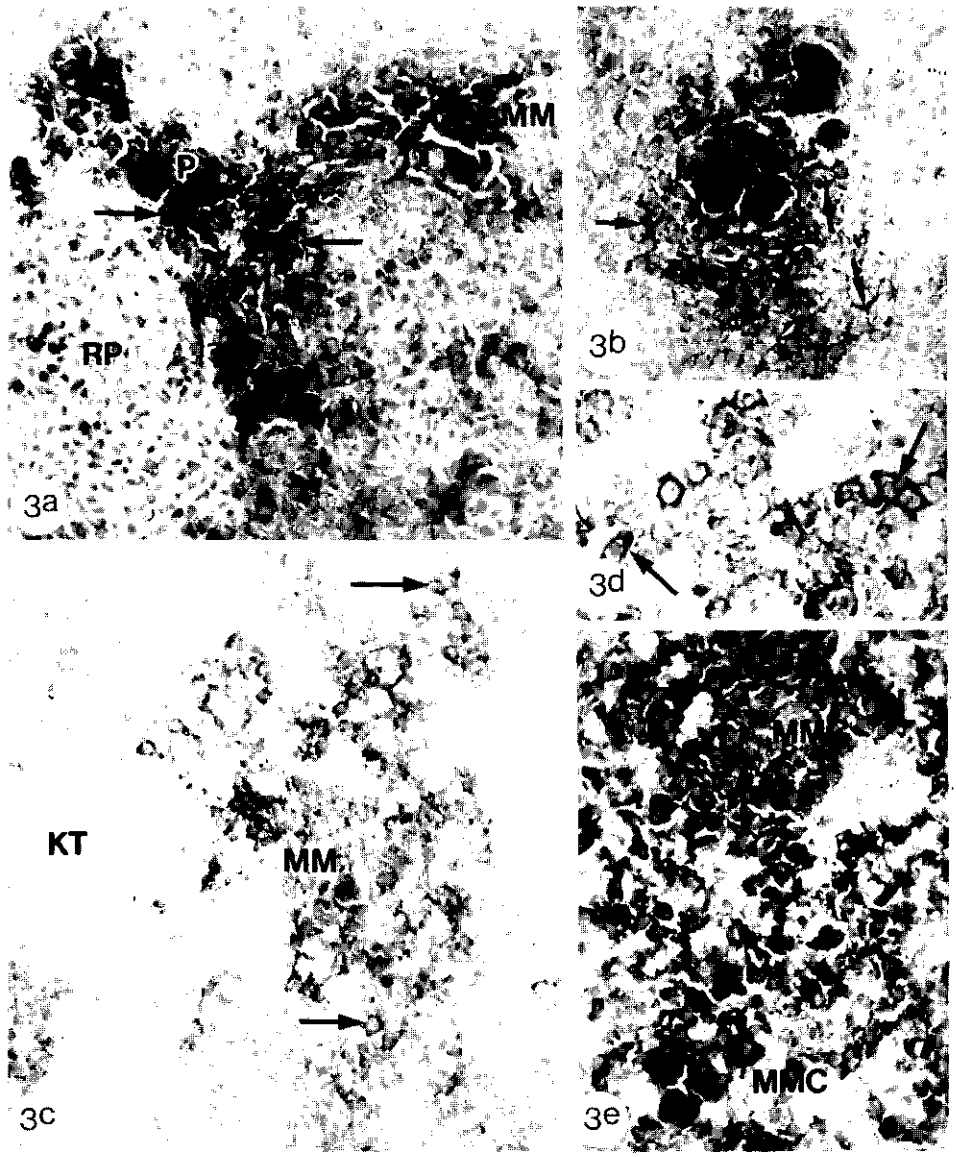

FIGURE 3. Methylgreen and Pyronin staining of sections of carp lymphoid organs after i.m. injection of *A. hydrophila* bacterin; a) spleen at day 7 after injection: ellipsoid (E) with several positive cells and a melano-macrophage (MM); note the cells that show a small positive rim of cytoplasm (arrow) and that in the red pulp positive cells are almost lacking (P = plasma cell, RP = red pulp). x 600; b) spleen at day 45, showing a cluster of melano-macrophages surrounded by a group of small cells with a faintly stained rim of cytoplasm (arrow). x 600; c) trunk kidney at day 45, showing haemopoietic parenchyma between the kidney tubules with several positive cells, some are probably plasma cells (arrow); also some melano-macrophages are present. x 490;

DISCUSSION

In this study both physiological and histological parameters, involved in the primary immune response in carp against *A. hydrophila* Ag, were monitored simultaneously. The effect of emulsifying the Ag in adjuvant was also considered.

Following intramuscular injection of *A. hydrophila* Ag in carp, its processing and the Ab production were influenced by the use of adjuvant, however, the pyroninophilic response and distribution of tissue bound Ig were not affected. Adjuvant may considerably stimulate the uptake of soluble Ag by fish macrophages (Serero & Avtalion, 1978); however, Secombes & Manning (1980) observed little effect of adjuvant on processing of particulate Ag after intraperitoneal (i.p.) injection.

The localization of *A. hydrophila* Ag is extensively described by Lamers & De Haas (1985). However, in the present study the sequence of the processes was slightly retarded especially when adjuvant was used; at day 45 some Ag was still present in splenic ellipsoids and solitary phagocytic cells which had disappeared by that time in the former experiment. The prolonged presence of Ag at the initial localization sites in the adjuvant group was probably due to the gradual release of Ag from the water-oil emulsion. The difference, concerning Ag localization, between observations in this paper and those by Secombes & Manning (1980) might be attributed to the different injection routes. Lamers & De Haas (1985) concluded that Ag processing in carp bears resemblances to the 2 phased process in mammals (Nossal & Ada, 1971; Van Rooijen, 1980). Initially *A. hydrophila* Ag was immobilized and phagocytized in splenic ellipsoids and by cells spread all over the splenic and nephric tissue. Subsequently the Ag became localized extracellularly on the surface of cells in and around MMCs and here it remained for a long time (up to 12 months). In view of the similarities between the long-term

FIGURE 3. (continued) d) head kidney at day 9, showing several positive cells from weakly to strongly stained; some plasma cells are evident (arrow). x 600; e) head kidney at day 45, showing several positive cells; two small clusters of melano-macrophages (MMC), one is surrounded by small densely packed cells, displaying a small rim of faintly stained cytoplasm (arrow). x 600.

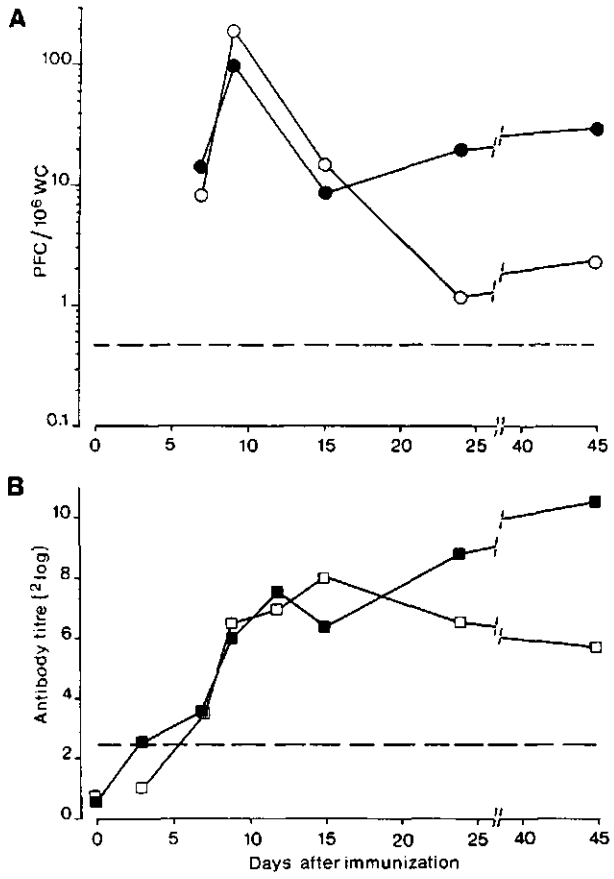

FIGURE 4. a) Kinetics of the primary anti *A. hydrophila* plaque forming cell (PFC) response in the head kidney of carp. Fish were i.m. injected with 10^9 bacterial cells suspended in saline (○) or emulsified in FCA (●). ---- is the PFC level in control fish. PFC/10^6 WC = Plaque Forming Cells per 10^6 White cells. Each point is the mean of 2 animals and each test was performed in duplicate; b) Kinetics of the primary anti *A. hydrophila* serum agglutinin response. Fish were i.m. injected with 10^9 bacterial cells suspended in saline (□) or emulsified in FCA (■). Agglutinin titres are expressed in ^{2}log of the highest serum dilution still giving a clear agglutination. Each point is the mean of 2 animals and each test was performed in duplicate. ---- is the mean of the control fish sampled during the experiment (titre 2.5 ± 1.4; mean ± S.D.; n = 13).

extracellular localization in MMCs and the long-term immune complex retention in mammalian germinal centres, it was speculated that immune complexes might be involved in the long-term Ag retainment in carp and that MMCs possibly have a function in the immune response regulation (Lamers & De Haas, 1985). Results of the present paper

support this supposition as localization in MMCs was not observed untill Ab titres had reached high levels (day 15). This will be discussed in more detail later on.

The PFC response and the Ab production against *A. hydrophila* Ag, injected in saline, are in agreement with former observations (Van Muiswinkel et al., 1985; Lamers & Van Muiswinkel, 1985) and clearly demonstrate that the serum Ab level is the cumulative resultant of the PFC response. Carp which received the Ag in adjuvant gave a characteristic PFC response in the head kidney. After the initial PFC peak at day 9 a second rise in PFC numbers occurred at a later phase of the response and at that time also the serum Ab level was higher than in fish which received the Ag in saline. French et al. (1970) reported a similar phenomenon in birds injected with Ag in adjuvant; after an initial primary response peak (at day 8-12) a second rise in Ab production occurred from day 21 onwards (peak at day 42). They suggested that during the first phase Ab was produced in the lymphoid organs, whereas in the second phase it was released from the granuloma developed at the site of injection of the water-in-oil emulsion. Although we did not trace Ab production at the site of injection in our study, it is clear that in carp a prolonged Ab production took place in the lymphoid organs, which might be induced by a continuous stimulation, because of a gradual release of Ag from the injection spot.

Staining for Ig, using monoclonal Ab raised against carp Ig, showed an increase in the number of solitary cells with cytoplasmic fluorescence around the peak of the PFC response. When serum Ab levels had increased, a slight increase was also seen in the amount of Ig bound to the reticular sheath of the ellipsoids and to the outer surface of melano-macrophages, although ellipsoidal bound Ig did not coincide with staining for Ag in the present study. Secombes et al. (1982b) have demonstrated, using a soluble Ag (human gamma globulin, HGG), that immune complexes may localize in splenic ellipsoids. Whereas Secombes et al. (1982b) did not observe localization of Ag in MMCs using HGG, Ellis (1980) mentioned that a different soluble Ag, bovine serum albumin (BSA), localized in both the splenic ellipsoids and MMCs of plaice and that Ig was present at the same sites. In our study, with the particulate Ag *A. hydrophila*, Ig

is present in both the ellipsoidal reticular sheath and at the outer membrane of MMCs, however, it only coincided with Ag in the MMCs (Lamers & De Haas, 1985). This simultaneous presence of Ag and Ig at the surface of MMCs indicates, but does not truly prove, the presence of immune complexes. A further indication is given by the fact that Ag remained present in that quality for a long period, which in mammals is only possible if Ag is complexed with Ig (Tew et al., 1980; Mandel et al., 1980). Follicular retained immune complexes in mammals were required for the maintenance of the immune response and the formation of immunological memory (Tew et al., 1980; Klaus et al., 1980). In this respect it is worth mentioning, that at 12 months after injecting carp with *A. hydrophila*, the Ag could still be detected in the lymphoid organs (Lamers & De Haas, 1985), a clear Ab level was still present and immunological memory could still be demonstrated (Lamers et al., 1985).

Whereas immunization leads to distinct observations on Ab production, Ag localization and Ig distribution, it only induced a weak pyroninophilic reaction. In the spleen pyroninophilia was associated with ellipsoids and in head and trunk kidney it was concentrated around blood vessels. Especially in spleen groups of cells appeared associated with MMCs, displaying a small rim of pyroniniphilic cytoplasm. According to the moment of their appearance, they might be the same cells which appear around MMCs at the time Ag gets concentrated there, and which have Ag bound to their cell surface (Lamers & De Haas, 1985). These cells were also observed by Ellis (1980), but their identity and significance are not known.

In amphibia and reptilia the proliferative and pyroninophilic reaction following immunization starts at the site of the first Ag localization (Manning & Horton, 1982; Muthukkaruppan et al., 1982). In the present study this phenomenon was only seen in spleen, where both Ag localization and the pyroninophilic reaction were associated with the ellipsoids. However, in mammals in which a clear compartmentation of the white pulp is established these processes are structurally separated (Langevoort, 1963; Nossal & Ada, 1971; Van Ewijk et al, 1977).

The pyroninophilic reaction in our experiment and that described by Secombes et al. (1982a) using *A. salmonicida* to a large extent

are similar, namely there are only weak histological changes. On the contrary injections with HGG in adjuvant induced large pyroninophilic cell clusters (PCCs), especially in head kidney (Secombes et al., 1982a). With the particulate bacterial Ag three injections of Ag in adjuvant were needed to induce similar changes (Secombes et al., 1982a). Bogner & Ellis (1977) induced the formation of comparable pyroninophilia in spleen and kidney of rainbow trout by injecting the B-cell mitogen LPS, which suggests that the pyroninophilic cell clusters are due to strong clonal expansion of a group of precursor cells. Data obtained by Secombes et al. (1982a) truly indicate to what extent the lymphoid organs are able to react after extreme stimulation, but the question arises as to the role of these processes in relation to the immune response. It is obvious from our experiments that a distinct humoral immune reaction is not always accompanied by clear histological changes.

Melano-macrophages, whether or not clustered in centres, are prominent in the lymphoid tissue of all teleosts. The identified pigments are melanin, lypofuscin and haemosiderin, probably resulting from melanosome ingestion, peroxidation of unsaturated lipids and haemoglobin breakdown (Agius, 1985; Agius & Agbede, 1984). From a series of studies by Agius (reviewed in Agius, 1985), it is obvious that these cells execute a multiplicity of functions, from scavengers or depot of e.g. iron to a possible involvement in the immune regulation. The present paper shows that Ig is present at the surface of these cells, and that long-term retention of Ag (probably complexed with Ig) also takes place there. The last indicated function of melano-macrophages might in higher vertebrates be taken over by a more specialized type of cell, the dendritic cell (Van Rooijen, 1981). However, cells with a scavenger function are still present in the white pulp compartment of higher vertebrates, especially in their germinal centres. In mammals they are called "tingible body macrophages" and are responsible for clearing away cellular debris (Veerman & Van Ewijk, 1975) and they accumulate indigestible substances (Chen et al., 1978).

Secombes et al. (1982a) speculated about the analogy of PCCs in carp with mammalian germinal centres. In mammals the immuno-regulatory functions related to germinal centres require the retainment

of Ag in the centres (Klaus et al., 1980; Mandel et al., 1980). Although Secombes & Manning (1980) demonstrated the association of some Ag with pyroninophilic areas, other authors clearly showed that MMCs are involved in Ag localization (Ellis, 1980; Lamers & De Haas, 1985) and postulated the analogy of MMCs with mammalian germinal centres (Ellis & De Sousa, 1974; Roberts, 1975; Ellis, 1980; Lamers & De Haas, 1985). The arguments in favour of the MMCs, as discussed above, are in short: extracellular Ag retention in MMCs, which is probably in the form of immune complexes; the long-term presence of Ag in the MMCs (Lamers & De Haas, 1985) correlated with the long-term serum Ab levels and the presence of immunological memory (Lamers et al., 1985). Ellis & De Sousa (1974) showed, by means of a cell transfer experiment followed by a radioautographic study, that small, metabolically active, lymphocytes migrate through MMCs, which might provide a suitable site for lymphocyte induction by the retained Ag.

Despite several indications in favour of MMCs in fish being analogous to mammalian germinal centres, further experiments are needed to give the conclusive evidence.

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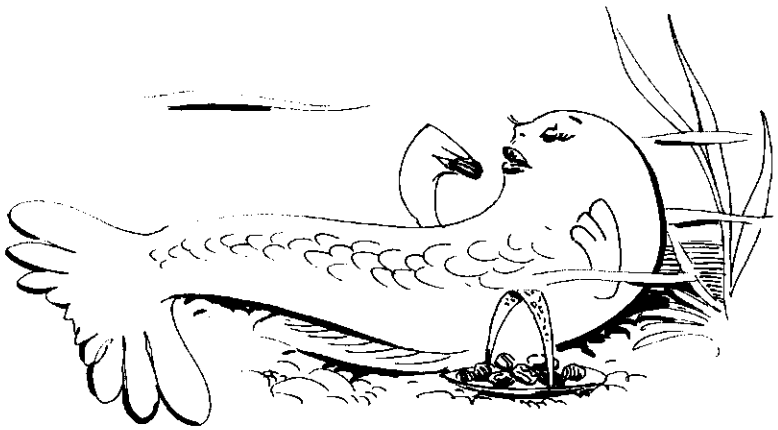

ORAL VACCINATION

APPENDIX PAPER IX

UPTAKE AND TRANSPORT OF INTACT MACROMOLECULES IN THE INTESTINAL EPITHELIUM OF CARP (*CYPRINUS CARPIO* L.), AND THE POSSIBLE IMMUNOLOGICAL IMPLICATIONS[a)]

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SUMMARY

Two protein antigens, horseradish peroxidase (HRP) and ferritin, have been administered to the digestive tract of carp. Electron microscopical observations reveal considerable absorption of both antigens in the second segment of the gut (from 70 to 95% of the total length) and also, although to a lesser extent, in the first segment (from 0 to 70% of the total length). Even when administered physiologically with food, a large amount of ferritin is absorbed by enterocytes in the second gut segment. HRP and ferritin are processed by the enterocytes in different ways. HRP seems to adhere to the apical cell membrane, probably by binding to receptors, and is transported in vesicles to branched endings of lamellar infoldings of the lateral and basal cell membrane. Consequently, most of the HRP is released in the intercellular space where it contacts intra-epithelial lymphoid cells. Only small amounts of HRP become localized in secondary lysosomes of enterocytes. Ferritin does not bind to the apical cell membrane; after uptake by pinocytosis, it is present in small vesicles or vacuoles that appear to fuse with lysosome-like-bodies. In the second segment, intact ferritin ends up in the large supranuclear vacuoles (after 8h), where it is digested slowly. Although no ferritin is found in the intercellular space, ferritin-containing macrophages are present between the epithelial cells, in the lamina propria and also to a small extent in the spleen. The transport of antigens from the intestinal lumen, through enterocytes, to intra-epithelial lymphoid cells or macrophages may have immunological

a) Cell and Tissue Research 239, 1985 (in press).

tions, such as induction of a local immune response and prospectives for oral vaccination.

INTRODUCTION

During their larval stage most teleost fish have a stomachless digestive tract. This feature is still present in about 1.5% of the adult fish, including all cyprinids (Jacobshagen 1937). The intestinal tract of teleosts shows a regional differentiation (Yamamoto, 1966; Iwai, 1969; Gauthier & Landis, 1972; Noaillac-Depeyre & Gas, 1976; Stroband & Debets, 1978; Stroband et al., 1979): a first or proximal segment (60-75% of the gut length) containing enterocytes with morphological characteristics associated with lipid absorption, a second or middle segment (20-25% of the gut length) in which the epithelial cells have the ability to absorb proteins by means of pinocytosis and a third or distal segment (5-15% of the gut length) containing enterocytes with characteristics of water-and ion-transporting cells. It is postulated by the above mentioned authors that absorption of macromolecules in the second segment might be related to the lack of a stomach and, as a consequence, to inadequate protein digestion. These phenomena have also been described in the immature intestine of suckling mammals (cf. Walker, 1981), thereby supporting this theory. However, stomach-containing teleosts still possess a second gut segment with the ability to absorb macromolecules (cf. Stroband & Kroon, 1981). In addition Shcherbina et al. (1976) and Stroband & Van der Veen (1981) have found in cyprinids that 80% of the absorption of proteins takes place in the first segment. Therefore, no quantitative function for protein absorption can be attributed to the second segment of the gut of adult teleosts. Other possible functions have been suggested by Stroband & Van der Veen (1981) and Davina et al. (1982): the second segment may have a "stand-by" function when large amounts of food are suddenly available or it may be of immunological importance. Some evidence for the latter possibility is available many leukocytes, including lymphoid cells, are situated between the epithelial cells and in the lamina propria throughout the intestine (Bullock, 1963; Pontius & Ambrosius, 1972; Zapata, 1979; Davina et al., 1980, 1982).

In addition to solitary lymphoid cells, the gut associated lymphoid tissue (GALT) of mammals consists of lymphatic follicles (Peyer's patches) located in the mucosa of the small intestine. The epithelium overlying these Peyer's patches contains specialized cells (membranous cells or M cells) that can take up macromolecules by means of pinocytosis, even when they are present in small quantities (Bockman & Cooper, 1973; Owen & Jones, 1974; Owen, 1977; Walker, 1981). M cells seem to be specially adapted for antigen transport, for they rapidly release macromolecules into the intercellular space, where they may contact antigen-processing cells and lymphoid cells. As the pinocytotic capacity of enterocytes of the second gut segment of teleosts resembles that of M cells, it seems worthwhile to investigate whether the epithelium of this gut segment can transmit intact antigens to the intercellular space where leucocytes are abundant.

The present study reports the uptake and transport of two different antigens in the intestinal epithelium of carp: 1. *Horseradish peroxidase* (HRP; MW: 40.000), which can be detected histochemically either by light or electron microscopy, 2. *Ferritin* (MW. about 500.000), which can be recognized with the electron microscope because of its electron density. These antigens can be administered physiologically as a food component or by means of oral or anal intubation. Because the stomach is an important barrier for antigens, a stomachless fish has been used in this study.

MATERIALS AND METHODS

Animals

Common carp (*Cyprinus carpio* L.) of approximately 50 g (standard length about 10-12 cm) were used. The animals were reared at our laboratory at 23°C and fed with K30 pellets (Trouw & Co., Putten, Holland). The antigens were administered after a fasting period of 3 days.

Administration of peroxidase

Carp received HRP (type II, Sigma, St. Louis) by three different routes:

a. Oral intubation into the intestinal bulb of 0.1-0.2 ml of a HRP-solution (1-4% in phosphate buffered saline, PBS), using a syringe, the end of which was attached to a polyethylene tube.
b. Anal intubation directly into the second gut segment of 0.1 ml of a HRP-solution (1-4% in PBS).

c. Feeding 0.5-1 g pellets containing 0.1% HRP; pellets were made from K30 meal.

At various times (5 min, 2h, 16h) after administration of HRP, carp (at least two specimen for each time) were killed by decapitation and parts of the gut (at 10, 40 and 85% of the gut length) fixed for 1h at 4°C in 2% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.2) and rinsed for 15 min in 0.05 M Tris-HCl (pH 7.6). For demonstration of HRP, small pieces of the gut were incubated for 1h at room temperature in 0.5% 3.3-diaminobenzidine-tetrachloride (DAB, grade II, Sigma, St. Louis) buffered in 0.05 M Tris (pH 7.6), followed by incubation in the same solution containing 0.01% H_2O_2 for 1h. After rinsing for 10 min in 0.05 M Tris (pH 7.6) and 10 min in 0.1 M cacodylate-buffer (pH 7.2) tissue was postfixed for 1h at 4°C in 1% OsO_4 buffered in 0.1 M cacodylate (pH 7.2). The tissue of two control animals, which received only PBS (pH 7.2), was treated similarly to test for endogenous peroxidase activity.

Administration of ferritin

Ferritin (horse spleen; Merck, Darmstadt) was administered in the same way as described for HRP. A 10% ferritin solution was used for intubation, while for feeding pellets containing 2.5% ferritin were used. At various times (1/2,1,4,8,16,24,36,48,72 and 144h) after administration of ferritin, carp were killed by decapitation and parts of the gut (at 10, 40 and 85% of the gut length) and spleen were fixed for 1h at 4°C in a mixture of 2% glutaraldehyde, 2% OsO_4 and 1% potassium dichromate buffered in 0.1 M cacodylate (pH 7.2). At least two specimen were used for each time.

Electronmicroscopy

Fixed tissue was dehydrated and embedded in Epon 812. Sections were cut on a Reichert OMU IV ultra-microtome using a Diatome diamond knife. Most of the ultra-thin sections were examined unstained with a Philips EM-400 electron microscopy. Some sections were faintly stained for 3 min in uranyl acetate saturated in metanol.

RESULTS

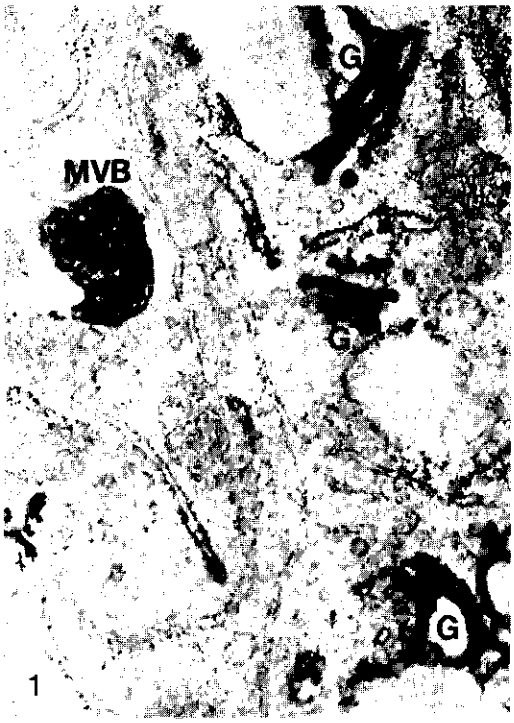
Absorption of Horseradish Peroxidase (HRP)

Untreated animals were examined in order to trace the presence of endogenous peroxidase activity. animals were examined which had

FIGURE 1. Endogenous peroxidase activity in Golgi (G) and multivesicular body (MVB) of an enterocyte in the first segment of a control animal. x 28,000.

FIGURE 2. HRP-uptake in an enterocyte of the first segment, 5 min after oral intubation of HRP. Note the binding of HRP to the apical cell membrane (arrow) and the presence of HRP-containing vesicles (V). x 38,000.

FIGURE 4. Middle part of an enterocyte in the first segment, 2h after oral intubation of HRP. HRP is present in the intercellular space (IS) and in the lamellar infoldings (LI), which are often oriented parallel to the long axis of the cell. Note the fenestrations of the lamellar infoldings (arrows). N nucleus. x 29,000.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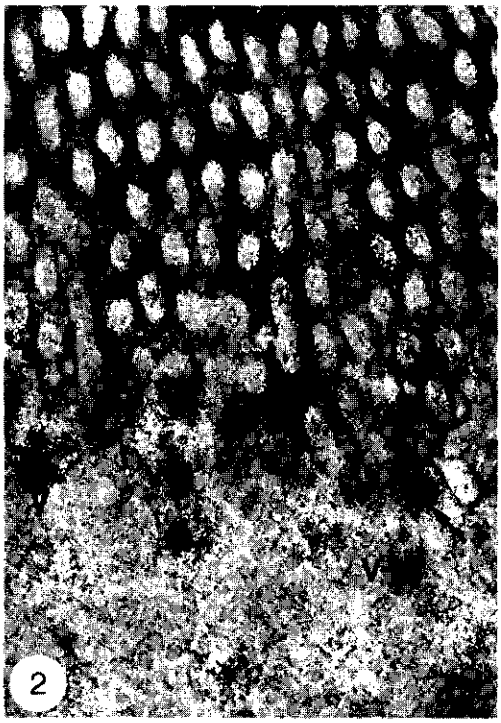
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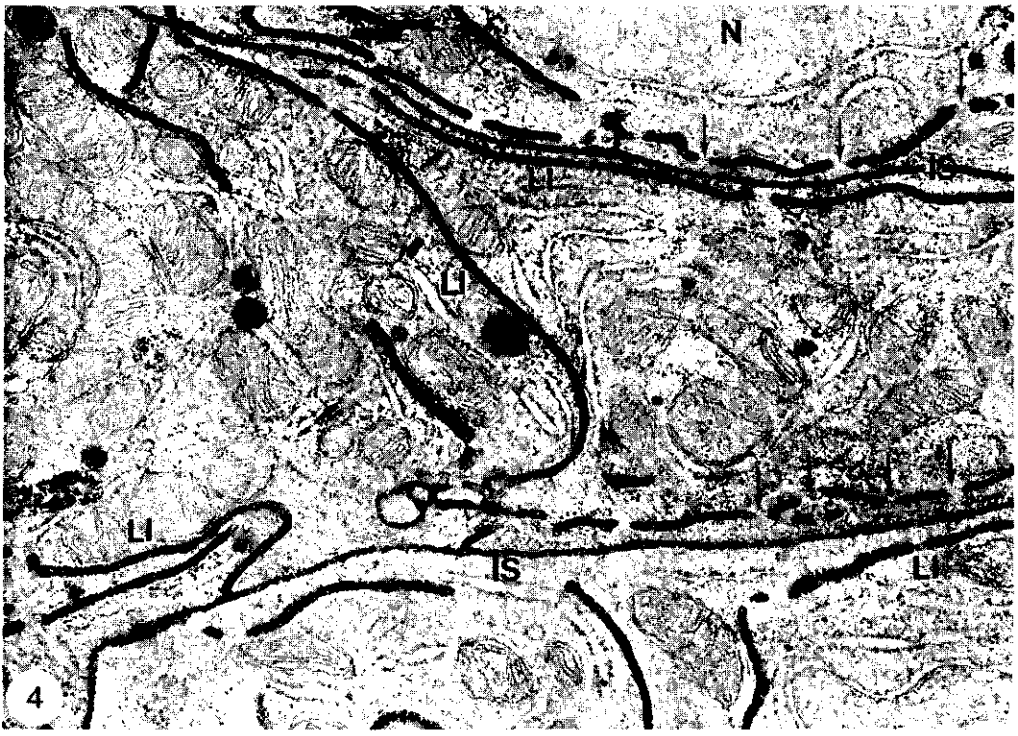
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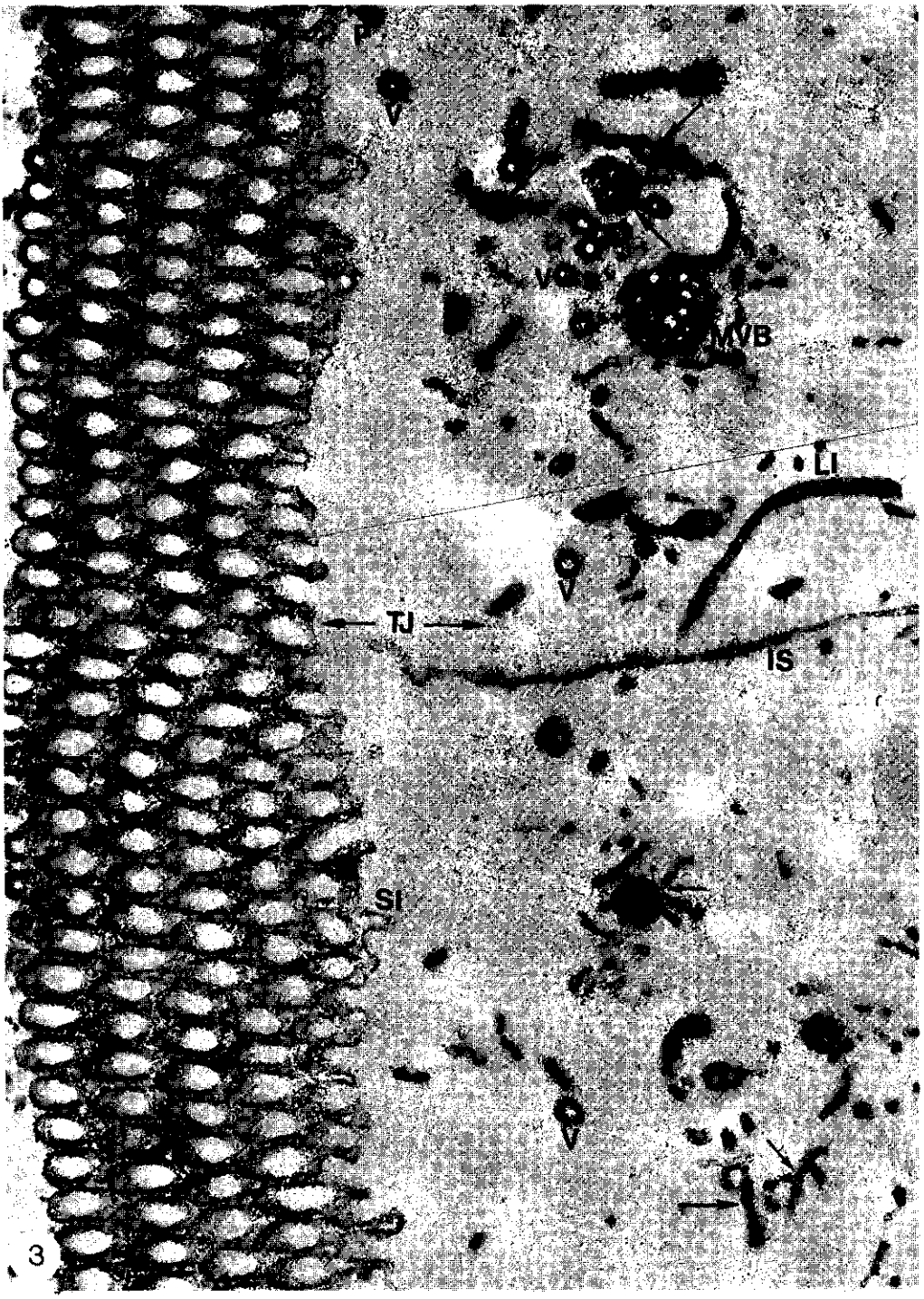
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not received HRP. Considerable differences in activity were found between control animals and even between intestinal folds of one fish. Distinct endogenous peroxidase activity was observed in erythrocytes and in most granulocytes and macrophages. Enterocytes regularly showed faintly stained small vacuoles and multivesicular bodies in the apical part of the cell; occasionally the Golgi cysternae were clearly stained (Fig. 1).

As carp refused to eat HRP-containing pellets the absorption of HRP in the intestine was studied only after oral or anal intubation.

First segment. Immediately after oral administration of HRP, peroxidase activity was localized predominantly in the glycocalyx and in some apical vesicles of the enterocytes of the first segment (Fig. 2). HRP seemed to be bound to the apical cell membrane, which formed pits or short saccular invaginations at the basal site of the micro-villi. HRP-containing vesicles were pinched off (pinocytosis) from these structures (Fig. 3).

Two hours after oral administration, HRP was less concentrated in the glycocalyx and was present in vesicles and intensely stained multivesicular bodies located in the apical part of the cytoplasm (Fig. 3). Although HRP did not pass through the tight junctions, considerable amounts of HRP could be observed in the intercellular space and in long lamellar infoldings from the lateral and basal plasma membrane (Fig. 3-4) these infoldings, which are probably fenestrated plates when seen in three dimensions, were often oriented parallel to the long axis of the cell and seemed to be strongly branched in the apical part of the cytoplasm. HRP-containing pinocytotic vesicles fused with branches of the infoldings and with multivesicular bodies (Fig. 3). Fusion of HRP-containing vesicles with the lateral cell membrane was never observed. No HRP was found in the subapical and basal parts of the enterocytes, with the exception of some HRP-contaning multivesicular bodies and the infoldings of

FIGURE 3. Enterocytes of the first segment, 2h after oral intubation of HRP. Note the presence of HRP in a pit (P), saccular invagination (SI), vesicles (V), multivesicular bodies (MVB), lamellar infoldings of the plasma membrane (LI) and intercellular space (IS). HRP is absent from the tight junction (TJ). HRP-containing vesicles appear to fuse with branches of the lamellar infoldings and with multivesicular bodies (arrows). Note also the branching of the lamellar infoldings (small arrows). x 35,000.

the plasma membrane. Small amounts of HRP were taken up by leucocytes resembling lymphoid cells (Fig. 5).

After 16h, HRP was seldom present in the apical cytoplasm and intercellular space, although more HRP-containing lysosome-like bodies were found throughout the cell.

Second segment. When HRP was given orally, of the enzyme was taken up by the enterocytes of the second segment (16h). However, after direct administration into the second segment, the absorption of HRP was more pronounced.

The pathway for HRP-absorption in this segment was generally the same as described for the first segment, but the number of HRP-containing pits was considerably larger and as a consequence many HRP-containing vesicles could be found (Fig. 7). Moreover, distinct long saccular invaginations were present in this segment. Small HRP-containing vacuoles were observed in the apical cytoplasm (Fig. 7) but the large supranuclear vacuoles rarely showed peroxidase-activity. Clear HRP containing multivesicular bodies as described in the first segment were scarce in this segment. Again, much HRP was found in the lamellar infoldings of the plasma membrane and in the intercellular space (Fig. 7, 9). Because these infoldings appeared to branch near the tubulo-vesicular network (cf. Noaillac-Depeyre and Gas 1973, 1976), the organization of the apical cytoplasm was rather complicated (Fig. 7). The HRP-containing vesicles and small vacuoles probably fused with branches of the lamellar infoldings (Fig. 7, 9). HRP-containing leucocytes (Fig. 6) were also found in this segment. However, as granulocytes and macrophages exhibited endogenous peroxidase activity, it was difficult to ascertain, whether they had taken up HRP. Finally, there was considerable variation in the

FIGURE 5. A leucocyte resembling a lymphoid cell in the epithelium of the first segment, 2h after oral intubation of HRP. HRP is present in small vesicles (arrows) in the cytoplasm. N nucleus. x 23,000.

FIGURE 6. Part of a macrophage (MØ) in the epithelium of the second segment, 2h after anal intubation of HRP, showing peroxidase activity (HRP?) in small vacuoles (arrows). Note the presence of a membraneous body (MB). x 23,000.

FIGURE 7. Enterocytes of the second segment, 2h after anal intubation of HRP. Most of the HRP is absorbed from the lumen and present in vesicles (V), small vacuoles (SV), tubular-like structures, which probably originate from lamellar infoldings (LI) and in the intercellular space (IS). Note the fusion of vesicles and small vacuoles with these tubular-like structures (arrows). SI saccular invagination. x 35,000.

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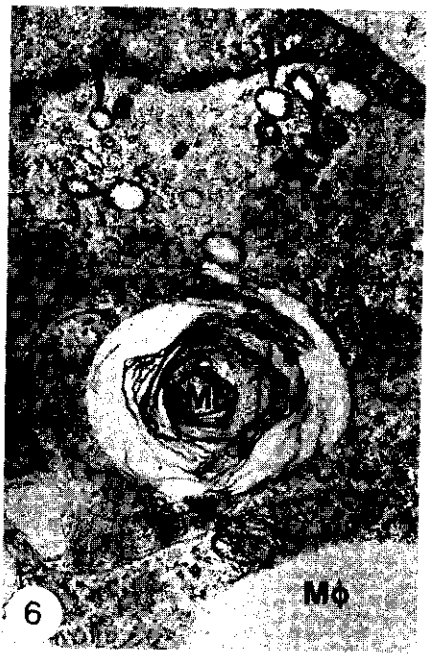

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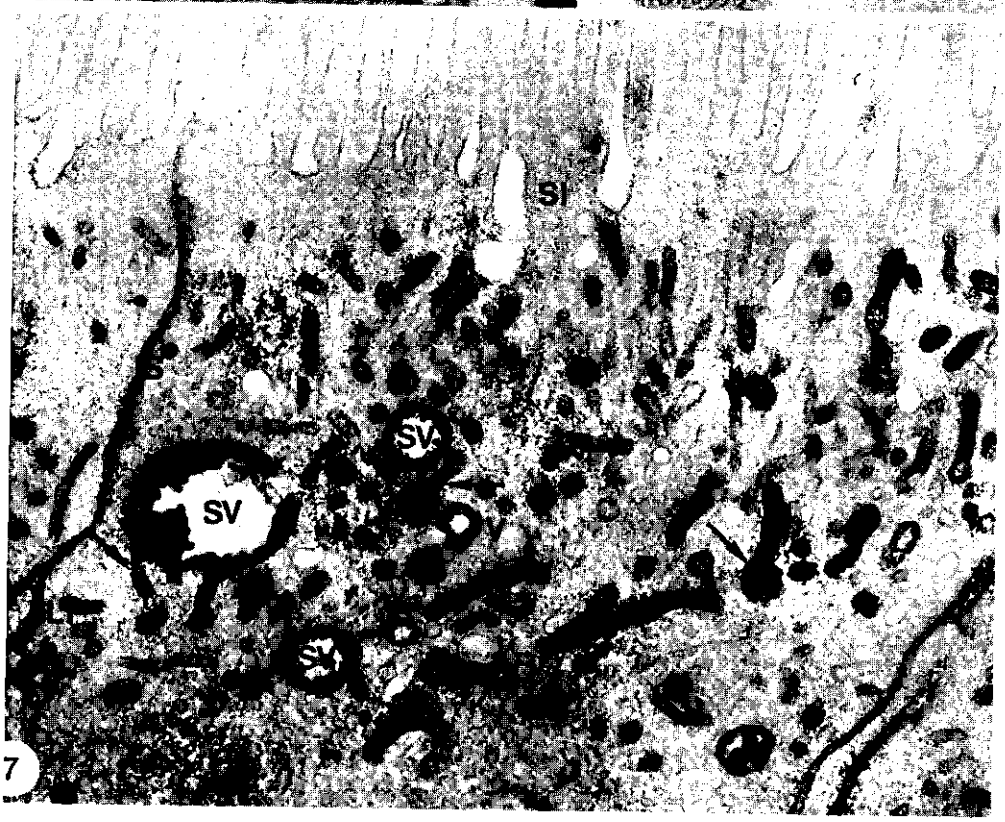

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amount of HRP absorbed by different enterocytes, even in neighbouring cells. Between 2 and 16h after administration, HRP completely disappeared from the cytoplasm, although small amounts were still present in the intercellular space and lamellar infoldings.

Absorption of ferritin

Ferritin was detectable in unstained sections with the electron microscope. This macromolecule was not found in gut or spleen of animals that had not received ferritin. In contrast to HRP-containing pellets, ferritin-containing pellets were readily eaten by carp; consequently information was obtained on antigen-uptake under physiological conditions.

First segment. One hour after administration, ferritin had already been taken up into small vacuoles in the apical part of the cytoplasm (Fig. 10). As these vacuoles frequently contained vesicular or membranous inclusions, they were possibly secondary lysosomes. In carp fed with ferritin-containing pellets, an increasing amount of lipid-containing vacuoles and chylomicrons was found up to 8h after administration. Although ferritin and chylomicrons were usually observed in different vesicles or vacuoles (Fig. 11), a few lipid-containing vacuoles also contained a small amount of ferritin. After 24h, when chylomicrons and lipid-containing vacuoles had almost disappeared, ferritin was still present in lysosome-like bodies

FIGURE 8. Enterocytes of the same part of the gut as shown in Fig. 7; small amounts of HRP are present at the apical cell membrane. Note again the branching of a lamellar infolding (arrow) and a HRP-containing pit (P). x 46,000.

FIGURE 9. Enterocyte of the same part of the gut as shown in Figs. 7 and 8 showing fusion of a small vacuole with a lamellar infolding (arrow). x 35,000.

FIGURE 10. Enterocyte of the first segment, 1h after feeding with ferritin-containing pellets. Ferritin has accumulated in small vacuoles (arrows), that are possible lysosome-like structures. Note the presence of some chylomicrons (C). x 47,000.

FIGURE 11. Enterocyte of the first segment, 8h after feeding with ferritin-containing pellets. Ferritin has accumulated in lysosome-like structures (arrows). Note the presence of many chylomicrons (C) in the endoplasmatic reticulum. L. lipid-containing vacuole. x 46,000.

FIGURE 12. Enterocyte of the first segment, 24h after feeding with ferritin-containing pellets. Chylomicrons have disappeared but ferritin is still present in lysosome-like structures (arrows). L lipid-containing vacuole. x 35,000.


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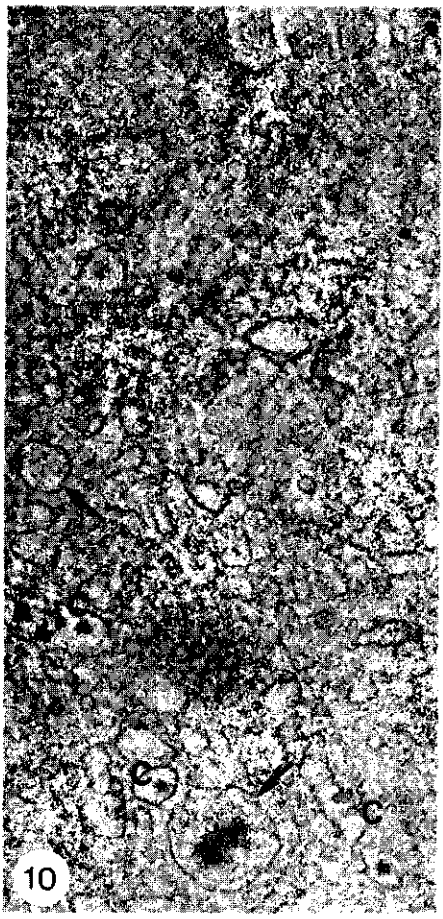
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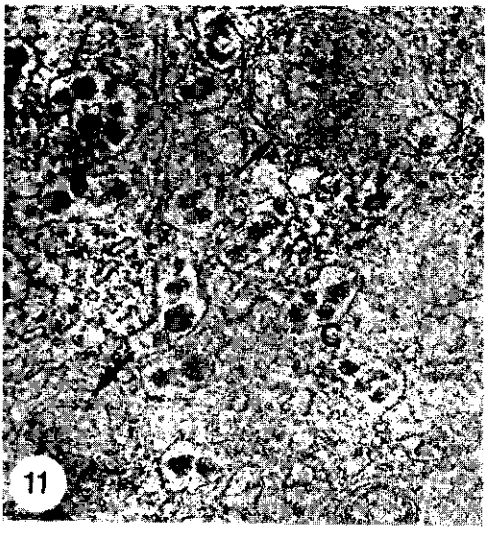
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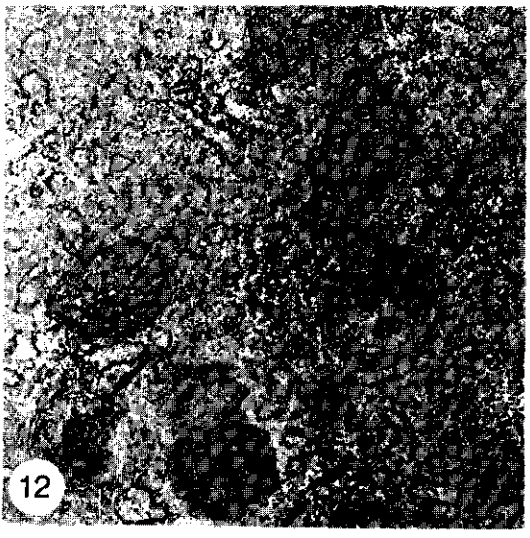
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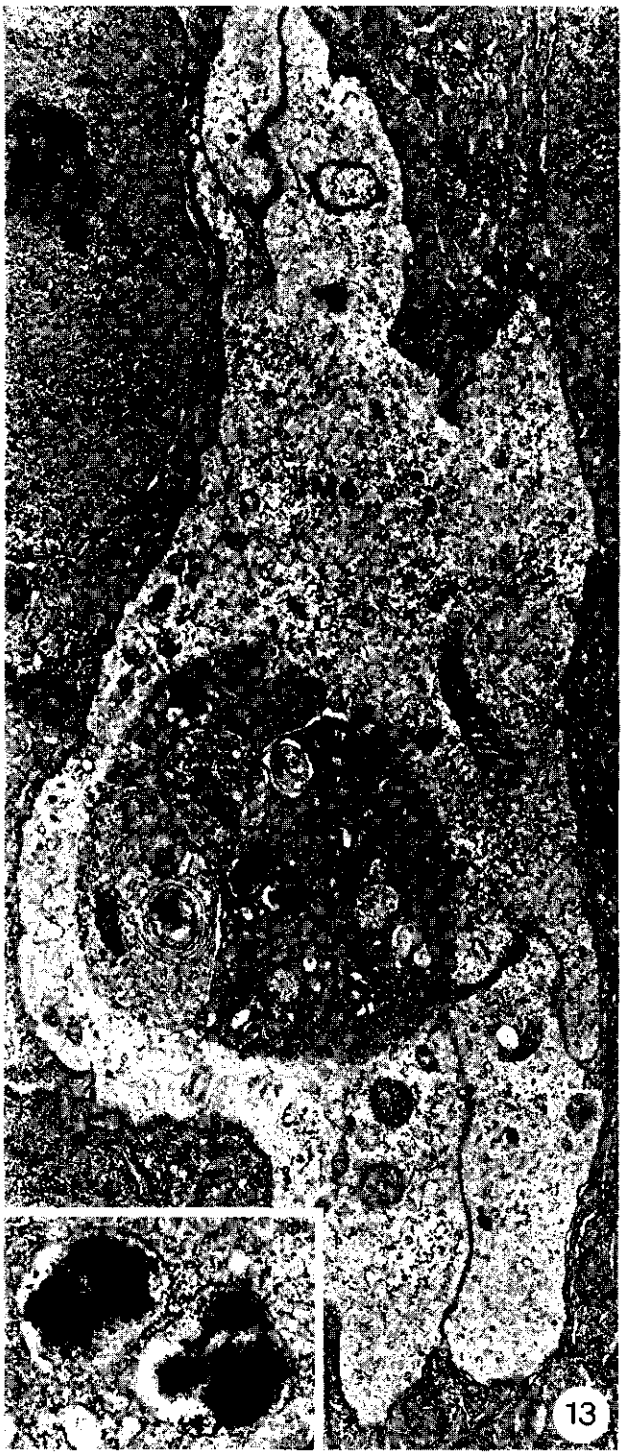
in all parts of the cytoplasm (Fig. 12). Macrophages contained ferritin whereas at that time no ferritin was observed in the intercellular space (Fig. 13). The size of the ferritin-containing vacuoles was similar in enterocytes and macrophages. From 24h, onwards the amount of ferritin present in the enterocytes decreased gradually but was still present 3 days after administration. No ferritin was detected in the intra-epithelial lymphoid cells.

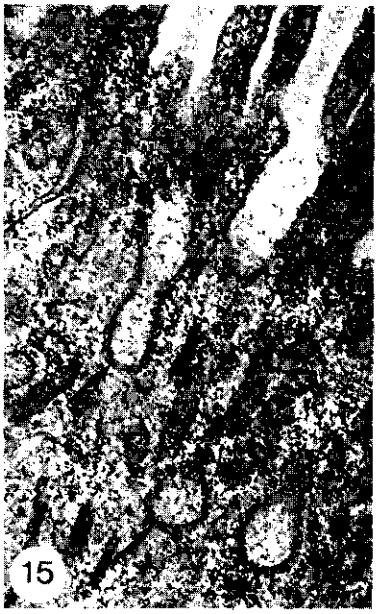
Second segment. A larger amount of ferritin was taken up in this segment, as was the case when ferritin was administered orally. Although ferritin did not adhere to the cell membrane, it could be found in coated pits and vesicles (Fig. 14, 15). During the experiment (Table 1), ferritin seemed to be transported from the lumen through small vesicles and vacuoles towards the supranuclear vacuoles (Fig. 16): in these vacuoles a strong accumulation of ferritin could be observed. After 24h, intact ferritin generally had disappeared from the lumen. However, occasionally still some ferritin-containing cellular material could be found in the lumen; this probably originated from discarded enterocytes or macrophages. After 6 days, small amounts of intact ferritin were still present in the supranuclear vacuoles. In contrast with HRP, no demonstrable release of ferritin into the lamellar infoldings or the intercellular space was observed. Nevertheless, considerable amounts of ferritin were present in large intra-epithelial macrophages (Fig. 16). Most of these macrophages contained large phagosomes with cellular inclusions (e.g. vacuoles) and frequently also membranous bodies (Fig. 6, 16). It is noteworthy that the size of the ferritin-containing vacuoles was similar in enterocytes and macrophages. Smaller ferritin-containing macrophages were found in the lamina propria and around capillaries in the spleen (Fig. 17). Ferritin-containing macrophages represented a minority of the macrophage population of the spleen

FIGURE 13. Macrophage (MØ) in the epithelium of the first segment, 24h after feeding with ferritin-containing pellets x 17,000. Inset: ferritin is found in small vacuoles, present in a large phagosome (P). x 58,000.

FIGURE 14. Enterocyte of the second segment, 8h after oral intubation of ferritin. Note the fluid phase uptake of ferritin (arrows). x 47,000.

FIGURE 15. Enterocyte of the second segment, 4h after anal intubation of ferritin. Note the clathrin coat (arrows) at the cytoplasmic site of pits or vesicles. The ferritin although present is hardly recognisable in this stained section. x 46,000.





and usually contained less ferritin than the gut macrophages. In one carp, which had erroneously received ferritin in the coelomic cavity the majority of macrophages in the spleen contained large amounts of ferritin. Apparently macrophages which have phagocytized antigen in the gut may migrate to the spleen.

DISCUSSION

Absorption of macromolecules

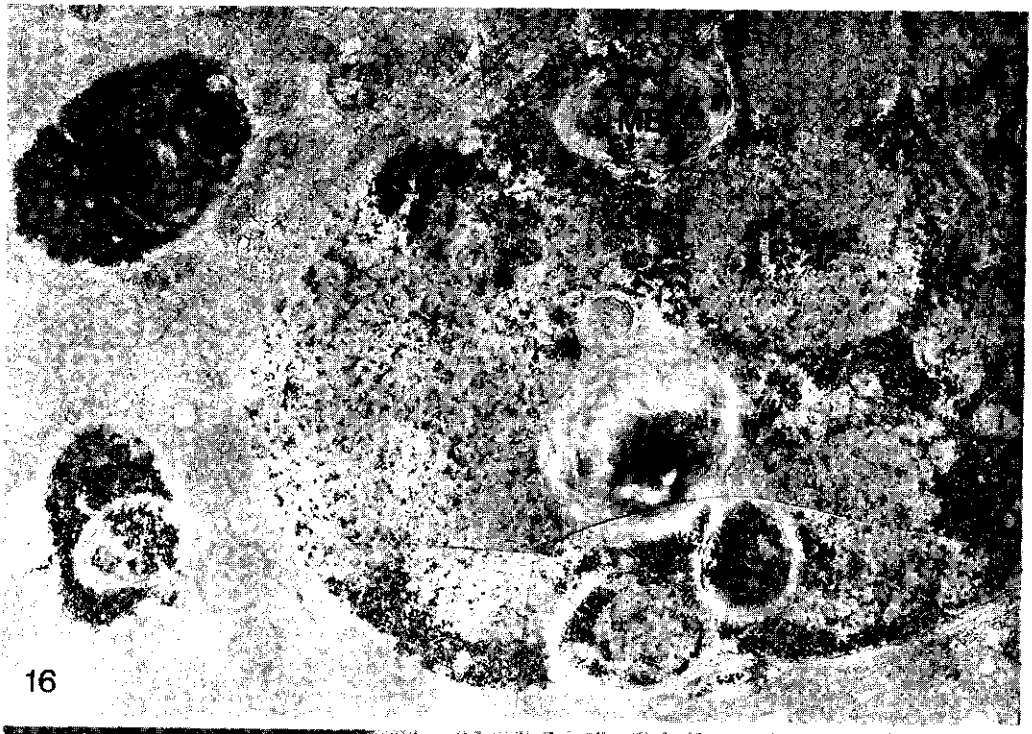
Our study shows that considerable amounts of proteins, such as peroxidase and ferritin, can be absorbed by enterocytes of both the first and second gut segment of carp when administered under physiological conditions. Several authors have described this phenomenon for the distal part of the gut for different adult teleost fish (Gauthier & Landis, 1972; Noaillac-Depeyre & Gas, 1973, 1976; Stroband et al., 1979), but none of them administered the proteins with food. The digestive capacity of the gut in stomachless fish is not sufficient to degrade all proteins and consequently intact proteins can be taken up by pinocytosis.

The present study demonstrates for the first time that intact protein can be taken up by the first segment of the gut of teleosts, although, to a smaller extent than by the second segment. Some of the above mentioned authors have described slight pinocytotic activity in the proximal segment.

The ingestion of intact macromolecules by enterocytes is a common feature in the mammalian small intestine, especially in the intestine of neonates, where it appears to be important for the passive immunization by maternal colostrum (cf. Walker, 1981).

FIGURE 16. Macrophage in the epithelium of the second segment, 24h after anal intubation of ferritin. Note the large phagosome (P) and many ferritin-containing vacuole-like structures. The ferritin-containing supranuclear vacuole (SNV) in the neighbouring enterocyte is of the same size as the vacuole-like structures of the macrophage. A membraneous body (MB) is also loaded with ferritin. x 23,000.

FIGURE 17. Ferritin-containing macrophage (MØ) in the spleen, 24h after anal intubation of ferritin. It lies between an erythrocyte (E) and a granulocyte (G). N nucleus. x 35,000.



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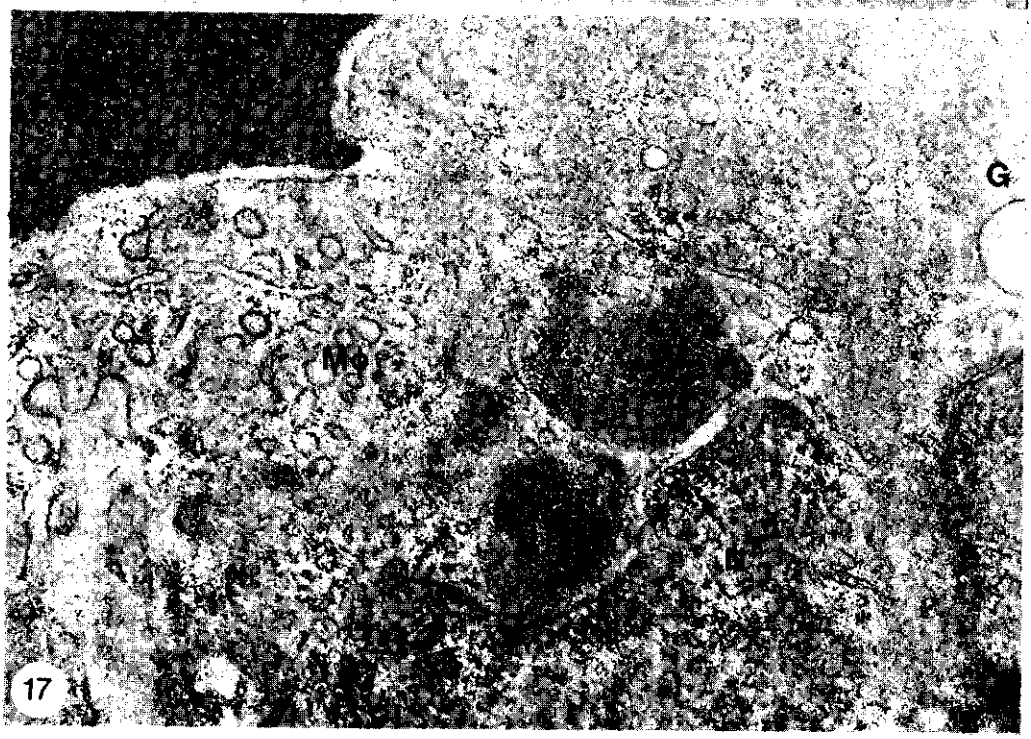

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TABLE 1. Transport of anally[a] administered ferritin through the intestinal epithelium of the second segment with time.

	5'	30'	1h	4h	8h	16h	24h	36h	2d	3d	6d
Intestinal lumen	+	+	+	+	+	-	±	-	-	-	-
Pinocytotic vesicles	+	+	+	+	+	-	-	-	-	-	-
Small vacuoles	±	+	+(+)	++	++	+	+	+	+	-	-
Supranuclear vacuoles	-	-	-	±	+(+)	+	+(+)	+	+	+	-(+)
Macrophages (gut)	-	-	-	-	-	-(+)	+(+)	++	++	+	+
Macrophages (spleen)	nd	nd	nd	nd	nd	-[b]	+	-(+)	-[b]	-(+)	+

nd = not done; -, ±, +, ++: indication for the amount of ferritin present: +(+) variation between animals;

a) oral administered ferritin reached the second segment after 4h

b) only a small part of the spleen is studied

The enterocytes of carp, especially those of the first segment, seem to be adapted for the transport of macromolecules. In the apical cytoplasm, lammellar infoldings of the lateral and basel cell membrane appear to be strongly branched; this is represented diagrammatically in Fig. 18. Lamellar infoldings have been described in enterocytes of several teleost species (Yamamoto, 1966; Noaillac-Depeyre & Gas, 1976; Stroband & Debets, 1978; Ezeasor & Stokoe, 1981; Rombout et al., 1984). Untill now, only Yamamoto (1966) has described these structures as "ribbon-like seets, with fenestrations and finger-like extension which are often accompanied by vesicles near their tip". This definition fits very well with our observations of the fusion of HRP-containing vesicles or vacuoles with branches of the lamellar infoldings. Although many of the authors mentioned have suggested that minarals and water exchange is related to the system of infoldings, an important function of these seems to be the transfer of macromolecules from the apical cytoplasm to the intercellular space. Chylomicrons also appear to be released through this system of infoldings (Noaillac-Depeyre & Gas, 1976; Ezeasor & Stokoe, 1981).

HRP and ferritin are processed differently in both the first and the second gut segment studied. HRP is probably transferred into the cell by formation of coated pits at the basal site of the microvilli and saccular invaginations; these pits pinch off to form coated vesicles. Most of the absorbed HRP appears to be transported through the system of lammellar infoldings to the intercellular space and hence to the blood capillaries in the lamina propria. According to Abrahamson & Rodewald (1981) and King (1982), such selective transfer of macromolecules is dependent on the presence of a clathrin coat on the macromolecule-containing vesicles; this coat protects these vesicles from fusion with lysosomes and is maintained only when receptors present in the cell membrane are occupied. Consequently HRP seems to be bound to receptors present in the apical cell membrane. However, in our study a number of HRP-containing vesicles fuse with multivesicular bodies; therefore it may be concluded that not all HRP-containing vesicles are coated. Whether these HRP-containing vesicles are pinched off without such a coat, or have lost their coat during transport, or both (as suggested by King (1982), studying IgG-transport in rabbit yolk sac endoderm),

remains to be investigated. In an electron microscopical study on HRP-absorption in the second segment of adult cyprinids Gauthier, & Landis (goldfish, 1972) and Noaillac-Depeyre & Gas (carp, 1973; tench, 1976) have shown uptake and transport to the intercellular space. However, they suggested that most of the HRP was transported to the supranuclear vacuoles, although no convincing pictures were shown. In larval or juvenile teleosts Stroband et al. (1979), Stoband & Kroon (1981) and Watanabe (1981, 1982) have described an accumulation of HRP in the supranuclear vacuoles of the second segment. The present study does not demonstrated much HRP in the supranuclear vacuoles in older animals. Preliminary studies in our department have shown that in larval carp HRP is transported to the supranuclear vacuoles and not to the intercellular space. From these data it may be assumed that the extent of selective transport of HRP across the intestinal epithelium increases with age. The contrasting results obtained by other authors may thus be explained by the use of animals of different ages rather than by differences between species.

Ferritin appears to be absorbed unselectively in both segments of carp intestine. Specific receptors for this macromolecule are apparently absent in the cell membrane of carp enterocytes. Ferritin seems to be taken up by fluid phase endocytosis and is accumulated in small vesicles or vacuoles that fuse with lysosome-like bodies. In the second segment, ferritin finally reaches the large supranuclear vacuoles. This unselective method of absorption is well known in transporting epithelia of mammals (Rodewald, 1973; Wild, 1976; Walker, 1981; Abrahamson & Rodewald, 1981; King, 1982); these authors suggest that the macromolecules are degraded in phagolysosomes (secondary lysosomes or dense bodies). Our results are comparable with those of Graney (1968), who has found unselective uptake of ferritin in the ileal absorptive cells of suckling rats; it appears in small vacuoles and finally in the large supranuclear vacuole that is only present in the neonatal stage. The absorbed macromolecules are supposed to be digested by strong lysosomal activity in this supranuclear vacuole. In all larval teleosts (stomachless) and in adult cyprinids, intracellular digestion of macromolecules is supposed to occur in the supranuclear vacuoles of the enterocytes of the second segment (Noaillac-Depeyre & Gas, 1973, 1976; Stroband et

al., 1979; Ezeasor & Stokoe, 1981; Watanabe, 1981, 1982). This is supported by the demonstration of intensive acid phosphatase activity in the supranuclear area of the enterocytes of the second segment (Gauthier & Landis, 1972). It is thus remarkable that we can find intact ferritin 6 days after its administration, whereas it disappears from the lumen during the first day. The same phenomenon was observed by Watanabe (1982) who has found that HRP is present in the supranuclear vacuoles of several larval teleosts as long as 15 days after administration. This slow rate of degradation indicates that the intracellular digestion of macromolecules is not the main function of the second segment.

The present study demonstrates for the first time the existence of two distinct pathways for macromolecular transport in the intestinal epithelium of teleosts. Similar distinct routes have been described for the proximal intestine of newborn mammals, in which selective transmission of IgG and unselective absorption of other undegraded proteins takes place (Waldman & Jones, 1976; Abrahamson & Rodewald, 1981; Telemo et al., 1982). Figure 18 represents our hypothesis for the transport of HRP and ferritin in the intestinal epithelium of carp.

Immunological relevance of absorbed macromolecules (antigens)

The second gut segment of cyprinids may not have an important quantitative function in protein absorption, as protein appears to be absorbed with a high efficiency (80%) in the first segment (Shcherbina et al., 1976; Stroband & van der Veen, 1981), and is digested rather slowly in the enterocytes of the second segment. The present study demonstrates that intact macromolecules can reach the second segment under physiological conditions. They are absorbed in considerable amounts and transferred either to the intercellular space (HRP) or to macrophages (ferritin). Although enterocytes of the second segment show a much stronger absorptive capacity than enterocytes of the first segment, the present study povides no evidence for augmented lysosomal activity in these enterocytes. Enterocytes of the second segment have may functions similar to mammalian M cells, which seem to be specially adapted for antigen transport because of their strong absorption capacity and paucity of lysosomes, glycocalyx and microvilli (Owen & Jones, 1974; Owen, 1977;

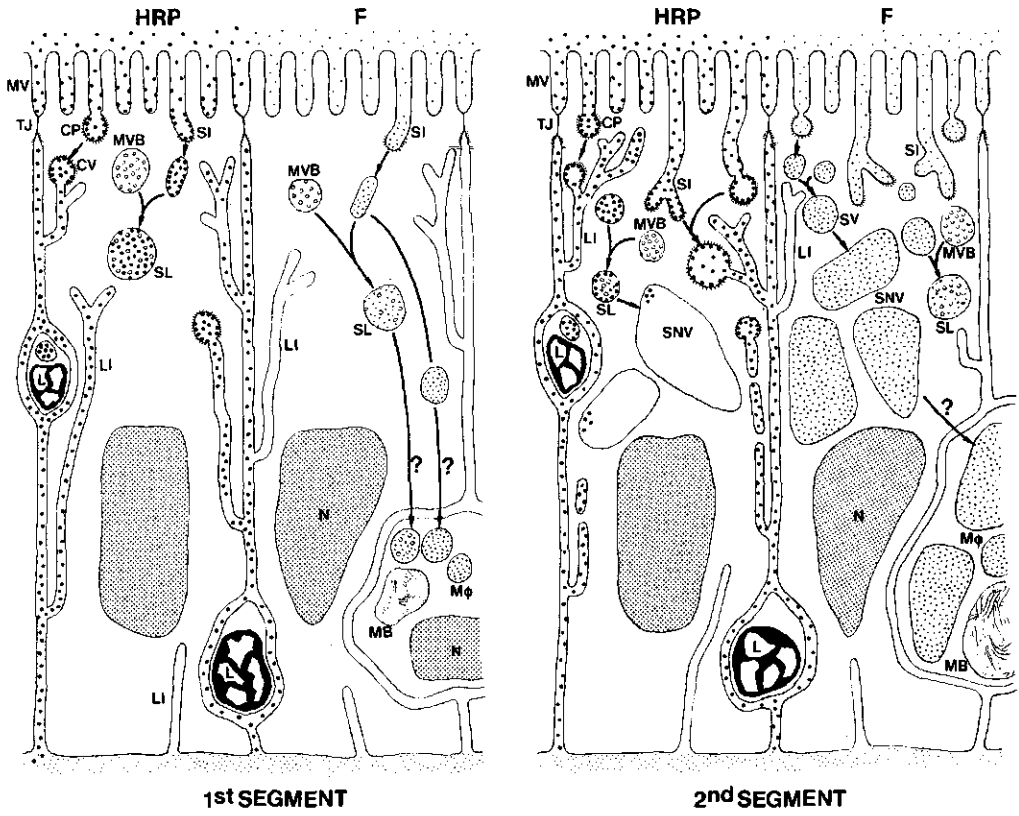

FIGURE 18. Schematic visualization of the uptake of HRP and ferritin (F) in the first and second segment of the gut of carp, based on data from Wild (1976); King (1982) and the present study. Two mechanisms can be suggested for the transfer of membrane bound HRP: 1. internalization via coated pits (CP) and coated vesicles (CV) and their fusion with lamellar infoldings (LI); 2. detachment of saccular invaginations (SI) from the surface, loss of the clathrin coat and fusion with multivesicular bodies (MVB) resulting in secondary lysosomes (SL). The fluid phase uptake of ferritin results in the accumulation of ferritin in secondary lysosomes and, in the second segment also in small vacuoles (SV) and supranuclear vacuoles (SNV). How ferritin finally ends in macrophages (MØ) is at present unknown. Note the branching of the lamellar infoldings in the apical part of the cytoplasm. L. leucocyte resembling a lymphoid cell, MB membraneous body, MV microvilli, N nucleus, TJ tight junction.

Walker, 1981). Owen (1977) has demonstrated the uptake of HRP by M cells and the transfer of this macromolecule to the intercellular space, from which it is transported to the underlying lymphoid tissue. In contrast to mammals, no lymphoid accumulation occurs in the

intestine of fish, but lymphoid cells are widely distributed in the epithelium and lamina propria throughout the intestine. Transfer of ingested HRP to leucocytes is also found in our experiments. However, selective HRP-uptake by specific receptors at the apical cell membrane of enterocytes is probably not the usual method. Two theories can be proposed to explain the selective HRP-uptake:

1. HRP resembles a protein (e.g. a carp digestive enzyme) that is normally present in the chyme and that is specifically reabsorbed; the process of reabsorption in fish of their own digestive enzymes has been suggested by Hofer (1982).
2. An HRP-like antigen has frequently been present in the food of our carp, leading to a state, unresponsiveness (tolerance). This phenomenon has been reported in mammals by several authors (cf. Mowat et al., 1982); in this state of tolerance suppressor T cells induced in the GALT seem to play an important role (Mattingly & Waksman, 1978; Ngan & Kind, 1978; Miller & Hanson, 1979).

During the acquisition of tolerance, specific receptors for food antigens may be introduced at the apical cell membrane of enterocytes; however, this remains to be investigated. This second theory is supported by the observation that the unselective uptake of HRP in larvae and juvenile fish changes into a selective uptake in adults (discussed earlier) and further by the increased absorption of ferritin in the intestine of sensitized hamsters (Bockman & Winborn, 1966). The second theory is very speculative and thus the first hypothesis is preferred.

The fluid phase uptake of ferritin is probably the more common route of antigen-uptake. It is therefore likely that most antigens are absorbed at random and transferred to vesicles or vacuoles that may fuse with lysosomes (often multi-vesicular bodies). In the second segment, they accumulate in the supranuclear vacuoles, where they appear to be degraded slowly. Some antigen reach macrophages present between the epithelial cells or in the lamina propria. Gas (1976) has described the transfer of a fraction of the ingested ferritin (she administered much larger amounts than were orally administered here) to the intercellular space of the second segment of carp. However, we have not been able to demonstrate any intact ferritin in the intercellular space and therefore consider it unlikely

that ferritin molecules are taken up from this space. Whole vacuoles or even larger parts of enterocytes may be pushed out and phagocytized by macrophages. Although this process has never been observed, the cellular inclusions and the size of the ferritin-containing vacuoles observed in the macrophages strongly support this supposition. In addition, the large macrophages that are predominant in the intestinal epithelium of the second segment frequently contain multi-membranous bodies. According to Agius & Agbede (1984), such bodies are frequently present in melano-macrophages, a type of cell that is common in haemopoietic tissue of fish. These authors have claimed that the multi-membranous bodies contain the pigment lipofuscin, a metabolic product derived from the degeneration of cellular components containing a high level of unsaturated fatty acids. The macrophages of the second segment resemble "tingible body" macrophages of mammals; these are found among others in Peyer's patches and are characterized by vacuoles containing cellular components (Bockman, 1981; Lause & Bockman, 1981). These authors have demonstrated that macrophages present in the epithelium overlying the Peyer's patches take up ferritin when it is introduced into the lumen and transferred to the intercellular space via the M cells. The important role of macrophages as antigen-presenting cells in the immune system is generally accepted (cf. Unanue, 1975; Varesio et al., 1980). Macrophages derade most of the phagocytized antigens but a small amount remains uncatabolized or is only partially degraded. These antigens may be displayed at the macrophage surface in order to interact with lymphocytes. Consequently, the transport of antigen in carp from the intestinal lumen, through the enterocytes (especially in the second segment), to the intra-epithelial macrophages, some of which appear to migrate to the spleen, may be of immunological importance. This hypothesis is supported by several studies showing that oral vaccination of fish can result in a certain level of protection (Ross & Klontz, 1965; Anderson & Ross, 1972; Prescott, 1977; Anders, 1978; Johnson & Amend, 1983; cf. Paterson, 1981). Moreover Davina et al. (1980, 1982) have described an increase in the number of intra-epithelial leukocytes, especially in the second segment, after the oral administration of *Vibro*-bacterin to carp and rosy barb (*Barbus conchonius*). In addition, *Vibrio*-immunoreactivity has been demonstrated in the supranuclear

vacuoles and intra-epithelial macrophages; this supports our observations.

In mammals the transfer of antigens through enterocytes to lymphoid tissue induces a local immune response, resulting in the production of secretory immunoglobulin (sIgA; cf. Walker, 1981). Some evidence is available for the presence of a local (secretory) immune system in fish; (secretory IgM has been demonstrated in skin mucus (Fletcher & Grant, 1969; Bradshaw et al., 1971; Ourth, 1980; Lobb & Clem, 1981a), gut mucus (Fletcher & White, 1973; Lamers, unpublished) and bile (Lobb & Clem, 1981b). Fletcher & White (1973) have detected in plaice (*Pleuronectes platessa*), provided with food containing heat-killed *Vibrio anguillarum*, a higher level of specific immunoglobulin in intestinal mucus than in serum. In addition, Lamers et al. (1985) have found that in carp immunized by bathing, a secondary immune response could only be demonstrated after a mucosal booster (bath) and not after boosting by injection. Moreover, Noaillac-Depeyre & Gas (1979) and Zapata (1979) have described plasma cells in or below the intestinal epithelium of fish. These results, together with the earlier discussed functional resemblance of the enterocytes of the second segment to the M cells of mammals, indicate the existence of a local immune system in the intestine of teleosts. However, in contrast to mammals IgM is secreted in mucus of fish, but it also appears to be secreted at the mucosal surface in IgA-deficient mammals (cf. Walker, 1981).

In conclusion, the present study, combined with the results of others, suggests the existence of a local immune system in the intestine of teleosts, although more data are necessary to support this supposition. Physiologically administered antigens reach macrophages, which can present them intact to lymphoid cells, probably resulting in a (local) immune response. As most of the antigens are transmitted in the second segment, this part of the gut may have an important immunological function.

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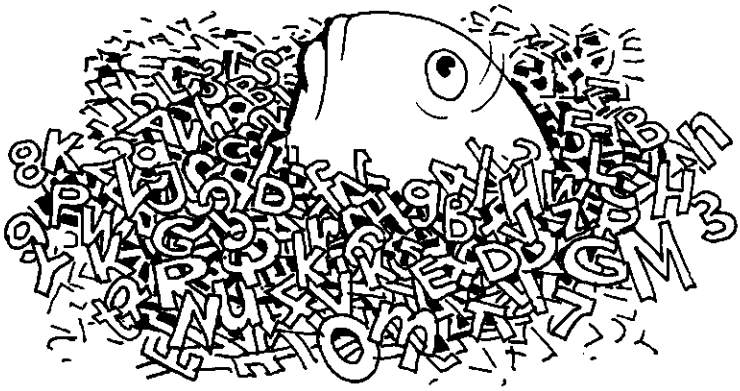

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