Immunological aspects of oral vaccination in fish

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Abstract

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In this thesis immunological consequences of oral vaccination in fish have been described. The efficacy of oral vaccination can be increased by protection of the antigen against degradation in the foregut, in order to reach the hindgut in sufficient quantities for uptake and subsequent activation of the mucosal and systemic immune system. Using a specific monoclonal antibody, in addition to mucosal B cells, a distinct mucosal T cell population was demonstrated, which may play an important role in local immunity. Furthermore, two approaches to protect antigens against digestive degradation are described: bioencapsulation and microencapsulation. For the first approach antigen is encapsulated in living food, and subsequently fed to juvenile carp and seabream. In carp, oral vaccination at 2 and 4 weeks old resulted in immunological tolerance. However, in older carp (8 weeks old) and seabream (8 and 10 weeks old), immunological memory was induced. It can be concluded that oral vaccination with bioencapsulated bacterial antigens is effective for oral vaccination of juvenile fish, when applied at the right age. For microencapsulation an alginate microparticle system was studied, which may be more suitable for vaccination of older fish. The supernatant appeared to be the most immunogenic fraction of a bacterin, which is taken up in the hindgut and evokes best memory formation. This fraction was encapsulated in alginate microparticles and fed to adult carp and trout. Different microparticle preparations, with respect to release time and antigen concentration, were needed for immunological memory formation in each fish species. Therefore, oral vaccination with bacterial antigens in alginate microparticles can be effective. Oral tolerance against protein antigens was demonstrated in animals fed with ferritin or recombinant VHS G protein. However, the immune response to ovalbumin appeared to be carp strain dependent. A carp strain that produced specific antibodies after injection with OVA was selected and repeated feeding of OVA, prior to injection, resulted in increased antibody titres in serum. Oral tolerance induction in fish therefore appeared to depend on the protein and possibly also on genetic factors.

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Stellingen

1. Orale toediening van bacteriële vaccins, als deze beschermd worden tegen afbraak in de darm, resulteert in immunologisch geheugen en is daarom veelbelovend voor orale vaccinatie.
   Dit proefschrift.

2. Er zijn voldoende aanwijzingen om aan te nemen dat vissen over een mucosaal immuunsysteem beschikken.
   Dit proefschrift.

3. Wanneer bescherming van vissen tegen een ziekteverwekker gebaseerd is op mucosale afweerreacties, zal orale vaccinatie zinvoller zijn dan toediening via injectie.
   Dit proefschrift.

4. Gezien de grote verschillen tussen vissoorten moet men bij het doen en interpreteren van uitspraken over vissen in het algemeen, op basis van gegevens van één vissoort, de nodige voorzichtigheid in acht nemen.

5. Zoals in de eerste periode van het leven immunologische tolerantie kan ontstaan, zou ook de aanwezigheid van allochtonen in de leefomgeving op jonge leeftijd kunnen leiden tot tolerantie door herkenning als eigen.

6. Met het oog op de steeds lagere financiële waardering van jonge onderzoekers door universiteiten, zal ook hier gelden: "Wie peanuts betaalt, krijgt apen in dienst."

7. Uitbreiding van varkenspest kan beter voorkomen worden door vaccinatie van de varkensstapel dan door te trachten het virus te elimineren.

8. Met steeds kortere studieduur en promotiefase heeft de toekomstige academicus ook minder tijd voor de ontwikkeling van maatschappij-kritisch denken, waarmee het maken van de stellingen bij het proefschrift beter afgeschaft kan worden.

9. Drukfouten ontdekken is niet moeilijk, voor het ontdekken van denkfouten is meer nodig.

10. Italiaans ijs is eigenlijk geen italiaans ijs als het beste italiaanse ijs in Nederland gemaakt wordt.

Stellingen behorende bij het proefschrift
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Contents

Chapter 1

General introduction

General introduction

1 The immune system of fish.

Fish, like other 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 material recognized as non-self, including pathogens and malignant cells. Physical (e.g. mucosal surfaces) and chemical barriers (e.g. lysozyme) prevent penetration of the pathogen into the host. The immune system can be divided in innate (non-specific) and adaptive (specific) immunity. The innate immune system includes phagocytosis, natural killer cell activity by non-specific cytotoxic cells (NCC) and inflammatory reactions as well as soluble substances, i.e. CRP (C-reactive protein), interferon, transferrin, lysozyme, lectins and complement. These processes are more or less inducible upon infection and should eliminate the pathogen immediately after penetration. Non-specific responses do not result in immunological memory (Lamers, 1985; Manning, 1994). When the non-specific defence does not prevent invasion by the pathogen the adaptive immune system is activated. Adaptive immunity is based on two properties: specificity and memory, and can be divided in humoral and cell mediated immunity.

1.1 Humoral immune response

The humoral immune response involves antigen presenting cells (APC), T and B lymphocytes. T lymphocytes develop in the thymus. B lymphocytes originate from the bursa of Fabricius in birds, bone marrow in mammals and in teleosts probably from the kidney (Zapata, 1979; Kaattari, 1992). Exposure to an antigen results in the stimulation of a small proportion of lymphocytes, which possess specific receptors for this antigen. Micro-organisms have many different antigens on their surface and each antigen can be recognized by a different clone of lymphocytes. Fish have a polyclonal response against a particular antigen, but often recognize less epitopes than mammals, resulting in a limited number of different antibodies (Wilson & Warr, 1992; Manning, 1994). T cells can bind to processed antigen which is bound to molecules of the major histocompatibility complex (MHC) by APC. B lymphocytes will become activated after exposure to an antigen, with the help of cytokine producing T lymphocytes. This results in proliferation and differentiation of the stimulated B lymphocytes into plasma cells or into memory B cells. Plasma cells will start to produce specific antibodies against the antigen. At the same time, T lymphocytes will proliferate and produce effector and memory T cells. After a second activation by the same antigen memory B cells differentiate in antibody producing cells, with the help of increased numbers of memory T cells. This leads to a faster and enhanced response at second contact, due to higher numbers of antibody producing cells (Miller & Clem, 1984; Arkoosh & Kaattari, 1991; Kaattari, 1992; 1994). In fish, a 10 to 50 fold

improvement over the primary response has been described, in mammals a 100 fold increase or more is possible (Lamers, 1985; Manning, 1994). In mammals, upon secondary activation B lymphocytes produce antibodies with a higher affinity, and in general of a different immunoglobulin class. This affinity maturation is not observed in fish (Arkoosh & Kaattari, 1991; Kaattari, 1992). A switch from IgM to a low molecular weight antibody class (IgG in mammals), observed in higher vertebrates after a second contact with the antigen, is also not found in fish (Kaattari, 1992). The immunoglobulin of fish appears to be a tetrameric molecule with a molecular weight similar to mammalian IgM, although low molecular weight Igs have also been found (Lobb & Clem, 1981; Wilson & Warr, 1992; Manning, 1994; Warr, 1995). Structural variants have been shown to occur in different fish species (Wilson & Warr, 1992) and at different anatomical sites within a fish (skin mucus, gut mucus, bile). Although teleosts lack the characteristic switch to low molecular weight antibody and affinity maturation for memory in mammals, fish clearly give a significantly elevated and faster antibody response upon second or repeated challenge with a given antigen (Arkoosh & Kaattari, 1991; Wilson & Warr, 1992; Kaattari, 1994).

1.2 Cell mediated immune response

The specific cell-mediated immune response involves T cells. These cells can act as regulatory (helper) or cytotoxic T cells. Cytotoxic T cells lyse the recognized target cell through direct cell contact, whereas helper T cells secrete soluble mediators (cytokines) that influence cytotoxic T cells and B cells in their function. In fish this T cell population has only functionally been described to react as mammalian T cells. Functional T cells can be demonstrated by Mixed Lymphocyte Reaction (Caspi & Avtalion, 1984; Kaattari & Holland, 1990), Delayed Type Hypersensitivity (Stevenson & Raymond, 1990) or transplantation immunity (Rijkers & van Muiswinkel, 1977). Furthermore, stimulation with PHA or Con A, mammalian T cell stimulators, results in proliferation (Etlinger *et al.*, 1976). Cooperation between T helper and B cells was demonstrated by hapten carrier assays (Yocum *et al.*, 1975; Ellis, 1982).

Fish T and B lymphocytes can not be distinguished by their morphology. Markers for the T cell population in fish are scarce (Manning, 1994). However, monoclonal antibodies (mAbs) which are reactive with IgM, have been developed for different fish species to distinguish IgM^+ lymphocytes (B cells and plasma cells) from IgM^- lymphocytes (DeLuca *et al.*, 1983; Secombes *et al.*, 1983; Sizemore *et al.*, 1984). Not all IgM^- lymphoid cells can be defined as T cells, because in fish thrombocytes, monocytes and NCC can not be distinguished from lymphocytes. Subpopulations of T cells, like mammalian $CD4^+$ and $CD8^+$ T lymphocytes, with distinct helper and cytotoxic functions, have not yet been identified in fish. However, the presence of

MHC class I and class II genes/ molecules (Stet *et al.*, 1996) strongly suggests the existence of these T cell subpopulations. Some recent reports describe mAbs reactive with T cell (sub)populations. In seabass a mAb (DLT15) is described reactive with the majority of thymocytes and some cells in blood, head kidney and spleen (Scapigliati *et al.*, 1995). Also a mAb (WCL9) reactive with a subpopulation of carp thymocytes was produced. It is suggested to be a marker for early thymocytes present in the cortical region of the thymus (Rombout *et al.*, in press). For channel catfish, a mAb against IgM^- cells was described, and immunopositive cells showed a better responses to Con A than to LPS. However, further characterization was not performed (Ainsworth *et al.*, 1990). In catfish a mAb was produced reactive with a T cell antigen, which was expressed on most thymocytes and a subpopulation of the lymphoid cells (T cells) in blood and other lymphoid organs. The monoclonal was not reactive with erythrocytes, thrombocytes, myeloid cells, B cells and macrophage cell lines (Passer *et al.*, 1996). Both the α and ß chain of the T cell receptor of rainbow trout have been isolated and characterized recently (Partula *et al.*, 1995; 1996). This may lead to a full characterization of the T cell receptor molecule, which will give possibilities for the recognition and isolation of T lymphocytes.

2 Vaccination of fish

With intensification of aquaculture, disease problems in fish are growing, causing considerable economic losses (Leong & Fryer, 1993). Due to crowding, monoculture conditions, handling and transport, fish in aquaculture are more susceptible to diseases (Newman, 1993). Once a disease outbreak occurs it will spread fast, because of the easy transmittance in an aqueous environment. Therefore control of fish diseases is of great concern in aquaculture (Dunn *et al.*, 1990) and can be achieved by different methods, including exclusion, chemotherapy, genetic selection of stress and disease resistant strains and vaccination (Newman, 1993). Exclusion of the causative agent from certain areas can be achieved by a legislative policy (Ellis, 1988). Antibiotics can be used to eliminate bacteria before or during disease outbreak. However, when administered on a regular basis, bacteria can become resistant to antibiotics. In addition, antibiotics can accumulate in fish and can have a very long half life (Bernoth, 1991). Chemicals have to be administered with great care, because the difference between a therapeutic and toxic dose is usually small (Ward, 1982). Moreover, a growing concern about the possible effects of the use of large quantities of antibacterial drugs in aquaculture on the environment, public health and target animals is observed (Michel & Alderman, 1992) and reduction of the use of chemicals and drugs is desired. Selection of strains that are genetically more resistant to disease

can be an alternative (Wiegertjes, 1995). Prevention of disease by vaccination will reduce the need for antibacterial drugs and is much more desirable than intervention to stop and reverse disease processes once they have started (Newman, 1993).

2.1 Principle of vaccination

Experiments on vaccination as prevention against disease were first described in the 19th century by Edward Jenner. It was known that animals developed resistance to a disease once they had survived an infection. The resistance seemed to be specific for a pathogen and persisted for a long period. At that time, the phenomenon was not understood but vaccination was successful (Martin, 1994). Nowadays, it is known that the principle of vaccination is based on memory formation by the immune system. Vaccines are preparations of antigens derived from pathogenic organisms, rendered non-pathogenic by various means, which will stimulate the immune system in such a way that the animal becomes more resistant to a specific disease from subsequent infection by the pathogen (Ellis, 1988). Using modern molecular technology, only the immunogenic part of a pathogen (epitope) can be produced and recombinant vaccines are being developed (Leong & Fryer, 1993). This may increase the efficacy and safety of a vaccine. These recombinant proteins or synthetic peptides can be produced in large quantities by recombinant DNA technology (Dunn *et al.*, 1990). After a first contact the animal will respond more adequate to an infectious agent because the immune system recognizes it immediately and mount a higher and faster response. Although vaccination is widely used in human and livestock, the first attempt to vaccinate fish was reported relative recently (Duff, 1942). From that time knowledge on the immune system of fish has improved considerably (van Muiswinkel, 1995), which is important for a better approach of fish vaccine development. Furthermore, different methods for mass vaccination of fish have been under development (Ellis, 1988).

2.2 Factors affecting the development of protective immunity

The development of a protective immune response upon vaccination can be influenced by different factors. *Temperature* is known to have an effect on induction of immunity. Immune responses in ectothermic animals should function over a wide range of environmental temperatures in order to protect adequately against infectious diseases. Higher environmental temperatures, within the physiological range, usually enhance immune responses, i.e. the production of antibody is faster and of a higher magnitude than at lower temperatures (Rijkers *et al.*, 1980a; Ellis, 1982; Bly & Clem, 1992). In general, low environmental temperatures tend to inhibit immune responses (Avtalion, 1981; Bly & Clem, 1992) and/or development of protective immunity is

slowed down (Horne & Ellis, 1988). Especially helper T cells functions are influenced by low temperatures (Whiskovsky & Avtalion, 1982; Bly & Clem, 1992). It has been reported that responses to T independent antigens were not inhibited at lower temperatures (Azzolina, 1978; Bly & Clem, 1992). It seems that mature T cell functions are not suppressed, but the generation/activation of virgin T helper cells may be inhibited rather specifically (Bly & Clem, 1992). It is still not clear, whether it is possible to vaccinate fish at low temperatures, even when a much longer time is taken in account to build up a significant level of immunity (Horne & Ellis, 1988; Lillehaug *et al.*, 1993) or that immunosuppression is induced during vaccination at lower temperatures, resulting in higher susceptibility to a disease (Wishkovsky & Avtalion, 1982; 1987). However, the onset of infectious diseases has often been related to changes in environmental temperature. A cause and effect relationship between low temperature and *in vivo* immune competence is frequently inferred, but not proven (Bly & Clem, 1992).

The *route of antigen administration* is important for the magnitude of the primary and secondary response. The route of exposure has a direct influence on the level and type (i.e. sites) of protective immunity that develops. Best immune responses seem to develop when the vaccination and booster are given by the same route (Lamers, 1985). Oral and immersion vaccination may result in local immune responses, which will only be protective when the second contact occurs via the same route as the first (Ellis, 1988; Estevez *et al.*, 1994). The amount of antigen finally reaching the systemic immune system by the different routes, may have an effect on the development of circulating antibodies (Pyle & Dawe, 1985).

The *dose of antigen* seems to influence the development of memory. Primary agglutination titres increased exponentially with an increasing dose of intramuscularly injected bacteria (Lamers, 1985; Pyle & Dawe, 1985). However, high doses which are optimal for primary responses, might be suboptimal for induction of immunological memory, which seems to be induced better at lower doses (Rijkers *et al.*, 1980b); best memory formation was achieved with equal priming and booster doses (Lamers, 1985).

Moreover, not all antigens are effective immunogens in fish. The *form of the antigen* can have an effect on the outcome of immunisation. Pyle & Dawe (1985) compared immune responses in channel catfish against a soluble antigen derived from *Tetrahymena pyriformis*, with a particulate bacterial antigen (formalin killed bacterin, *Brucella*). Higher immune responses were induced with the particulate antigen, independent of the route of administration. Rombout *et al.* (1989c) describes different primary and secondary serum immune responses against particulate (*V. anguillarum*) and soluble antigen (ferritin). Higher serum antibody levels were found for ferritin.

However, secondary responses were observed with bacteria, and not with soluble antigen. It is discussed that immunosuppression may be due to the dose of ferritin or to differences between particulate and soluble antigen. Mughal *et al.* (1986) found that in young carp (4 weeks old) immunosuppression was induced after injection of soluble as well as particulate HGG (human gamma globulin). However, an immune response was detected after immunisation with *Aeromonas salmonicida*.

The use of *adjuvants/immunostimulants* can also influence the outcome of vaccination. Immunostimulants can elevate the non-specific defense mechanisms or the specific immune responses. They can, for example, act as reservoirs and depots for the antigen or induce inflammation at the site of antigen entry. However, although most mechanisms remain speculative, immunostimulants may allow a lower dose of vaccine (Anderson, 1992).

Some *antibacterial drugs* have been shown to modulate the immunological defence mechanisms (Anderson *et al.*, 1984; Grondel *et al.*, 1985; Siwicki *et al.*, 1994; Van der Heijden, 1995; 1996), resulting in immunostimulation or immunosuppression.

Furthermore, the state of *immune maturation* of the fish at the time of vaccination may be important. It is difficult to define when maturation of the fish immune system has been achieved. The earliest age that fish can be vaccinated seems to depend on the fish species and the vaccine used. When very young fish are exposed to antigen, immune tolerance can be induced (van Muiswinkel, 1985; Mughal *et al.*, 1986; Ellis, 1988). The development of protective immunity has been correlated with the existence of a critical number of lymphocytes. Although lymphocytes are present early in development their functional maturation takes more time before they are capable of executing an immune response. The ability to produce antibodies to thymus-independent antigens develops earlier than to thymus-dependent antigens (Mughal *et al.*, 1986). It seems that B cells and cytotoxic T cells are functionally mature at an earlier age than T helper and memory cells (Ellis, 1988).

In addition, *stressors* appears to suppress immune functions. It is often reported that a disease outbreak occurs after stressful events, such as transport, vaccination, handling or crowding (Barton & Iwama, 1990). The increased secretion of cortisol caused by stressful events is thought to inhibit the immune response in fish (Wendelaar Bonga, 1997). The mechanism of action for stress-induced reduction in disease resistance in fish appears to be a suppressive effect on both numbers and function of leucocytes (Barton & Iwama, 1990; Ottaviani & Franceschi, 1996).

2.3 Vaccination methods in fish

In principle, three different methods for vaccination of fish are used in aquaculture: injection, immersion and oral delivery.

2.3.1 Injection vaccination

Injection has proven the most effective method for vaccination of fish. Often, good protection is developed after injection of a vaccine. Furthermore, it allows the use of adjuvants which can enhance the development of protective immunity. However, for injection fish have to be handled and anaesthetized. Injection of fish is therefore very labour intensive and it can be stressful to fish (Espelid *et al.*, 1996), which may result in decreased resistance and in lesions decreasing the economic value of the fish, especially when an adjuvant is used. However, since the introduction of injecting machines injection vaccination has become less labour intensive and less dangerous for the injector (Newman, 1993) and therefore better applicable for mass vaccination. In addition, fish need to have a certain size for injection (>15g), while the stock needs to be protected as young as possible (Ellis, 1995).

2.3.2 Immersion vaccination

Immersion is an effective and more practical method for vaccination. Handling is minimized and this method can be applied to fish at a smaller size than injection. Immersion vaccination includes dip vaccination and bath vaccination. In dip vaccination the fish are caught in a net and dipped in a diluted vaccine in large quantities (450 g fish.l^{-1} of diluted vaccine). The handling procedure, crowding and netting, required by dip vaccination are potentially stressful and may cause scale loss and a temporary decrease of fitness. Netting or handling are limited with bathing methods in which vaccine is added directly to the holding tank (bath vaccination). However, much more vaccine is needed for this method (Horne & Ellis, 1988). The amount of antigen taken up is linearly correlated to the immersion time (Ototake *et al.*, 1996). The route of entry in fish is still obscure. Possibilities are skin, gills (Smith, 1982; Zapata *et al.*, 1987; Al-Harbi & Austin, 1992), lateral line (Amend & Fender, 1976) and intestine (Robohm & Koch, 1995). Uptake of BSA (bovine serum albumin) was reported predominantly in the skin and gills after immersion. Phagocytes and epithelial cells are involved in this process, mainly in the skin (Ototake, 1996). It was also reported that particulate antigens are better taken up than soluble antigens (Smith, 1982). Antibodies are not always detectable in the serum after immersion vaccination and a detectable antibody level is not always correlated with protection (Thuvander *et al.*, 1987; Lobb, 1987; Magariños *et al.*, 1994). However, antibodies are often

detected in skin mucus and bile. Possibly the cell-mediated and the mucosal (local) immune system play an important role in protection after immersion vaccination.

2.3.3 Oral vaccination

Potentially the best method to vaccinate fish is oral vaccination. The animals need not to be handled, the treatment does not require extra labour, large masses of fish can be vaccinated simultaneously, and the vaccine can easily be applied to young fish. However, until now oral vaccination was less effective than immersion or injection vaccination. Usually multiple doses of vaccine and more vaccine is needed, probably because the antigen is introduced in the digestive environment of the alimentary canal. It has been shown in fish that anal administration of a vaccine resulted in better responses or protection than oral administration (Johnson & Amend, 1983; Rombout *et al.*, 1986). These data indicate that antigen given orally, can be degraded during transport through the anterior part of the digestive tract and hence is not able to reach the immune system in sufficient quantities. In carp, the posterior part of the gut showed a higher capacity for uptake of macromolecules (Rombout *et al.*, 1986). Antigens taken up in this part are transported to intestinal macrophages and probably activate the immune system (see § 3.1). Thus protection of the antigen against degradation might increase the efficacy of a vaccine. Another important aspect of oral vaccination is the induction of a mucosal immune response. Indications for a mucosal immune system in fish will therefore be summarized.

3 Mucosal immunity in fish

Especially for fish living in a pathogen-rich aquatic environment, the existence of a mucosal immune system may be very important. In contrast to higher vertebrates their skin forms an extra mucosal barrier. The mucosal surfaces of gut, skin and gills are protected by both humoral and cellular mechanisms. Non-specific protective agents, e.g. lysozyme and complement are found in mucus.

3.1 Gut associated lymphoid tissue (GALT)

Although fish lack clear lymphoid accumulations in their mucosae, many lymphoid cells and macrophages can be found in their intestine (Davina *et al.*, 1980; Temkin & McMillan, 1986; Rombout *et al.*, 1993a; Doggett & Harris, 1991) and skin (St. Louis-Cormier *et al.*, 1984; Lobb, 1987). Up to this moment, most attention has been paid to the intestine of carp. Therefore, data from this species will be described and when necessary important differences observed in other species will be mentioned. In carp, many IgM^+ cells (B cells and plasma cells) can be found in the

lamina propria throughout the intestine, while most IgM$^+$ cells and large Ig-binding macrophages were observed in the intestinal epithelium (Rombout *et al.*, 1993a). This situation is comparable with higher vertebrates, although Peyer's patches(PP)-like structures are absent, which is not surprising because fish lack lymph nodes or germinal centre-like structures (Van Muiswinkel *et al.*, 1991). Fish seem to have a more diffuse intestinal immune system. To our knowledge, all fish have a so called second segment in their hindgut (20-25 % of the gut length), which contains epithelial cells with a much higher endocytotic capacity and with more and larger intracellular vacuoles compared to the first gut segment (50-75% of the gut length, depending on the species; Rombout *et al.*, 1985). The second gut segment appears to be more adapted for absorption of digested molecules. Enterocytes in this segment can transport antigens from the lumen to the lymphoid cells and macrophages in the mucosal tissue (carp: Rombout *et al.*, 1985, 1989a; trout: Georgopoulou *et al.*, 1986a; tilapia: Doggett & Harris, 1991). Specialized cells for uptake of particulate antigens, M cells, as described in mammals, cannot be found in fish. In carp, much more and larger intraepithelial macrophages are present in this part of the gut compared to the first segment (Rombout *et al.*, 1989b). After oral or anal administration of antigens intestinal epithelial cells appear to take up macromolecules by endocytosis and transport them to these large macrophages (Georgopoulou *et al.*, 1986b; Rombout *et al.*, 1989b), which at least in carp appeared to have an Ig-binding capacity (Koumans-van Diepen *et al.*, 1994) and an antigen presenting function (Rombout *et al.*, 1986; 1989a). The combination of an antigen transporting epithelium and the presence of antigen processing macrophages, next to abundant Ig$^+$ (B) and Ig$^-$ (T) cells make the second segment an important candidate for the induction of a mucosal immune response. Although not much is known about antigen processing in gills and skin, indications are available for antigen uptake by these epithelia and for the presence of leucocytes at these locations (Lobb, 1987; Rombout *et al.*, 1993a; Iger *et al.*, 1994).

3.2 Mucosal immune responses

An important criterion for the existence of a mucosal immune system is the secretion of antigen-specific antibodies at mucosal surfaces. Antigen-specific antibodies could be detected in skin mucus, bile or intestine after oral administration of a variety of antigens to several fish species (plaice: Fletcher & White, 1973; ayu: Kawai *et al.*, 1981; carp: Rombout *et al.*, 1986, 1989c), while small amounts of antigen-specific antibodies were found in serum. Similar results were obtained after bath vaccination (channel catfish; Lobb *et al.*, 1987) suggesting that mucosal responses can also be induced through skin and/or gills. On the other hand, intraperitoneal or intramuscular immunisations only revealed high titres of antibodies in serum and not at the mucosal

surfaces. However, these results are difficult to explain when fish only have one IgM-like molecule, which is stated by most fish immunologists. However, mAb could be produced against skin mucus IgM, which reacted with mucus IgM and not with serum IgM (Rombout *et al.*, 1993b). These mAb reacted specifically with a subpopulation of B cells and with skin epithelium and bile capillaries, the sites were mucosal IgM can be expected. Indications for another type of mucus IgM (including a secretory component) are also available for sheepshead (Lobb *et al.*, 1981). These data clearly suggest that fish have an IgA-homologue, although, at least in carp, it has to be considered as an IgM subtype. For a complete prove, more attention has to be payed to the molecular differences between both IgM molecules and to mechanisms behind the homing of mucus IgM producing B cells and plasma cells.

3.3 Oral tolerance

Another important aspect of a mucosal immune system is the induction of tolerance. The phenomenon by which oral administration of protein antigens results in specific immunosuppression to the same antigen administered parenterally is termed oral tolerance (MacDonald, 1994). Indications are available that a similar phenomenon as found in mammals can be observed in fish. In carp (Rombout *et al.*, 1989c) and trout (Davidson *et al.*, 1994) induction of oral tolerance has been described when fish were fed repeatedly with protein antigens (in trout only after simultaneous i.p. injection of the antigen). For mammals it has been suggested that T cells migrating from the gut to other lymphoid organs may suppress the systemic immune responses by producing inhibitory cytokines (Friedman *et al.*, 1994). Another possibility is that peptides, taken up in the gut by enterocytes, enter the systemic circulation and alter T cell responses locally or dendritic cells migrate from the gut to present the tolerogenic peptide to T cells in other tissues (McDonald, 1994). Whether an antigen induces a tolerogenic signal seems to depend on the dose, the type and structure of the antigen and the route of entry (Mowat, 1994; Friedman *et al.*, 1994; Weiner *et al.*, 1994). Inhibitory cytokines are thought to be produced by $CD8^+$ lymphocytes, which are activated by $CD4^+$ T lymphocytes (Friedman *et al.*, 1994; Weiner *et al.*, 1994). Intraepithelial T cells possess a number of membrane molecules that are distinct from T cells present in other lymphoid tissues. This may result from organ specific homing, resulting in functionally distinct T lymphocyte populations in the organs (Friedman *et al.*, 1994).

The antigen transporting capacity of the hindgut of fish, the abundant but diffuse presence of immunocompetent cells in this part of the gut, the detection of specific antibodies in skin mucus, bile or intestine only after intestinal or bath immunisation, the presence of a distinct mucosal IgM subtype strongly indicate that fish indeed have

a mucosal immune system. Moreover, fish also seem to be tolerised by repeated feeding of protein antigens, which can be considered as another aspect of the mucosal immune system.

4 Strategies in oral vaccine development

4.1 Delivery methods

More effective oral vaccination may be achieved when antigens can be protected against degradation in the stomach and anterior intestine. Various vaccine (and drug) delivery forms to protect the vaccine from degradation in the anterior intestine have been studied in mammals. It has been shown that entrapment of the antigen in particulate carriers, such as biodegradable microspheres (Eldridge *et al.*, 1991; O'Hagan *et al.*, 1993; Morris *et al.*, 1994), liposomes (Michalek *et al.*, 1994; Chandrasekhar *et al.*, 1994) and ISCOMs (Immune Stimulatory Complexes; Mowat *et al.*, 1991) can prevent antigen degradation and enhance delivery to the M cells and antigen presenting cells. Increased antibody production at mucosal surfaces (IgA) and/or in the serum (IgA and IgG) were reported (O'Hagan *et al.*, 1993; Michalek *et al.*, 1994). Besides a more effective delivery, these effects can also be due to adjuvant properties of the particles or the result of sustained release by storage of microparticles in the PP and lymph nodes (Eldridge *et al.*, 1991; Morris *et al.*, 1994; Michalek *et al.*, 1994).

Some studies have been dedicated to oral application of protected vaccines in fish (Ellis, 1995). Lillehaug (1989) tried two different methods for protection of the antigens: lyophilized *V. anguillarum* vaccine was incorporated in slow-release pellets (matrix of saturated long chain fatty acids) or was coated with an acid-resistant film. However, feeding of unprotected vaccine to rainbow trout resulted in better protection after challenge than feeding with protected vaccine. Possibly the antigen was protected in a way that it could not be absorbed by the intestine. Wong *et al.* (1992) tested dextrose beads coated with *V. anguillarum* and Eudragit L-30D (acid-resistant coating). These enteric coated antigen microspheres (ECAMs) were fed to coho salmon for 30 days. Better protection and higher antibody titres were found in serum and mucus compared to controls. ECAMs coated with TNP-LPS also induced increased antibody titres after feeding compared to feeding with only ECAMs, in contrast to feeding of ECAMs coated with TNP-KLH (Piganelli *et al.*, 1994). Poly(D,L-lactide-co-glycolide) microparticles containing HGG were orally intubated and localization of HGG in the body tissues and serum were studied in Atlantic salmon. Oral delivery of HGG in microparticles resulted in HGG being detectable in serum from 15 min to

two days with two peaks after six days and 5 weeks, while free HGG was detectable from 15 min to three days. In the posterior intestinal epithelium HGG was detected for up to 3 days and in the kidney for up to 5 weeks after administration of HGG in microparticles, which was considerably longer (2 days and 7 weeks respectively) than after administration of free HGG (O'Donnell et *al.*, 1996). Furthermore, the release of BSA and *V. anguillarum* from chitosan-alginate capsules was tested *in vitro* and possible applications in vaccine delivery for aquaculture were discussed, although *in vivo* studies were not done (Polk et *al.*, 1994). LPS incorporated in liposomes was orally intubated in rainbow trout and uptake and distribution in several organs were studied. The oral route resulted in the lowest antigen uptake in all organs compared to injection of liposomes (Nakhla et *al.*, 1994). Unfortunately, uptake in the intestine was not studied. For vaccination of juvenile ayu Kawai et *al.* (1989) tested encapsulation of *V. anguillarum* in plankton (rotifer and water flea). Challenge with *V. anguillarum* after feeding with this enriched plankton for 22 days resulted in survival of 92.4 % versus 64.2 % in the control group (Kawai et *al.*, 1989). A combination of liposomes and delivery by live food (*Artemia* nauplii) was developed, which increased the concentration of liposomes in the *Artemia* nauplii and thus would increase antigen delivery to young fish. The preparation was, however, not tested *in vivo* (Hontoria et *al.*, 1994).

4.2 Oral adjuvants

Adjuvants are usually mixed and injected with antigen to enhance the immune response to vaccination. The mechanisms by which they act are not well understood. The possible effects of a given adjuvant are complex, may overlap, and are likely to be multiple (Anderson, 1992). Some of them have been demonstrated, in mammals, to enhance antigen uptake from the intestinal tract, which might result in more effective oral immunisation. Several adjuvants were tested in tilapia (Jenkins et *al.*, 1991; 1992; 1994a; 1994b). HGG was administered by intraperitoneal injection, anal and oral intubation with or without Quil-A in several formulations. The delivery of HGG with Quil-A raised the levels of antigen absorbed across the intestine of *Oreochromis mossambicus* after oral and anal delivery (Jenkins et *al.*, 1991) and also the levels of HGG absorbed into the plasma were increased (Jenkins et *al.*, 1992). The antibody levels in the plasma, bile and cutaneous mucus were increased after both oral and anal administration of HGG with Quil-A, compared to administration of HGG alone (Jenkins et *al.*, 1994a). In further experiments, HGG was mixed with cholera toxin ß subunit (CTB), aluminium hydroxide, or ammonium chloride (Jenkins et *al.*, 1994b). In the same experimental design as described above, CTB increased the levels of HGG transcytosed into the plasma over levels observed after HGG delivery alone or with

either $Al(OH)_3$ or NH_4Cl. The enteric immunisation with HGG plus CTB or $Al(OH)_3$ resulted in increased antibody titres, compared to HGG alone or with NH_4Cl. Antibody titres after intraperitoneal immunisation were not increased with each of the three adjuvants compared to HGG alone (Jenkins *et al.*, 1994b).

4.3 Alginate microparticles

Alginate microparticles can be used for the protection of an oral vaccine. Sodium alginates are polysaccharides extracted from brown seaweeds, which consist of chains of ß-D-mannuronic (M) and *α*-L-guluronic (G) acids. The two monomers are linked together to form a copolymer with G, M and GM blocks. The sodium alginate molecules form a gel in contact with Ca^{2+}, forming a Ca^{2+} alginate matrix, in a model of an egg box (Figure 1). The M/G ratio is important for the stability of the matrix, since the dimerisation is a linkage of calcium with the G acid segments. More M makes the alginate network more soluble.

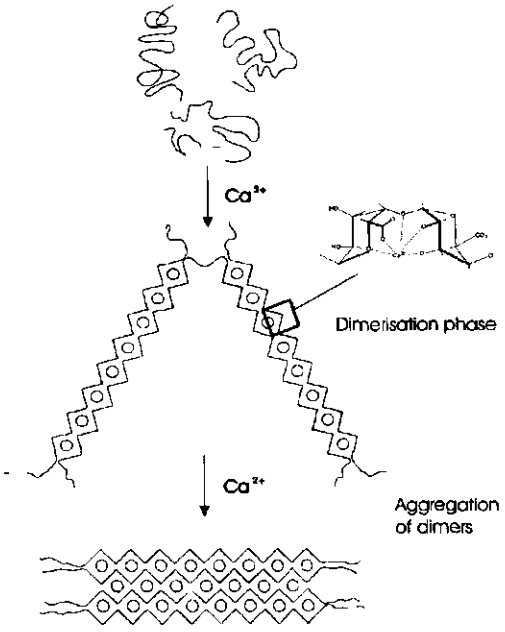

Figure 1. Mechanism of alginate gelification. The sodium alginate molecules form a gel in contact with Ca^{2+}, making a Ca^{2+} alginate matrix, in a model of an egg box.

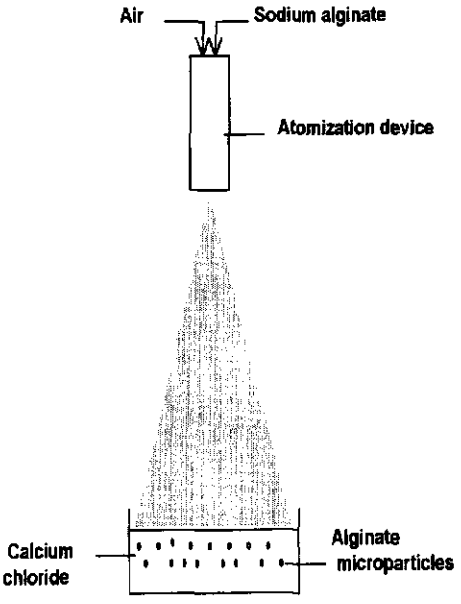

Figure 2. Schematic diagram of the atomization technique for production of alginate microparticles. Sodium alginates are sprayed, under air inflow, to form droplets, which are stabilized in a $CaCl_2$ solution to form microparticles.

Production of alginate microparticles is a quick and easy method, without causing denaturation of the antigen, which can be easily entrapped in the matrix during production of microparticles. With the atomization technique sodium alginates are sprayed with air inflow, to form droplets, that are stabilized in a calciumchloride ($CaCl_2$) solution to form microparticles (Figure 2). These microparticles are spherical (without aggregates) and have a diameter dependent on air pressure, flow rate, concentration of $CaCl_2$ and concentration of alginate. Characteristics of the particles are dependent on formation, M/G ratio and concentration of alginate. With an air pressure of 2.5 bar and flow rate of 2.3 ml/minute, microparticles can be produced with an average diameter of 4-5 μm, with a range from 0 to 20 μm. The concentration of alginate and $CaCl_2$ is important for the amount of antigen in the microparticles and the release.

The alginate microparticles are protective in an acid environment, but erode and swell in an alkaline environment, resulting in release of its content. The pH dependent release, non-toxicity and biodegradability would make alginates good polymers for protection of antigen in the anterior digestive tract and delivery in the hindgut of fish. Important factors for vaccine delivery are swelling capacity, release capacity, pH dependency and production procedure.

5 Aim and outline of this thesis

Oral application of vaccines is an attractive way of inducing protection against diseases. Especially oral mass vaccination of fish would be of economic importance. However, degradation of the antigen in the digestive tract seems to be the main reason for a lower efficacy of oral vaccination. Protection of the antigen against degradation is important to deliver the antigen in the hindgut, where it can be taken up by intestinal cells and reach the immune system undegraded. Since fish live in an aquatic pathogen-rich environment, it may be important to vaccinate them as early as possible. Moreover, less vaccine would be needed at a smaller body weight. The aim of this thesis was to study the induction of immunological reactions after oral vaccination and the possibilities to enhance the efficacy of an oral vaccine by protection of the antigen during transit in the digestive tract.

In **chapter 2** a bioencapsulation method (i.e. encapsulation in *Artemia* nauplii) for protection of a *V. anguillarum* bacterin is studied in juvenile carp and seabream. Immune responses at different ages are studied to investigate the earliest age to effectively vaccinate fish against a bacterial disease. In order to develop a vaccine for oral vaccination it has to be clarified which fraction of a vaccine is most effective for induction of immunological memory.

In **chapter 3** experiments are described to investigate the most antigenic part of a commercially available bacterin. This most effective fraction would be the best

candidate to be used for oral vaccination, encapsulated in a microsphere to protect the antigen from degradation.

In **chapter 4** immune responses after oral vaccination with a *V. anguillarum* antigen encapsulated in alginate microspheres were studied in carp and trout. Memory formation was investigated by measurement of secondary serum antibody responses and mucosal immune responses by measurement of mucosal plasma cells.

Oral application of proteins in mammals is known to induce oral tolerance instead of immunity. This causes problems in development of an effective oral vaccine against viral diseases, where the antigenic determinant is often a protein. In **chapter 5** experiments are described to investigate the possibility of oral tolerance induction in fish.

In **chapter 6** a distinct mucosal Ig^- lymphocyte population in carp is described using a monoclonal antibody, reactive with Ig^- lymphocytes (T cells) in intestinal epithelium and gills. Their role in the mucosal immune system is discussed.

In the final chapter, **chapter 7**, the observations described in this thesis and their possible implications for the design of vaccines are discussed.

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Chapter 2

Oral vaccination of juvenile carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) with bioencapsulated *Vibrio anguillarum* bacterin.

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Abstract

In this study *Artemia* nauplii were used as an antigen delivery system for oral vaccination of young fish with a *Vibrio anguillarum* bacterin. Juvenile carp of 15-, 29- and 58- days old (stomachless fish) and gilthead seabream of 57- and 69- days old (stomach-containing fish) were fed with this bioencapsulated bacterin. Antigen transport in the hindgut was studied using immunohistochemical techniques. In carp of 29 and 58 days old antigenic determinants of *V. anguillarum* were transported from lumen to supranuclear vacuoles in carp and finally to intraepithelial macrophages. In carp of 15 days old a similar transport was observed, only macrophages could not be found. In gilthead seabream a different (more diffuse) transport was observed and no macrophages were found. Ten weeks after oral vaccination fish of both species received an intramuscular booster. Three weeks later, serum was collected and measured for specific antibodies by ELISA. Carp orally vaccinated at 15- or 29-days old showed a significantly lower response after the booster, compared with non-vaccinated controls. However, carp orally vaccinated at 58 days old tended to have a higher antibody response compared with the control. Seabream orally vaccinated at 57 or 69 days showed significantly higher secondary responses compared with the control. It is concluded that oral vaccination with bioencapsulated vaccines is very promising when applied at the right age. Application at too young an age seems to induce immunosuppression, instead of immunisation.

Introduction

Control of fish diseases is of great concern in aquaculture, because of the large populations of fish and the high risk of disease transmission in an aqueous environment (Dunn *et al.*, 1990). Prevention of diseases can be established through vaccination. Potentially the most useful method for fish will be oral vaccination, as it is non-stressful, requires no extra labour and can be applied on a large scale, even to young fish. However, till now oral immunisation of fish has not been very successful (Ellis, 1988). Compared with injection or immersion methods lower protection and/or lower antibody levels in serum were observed (Amend & Johnson, 1981; Johnson & Amend, 1983; Rombout *et al.*, 1986; Dec *et al.*, 1990) and larger quantities of bacterin are required to be effective (Ward, 1982; Ellis, 1988). On the other hand, Johnson & Amend (1983) showed that anal vaccination against vibriosis and yersiniosis resulted in good protection, while oral vaccination only gave a limited protection. It was suggested that most of the antigen was degraded before it reached

the hindgut, which was recently proved to be the most important antigen-transporting area in the digestive tract of fish (Rombout et *al.*, 1989 a,b,c; 1993). Consequently for more effective oral vaccination, it might be necessary to protect antigens against acid (in a stomach-containing fish) and proteolytic degradation in the foregut in such a way that it reaches the hindgut in sufficient quantities to provoke a protective immune response. In carp, antigens appear to be taken up by pinocytosis and are finally transported to intraepithelial macrophages (Rombout et *al.*, 1985, 1986, 1989a). This feature together with the occurrence of many mucosal lymphoid cells (Rombout et *al.*, 1989b, 1993) may explain the induction of a mucosal immune response after intestinal immunisation (Rombout *et al.*, 1986, 1989). In addition, orally or anally administered antigens can also induce a systemic immune response (Rombout et *al.*, 1986, 1989c). Consequently, when antigens can be delivered to the hindgut in sufficient quantities, both systemic and mucosal immune responses can be achieved by oral immunisation.

In this paper we report an oral administration procedure of a *Vibrio anguillarum* bacterin using brine shrimp (*Artemia*) nauplii as a biological antigen carrier. Brine shrimps are generally used as food for juvenile fish and are able to incorporate particulate antigens by filterfeeding (Campbell *et al.*, 1993). This method of bioencapsulation would give possibilities for oral vaccination of juvenile fish, at an age when the immune system of young fish is well developed. In this study, the effectiveness of this delivery method was investigated. The uptake of antigen and the formation of systemic immunological memory in juvenile carp (stomachless fish) and gilthead seabream (stomach-containing fish) after oral vaccination were studied.

Materials and methods.

Animals

Carp, *Cyprinus carpio* L., and gilthead seabream, *Sparus aurata* L., were used. Carp were reared in our laboratory in Wageningen and kept at 23 ± 1°C in recirculating, filtered and UV treated water. Gilthead seabream were obtained from Sepia International (Gravelines, France), kept at 21 ± 1°C in recirculating, filtered and UV treated artificial seawater (36 ppt). At the start of the experiment carp were 15-, 29- and 58- days old and seabream were 57- and 69 days old. Until vaccination fish were fed ad lib. with brine shrimp (*Artemia franciscana* nauplii, Sanders Brine Shrimp Company, Ogden UT-USA). After vaccination, feeding with pelleted dry food commenced (Trouvit 00; Trouw and Co, Putten, The Netherlands for carp and Trofic M-8; Trofic SA, Spain for gilthead seabream) amounting to 5 % of their body weight

per day.

Antigen

A commercial *V. anguillarum* bacterin (Aquaculture Vaccines Ltd., Essex, UK) was used. This bacterin contains 10^9 bacterial cells ml^{-1} suspension of formalin-killed *V. anguillarum*. The same lot was used for all vaccinations.

Bioencapsulation of antigen

Brine shrimp were hatched in artificial seawater (36 ppt) at 29 ± 1°C, under continuous light and sufficient air. After 16 h, hatched brine shrimp nauplii were collected, adjusted to a concentration of 360 brine shrimps ml^{-1} seawater and incubated in a 1:10 dilution of the bacterin for 3 h.

Immunisation procedure and blood sampling

Directly after bioencapsulation, the brine shrimp were fed to the fish. Depending on the age, fish were fed an average of 150, 200 or 300 bacterin-containing brine shrimp nauplii per fish. During feeding the waterflow was stopped. After 2.5 h, animals were fed again with these brine shrimp nauplii. Control fish were fed with non-treated brine shrimp nauplii. After ten weeks vaccinated and non-vaccinated fish received a secondary immunisation by intramuscular injection of 10^8 washed bacteria (of the same bacterin) in 10 µl PBS. Three weeks later, fish were anaesthetized in 0.03% TMS (Tricaine methane sulphonate, Crescent Research Chemicals, Phoenix AZ-USA) and blood was taken by caudal vein puncture. Blood samples were allowed to cloth at room temperature for 2 h, then stored overnight at 4°C and centrifuged for 10 min at 700 g and 4°C. Serum was collected and stored at -20°C until measurements were made.

Immunohistochemistry

At 1, 6 and 20 h after the first feeding with bacterin-containing brine shrimp, two fish of each group and of each species were killed by decapitation. Part of the first and second segment of the gut was dissected and fixed in 4% formol (overnight) and, after rinsing and dehydration, embedded in paraffin wax. Longitudinal sections (5 µm) were cut and mounted on poly-L-lysine coated slides. After deparaffination and rehydration, sections were incubated for 20 min in a 0.3% H_2O_2-containing methanol solution to inactivate endogenous peroxidase. Antigenic determinants of *V. anguillarum* were detected by indirect immunoperoxidase reaction using a rabbit-anti-*Vibrio* serum (1:100; kindly supplied by Dr. Tatner, Stirling, UK) and a horseradish peroxidase(HRP)-conjugated goat-anti-rabbit Ig serum (1:200; Biorad, Richmond CA-

USA). Conjugates were visualized with 0.05% DAB (3',3'-diaminobenzidine-tetrahydrochloride, Sigma, St Louis, MO-USA) with 0.01 % H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). Sections were slightly post-stained with haemalum. Standard controls, i.e. sections of non-vaccinated animals, preabsorption with bacteria and omission of specific antibodies were carried out.

ELISA

All steps of the reaction were carried out in 96-well flat bottom microtitre plates (Omnilabo, Breda, The Netherlands) in a moist chamber. Between the successive steps, plates were washed twice for 15 sec in tap water containing 0.05% Tween 20. Plates were coated with 100 μl of a suspension of 10^8 bacterial cells ml^{-1} washed bacteria in distilled water by drying overnight at 37°C. To prevent non-specific binding, plates were treated for 1.5 h at room temperature with 200 μl per well of a solution of 1% BSA (Bovine Serum Albumin), 0.05% Tween 20 in PBS (pH 7.2). Plates were incubated for 1.5 h at 37°C with 100 μl of ^{2}log serial dilutions of the test samples in PBS, using a hyperimmunised carp- or seabream-anti-*Vibrio* serum as standard. Next, plates were incubated for 1.5 h at 37°C with monoclonal antibodies specific for carp immunoglobulin (WCI-12; Rombout *et al.*, 1990) or gilthead seabream immunoglobulin (WSI-5; Navarro *et al.*, 1993) and subsequently treated with HRP-conjugated goat-anti-mouse IgG (1:5000; Biorad, Richmond, CA-USA) for 1,5 h at 37°C. A colour reaction was produced by incubating the plates for 30 min at room temperature with 100 μl of a solution of 0.04 % OPD (O-phenylenediamine dihydrochloride; Sigma, St Louis, MO-USA) and 0.02 % H_2O_2 in 0.1 M citric acid monohydrate/0.25 M Na_2HPO_4 (pH 5.0). The reaction was stopped after 30 min by addition of 50 μl of 2.5 M H_2SO_4 per well. The resulting absorbance (O.D.) at 492 nm was read using an Anthos reader 2001 (Anthos Labinstruments, Salzburg, Austria). Using data regression, antigen-specific antibody titres are estimated by calculating sample dilutions at an O.D. of 0.4 for carp and O.D. of 0.5 for gilthead seabream.

Statistical analysis

To evaluate the statistical significance of the differences in antibody titres between vaccinated and control fish, an unpaired Student's t-test was used to compare the means of each group.

Results

Immunohistochemistry

In carp of all groups antigenic determinants of *V. anguillarum* were transported in the second gut segment from the lumen to the supranuclear vacuoles (Fig. 1 a,b). Finally, immunoreactive intraepithelial macrophages could be found in carp of 29- and 58-days (Table 1, Fig. 1b). In carp of 15-days old intraepithelial macrophages could not be distinguished (Fig. 1a). In gilthead seabream, the mechanism of uptake appeared to be different from that in carp. Antigenic determinants were not found in vacuoles but were diffusely present in the cytoplasm. In seabream at both 57- and 69-days old, antigenic determinants were observed in the apical part of the enterocytes after 6 h and at the basal site of the epithelium after 20 h (Fig. 1 c,d). Intraepithelial macrophages could not be detected either age (Table 2). All controls were found to be negative for both species used.

Table 1. Immunoreactivity in the second segment of the gut of carp at 1, 6 and 20 h after oral vaccination with *V. anguillarum*-containing brine shrimps.

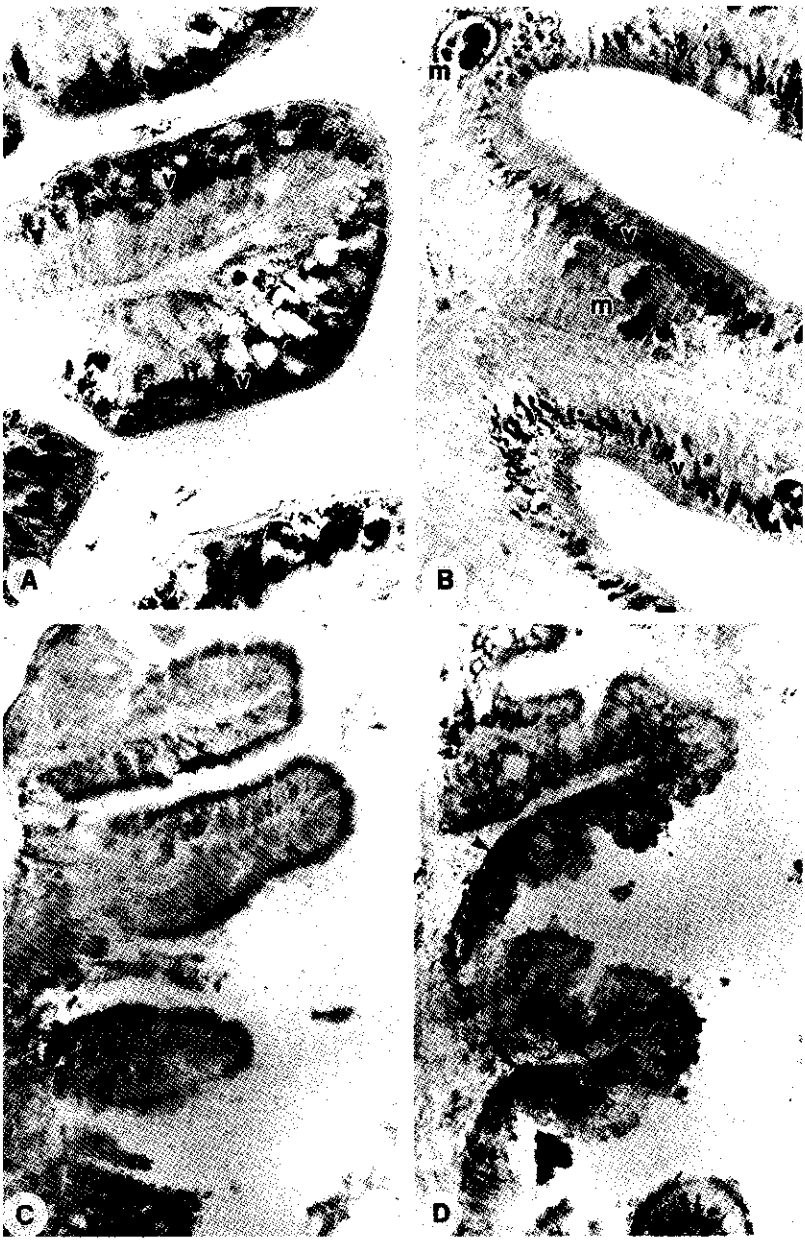
Age (days)	15			29			58		
Time (h)	1	6	20	1	6	20	1	6	20
Lumen	+	+	+	+	+	+	+	—	±
Enterocytes	—	±	++	+	+	++	+	±	++
Macrophages	NF	NF	NF	±	+	++	—	±	++

Each estimation is the average of two animals.
NF: Not found, — unstained, ± weak, + medium, ++ strong.

Table 2. Immunoreactivity in the second segment of the gut of seabream at 1, 6 and 20 h after oral vaccination with *V. anguillarum*-containing brine shrimps.

Age (days)	57			69		
Time (h)	1	6	20	1	6	20
Lumen	—	±	±	±	+	+
Enterocytes A	—	±	±	—	+	++
Enterocytes B	—	+	++	—	±	±

Each estimation is the average of two animals.
A: apical part, B: basal part, – unstained, ± weak, + medium, ++ strong.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Systemic memory formation

Serum antibody titres of carp orally vaccinated at 58-days old tended to be higher after intramuscular injection 10 weeks later, when compared with control carp, which only received the booster. However, in younger carp (vaccinated at an age of 15 or 29 days) serum antibody titres were significantly ($p<0.05$) lower for the orally vaccinated group compared to the control. Comparison of serum antibody titres of control carp between the groups clearly showed an age-dependent increase in the response (Fig. 2a). The antibody titres of non-vaccinated controls, measured after i.m. injection, increased significantly with age. Antibody titres of the oldest control group were significantly higher ($p<0,02$) than the younger groups. Also the difference between these two youngest groups was significant ($p<0.01$). In gilthead seabream significantly ($P<0.05$) higher serum antibody titres were observed in the orally vaccinated fish after an intramuscular booster 10 weeks later compared with the controls at both ages (Fig. 2b).

Discussion

This study demonstrates the transport of antigenic determinants of *V. anguillarum* from the lumen to the supranuclear vacuoles and finally to the intraepithelial macrophages in the hindgut of juvenile carp after oral immunisation, using *Artemia* nauplii as an antigen delivery system. The uptake appeared to be more or less comparable to that described for older carp after anal intubation with a similar bacterin (Rombout *et al.*, 1986, 1989a) and with ferritin in carp (Rombout *et al.*, 1985, 1989a,) and adult trout (Georgopoulou *et al.*, 1985). However, gilthead seabream showed a different uptake mechanism of the bacterial antigens. Antigenic determinants were not found in vacuoles but diffusely distributed in the cytoplasm of the enterocytes. Between 6 and 20 h after oral immunisation antigenic determinants were gradually transported from the apical to the basal part of the enterocytes and

Figure 1. Histological section of the second gut segment of carp or seabream after oral vaccination with bioencapsulated *Vibrio anguillarum*, immunohistochemically stained for *V. anguillarum* determinants by an indirect peroxidase reaction.
(**A**) 15 days old carp, 20 h after vaccination. Immunoreactive vacuoles (V), macrophages were not detected. (**B**) 58 days old carp, 20 h after vaccination. Immunoreactive vacuoles (V) and positive macrophages (M). (**C**) 57 days old gilthead seabream, 6 h after vaccination. Immunoreactivity in apical part of the enterocytes (arrows). (**D**) 57 days old gilthead seabream, 20 h after vaccination. Immunoreactivity in the basal part of the enterocytes (arrows). Magnification 400 x.

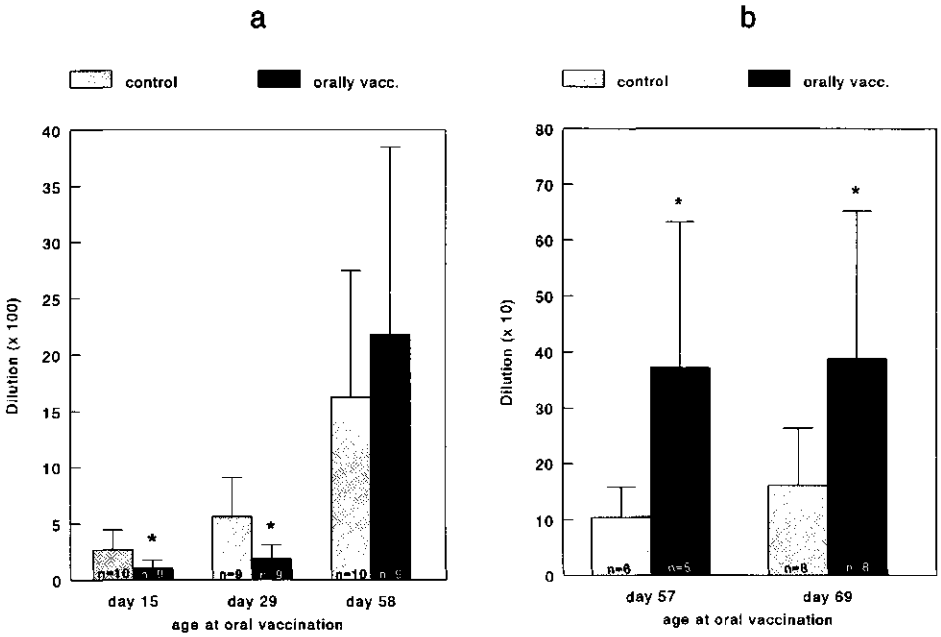

Figure 2. (**a**) Serum dilution (+ S.D.) at O.D. 0.4 in orally vaccinated carp 21 days after intramuscular booster. (**b**) Serum dilution (+ S.D.) at O.D. 0.5 in orally vaccinated gilthead seabream 21 days after intramuscular booster. * significant for $P<0.05$; n = number of fish per group.

possibly subsequently released into the blood stream (though not tested in this study). This type of transport has also been described for HRP in trout (Georgopoulou *et al.*, 1985), but has been considered as an additional uptake mechanism subsidiary to the transport to vacuoles. In contrast to carp, intraepithelial macrophages were not observed in gilthead seabream. These results indicate differences in the local immune system of seabream and carp, at least in 2-month old animals. In carp, intraepithelial macrophages were already observed in 29- and 58-, but not in 15-day old animals, which suggests important changes in the local immune system of carp in the first month after hatching.

With respect to the systemic immune response our results show a significant increase in carp serum antibody titres between animals intramuscularly immunised at 85-, 99- and 128-days old (controls), which indicates that the immune system of carp is still developing during this period. These results are in agreement with those of Koumans-van Diepen *et al.* (1994) who suggested that the development of the immune system in carp was completed between 3 and 8 months of age, based on the numbers of B cells and plasma cells found. In addition, van Loon *et al.* (1981) had already suggested that the adult immunoglobulin levels of carp were reached between

5 and 8 months of age. Although similar data are lacking for the development of the immune system in gilthead seabream, we may suppose that their immune system is still developing at the ages tested here. The lower antibody titres found in gilthead seabream compared to carp of the same age may be explained by the use of different monoclonal antibodies rather than by differences in immune competence. For gilthead seabream, WSI-5, reactive with the immunoglobulin light chain (Navarro *et al.*, 1993), was used. Because differences in light chains are described for several fish species (Lobb *et al.*, 1984; Sánchez *et al.*, 1993; Van der Heyden *et al.*, 1994), only a limited proportion of specific antibodies might be monitored. On the other hand, WCI-12 probably recognizes most if not all immmunoglobulin molecules secreted (Koumans-van Diepen, 1993).

In the present study carp orally vaccinated with bioencapsulated bacterin at 58-days old, tended to have a higher and gilthead seabream orally vaccinated at 57- or 69- days old even showed a significantly higher systemic immune response after an intramuscular immunisation with the same bacterin 10 weeks later, compared to the controls. These results suggest the formation of systemic memory in both species after oral vaccination with a bioencapsulated bacterin. In the case of vaccination against *V. anguillarum*, the humoral response is strongly related to final protection against vibriosis (Viele *et al.*, 1980; Harrel *et al.*, 1975; Dec *et al.*, 1990). In addition, a mucosal immune response resulting in specific antibodies at mucosal surfaces may also be induced after oral vaccination (Rombout *et al.*, 1989c) and could contribute to a higher level of protection. Consequently, these results indicate that oral vaccination with bioencapsulated bacterin may result in good protection of young fish against vibriosis. Bioencapsulation of a bacterin into Artemia, as used in this study, seems to be a promising method for oral vaccination. Apparently the antigen reaches the hindgut and is taken up in sufficient quantities to reach the immune system. Whether this antigen delivery system also protects the antigen against degradation in the first part of the digestive tract is not clear at present. *Artemia* have to be digested in the first part of the gut before LPS becomes available for uptake in the second segment. Probably the bacteria will be (partly) degraded in the digestive tract of *Artemia* and hence LPS will be freed. Because whole bacteria can not be taken up by the enterocytes (not published yet) the release of higher amounts of LPS in the second gut segment may be an advantage of this antigen delivery system. Especially because LPS has been shown to evoke a better immune response and memory formation, compared with the use of bacteria (recent unpublished data from our laboratory). However, to which extend predigestion occurs and whether this results in a positive effect on the oral vaccination of fish remains to be investigated.

Earlier studies using the water flea as an antigen-delivery system, indeed showed a higher protection against vibriosis in juvenile ayu of 73-days old, compared to non-vaccination ayu (Kawai *et al.*, 1989), supporting the possibilities of such an antigen delivery system. However, in carp orally vaccinated at 15- or 29-days old, significant lower serum antibodies were observed after intramuscular injection 10 weeks later, compared with non-vaccinated control animals. So, immunisation of young carp seems to induce immunosuppression. These results are in agreement with those of Van Muiswinkel *et al.* (1985) who showed that carp of 4-5 weeks old did not show a plaque forming cell (PFC) response after intramuscular injection of sheep red blood

cells. A second injection 2-3 months later also showed the absence of a response, while non-primed carp gave a good PFC-response at that age. In carp primed at 4 months old, a second injection resulted in normal PFC-responses. On the other hand, Manning & Mughal (1985) reported that carp of 4 weeks old had already developed an immune response and memory formation after priming by injection or direct immersion with *Aeromonas salmonicida*. Similar results were also obtained for rainbow trout (Manning *et al.*, 1982) The discrepancy between these and our results for 4 week old carp may be explained by the differences in antigen, immunisation routes and/or culture conditions. Mughal *et al.* (1986) found that carp immunised at 4 weeks old with *A. salmonicida* developed memory, while they became tolerant after immunisation with human gamma globulin (HGG). It has been concluded that carp can respond earlier against T cell-independent antigens, like *A. salmonicida*, than against T cell-dependent antigens, like HGG. Data are not yet available on the development of the immune response in gilthead seabream.

In conclusion, vaccination of fish at too young an age appears to give an undesired effect of immunosuppression. The right age for vaccination has to be estimated for each fish species, vaccine and immunisation route. For carp and gilthead seabream, 2-month old animals appear to be suitable for oral vaccination with bioencapsulated bacterins.

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Chapter 3

Anal immunisation of carp and rainbow trout with different fractions of a *Vibrio anguillarum* bacterin

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Abstract

Carp and rainbow trout were anally intubated with different fractions of a *Vibrio anguillarum* bacterin in order to determine which fraction of the bacterin results in optimal antigen transport and induces immunological memory following application to the gut. Antigen uptake in the hindgut was demonstrated in fish intubated with complete vaccine, supernatant of the vaccine and LPS extracted from the bacterin, while whole bacteria were not taken up. Fish received a secondary immunisation by intramuscular injection with complete vaccine 10 weeks after anal intubation. Primary antibody titres were significantly increased in carp 21 days after intubation with complete vaccine or supernatant of the vaccine. Secondary antibody titres were only significantly increased in carp which were intubated with supernatant of the bacterin. Furthermore, antibody titres in carp intubated with complete vaccine or LPS tended to be increased. In contrast to carp, trout required 10 x more supernatant to obtain similar results. In conclusion, the supernatant seemed to contain the most immunogenic part of the bacterin. The significance of these results for oral vaccination will be discussed.

Introduction

Oral delivery of vaccines has been described as the most desirable method for fish vaccination (Ellis, 1988). Oral vaccines can easily be administered to large numbers of fish without inducing stress and can even be used for vaccination of young fish. Although positive results with oral vaccination have been achieved, oral vaccination is still less effective compared to injection or immersion methods and more vaccine is needed (Johnson & Ahmend, 1983; Pyle & Dawe, 1985; Rombout *et al.*, 1986; Lillehaug *et al.*, 1989; Dec *et al.*, 1990). Degradation of the vaccine in the digestive tract before uptake by enterocytes, and hence lower amounts of antigen reaching the immune system of fish, is suggested to be a main reason (Johnson & Ahmend, 1983; Lillehaug *et al.*, 1989; Bøgwald *et al.*, 1994). Uptake of antigen by the epithelial cells of the gut, transport to antigen-processing cells and stimulation of lymphocytes is required to obtain immune responses and memory formation after oral vaccination. Uptake in the posterior part would only induce local immune responses. However, it is difficult to distinguish whether the transport in the anterior or posterior part was responsible for the induction of the immune response. In the hindgut (the second segment) of carp and trout a diffuse gut associated lymphoid tissue has been described and considerable amounts of antigen are transported from the lumen to intraepithelial macrophages (Rombout *et al.*, 1985, 1986, 1989a; Georgopoulou & Vernier, 1986). A certain proportion of macromolecules

may escape intracellular hydrolysis and pass directly into the lamina propria by exocytosis (Noaillac-Depeyre & Gas, 1973; Rombout *et al.*, 1985; Georgopoulou *et al.*, 1988). Although previous reports suggest that the posterior part of the gut is important to induce a sufficient immune response, uptake of LPS in the anterior part of the gut of brown trout has been demonstrated and it is suggested that this can induce a systemic immune response (O'Donnell *et al.*, 1994). Anally and orally administered antigens can induce mucosal as well as systemic immune responses (Rombout *et al.*, 1986, 1989b). Consequently, when antigens can be delivered to and taken up by the hindgut in sufficient quantities, better immunological memory may be achieved by oral administration. However, the antigen also has to reach the hindgut in a suitable form prior to uptake. Encapsulation of the antigenic part of a bacterin in microparticles which protect the antigen against degradation during transit might give possibilities for oral vaccination. In order to establish which fraction of a bacterin is the most suitable for oral vaccination, the uptake of antigen and formation of immunological memory in carp and trout were investigated after anal intubation.

Materials and Methods

Animals

Carp (*Cyprinus carpio* L.) were reared in our laboratory and kept in recirculating, UV treated water at 23 ± 1 °C. Rainbow trout (*Oncorhynchus mykiss*, Walbaum) fertilized eggs were obtained from Seven Springs Trout hatchery, Larne, Northern Ireland, hatched and were kept in our laboratory at 13 ± 1°C in UV treated, recirculating water. Carp of 5 months old (± 30 g), and rainbow trout of 6 and 8 months old (± 60 g) were used. Both species were fed with pelleted food (Trouvit; Trouw & Co, Putten, The Netherlands) at 2 % of their body weight per day.

Antigen

Different fractions of a commercial *Vibrio anguillarum* bacterin (10^{10} formalin killed bacteria ml^{-1}; Biovax 1300, BIOMED, Seattle, WA, USA) were prepared: 1. complete vaccine (vacc), 2. supernatant after centrifugation (sup; 15 min at 1500 *g*), 3. whole bacteria (bact; pellet of fraction 2 resuspended in PBS (Phosphate Buffered Saline, pH 7.3), 4. sonicated bacteria (son; 5 x 30 sec. on ice, output: 5, 100%, Branson sonifier 250, Danbury, CT, USA), 5. purified lipopolysaccharide (LPS; 3.5 mg ml^{-1}) in PBS. 6. PBS (con). LPS was extracted as described by Westphal & Jann (1965), from the pellet of the *V. anguillarum* vaccine. These fractions were used in experiment I. For experiment II, fractions 2 (sup) and 3 (bact) were 10 x concentrated for immunisation of trout. For carp

fractions 2 (sup), 3 (bact) and 4 (son) were 10 x concentrated. Ten mg LPS ml^{-1} was used in this experiment. Supernatant was concentrated by Speedvacc Concentrator (Savant instruments Inc., Farmingdale, NY, USA).

Immunisation procedure and blood sampling

Fractions (100 μl per animal) were anally intubated into the hindgut of carp and trout using a flexible tube (diameter 1 mm) attached to a 1 ml syringe. Each carp (30 g) received LPS anally at a dose of 11.67 mg kg^{-1} and 33.3 mg kg^{-1} body weight in experiment I and II, respectively, and 16.67 mg kg^{-1} body weight for trout. Following this, 50 μl of vaselin (Chesebrough Pond's Inc., USA) was intubated to prevent leakage of antigen from the intestine. Each fraction was administered to 12 fish. Before treatment, the animals were starved for 48 h and anaesthetized in TMS (0,2 g l^{-1}; Tricaine Methane Sulphonate, Crescent Research Chemicals, Phoenix, AZ, USA). Blood samples were taken by caudal vein puncture 3 weeks after anal intubation. Ten weeks after intubation all carp received an intramuscular injection of 100 μl complete vaccine. Trout received the booster after 10 weeks intraperitoneally in the first or intramuscularly in the second experiment. Three weeks after the booster, blood samples were taken again and allowed to clot at room temperature and stored overnight at 4°C. Serum was collected and stored at -20°C until used.

Immunohistochemistry

Six and 24 hours after anal intubation (experiment I) two fish of each group were killed in TMS and part of the hindgut was dissected and fixed in 4 % formaldehyde for further processing and staining according to Joosten *et al.* (1995). Sections were mounted on albumen/glycerin coated slides and stained by indirect immunoperoxidase reaction using a polyclonal rabbit-anti-*Vibrio* serum (1:100; produced in our laboratory) and horseradish peroxidase conjugated goat-anti-rabbit IgG (GAR-HRP, Biorad, Richmond CA, USA; 1:200 for carp, 1:400 for trout). Standard controls, i.e. preabsorption of polyclonal with bacteria and omission of specific antibodies were carried out.

ELISA

Vibrio-specific antibody (Ab) titres were measured as described by Joosten *et al.* (1995). Briefly, plates (Greiner Labortechniek, Alphen a\d Rijn, The Netherlands) were coated overnight at 37°C with a suspension of washed *V. anguillarum* bacteria (5 x 10^8 bacteria ml^{-1} distilled water, 100 μl per well) and subsequently treated with PBS containing 1% BSA (type IV, Boehringer, Mannheim, Germany) and 0.05 % Tween 20. Plates were incubated with the test sera (^{2}log serial dilution in PBS), with monoclonal antibodies specific for carp immunoglobulin (WCI-12, 1:200; Secombes *et al.*, 1983) or

for rainbow trout immunoglobulin (1-14, 1:100; DeLuca *et al.*, 1983) and HRP-conjugated goat-anti-mouse IgG (GAM-HRP, Biorad, Richmond CA, USA; 1:3000 for carp, 1:2000 for trout). Plates were incubated with OPD (O-phenylenediamine dihydrochloride; Sigma, St Louis, MO, USA) for 30 min before 2.5 M H_2SO_4 was added to stop the reaction. Between the successive steps plates were washed 2 x 15 sec in tap water containing 0.05% Tween 20. Optical density (O.D.) was determined at 492 nm (reference 690 nm). Using data regression, antigen-specific antibody titres for carp and trout are estimated by calculating dilutions at an O.D. of 0.4.

Statistical analysis

To evaluate the statistical significance of differences in mean antibody titres between treatment groups, an unpaired Student's *t* test was used.

Results

Immunohistochemistry

At 6 and 24 h after anal intubation uptake of antigen in the hindgut was observed in carp and trout which had received complete vaccine, supernatant of the vaccine or LPS (Table 1). The highest uptake was demonstrated in carp which received supernatant or LPS, and in trout after intubation of supernatant (Fig. 1). Transport to intraepithelial macrophages was only observed in carp. Controls were negative for both species used.

Table 1. Immunoreactivity in the second gut segment of carp and trout at 6 and 24 h after anal intubation (a.i.) of different fractions of a *V. anguillarum* bacterin (n = 2).

Species	Carp		Trout	
time a.i.	6 h	24 h	6 h	24 h
vacc	+	+	+	±
sup	+	++*	++*	++*
bact	—	—	—	—
son	±	±	—	—
LPS	++	+	+	+

vacc, complete vaccine; sup, supernatant; bact, bacteria; son, sonicated bacteria; LPS, purified LPS. —, unstained; ±, weak; +, medium; ++, strong immunostaining
* Differences (+ and ++) were observed between the two fish.

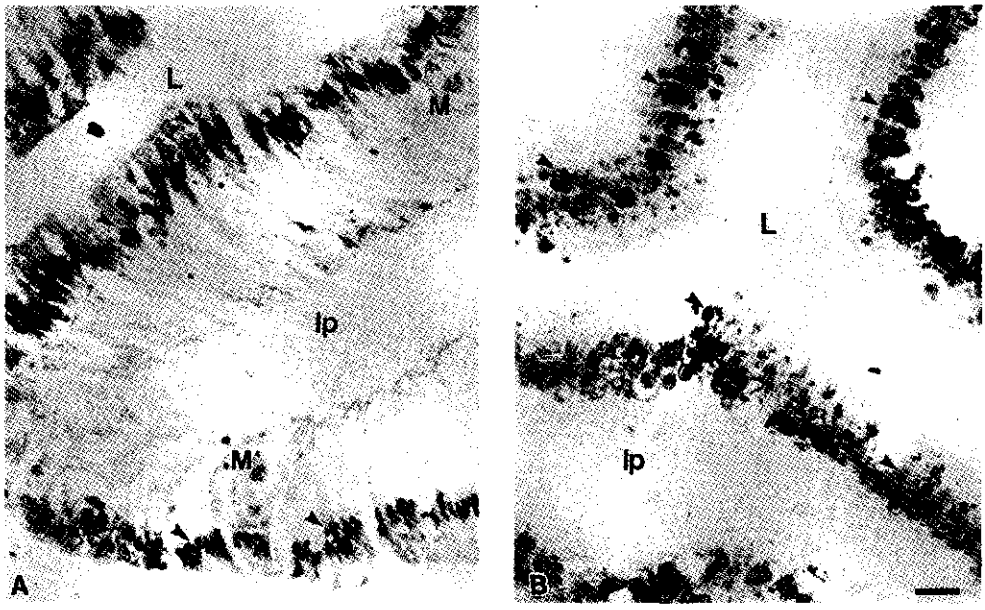

Figure 1. Histological section of the second gut segment of (**a**) carp, 6 h, or (**b**) trout, 24 h after anal intubation of supernatant of *V. anguillarum* bacterin, immunohistochemically stained for *V. anguillarum* determinants by an indirect peroxidase reaction. L, lumen; lp, lamina propria; M, macrophage; arrowheads, immunoreactive vacuoles in the enterocytes. Bar = 10 μm.

Systemic memory formation

Figure 2 shows mean values of primary and secondary Ab titres in serum of carp. The primary Ab titres were significantly higher in fish intubated with complete vaccine or with supernatant compared to control fish. Only in animals which received supernatant was a significantly increased secondary Ab titre found compared to the control (anally intubated with PBS). Additionally, secondary Ab titres in carp which were primed with vaccine or LPS tended to be higher compared to the control. In trout neither the primary nor the secondary Ab titres showed any (significant) increase compared to the control (data not shown).

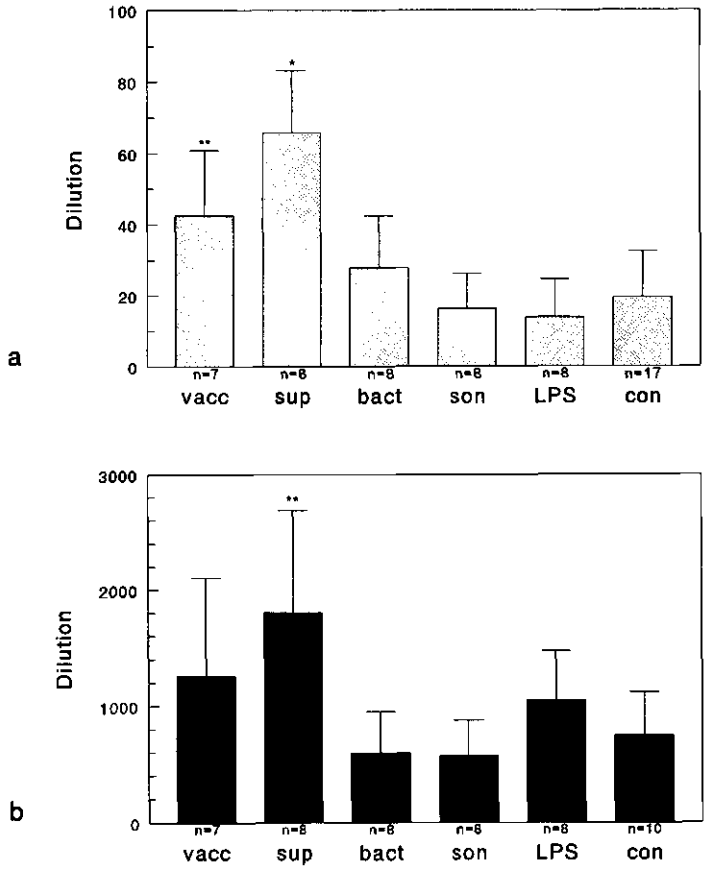

Figure 2. Mean serum dilution (+ S.D.) at O.D. 0.4 in carp. (**a**) Primary response 21 days after anal intubation with different fractions of a *V. anguillarum* bacterin. (**b**) Secondary responses 21 days after intramuscular booster injection with *V. anguillarum* bacterin. *, $P < 0.001$; **, $P < 0.01$, significantly different from controls; n = number of fish per group.

Primary and secondary Ab titres after immunisation with 10 x concentrated fractions are shown in Figure 3 for carp and Figure 4 for trout. In carp, primary Ab titres were significantly increased in fish immunised with supernatant and LPS in comparison with the PBS intubated control. Again the supernatant group showed the highest mean

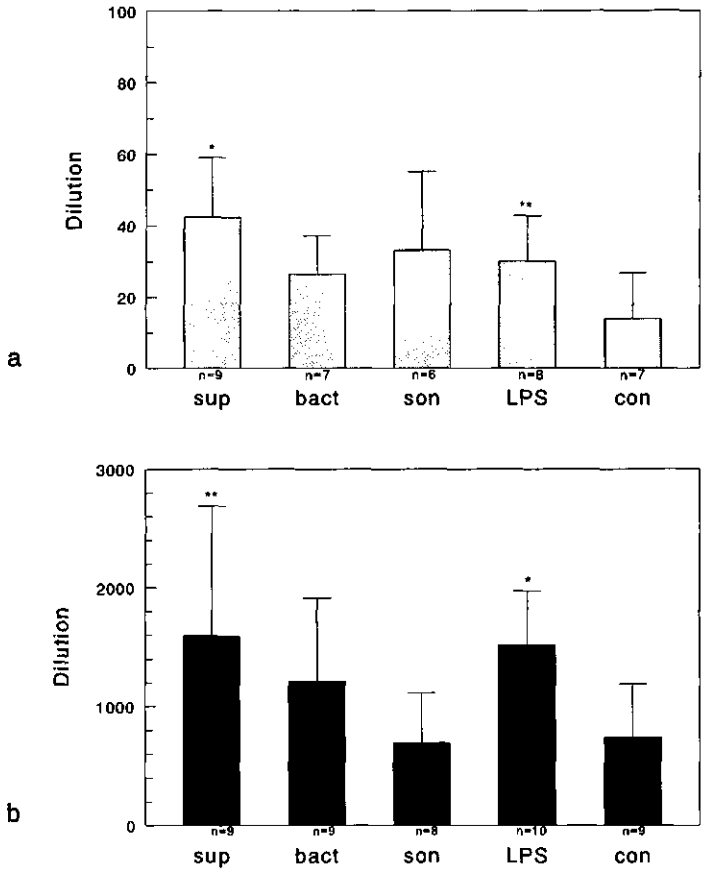

Figure 3. Mean serum dilutions (+ S.D.) at O.D. 0.4 in carp. (**a**) Primary response 21 days after anal intubation with 10 x concentrated fractions of a *V. anguillarum* bacterin. (**b**) Secondary responses 21 days after intramuscular booster injection with *V. anguillarum* bacterin. *, $P < 0.01$; **, $P < 0.05$, significantly different from controls; n = number of fish per group.

Ab titre. Secondary Ab titres were also significantly increased in supernatant and LPS intubated carp compared to the control. In trout, primary Ab titres were increased in the supernatant intubated group. The secondary Ab titre, after intramuscular booster injection, of the supernatant intubated trout tended to be higher than in the control fish (Fig. 4).

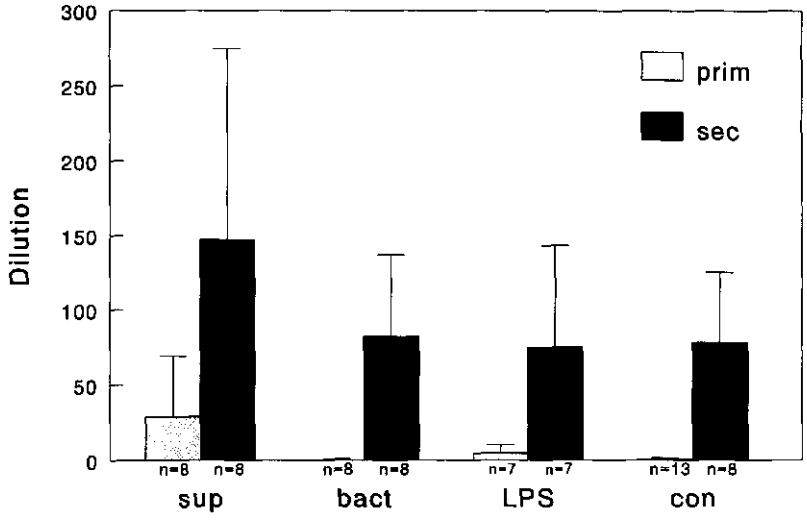

Figure 4. Primary and secondary mean serum dilutions (+ S.D.) at O.D. 0.4 in trout, anally intubated with 10 x concentrated fractions of a *V. anguillarum* bacterin. Booster by intramuscular injection. n = number of fish per group.

Discussion

As found in previous studies (Rombout *et al.*, 1986, 1989a), *V. anguillarum* bacterin was taken up by the enterocytes in the hindgut after anal intubation. In addition, the present investigation shows that soluble factors present in the bacterin, and not whole bacteria (bact), are taken up by the enterocytes. These results indicate that the antigens are only taken up by pinocytosis and not by phagocytosis. Previous studies indeed showed that macromolecules can be taken up in the hindgut by pinocytosis (Noaillac-Depeyre & Gas, 1973; Rombout *et al.*, 1985; 1989a) and Bøgwald *et al.* (1994) stated that degradation of bacteria may be a prerequisite for absorption of bacterial antigens by the gut. Phagocytosis and uptake of dead formalised bacteria did not occur. It can be assumed that the sonicated fraction mainly contains outer membrane proteins and bacterial fragments that might lead to a higher degree of uptake, compared to whole bacteria. However, a better uptake of this fraction by the enterocytes after anal intubation was not observed. Although sonication took place on ice antigenic determinants may be damaged by a short elevation of the temperature during the procedure and the antigenicity may be lost (Al-Harbi & Austin, 1993).

Uptake was observed in the enterocytes of carp and trout after anal intubation of supernatant or LPS. The rabbit-anti-*Vibrio* serum used in these experiments was raised against whole bacteria and appears to react very well with both the supernatant and LPS fraction in the tissue. Moreover, the rabbit-anti-*Vibrio* polyclonal was shown to be reactive with supernatant in a dot blot assay (data not shown). Evelyn and Ketcheson (1980) found that immunogenicity of *Vibrio* was due to a substance occurring in both free and cell-associated form. Furthermore, Chart & Trust (1984) reported that LPS is the major surface antigen of *Vibrio* bacteria. It is assumed that infection with *Vibrio* results in production of antibodies predominantly directed to LPS-structures in fish and mammals (Chart & Trust, 1984; Bøgwald *et al.*, 1992). LPS derived from different bacteria was shown to induce specific protection against disease when applied to fish (Kawai & Kusuda, 1983; Salati *et al.*, 1984; Baba *et al.*, 1988; Velji *et al.*, 1990).

From the present study it can be concluded that formalin-inactivated bacteria were not able to elicit an immune response after anal intubation. However, in carp primary immune responses were induced after anal intubation with supernatant or complete vaccine. In both groups memory formation could be observed after an intramuscular booster 10 weeks later. In fish immunised with LPS secondary antibody titres tended to be higher, while primary titres were not induced in this group. Thus, it can be concluded that the supernatant seemed to contain the most antigenic part (probably the LPS) of the bacterin.

The lower response against anally intubated LPS may be due to the purity of the LPS fraction. Some reports indicate that LPS alone is not enough to elicit memory formation. According to Westphal & Jann (1965), our fraction contains 97% LPS, the rest being RNA. Crude LPS of *Edwardsiella tarda* (50-60% LPS, 40-50% RNA) injected intramuscularly gave higher agglutination titres in eel than pure LPS or lipid A. Pure LPS and lipid A did not result in protection against *Edwardsiella tarda* infection (Salati *et al.*, 1984). Intraperitoneal injection of a LPS-protein complex surface layer antigen (VS-P1) of *Vibrio salmonicida* resulted in protection that was superior to injection with purified LPS (Bøgwald *et al.*, 1992). This might indicate necessity of a more complex antigen which may also stimulate T lymphocytes, instead of only LPS which is assumed to be a T cell independent antigen. On the other hand, administration of higher amounts of LPS (in experiment II; 2.85-fold higher dose of LPS) resulted in higher antibody titres. Apparently more LPS is needed for anal administration compared to other routes. Much less LPS seems to be sufficient to induce immune responses when administered by injection (Saeed & Plumb, 1987; Salati *et al.*, 1989; Velji *et al.*, 1990; Velji *et al.*, 1991) or immersion (Velji *et al.*, 1990).

With the other fractions concerned, immunisation of carp with 10-fold higher doses of antigen resulted in the same observations as described above. In trout, differences in

immune responses between the different treatment groups could only be measured after administration of 10 times more antigen than the amount which resulted in significant responses in carp. In trout apparently more antigen is needed to achieve significant antibody responses. However, as in carp, an intubation of 10 times concentrated supernatant in trout also resulted in increased Ab titres, compared to the control. Trout which received 10 times concentrated supernatant also developed immunological memory, however the differences were not significant due to the high standard deviations. The differences found between these two species may be causes by functional differences in the digestive tract, especially different uptake in the second segment (McLean & Donaldson, 1990). In trout, intestinal macrophages that are thought to be important in antigen-processing in the gut (Georgopoulou & Vernier, 1986; Rombout *et al.*, 1989a), were not observed.

Several authors have found that Ab titres against *V. anguillarum* are strongly correlated with protection (Harrel *et al.*, 1975; Viele *et al.*, 1980; Dec *et al.*, 1990; Velji *et al.*, 1990). The memory formation observed in these experiments was promising with respect to oral vaccination and subsequent protection against *Vibrio anguillarum* infection.

In conclusion, the most immunogenic determinant of the *Vibrio anguillarum* bacterin is present in the supernatant. Memory formation occurs after anal administration of this fraction. Consequently, the supernatant of this bacterin seems to be the best candidate for microencapsulation for an oral vaccine. It will be important to produce microparticles that release the antigen in sufficient quantities in the hindgut of fish.

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Chapter 4

Oral vaccination of fish against *Vibrio anguillarum* using alginate microparticles

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Abstract

In this study the 800 *g* supernatant fraction of a *Vibrio anguillarum* bacterin was encapsulated in alginate microparticles to protect the vaccine against degradation in the anterior part of the digestive tract. Microparticles can be optimised for different fish species with respect to differences in the digestive tract. Two types of microparticles were tested in carp (stomachless) and trout (stomach-containing). For oral vaccination alginate microparticles with or without antigen were added to food or food with non-encapsulated antigen was fed. Uptake of antigen in the hindgut, development of immunological memory and mucosal immune responses were studied. After feeding with encapsulated antigen a better uptake was observed compared to feeding with the same amount of non-encapsulated antigen. Antibody titres were measured 3 weeks after oral vaccination and 3 weeks after an intramuscular booster, given 10 weeks after oral vaccination. Best memory formation in carp was found when fed with encapsulated antigen in microparticles of type I, whereas type II microparticles gave better results in trout. The presence of mucosal plasma cells after repeated oral vaccination of carp with encapsulated antigen was studied with ELISPOT using a mucus-IgM-specific monoclonal antibody. Specific mucosal plasma cells appeared to be present mainly in gut and gills after oral vaccination and absent after intramuscular injection. These results indicate that oral vaccination with encapsulated antigens evokes systemic memory and induces mucosal immune responses in fish. Consequently, oral vaccination appears to be a promising method to control bacterial diseases.

Introduction

Diseases can cause great losses in aquaculture, due to the large populations of fish, crowding and easy transmittance of infections in an aquatic environment. Control of fish diseases is of great concern in aquaculture (Dunn *et al.*, 1990). Fish vaccination is becoming more important, since prevention of diseases is preferable to treatment with antibiotics or chemicals. Although at present injection and immersion are used as beneficial vaccination strategies, oral immunisation would be an ideal alternative (Hart, 1988; Dunn et *al.*, 1990). It is less time and labour intensive, less stressful for fish and allows mass vaccination of fish of any size (Lillehaug, 1989; Ellis, 1988). However, till now oral vaccination is not as effective as injection or immersion vaccination.

Although fish lack Peyer's patches and antigen-transporting M cells, the enterocytes in the hindgut of fish show an antigen-transporting capacity; many lymphoid cells and macrophages are diffusely present between the epithelial cells and in the lamina propria

(Georgopoulou & Vernier, 1986; Temkin & McMillan, 1986; Rombout *et al.*, 1989a,b; 1993a; Dogget & Harris, 1991). Systemic immune responses have been detected after anal immunisation (Georgopoulou & Vernier, 1986; Rombout *et al.*, 1986, 1989c; Joosten *et al.*, 1996). Oral or anal administration resulted in antigen-specific antibodies in skin mucus, bile or intestine in several fish species (Fletcher & White, 1973; Kawai *et al.*, 1981; Rombout *et al.*, 1986, 1989c). The obvious presence of a mucosal immune system makes oral vaccination more promising because the first contact with pathogens mostly occurs through mucosal surfaces (Manganaro *et al.*, 1994). To improve the effectiveness of oral vaccination, development of an efficient delivery method, which protects the antigen against digestive degradation in the anterior part of the digestive tract is of major importance (Johnson & Amend, 1983; Rombout *et al.*, 1986, 1989c; Lillehaug, 1989; Dunn *et al.*, 1990).

In this study a microparticle system for protection of the antigen and efficient oral vaccine delivery to the second gut segment of fish was tested in carp (stomachless) and trout (stomach containing). The 800 *g* supernatant of a *V. anguillarum* bacterin, found to result in a good memory formation after anal intubation (Joosten *et al.*, 1996), was encapsulated in microparticles. Uptake of antigens by enterocytes after feeding the microparticles and memory formation were studied. In addition, mucosal immune responses were monitored in carp using a monoclonal antibody specific for mucosal IgM (Rombout *et al.*, 1993b) in an ELISPOT assay.

Materials and Methods

Animals

Carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) were used. Carp were reared in our fish facilities ("De Haar" vissen) in Wageningen and kept at 23 ± 1°C in recirculating, filtered and UV treated water. Rainbow trout eggs (SPF) were obtained from Seven Springs trout hatchery, Larne, Northern-Ireland and raised in our fish facilities. They were kept at 14 ± 1°C in recirculating, filtered and UV treated water in circular black plastic tanks. At the start of the experiments fish were 6 months old with a mean body weight of 60 and 120 g for carp and trout, respectively. Cloned carp (E4 x E6; Komen *et al.*, 1991) of 14 months old with a mean body weight of 200 gram were used for the detection of mucosal immune responses by ELISPOT. Fish were fed with Trouvit (Trouw & Co BV, Putten, The Netherlands) amounting to 2 % of their body weight per day.

Vaccine

A commercial *V. anguillarum* bacterin (Biovax 1300, BIOMED Inc., Washington, USA), which contained formalin killed cells, was centrifuged for 15 min at 800 *g* to obtain a supernatant of the bacterin. This supernatant (*V.a.*sup) was lyophilised and encapsulated in microparticles or added to the food.

Microparticles

Two different types of alginate microparticles (type I and II) were prepared with and without the *V.a.*sup. The *in vitro* characterisation of these microparticles was realized by optic microscopy (Zeiss Axiophot) and a Coulter Multisizer in order to evaluate the diameter. The influence of pH on the diameter of the microparticles was studied in buffer with different pH (pH 4.8, 8 and 10). The increase in diameter (swelling) of both microparticle types and the release of antigens were studied in a phosphate buffer of pH 8 (20 mM KH_2PO_4). The amount of encapsulated *V.a.*sup and release from microparticles were tested using a sandwich-ELISA. In type II microparticles approximately 4.4 x more *V.a.*sup was encapsulated than in type I microparticles.

Food preparation for oral vaccination

Distilled water was added to the food to obtain a paste, which was subsequently mixed with *V.a.*sup or microparticles. Four different food preparations were made: food containing 18 % of empty microparticles (m), food containing 18 % of microparticles with *V.a.*sup (ms), food containing the same amount of non-encapsulated *V.a.*sup (s), and food containing 10 x the amount of *V.a.*sup (10s). Pellets were made by pressing the paste through a 50 ml syringe, dried for 48 h at room temperature and cut into pellets. Type I food contained 2.4 mg lyophilised *V.a.*sup g^{-1} while type II food contained 10.6 mg lyophilised *V.a.*sup g^{-1}.

Immunisation procedure

In the experiment with type I microparticles 10 fish/group and in the experiment with type II microparticles 18 fish/group were fed once with one of the above mentioned food preparations. Carp and rainbow trout were fed 16.67 g food kg^{-1} body weight. Three weeks later, fish were anaesthetized in 0.03% TMS (Tricaine Methane Sulphonate, Crescent Research Chemicals, Phoenix AZ, U.S.A.) and blood was taken by caudal vein puncture for measurement of the primary antibody response. Blood was allowed to clot at room temperature for 2 h, stored overnight at 4°C and subsequently centrifuged for 5 min at 700 *g*. Serum was collected and stored at - 20°C until measurements were done. Ten weeks after primary immunisation fish received an intramuscular injection (i.m.) of

150 µl of the complete *V. anguillarum* bacterin. Blood was sampled 3 weeks later as described above to measure secondary immune responses in serum .

In another experiment a group of 6 cloned carp was orally vaccinated as described above. For detection of mucosal plasma cells, carp received a second oral vaccination 10 weeks later. Nine days later lymphoid cells were isolated from blood, head kidney, spleen, gut and gills and used in an ELISPOT assay. A monoclonal antibody against mucosal IgM (WCIM2; Rombout *et al.*, 1993b) and against serum IgM (WCI12; Secombes *et al.*, 1983) were used to demonstrate mucosal and systemic plasma cells. Control animals received an i.m. priming and booster with 100 µl of *V.a.*sup.

Immunohistochemistry

In the experiment with type I microparticles 2 times (16 and 36 h for carp and 24 and 48 h for trout) after feeding, two fish of each group were killed by an overdose of TMS and part of the first and second segment of the gut was dissected and fixed in 4 % formaldehyde (overnight). Tissue was further processed as described by Joosten et al. (1996), but non-specific binding was blocked with 10 % Newborn Calves Serum (NCS) for 30 min and all antibodies used in the procedure were diluted in PBS with 1% NCS. Sections were slightly post-stained with heamalum. Preabsorption with *V. anguillarum* bacteria and omission of specific antibodies were carried out as controls.

ELISA

The ELISA used for the detection of *V. anguillarum*-specific antibodies was done as described by Joosten *et al.*, 1995, with some modifications. Plates (Greiner Labortechniek, Alphen a/d Rijn, The Netherlands) were coated with 100 µl of a suspensions of 10^{10} washed *V. anguillarum* bacteria ml^{-1} distilled water by drying overnight at 37°C and subsequently wells were treated with 200 µl of a solution of 1% skimmed milk (ELK, Campina B.V., Eindhoven, The Netherlands) and 0.05 % Tween 20 in PBS (pH 7.2) for 90 min at room temperature. GAM-HRP was used in a dilution of 1:2000. To measure the amount of antigen encapsulated in the microparticles and the release from microparticles a sandwich ELISA was used. In this case 96 wells microtitre plates were coated with 100 µl Rabbit-anti-Vibrio (RAV; 1: 5000 in coating buffer pH 9.6) overnight at 4°C and after blocking, incubated with the samples and washed as described earlier for ELISA. Subsequently plates were incubated with 100 µl RAV conjugated to HRP (1:500 in PBS) for 90 min at 37°C in a moist chamber and further treated as for the ELISA described earlier.

Leucocyte isolation

Fish were killed in TMS and blood was sampled with a syringe containing 1 ml of heparinised carp RPMI (cRPMI; RPMI 1640 with 10 % distilled water added) and centrifuged at 300 *g* and 4°C for 15 min. Supernatant, containing white blood cells, and the buffy coat on top of the erythrocyte pellet was used for isolation of leucocytes. Spleen, head kidney, gut and gills were dissected and cell suspensions were made by forcing the tissues through a 50 mesh nylon gauze filter while adding cRPMI. Cells were washed twice in cRPMI (680 *g* for 10 min at 4°C). Leucocytes were isolated by centrifugation on Lymphoprep (Nycomed, Oslo, Norway) at 700 *g* and 4°C for 30 min. Leucocyte suspensions were washed twice and resuspended in cRPMI with 1% BSA. The viable cell concentration was determined by trypan blue exclusion.

ELISPOT

A modification of the ELISPOT method described by Koumans-van Diepen (1995) was performed. A sheet of nitrocellulose (NC) was incubated in a 96-well dot blot apparatus (*minifold SRC-96; Schleicher & Schuell, Dassel, Germany*) with 100 *µl of V.a.*sup (diluted 1:50 in distilled water) per well for 15 min at room temperature. The NC sheet was then removed, blocked in PBS with 0.1 % BSA for 1 h at room temperature and returned to the dot blot apparatus with a sheet of parafilm beneath it to avoid leakage. Cell suspensions (100 µl in cRPMI) were added to the wells at four different concentrations (10^4, $3.3x10^3$, $1.1x10^3$ and $3,7x10^2$ cells per well). The cell suspensions were poured off after incubation for 5 h at 25 ± 1°C. Subsequently, the wells were washed 3 x with PBS and the sheet was incubated with WCI12 or WCIM2 (diluted 1:100 in PBS) overnight at 4°C. After 3 washings in PBS, the sheet was incubated in alkaline phosphatase conjugated goat-anti-mouse IgG (1:1500; Biorad, Richmond, CA, U.S.A.) in TBS with 0.1% BSA for 1 h at room temperature. Spots were visualized using substrate buffer with 0.1% NBT (Nitro-blue tetrazolium) and 0.04 % BCIP (5-bromo-4-chloro-3-indolyl-phosphate). The NC sheet was rinsed in distilled water, dried at room temperature and stored in the dark. Spots were counted under low magnification with a dissecting microscope.

Statistical analysis

Using data regression, antigen-specific antibody titres are estimated by calculating dilutions at an optical density of 0.4 for trout and 0.5 for carp, respectively. To evaluate statistical significance of the differences between vaccinated and control fish Students *t*-test was used to compare the means of each group for serum antibody titres (ELISA).

Results

In vitro ***characterisation of microparticles***

Figure 1a shows an alginate microparticle containing *V.a.*sup. The microparticles (type I and II) ranged in size from 1 to 20 μm with a mean diameter of 5 μm. Swelling of type II microparticles *in vitro* at different pH is shown in Figure 1b, as percentage increase in mean diameter, starting at the original size (0 %). The swelling is clearly pH dependent; faster and more swelling was observed at higher pH. Figure 1c shows swelling of both types of microparticles in a pH 8 buffer. Type II microparticles increased to 140 % of their original size (0%) while the diameter of type I only increased to 77 % of their original size at pH 8 after 48 h. After 8 hours in buffer of pH 8 approximately 40 % of entrapped *V.a.*sup was released from type I microparticles while 60 % was released from type II (Figure 1d). The release of *V.a.*sup from both microparticles reached the maximum after 36 h. About 70 % of entrapped *V.a.*sup was eluted from type II microparticles while 80 % was eluted from type I microparticles. The slow swelling of type I microparticles corresponded with a slow release of *V.a.*sup and faster swelling of type II corresponded with a more rapid release of encapsulated *V.a.*sup. However, more antigen is released from the type I microparticles after 36 h.

Antigen uptake

In the experiment with type I microparticles, immunostaining of vacuoles in enterocytes in the second gut segment of carp was observed 16 h after feeding ms and 10s, although less staining was observed in the 10s group (Figure 2). Immunoreactive macrophages were found in these groups after 36 h. Immunostaining was only slightly in the second gut segment of carp fed s and absent in carp fed m (Table 1). The second segment of trout fed ms showed many immunoreactive vacuoles at 24 h after feeding. The intestine of trout fed 10s showed slightly positive immunoreactive vacuoles, while an immunoreaction could not be detected in the s and m fed group. Only trout fed ms still showed some immunoreactivity in the second segment at 48 h after feeding. Immunoreactive macrophages were not observed in trout (Table 2).

Systemic immune responses

In the experiment with the type I microparticles, primary Ab titres in carp and trout did not rise above the control level (data not shown). After an intramuscular (i.m.) booster, however, only carp fed ms showed a significantly increased mean Ab titre compared to the control m (Fig. 3a). In trout, after feeding as well as after i.m. booster the Ab titres did not increase compared to the controls fed with only type I microparticles (data not shown). The results with type II microparticles are given in Figure 3b for carp

and Figure 3c for trout. Mean primary Ab titres were only increased in carp fed s (data not shown). After the booster, only carp fed 10 s showed a significant increase in mean Ab titre compared to the control m. In trout the mean Ab titre of the ms fed group was increased after the booster.

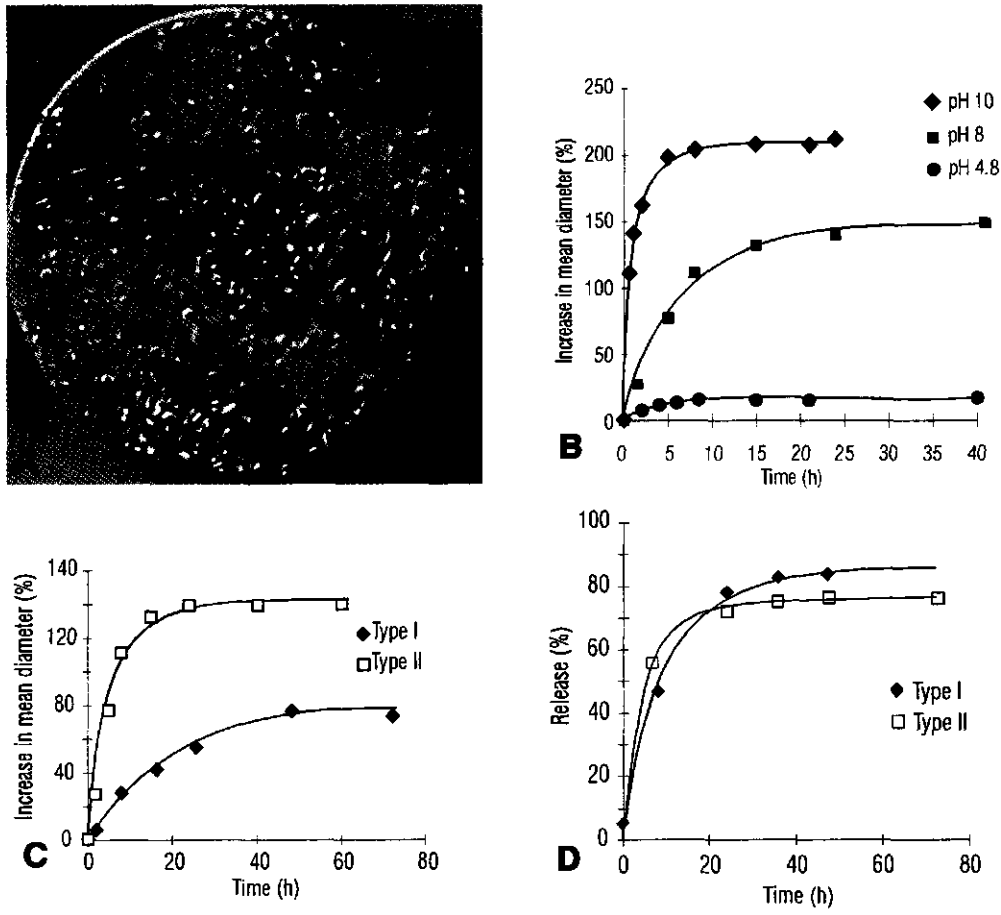

Figure 1. (**A**) Alginate microparticles containing *V. anguillarum* supernatant in pH 8 buffer. Bar = 2.2 μm. (**B**) Effect of different pH on swelling of type II alginate microparticles, showing that at low pH microparticles are resistant to swelling. (**C**) Swelling of type I and type II microparticles in pH 8 buffer, showing faster swelling of type II microparticles. 0 % increase in mean diameter is original size of the microparticles. (**D**) Release of encapsulated *V. anguillarum* supernatant from type I and type II microparticles in pH 8 buffer, showing a slightly slower but better release from type I microparticles.

Table 1. Immunoreactivity of antigenic determinants of *V. anguillarum* in the second gut segment of carp (n = 2), 16 and 36 h after feeding different food preparations with or without type I microparticles.

time (h)	16		36	
	EC	M	EC	M
m	—	—	—	—
ms	+++	+	+	++
s	±	—	—	—
10s	++	+	—	+

m = food containing empty microparticles, **ms** = food containing *V.a.*sup in microparticles, **s** = food containing non-encapsulated *V.a.*sup, **10s** = food containing 10 times the amount of *V.a.*sup; EC = epithelial cells, M = macrophages; +++, ++, +, ±, — = degree of immunoreactivity.

Table 2. Immunoreactivity of antigenic determinants of *V. anguillarum* in the second gut segment of trout (n = 2), 24 and 48 h after feeding different food preparations with or without type I microparticles.

time (h)	24		48	
	EC	M	EC	M
m	—	—	—	—
ms	++	—	+	—
s	—	—	—	—
10s	±	—	—	—

m = food containing empty microparticles, **ms** = food containing *V.a.*sup in microparticles, **s** = food containing non-encapsulated *V.a.*sup, **10s** = food containing 10 times the amount of *V.a.*sup; EC = epithelial cells, M = macrophages; +++, ++, +, ±, — = degree of immunoreactivity.

Figure 2. Histological section of the second gut segment of carp, 16 h after feeding microparticles containing *V. anguillarum* supernatant, immunohistochemically stained for *V. anguillarum* determinants by an indirect peroxidase reaction. L, lumen; lp, *lamina propria*; M, macrophage; arrowheads, immunoreactive vacuoles in the enterocytes. Bar = 10 μm.

Mucosal immune responses (ELISPOT)

WCIM2$^+$ *V. anguillarum*-specific spots were found in gut and gills of orally vaccinated and boosted fish, and a small number in blood and head kidney. In fish which received two i.m. injections with *V.a.*sup WCIM2$^+$ spots were absent in all organs studied. A low amount of WCI12$^+$ spots were found in orally vaccinated fish, while in head kidney and blood of i.m. immunised fish WCI12$^+$ spots were abundant, in contrast to gut and gills were they were absent (Table 3).

Table 3. Specific spots (representing plasma cells)/10^6 leucocytes in carp, 9 days after secondary oral or i.m. immunisation (± SD) in different organs measured with ELISPOT using monoclonal antibodies WCIM2 and WCI12.

WCI12	n	blood	head kidney	gut	gills
oral	6	0	240 ± 201	15 ± 30	58 ± 43
i.m.	2	3363 ± 2005	10070 ± 2956	0	0
WCIM2					
oral	6	56 ± 112	88 ± 165	1324 ± 979	5650 ± 2091
i.m.	2	0	0	0	0

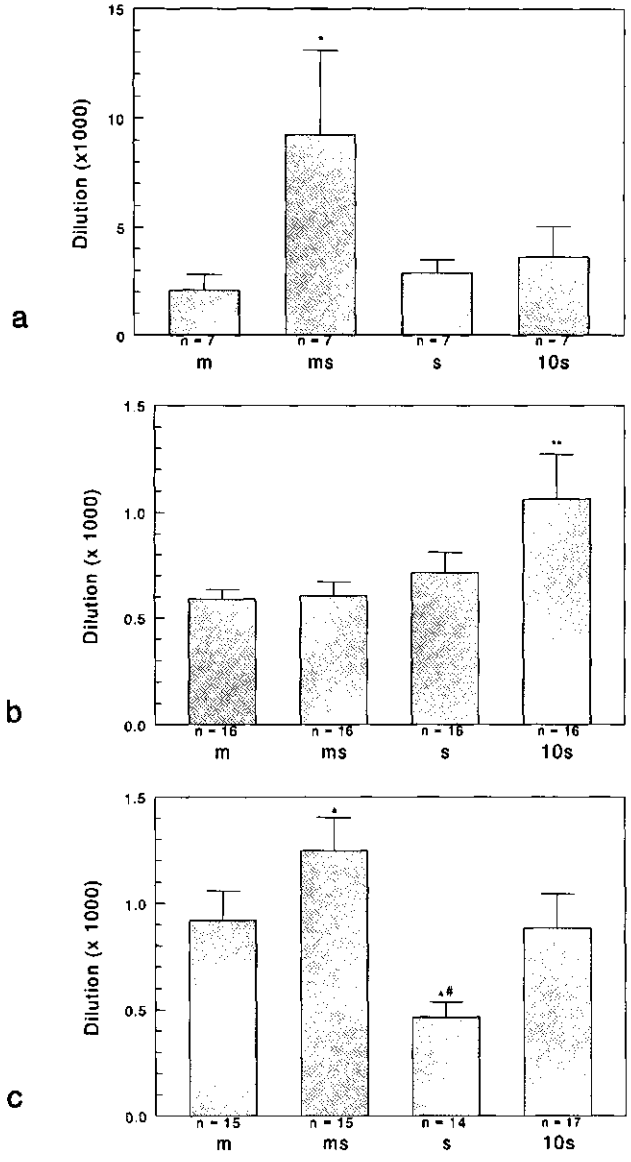

Figure 3. Mean secondary serum antibody titres (± SE) after feeding different food preparations: (**a**) Type I microparticles fed to carp (**b**) Type II microparticles fed to carp (**c**) Type II microparticles fed to trout. *, $P < 0.1$; **, $P < 0.05$; #, $P < 0.01$, significantly different from **m**; ▲, $P < 0.001$, significant difference between **ms** and **s**; n = number of fish per group.

Discussion

This study demonstrates that encapsulation of vaccine in alginate microparticles for oral delivery can be a solution for more effective oral vaccination of fish. Few studies have been reported on encapsulation of antigen in particles for oral vaccine delivery to fish. The use of "prills" and acid-resistant coating to prevent degradation, studied by Lillehaug 1989, was less effective than unprotected *V. anguillarum* in the food of rainbow trout. Enteric coated antigen microspheres, i.e. dextrose beads coated with *V. anguillarum* and an acid resistant film, were studied by Wong *et al.*(1992). After feeding protected antigen to coho salmon for 30 days, survival was not increased with coated compared to uncoated antigen, although surviving fish did have higher serum and mucus antibody titres compared to negative controls and fish fed with uncoated antigen. Piganelli *et al.* (1994) also used ECAMs for oral administration of two different antigens (TNP-LPS and a protein antigen, TNP-KLH) at different concentrations to coho salmon. TNP-LPS at a dose of 10 μg was found to increase serum Ab titres after oral vaccination, while lower doses did not. With TNP-KLH only the lowest dose (0.5 μg) induced a serum antibody response. However, the effectiveness of the ECAMs was not compared to administration of the antigen without protection.

The microparticles used in the present study seem to protect the antigen against degradation due to low pH and proteases in the anterior part of the digestive tract, permitting uptake of antigens in the posterior part. Uptake of *V.a.*sup from the microparticles in enterocytes of the posterior part of the digestive tract was demonstrated using a polyclonal antibody against *V. anguillarum*, indicating that antigenic determinants of *V. anguillarum* are still intact. In carp, more antigen was detected in enterocytes of fish fed with encapsulated *V.a.*sup compared to non-encapsulated *V.a.*sup. Only when 10 x more non-encapsulated *V.a.*sup was fed, a reasonable uptake by the enterocytes could be detected. In trout uptake was only visible in fish fed with encapsulated *V.a.*sup (ms). In mammals M cells can phagocytose microparticles and accomplish sustained or controlled release of its content in the Peyer's patches, thereby stimulating the immune system for a prolonged period (O'Hagan *et al.*, 1993; Morris *et al.*, 1994; Hodges *et al.*, 1995). In fish, enterocytes are not able to phagocytose particles (Dalmo *et al.*, 1995; Joosten *et al.*, 1996), but in the second gut segment they take up macromolecules by means of pinocytosis (Rombout *et al.*, 1986, 1989; Georgopoulou *et al.*, 1986). Consequently the antigen has to be released in a soluble form from the carrier in the lumen of the posterior part of the gut to ensure uptake and transport of antigen by the intestinal epithelial cells (Lillehaug, 1989; Dunn *et al.*, 1990; Bøgwald *et al.*, 1994; Dalmo *et al.*, 1995). In this study alginate microparticles were found to swell *in vitro* at increased pH (pH $>$ 5) and subsequently release *V.a.*sup. Consequently, the slightly

alkaline pH in the posterior part of the digestive tract may result in swelling of the microparticles and release of antigen. Differences in alginate microparticle production are shown to result in different *in vitro* release rates and times. Consequently, alginate microparticles can be produced for each fish species, dependent of differences in morphology and physiology of the digestive tract; i.e. presence of a stomach, pH changes, length of the digestive tract, transport time and uptake capacity are important factors (McLean & Donaldson, 1990). Probably due to these differences between the fish species used, uptake from type I microparticles was better in carp compared to trout, while type II microparticles seem to deliver the antigen better in trout. Probably as a consequence the use of type I and not type II microparticles seems to evoke immunological memory in carp, while type II microparticles appeared to be more effective to induce immunological memory in trout. On the other hand, type II microparticles seems to be ineffective in carp since the Ab response was not increased after feeding encapsulated compared to non-encapsulated antigen. When the antigen is fed unprotected in larger quantities (10s), oral vaccination can also result in significant memory formation. In the experiment with type II microparticles the amount of antigen in the food was 4.4 times higher than in the experiment with type I microparticles. This explains the higher effectiveness of the 10s food in carp in the experiment with type II microparticles compared to the 10s food in the experiment with type I microparticles. Consequently, the amount of antigen in food 10s is 44 times higher compared to the ms group of the type I experiment. It may be concluded that the use of at least 44 times more *V.a.*sup is needed to evoke a similar memory formation in carp as found with the encapsulated antigen. The better response in trout may be due to the larger amount of antigen in type II microparticles and a higher release rate (till $\pm$ 20 h) compared to type I microparticles. A more efficient delivery will result in a higher amount of antigen available for uptake. It is demonstrated earlier that trout needs higher amounts of antigen to elicit systemic immune responses (Joosten *et al.*, 1996). Moreover, in trout the antigen might have left the digestive tract before the *V.a.*sup is released from type I microparticles, because release will start after passage through the stomach. In carp a low pH does not occur and hence the swelling may start already in the anterior part of the gut, and consequently slower release might give better protection against degradation. Lower efficacy of a protected vaccine after oral vaccination compared to unprotected vaccine was reported by Lillehaug (1989), because the antigen in the slow-release pellets was not only protected against degradation, but may also have been protected from being absorbed by the encapsulation procedures. It was suggested that LPS was stable enough in the digestive tract and could consequently result in a better efficiency than protected LPS (Lillehaug, 1989). Our results show that encapsulation of *V.a.*sup, although probably containing LPS as the most important immunogenic molecules (Joosten *et al.*, 1996),

resulted in better immune responses compared to non-encapsulated antigen. As Ab titres against *V. anguillarum* are strongly correlated with protection against vibriosis (Harrel *et al.*, 1975; Viele *et al.*, 1980; Dec *et al.*, 1990), these results indicate a better effectiveness of encapsulated vaccines.

In mammals specific mucosal (IgA) plasma cells are present at all mucosal sites after oral vaccination (Holmgren *et al.*, 1992). In fish the origin of IgM found in mucosal secretions after oral or bath vaccination is still not clear. Production of IgM at mucosal sites is suggested, based on presence of antigen-specific antibodies in mucus and bile, but not in serum, after oral or bath administration of antigen (Kawai *et al.*, 1981; Lobb *et al.*, 1987; Rombout *et al.*, 1989c), and on differences between mucus and serum IgM (Lobb *et al.*, 1981; Rombout *et al.*, 1993b). In carp a monoclonal against mucosal IgM has been described, which reacted with mucus IgM and not with serum IgM (Rombout *et al.*, 1993b). The location of specific mucosal IgM producing cells still was not demonstrated. Our results indicate the presence of specific mucosal plasma cells in mucosal organs of carp after oral vaccination and not after i.m. injection. These cells were not or only partly detected with WCI12, possibly because WCI12 reacts with a lower affinity with secreted mucosal IgM. This lower affinity of WCI12 for mucosal IgM was earlier suggested by Rombout *et al.* (1993b). In earlier studies, where WCI12 was used to measure mucosal IgM (Rombout *et al.*, 1986, 1989c) this lower affinity may have resulted in detection of lower antibody titres than were actually present in bile and mucus. The present results prove the production of mucosal IgM at mucosal sites. In addition, mucosal immune responses in fish seem to develop independent of systemic immune responses and protection against disease may even be established without the development of a systemic immune response.

In conclusion, although alginate microparticle preparations need to be adapted for each fish species, our results indicate that encapsulation appears to be a promising method for oral vaccination of fish. Furthermore, mucosal plasma cells seem to be responsible for production of IgM at mucosal sites and can be found in high numbers after repeated oral vaccination. The development of systemic memory together with the induction of mucosal responses indicate that oral vaccination can be applied to achieve a better protection against bacterial fish diseases.

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Chapter 5

Induction of oral tolerance in carp (*Cyprinus carpio* L.) after feeding protein antigens

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Abstract

Induction of oral tolerance against ferritin, recombinant surface glycoprotein of viral haemorrhagic septicemia virus (KLG18) and ovalbumin (OVA) was studied in carp. Feeding of ferritin or KLG18 resulted in lower Ab titres compared to unprimed controls when animals were intramuscularly (i.m.) injected with protein 10 weeks later and sampled 21 days after this injection. After administration of OVA by different routes (oral, anal, i.m.) and i.m. injection with OVA + Freund's incomplete adjuvant 2 months later, only a few fish responded to OVA as measured by serum Ab titres. Responsiveness to OVA appeared to be carp strain dependent. When an isogenic carp strain was selected for an optimal response to i.m. injection with OVA, this carp strain did not develop oral tolerance after feeding. In contrast, 6x feeding high doses of OVA on subsequent days, resulted in immunological memory formation. Oral tolerance can be induced in carp, but differences in tolerance induction may depend on the protein used. A possible role of genetic factors in the induction of oral tolerance in fish will be discussed.

Introduction

Oral vaccination seems to be a potentially good method for mass vaccination of fish (Ellis, 1988; 1995). It would be more practical and less stressful than parenteral injection or bath immunisation. However, oral vaccination is still less effective than these currently used vaccination methods. Antigen uptake and transport by epithelial cells of the hindgut to gut associated lymphoid tissue (GALT; Rombout *et al.*, 1985; 1989a; Georgopoulou & Vernier, 1986; Temkin & McMillan, 1986; Doggett & Harris, 1991; Rombout *et al.*, 1993) or to blood (Bøgwald *et al.*, 1994) were demonstrated after oral administration of different antigens. Although less effective than other vaccination methods, oral vaccination against bacterial antigens has been shown to result in systemic (Rombout *et al.*, 1986; 1989b; O'Donnell *et al.*, 1994; Loghothetis *et al.*, 1994; Ainsworth *et al.*, 1995: Joosten *et al.*, 1997) and mucosal immune responses (Fletcher & White, 1973; Kawai *et al.*, 1981; Rombout *et al.*, 1986; Loghothetis *et al.*,1994; Ainsworth *et al.*, 1995; Joosten *et al.*, 1997). Also specific protection against diseases was reported after oral administration (Johnson & Amend, 1983; Kawano *et al.*, 1984; Lillehaug, 1989; Dec *et al.*, 1990). These findings indicate the potential of oral vaccination of fish.

Although certain antigens seem to be able to induce immune responses via the oral route, in mammals oral administration of proteins can induce oral tolerance

(McGhee *et al.*, 1992). Oral tolerance is a state of specific immunological unresponsiveness to a subsequent parenteral injection of the specific antigen, induced by oral administration of antigen, that normally would be capable of inducing an active immune response (Mowat, 1994; Husby *et al.*, 1994). Induction of oral tolerance is relatively difficult in rabbits and guinea pigs, whereas ruminants do not develop significant tolerance to dietary materials at all (Mowat, 1994). In fish little attention has been paid to the possibility of oral tolerance induction, although it will have implications for oral vaccine development, especially against viral diseases. When oral tolerance can be induced in fish, vaccines containing recombinant proteins or peptides as immunogen will be unsuccessful (Mowat, 1994). Some studies indicate that induction of oral tolerance can occur in fish. Repeated feeding of ferritin to carp was found to result in a decrease of specific serum antibody titres during the period of feeding antigen and some kind of oral tolerance was suggested (Rombout *et al.*, 1986). Furthermore, simultaneous feeding and i.p. injection of HGG (human gammaglobulin) resulted in oral tolerance in trout, however, only feeding with HGG did not induce tolerance (Davidson *et al.*, 1994). The aim of this study was to investigate oral tolerance induction in fish after feeding different proteins. Ferritin, a recombinant surface glycoprotein of VHSV (Viral Haemorrhagic Septicemia virus; KLG18) and ovalbumin (OVA) were used. OVA is an antigen often used to induce and study oral tolerance in mammals.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L.) were used with a mean body weight of 100 g (age 10 months, exp. 1b), 50 g (age 6 months, exp. 1a and 2) and 35 g (age 5 months, exp. 3). Carp were reared in our fish facilities "De Haar vissen" and kept at 23 $\pm$ 1°C in recirculating, filtered and UV treated water. Fish were fed with pelleted food (Provimi, Zwolle, The Netherlands) amounting to 2% of their body weight per day. In experiment 1b an isogenic carp strain (E4 x R3R8; Bongers *et al.*, 1997) was used. These fish were kept at 25 $\pm$ 1°C.

Antigens

Ferritin (Horse spleen ferritin, 10 %; Mr 450-600 kD) and Ovalbumin (OVA of chicken egg, grade V; Mr 45 kD) were obtained from Sigma (St Louis, USA). KLG18 (Mr 60-62 kD, 10-20 $\mu g.l^{-1}$) is a recombinant protein (surface glycoprotein) of VHSV

and was kindly supplied by Dr. M. Thiry (Pharos, Seraing, Belgium). The proteins were incorporated in the food: OVA, 40 mg.g^{-1} of food; KLG18, 0.3-0.6 μg.g^{-1}; ferritin, 4.4 mg.g^{-1}. In addition, ferritin and KLG18 were also used encapsulated in alginate microparticles before incorporation in the food (Joosten *et al.*, 1997). Distilled water was added to the food to obtain a paste and this was subsequently mixed with protein or an amount of microparticles which contained the same amount of antigen. The food paste was pressed through a 50 ml syringe, dried for 48 hours at room temperature and subsequently cut into pellets.

Immunisation protocol

Experiment 1. OVA

Six groups of fish were used. Fish were treated after a fasting period of 24 h. Three groups were fed with an average of 1% of their body weight (0.5 g in experiment 1a and 1 g in experiment 1b) OVA-containing food per fish: group **1xor** was fed once, **2xor** fed twice with a week interval and group **6xor** fed six times on consecutive days. Group **1xim** received an i.m. injection with 0.2 mg OVA and FIA (1:1 v/v) in 50 μl, while group **2xan** was anally intubated (Joosten *et al.*, 1996) twice with a week interval with 40 mg OVA in 100 μl PBS. Group **con** (control) in experiment 1b was fed with PBS containing food on the same regime as 6xor. Fish received an i.m. booster injection with OVA and Freund's Incomplete Adjuvant (FIA; 0.2 mg/50 μl, 1:1 v/v) after 1 or 2 months, in experiment 1b and 1a respectively. Prior to injection or anal intubation fish were anaesthetized in 0.03% TMS (Tricaine Methane Sulphonate, Crescent Research Chemicals, Phoenix AZ, U.S.A.). Blood was sampled for measurement of antibody levels at day 20, 28 and 35 (in exp. 1b) and day 23 (in exp. 1a) after the booster.

Experiment 2. Ferritin

Three types of food were used: containing a) free ferritin (**fer**), b) ferritin encapsulated in alginate microparticles (**mp/fer**) and c) empty microparticles as a negative control (**mp**). Groups of 10 fish were fed once with one of these food types, at an average of 0.5 g of food per fish. Blood was sampled 21 days after feeding. Ten weeks after oral immunisation the fish received an i.m. injection with 0.1 mg ferritin in 100 μl PBS and blood was sampled 21 days later to measure serum antibody titres.

Experiment 3: KLG18

Three types of food were used: containing a) free KLG18 (**KLG**), b) KLG18 encapsulated in microparticles (**mp/KLG**) and c) empty microparticles as a negative control (**mp**). Groups of 15 fish were fed once with one of the food types, at an

average of 1 g food per fish. Ten weeks after oral immunisation the fish received an i.m. injection with 50 μl KLG18. Blood was sampled 21 days later to measure serum antibody titres.

Blood sampling

Fish were anaesthetized in 0.03% TMS prior to treatment. Blood was sampled by caudal vein puncture, allowed to clot at room temperature for 2 h, stored overnight at 4°C and centrifuged for 5 min at 700 g. Serum was collected and stored at -20°C until measurements.

ELISA

The ELISA used for the detection of specific antibodies was done as described by Joosten *et al.*, 1996. However, plates were coated with 100 μl of protein solution in coating buffer (pH 9.6): OVA 1 $\mu g.ml^{-1}$; KLG18 1:100; ferritin 5 $\mu g.ml^{-1}$. Hyperimmunised sera against the protein antigens were used as standard. GAM-HRP was used in a dilution of 1:2000 in PBS + 0.05% Tween-20. Using data regression, antigen specific antibody (Ab) titres are estimated by calculating dilutions at an optical density of 0.4 (OVA and KLG18) or 0.5 (ferritin).

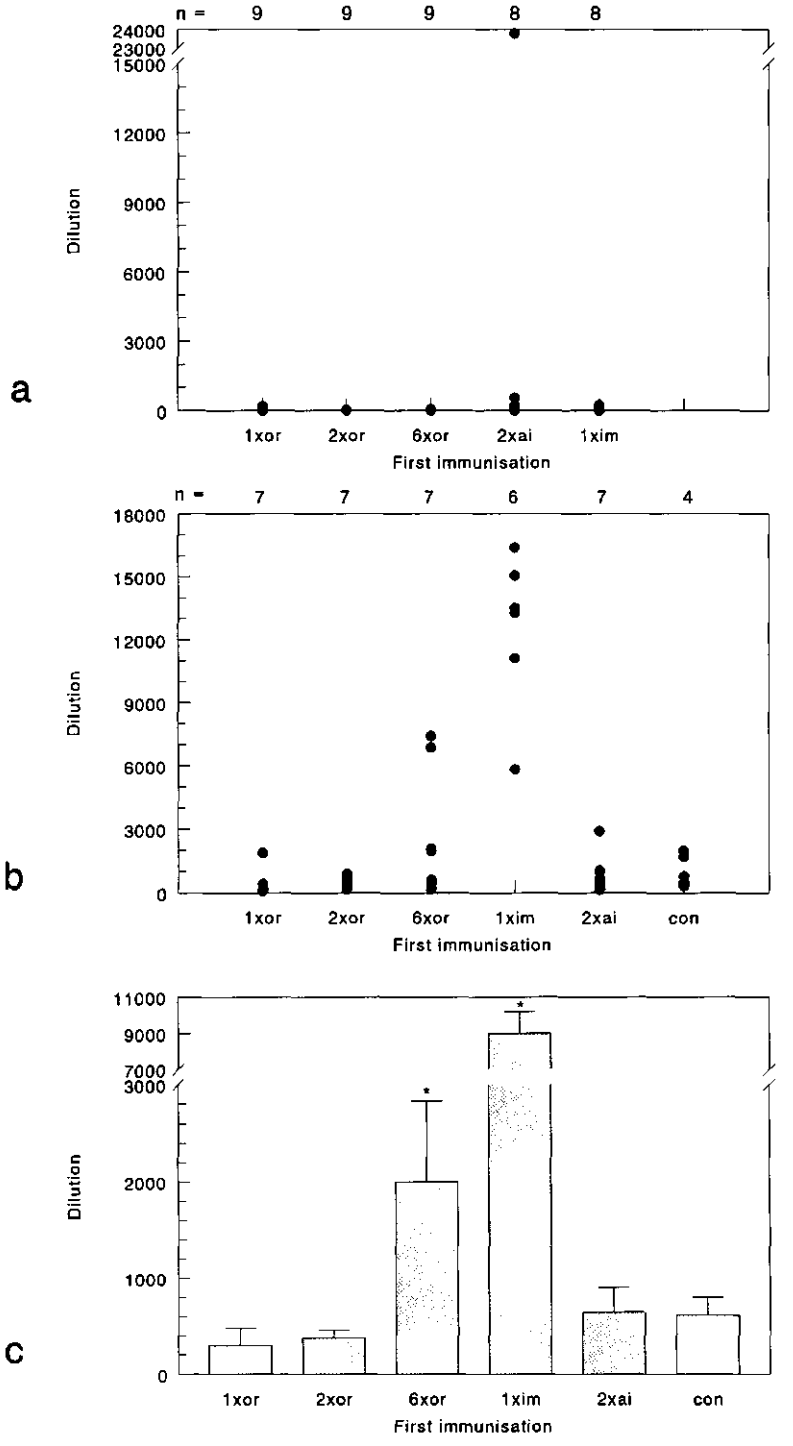
Statistical analysis

Students *t*-test was used to evaluate statistical significance of the differences in the mean serum Ab titres between treated and control groups.

Figure 1. Serum antibody titres of individual carp after treatment with OVA by various routes and i.m. injected with OVA + FIA (**a**) two months later and sampled 23 days after injection, or (**b**) 1 month later and sampled 20 days after injection. Each dot represents the serum Ab titre of one fish; n = number of fish per group

Figure 1c. Mean serum antibody titres (+ SE) of carp (n = 7) treated with OVA and i.m. injected with OVA + FIA 1 month later and sampled 20 days after injection.

1xor = 1x oral; 2xor = 2x oral with a week interval; 6xor = 6x oral on consecutive days; 1xim = 1x intramuscular injection, 2xai = 2x anally intubated with a week interval, con = control; *, $P < 0.1$; **, $P < 0.001$, significantly different from control.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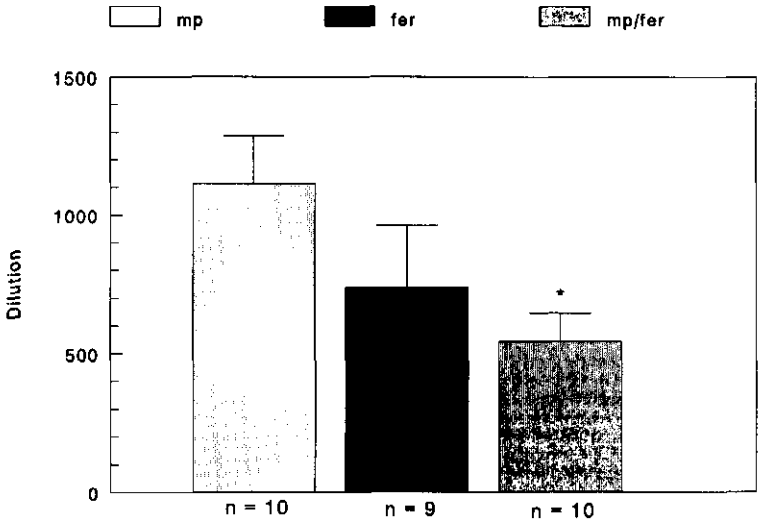

Figure 2. Mean serum antibody titres (+ SE) of carp 21 days after i.m. injection with ferritin, which were fed 10 weeks earlier with different food preparations with microparticles (mp), ferritin (fer) or ferritin-containing microparticles (mp/fer); *, $P < 0.01$, significantly different from mp; n = number of fish per group

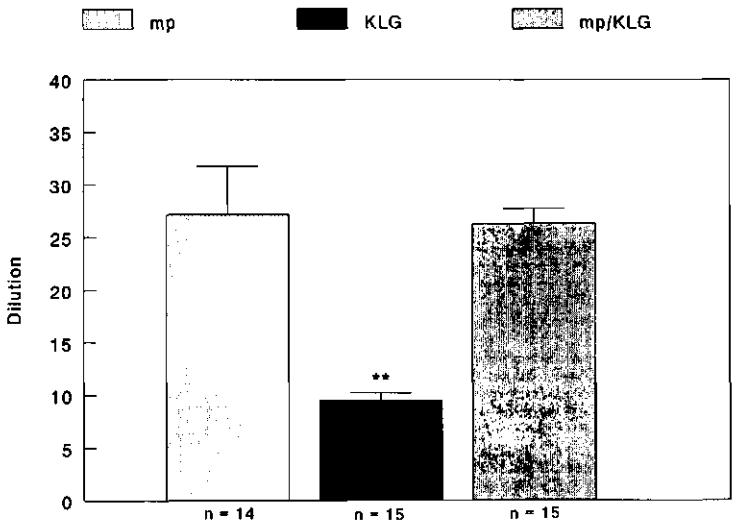

Figure 3. Mean serum antibody titres (+ SE) of carp 21 days after i.m. injection with KLG18 which were fed 10 weeks earlier with different food preparations with microparticles (mp), KLG18 protein (KLG18) or KLG-containing microparticles (mp/KLG18); **, $P < 0.001$, significantly different from mp and mp/KLG18; n = number of fish per group.

Results

OVA

Significant differences in mean Ab titres between the groups were not observed in experiment 1a (Fig. 1a). However, antibody titres of a few fish were above the average of all groups, while Ab titres were generally low, compared to Ab titres observed in experiment 1b. A significant increase in mean serum Ab titre to OVA was found in experiment 1b in the 1xim group, compared to the unprimed control (con; Fig. 1c). Also after repeated feeding of OVA (6xor), the mean Ab titre after i.m. injection were significantly increased compared to the unprimed control. However, no significant differences were observed between the mean Ab titres of the other groups. At the first sampling time (day 20 after booster injection) the Ab titres were higher than at later sampling times (day 28 and 35 after i.m. booster injection) in all groups (not shown). Ab titres per individual fish (of experiment 1b) are displayed in Figure 1b to illustrate variability between fish.

Ferritin

The mean Ab titre to ferritin in carp after feeding encapsulated ferritin was significantly increased compared to the Ab titre in carp fed with only microparticles (not shown). At 21 days after booster injection the mean Ab titres of both groups fed with ferritin containing food (fer and mp/fer) were lower, and in the mp/fer group significantly lower, than the control group (Fig. 2). The mean Ab titre of the control group (mp) can be regarded as a primary immune response to injection with ferritin, since this group did not receive any ferritin in the food (unprimed).

KLG18

Figure 3 shows the mean Ab titres of carp 21 days after booster injection with KLG18. Fish previously fed with free KLG 18 in the food (KLG) showed a lower mean serum Ab titre compared to the negative control, which only received an i.m. injection (mp). Mp/KLG feeding had no effect on the serum Ab titres after injection, compared to the control (mp). Ab titres against KLG18 were generally much lower, compared to the immunisation with the other protein antigens.

Discussion

In mammals oral tolerance to several antigens has been described (Mowat, 1994). The effect of feeding antigens on the immune response seems to depend on the type of antigen, dosage and immunisation schedule (Mowat, 1994; Husby *et al.*, 1994). Especially feeding of proteins can induce oral tolerance. Suppression of the humoral immune response seems to be more difficult than of cell mediated immunity (CMI). For tolerisation of the humoral response higher doses and often more feedings are necessary, while with low doses CMI can be tolerised (Mowat, 1994). B cells seem to recover much more quickly than T cells after tolerisation by feeding antigen (Vives *et al.*, 1980; Mowat, 1994; Weiner *et al.*, 1994). At doses of antigen immediately below those that induce tolerance, feeding antigen has no effect on

systemic immunity, whereas even lower amounts (1-50 μg OVA in mice) actually may prime the immune response (Mowat, 1994; Lamont *et al.*, 1989). Our results suggest that in carp prolonged feeding primes the immune response to OVA, although high doses are used. Rombout *et al.* (1989b), however, reported a decrease of the specific serum Ab titre in carp after prolonged feeding of ferritin. This may indicate tolerance induction after multiple feedings, although this was not investigated by measuring Ab titres after parenteral booster injection. In contrast to OVA, our results with ferritin and KLG18 demonstrate that a single feeding of protein can result in decreased Ab titres after i.m. injection 10 weeks later. This indicates that oral tolerance can be induced to certain protein antigens in fish. Systemic tolerance after a single feed of protein is also reported in mice (Challacombe & Tomasi, 1980; Mowat et *al.*, 1982; 1986; Mowat, 1994). Only one feed of a few mg of OVA is needed to induce oral tolerance (Challacombe & Tomasi, 1980; Mowat et *al.*, 1986). Oral tolerance can appear very soon after feeding and the maximum degree of unresponsiveness then occurs during the first weeks after feeding. In most experimental protocols a 1 to 2 week gap between feeding and challenge has been found most appropriate, although suppression of humoral immunity after antigen feeding has been shown to persist for at least a few months (Challacombe & Tomasi, 1980; Strobel & Ferguson, 1987; Mowat, 1994). In the experiment with OVA carp received an i.m. injection 30 days after oral immunisation, but tolerance was not observed. It is not likely that oral tolerance in carp develops much slower than in mice, because decreasing specific Ab levels against ferritin are already observed approximately 20 days after feeding (Rombout *et al.*, 1989b). In our experiments oral tolerance against ferritin and KLG18 persisted until 10 weeks after oral immunisation of carp, indicating that also in fish oral tolerance may persist for at least 2 months.

The dose required for optimal induction of tolerance may differ, perhaps reflecting size or charge related differences in uptake by the intestine as well as differences in immunogenicity (Mowat, 1994). For example, in mice it has been demonstrated that OVA is more tolerogenic than bovine gammaglobulin or HGG (Rios et *al.*, 1988). Even after oral delivery of multiple high doses (10 $mg.g^{-1}$), oral tolerance to OVA was not induced in trout (Davidson *et al.*, 1994). A suppressive effect of orally fed antigen in coho salmon or trout was only found with simultaneous i.p. injection with antigen (Udey & Fryer, 1978; Davidson *et al.*, 1994). In our study, repeated feeding (6xor) of fish with a high dose (40 mg/fish) of OVA did result in systemic memory formation to OVA, and one feeding with OVA did not affect the serum antibody titres compared to the unprimed control. In contrast with mice, OVA was not able to induce oral tolerance, although higher doses were fed than with ferritin and KLG18, which both were able to induce oral tolerance in carp.

KLG18 seems to be a protein with a low antigenicity, because the titres after i.m. priming were very low, compared to ferritin. It might be necessary to administer this protein with adjuvant to induce a higher immune response after parenteral injection. After oral administration of KLG18 in microparticles, Ab titres are not affected, while a decrease of the specific Ab titre is found with "free" KLG18. The absence of tolerance induction with encapsulated KLG18 may be the result of a limited release of the protein. Ferritin effectively induced oral tolerance with both food preparations, but the titre was lower when microparticles were used. Different

alginate formulations are used for ferritin and KLG18, which may explain the differences observed in tolerance induction. In earlier studies using *Vibrio anguillarum* antigens encapsulated in different alginate formulations, similar results were observed; type I and not type II alginate microparticles were effective for the release of bacterial antigen in carp (Joosten *et al.*, 1997). Probably, microparticles used for ferritin encapsulation were more suitable for protection of antigen in the gut, thus delivering more ferritin in the hindgut than after administration of free ferritin, resulting in a better tolerance induction.

Variability was observed between individual carp after administration of OVA, and most of them did not develop Ab titres. Only some fish were able to respond to OVA. In trout, after i.p. injection of OVA inconsistent reactions concerning the induction of Ab titres to OVA were also reported (Whittington *et al.*, 1994). In a pilot experiment differences in the ability to react to OVA were found between different fish strains (not published). The response to OVA seemed to be genetically determined. We have selected an isogenic carp strain because of its ability to produce Ab to OVA after i.m. injection. Responses to OVA also have been shown to be strain dependent in mice. However, although low responder mouse strains can be tolerised by mucosal pretreatment more easily than high responder strains, high responsiveness per se is not a condition for resistance to tolerance induction (Vaz *et al.*, 1987). Balb/c mice were more susceptible to oral tolerance induction to HGG than other strains (Mowat *et al.*, 1987; Lamont *et al.*, 1988). Furthermore, Balb/B mice do not develop oral tolerance with OVA (Vaz *et al.*, 1987; Rios *et al.*, 1988; Lamont *et al.*, 1988). A possible role for MHC genes, in combination with other background genes, in the regulation of oral tolerance is suggested. Difference in uptake or processing of antigen may also be under genetic influence, because differences in uptake of OVA between different mouse strains was reported (Mowat *et al.*, 1987).

Even with isogenic carp, used in this study, differences in immune responses against OVA were found between individuals. Although a high dose of food and antigen were administered, differences in food uptake between the individuals will occur, resulting in variations in the antigen dose per fish. Also after i.m. injection with OVA intragroup differences were observed, indicating variability in the administration of antigen by injection; some leakage is unavoidable after i.m. injection with an antigen in Freund's adjuvant.

In conclusion, induction of oral tolerance is possible in carp. However, differences between proteins, possibly dependent on dose or chemical characteristics of the protein, were observed. Genetic factors may also be important in the susceptibility to induction of oral tolerance in fish, like demonstrated in mice. Especially when subunit vaccines or synthetic proteins are used for oral vaccines this may seriously influence the efficacy of oral vaccination.

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Chapter 6

Indications for a distinct mucosal T cell population in carp (*Cyprinus carpio* L.)

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Abstract

A monoclonal antibody against carp intestinal T cells (WCL38; of IgM class) was produced by immunisation of mice with isolated membrane molecules of carp intestinal epithelial cells, which predominantly consist of Ig^- lymphoid cells. Flow cytometric analysis showed that WCL38 reacted with 50-70% of the lymphoid cells isolated from intestine or gills and with less than 6% of lymphoid cells isolated from thymus, head kidney and spleen and with a negligible number of PBL. Immunogold labelling revealed that $WCL38^+$ cells can be considered as lymphoid cells and in gills a part of them as large granular lymphoid cells. Because WCL38 does not react with Ig^+ B cells or macrophages and the number of NCC cells seems to be negligible in IEL suspensions (< 2%), WCL 38^+ cells can be considered as T cells. In cryo-sections $WCL38^+$ lymphoid cells were abundantly found within the intestinal epithelium, but a limited number of cells could also be detected in the lamina propria. Many $WCL38^+$ cells were found in gills. In thymus, spleen and head kidney immunoreactive cells are scarcely present. Immunochemical analysis showed that WCL38 reacted with a 40 kD molecule. In conclusion, like higher vertebrates, carp seem to have a distinct mucosal T cell population. The significance of such a T cell population will be discussed.

Introduction

Cell-specific monoclonal antibodies (mAb) are indispensable to the identification of mammalian leucocytes, especially lymphocyte subpopulations. The panels of mAb to fish leucocytes are very limited and in addition most mAb are species-specific (Koumans-van Diepen *et al.*, 1993). In several species, including carp, mAb raised against immunoglobulin (Ig) are available and can be used to distinguish B cells from T cells and other Ig^- lymphoid cells (Koumans-van Diepen *et al.*, 1993). However, only a limited number of specific T cell markers have been described for fish: a mAb (DLT15) reacting with a T cell population of sea bass, *Dicentrarchus labrax* L. (Scapigliati *et al.*, 1995), a mAb (WCL9) reacting with early T cells of carp (Rombout *et al.*, 1997) and a mAb reacting with a T cell population in channel catfish (Passer *et al.*, 1996). In addition, a mAb (5C6) raised against channel catfish, *Ictalurus punctatus*, non-specific cytotoxic cells (NCC; Ig^- lymphoid cells) is described which appeared to react with a variety of vertebrate species NK cells (Evans *et al.*, 1988; Evans & Jaso-Friedman, 1992). Till now thymocytes have been used to produce T cell-specific mAb. However, intestinal cell suspensions of carp contain around 90% Ig^- lymphoid cells, mainly intraepithelial cells (IEL; Rombout *et al.*, 1993) and hence seem to be a good source of T cells. On the other

hand, the majority of mammalian intestinal T cells are clearly distinct from systemic T cells with respect to their membrane molecules. For instance most of them have a γδT cell receptor (TCR) and ααCD8, instead of αßTCR and αßCD8 (Lefrançois & Puddington, 1995; McGhee *et al.*, 1992). In this study cell lysates of carp IEL suspensions are used to immunise mice and a mAb specific for Ig⁻ IEL is selected and further analysed.

Materials and Methods

Animals

Carp (6-12 months old) were bred in our fish facilities, "De Haar vissen" and kept at 23°C ± 0.5°C in a recirculation system with filtered UV treated water. They were fed with pelleted food (Trouvit, Trouw Nederland BV, Putten, The Netherlands) at a daily ration of 2% of the body weight. Before each treatment, animals were anaesthetized with tricaine methane sulphonate (TMS; 0.3 g ml^{-1} water; Crescent Research Chemicals, Phoenix, AZ, USA).

Leucocyte isolation and flow cytometry

Before dissection of lymphoid organs fish were bled completely using a syringe filled with 0.5 ml carp RPMI (cRPMI: RPMI + 10% distilled water) containing 50 IU ml^{-1} heparin for each 2 ml blood. The mucosa of gills and intestine are scraped off the underlying tissue and subsequently, like other lymphoid organs, squeezed through a nylon gauze and suspended in cRPMI. Leucocytes from blood with a density < 1.077 g ml^{-1} were isolated using Lymphoprep (Nycomed, Oslo, Norway) and from thymus, spleen, head kidney, intestine and gills using a discontinuous Percoll gradient (Pharmacia AB, Uppsala, Sweden; densities of 1.02 and 1.07 g ml^{-1}) centrifugation for 30 min at 1500 x *g*. In most cases leucocytes were immunolabelled as described earlier (Koumans- van Diepen *et al.*, 1994) and subsequently analysed with a flow cytometer (FACStar, Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) using the FlowMATE data analysis package (Applied Cytometry Systems, Sheffield, U.K.).

Antigen preparation and mAb procedure

Approximately 10^9 IEL were isolated from one individual and resuspended in TBS (18.18 mM Tris, 0.82% NaCl, 0.73 mM $MgCl_2$, 0.18 mM $CaCl_2$) with 1% BSA (TBS^+), subsequently treated with 0.1 mM phenyl methyl sulphonyl fluoride + 1 mM ethylmaleinide in TBS and fragmented in a small Potter tube for 4 min on ice. Nuclei were separated by centrifuging for 5 min at 160 *g* at 4°C and then the supernatant was centrifuged for 1 h at 100,000 *g* at 4 °C. The membrane pellet was washed in TBS and

centrifuged again. Membranes were incubated in 1% CHAPS (3-[(3cholamido-propyl)-dimethylammonio]-1-propanesulphonate) in TBS^+ for 1h at 4°C and centrifuged again.

Balb/c mice were immunised subcutaneously with 200 µl of the membrane lysate mixed with Freund's incomplete adjuvant (1:1 v/v) and boostered intraperitoneally with only 100 µl membrane lysate 30 days later. Mouse spleen cells were isolated 3 days after the booster, fused with SP2/0-Ag-14 myeloma cells and cultured as described earlier (Rombout *et al.*, 1993b; Rombout *et al.*, 1996). Fast growing hybridoma cultures supernatants (n = 230) were first screened on IEL suspensions with the flow cytometer and 19 positive clones again on IEL, thymocyte and PBL suspensions. For both procedures fluorescein isothio-cyanate labelled rabbit-anti-mouse Ig serum (RAM-FITC; Dakopatts, Glostrup, Denmark; 1:100) was used. Only 3 clones produced a mAb not reactive with most, if not all, PBL or thymocytes, of which one (WCL38, showing the highest affinity) was selected and characterised further in this study. Isotyping was done with mouse mAb isotyping strips (Boehringer, Indianapolis, USA) and revealed that WCL38 belongs to IgM class.

Immunocytochemistry

Immunocytochemical labelling was carried out on cell suspensions and cytocentrifuge preparations of leucocytes and on cryo-sections of different organs using WCI12 (mAb specific for carp IgM; Koumans-van Diepen, 1993; Rombout *et al.*, 1993b; Secombes *et al.*, 1983), WCL6 (mAb specific for carp thrombocytes; Rombout *et al.*, 1996), WCL9 (mAb specific for early T cells; Rombout *et al.*, 1997), 5C6 (anti-catfish NCC; Harlan Bioproducts for Science, Indianapolis, USA; Evans *et al.*, 1988; 1992) and the selected WCL38 as first mAb, and RAM-FITC or tetramethyl-rhodamine isothiocyanate labelled rabbit-anti-mouse serum (RAM-TRITC; Dakopatts, Glostrup, Denmark; 1:100) as second antibody. Cell preparations were studied with fluorescence microscopy or flow cytometry. Immunocytochemical double staining was carried out using an FITC-labelled goat-anti mouse IgG (Southern Biotechnology Associates, Inc.; 1:20) for detection of WCI12, followed by r-phycoerythrin labelled goat-anti-mouse IgM (Southern Biotechnology Associates, Inc.; 1:100) for detection of WCL38.

Immuno-electronmicroscopy

Electron microscopic identification of immunoreactive cells was performed by immunogold labelling according to the method earlier described (Van Diepen *et al.*, 1991). Briefly, leucocytes were labelled in suspension with WCL38 and, after rinsing, labelled with a goat-anti-mouse Ig conjugated to 25 nm gold particles (Aurion, Wageningen, The Netherlands) prior to fixation for electron microscopy. Ultra-thin Epon sections were, after treatment with uranyl acetate/lead citrate, investigated in a Philips

208 transmission electron microscope.

Immunochemistry

For immunoprecipitation IEL and PBL membrane lysates were analysed (reduced with ß-mercaptoethanol) in an ECL protein biotinylation module applying protocol D of the manufacturer (Amersham, Buckinghamshire, UK). ECL molecular weight standards were used (Amersham, Buckinghamshire, UK).

Results

WCL38 (of IgM class) appeared to react with 50-70% of IEL and gill leucocyte suspensions, with $<6\%$ of thymus and $<3\%$ of spleen and head kidney suspensions and a negligible number of PBL as shown on cytocentrifuge slides (Fig. 1) and with flow cytometry (Fig. 2). Table 1 summarizes the distribution of $WCL38^+$ lymphoid cells in different organs as measured with flow cytometry in comparison with other lymphoid cell subpopulations. $WCL38^+$ cells are the major population of lymphoid cells in IEL and gill cell suspensions; only a limited number of Ig^+ cells are found, whereas the number of

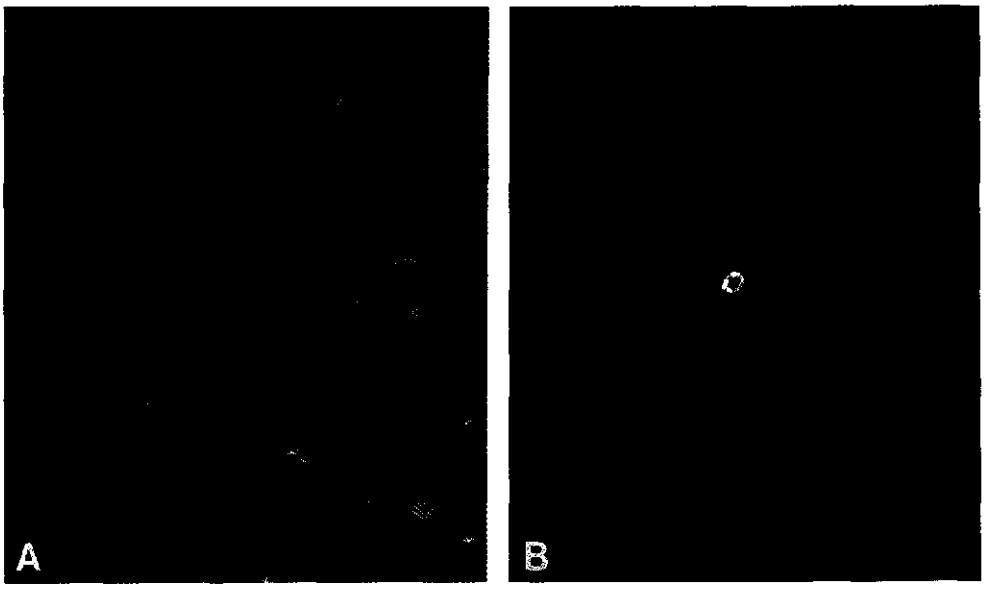


Figure 1. Fluorescence micrograph of a WCL38-labelled cytocentrifuge slide of (**a**) IEL, with a high proportion of positive cells and (**b**) PBL, with one rare positive cell. Magnification 320x.

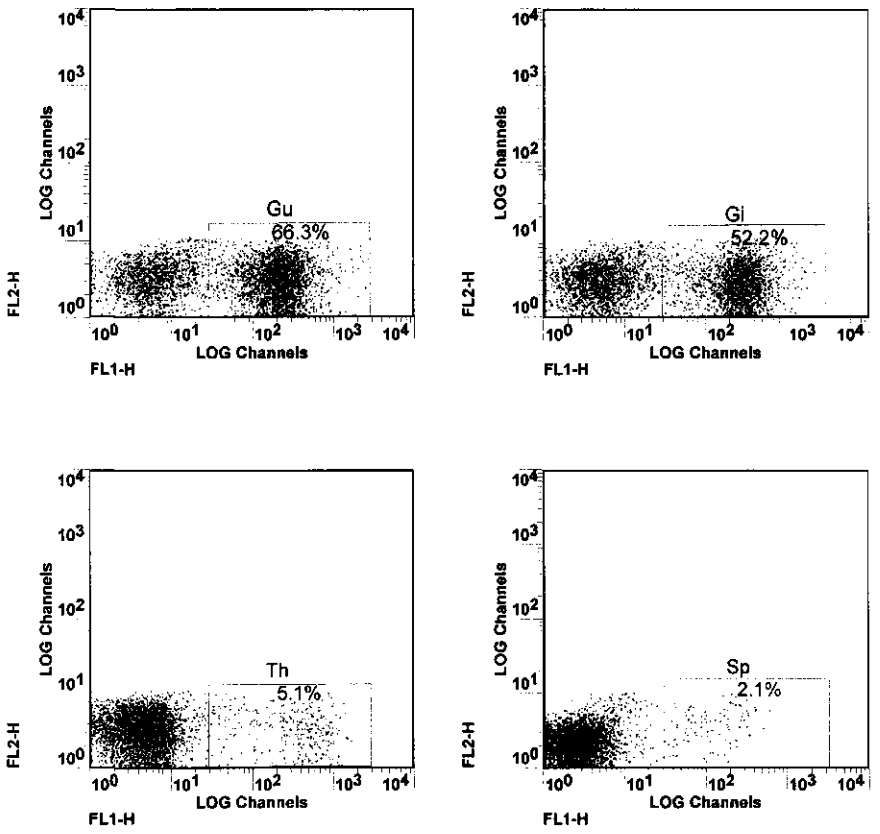


Figure 2. Flow cytometric dot blots of WCL38 labelled lymphoid cells (gated) of (**a**) gut, (**b**) gills, (c) thymus and (**d**) spleen. The proportion of WCL38$^+$ cells is 66.3, 52.2, 5.9 and 2.1%, respectively.

thrombocytes and 5C6$^+$ NCC are negligible. On the other hand, in other lymphoid organs the number of WCL38$^+$ cells is very limited, while much more other lymphoid cells (including thrombocytes) are detected. Double staining experiments demonstrated that WCL38$^+$ lymphoid cells do not react with WCI12 and can be considered as non-B cells (not shown).

In cryo-sections WCL38$^+$ lymphoid cells were abundantly found within the intestinal epithelium, but a limited number of cells could also be detected in the lamina propria (Fig. 3). Moreover, many WCL38$^+$ cells were detectable in gills (not shown). In thymus, spleen and head kidney a limited number of solitary WCL38$^+$ cells can be found throughout the tissue (Fig. 3).

Table 1. Proportion (%) of WCL38$^+$, WCI12$^+$ (B cells), 5C6$^+$ (NCC), WCL9$^+$ (early T cells) and WCL6$^+$ (thrombocytes) lymphoid cells[a] in gut (IEL), gills, thymus, spleen, head kidney (HK) and blood (PBL) cell suspensions as estimated by flow cytometry.

	Cells					
	IEL	Gills	Thymus	Spleen	HK	PBL
WCL38$^+$	50-70	50-70	3-6	1-3	1-3	<1
WCI12+	5-10	5-10	1-5	20-25	20-25	30-50
5C6$^+$	1-2	ND	1-2	2-11	2-16	1-6
WCL9$^+$	<1	<1	30-50	<1	<1	<1
WCL6$^+$	<1	<1	<1	5-15	<1	30-35

[a]) Variation in the proportion of immunoreactive cells in the lymphoid cell suspensions ($n \geq 8$) derived from a density between 1.020 and 1.070 g ml^{-1} and calculated from a gate containing the lymphoid cells. ND: not done.

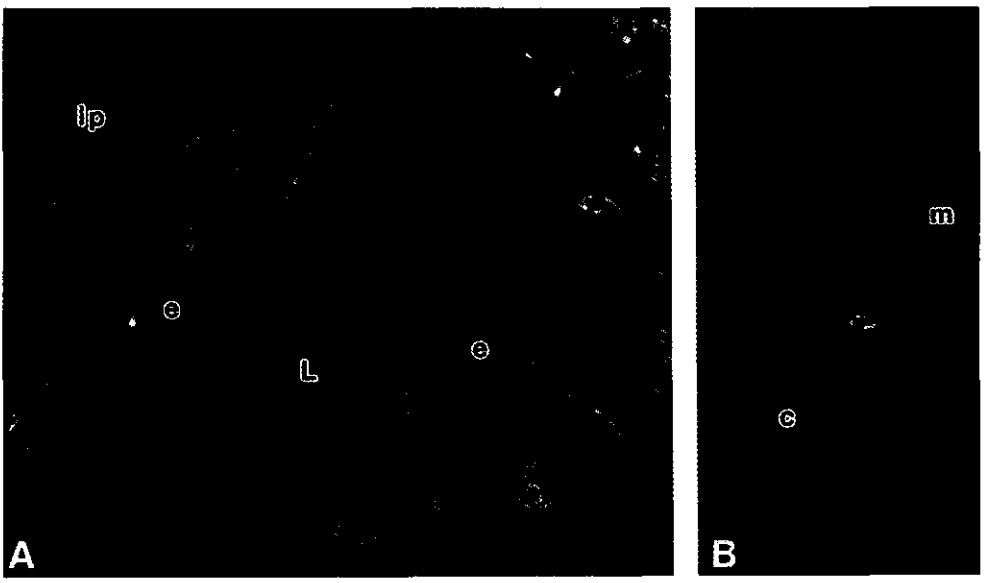


Figure 3. Fluorescence micrographs of cryo-sections of (**a**) gut of carp, showing high numbers of WCL38$^+$ cells in the epithelium (e) and only limited numbers in the lamina propria (lp) and (**b**) some clustered WCL38$^+$ cells at the transition of cortex (c) and medulla (m) in thymus of adult carp. L, lumen. Magnification 320x.

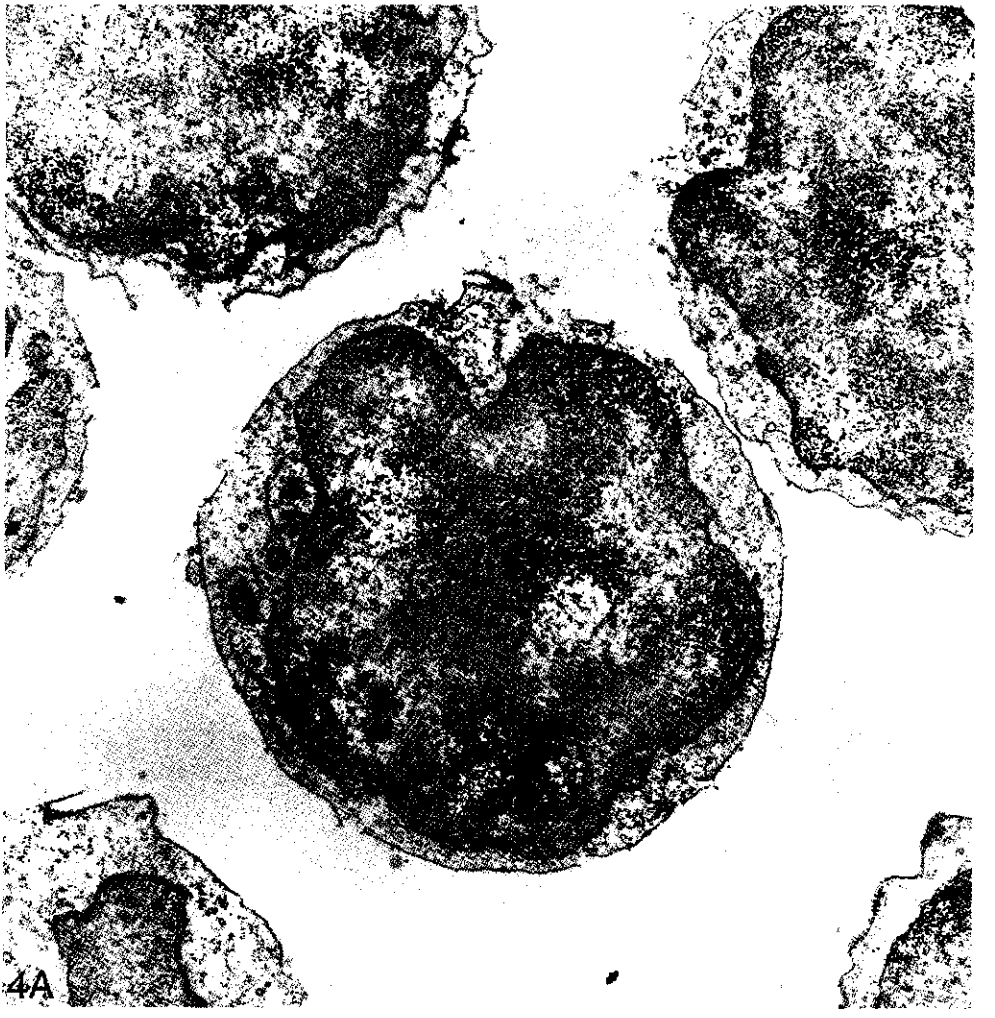
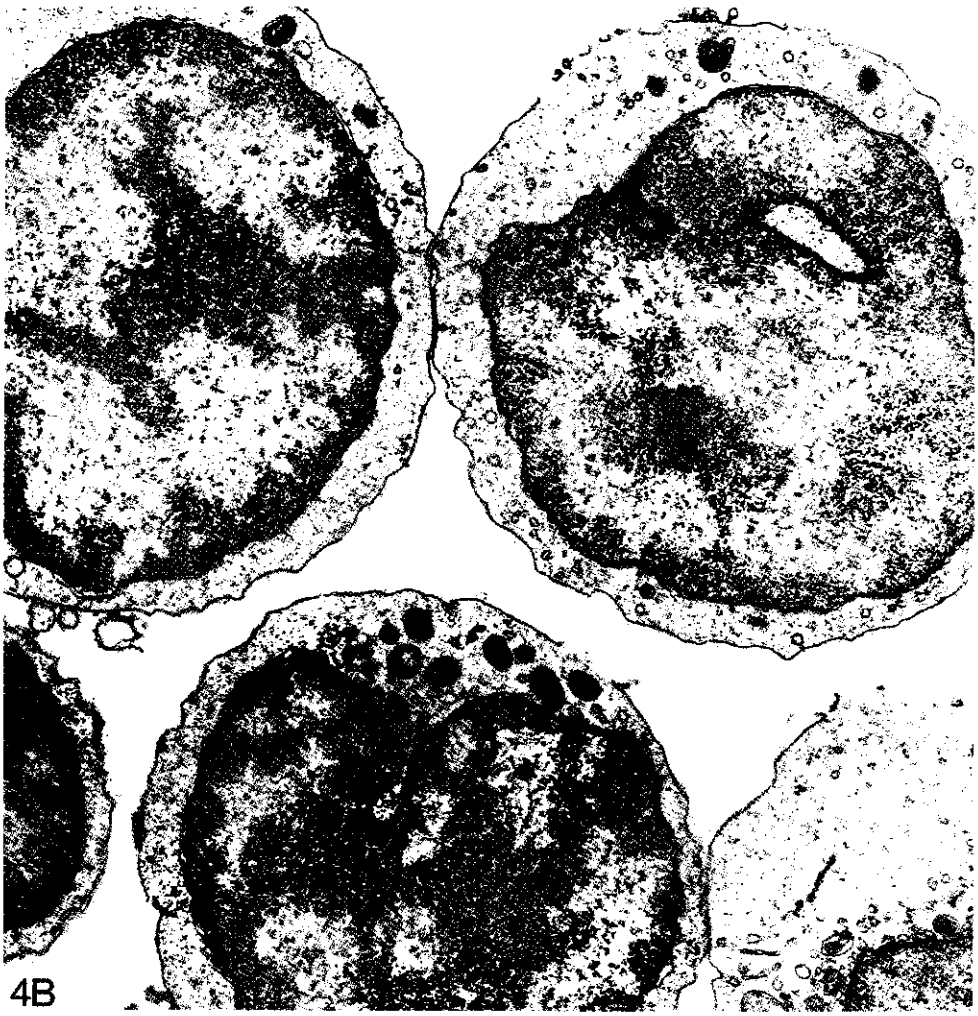
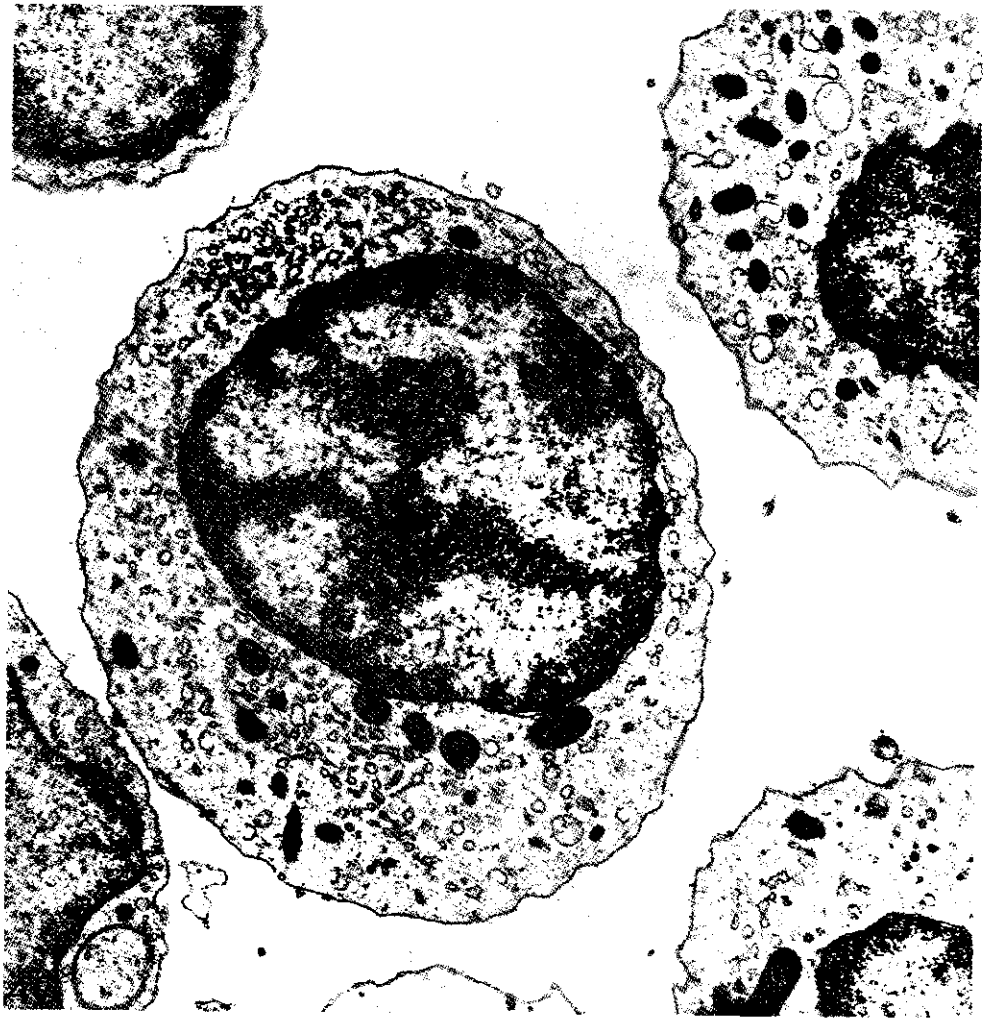


Figure 4. Electron micrographs showing WCL38$^+$ cells from (**A**) gut and (**B**,**C**) gills. All immunogold (25 nm) labelled cells could be considered as lymphoid cells, most of them having a high nucleus/cytoplasm ratio and a limited number of organelles, mainly mitochondria. Larger granular WCL38$^+$ lymphoid cells are frequently found in the gills (**C**). Magnification 16000x


4B



Immunogold labelling revealed that WCL38$^+$ cells can be considered as lymphoid cells but two morphologically different cells types can be distinguished:

1. small lymphocytes with a high nucleus/cytoplasm ratio and, with the exception of some mitochondria, a very limited number of other organelles (Fig. 4a,b).
2. larger granular lymphoid cells with a lower nucleus/cytoplasma ratio and a variable amount of electron dense lysosome-like granules (Fig. 4c).

All WCL38$^+$ IEL and the majority of WCL38$^+$ gill cells appeared to be of type 1. Approximately 20 % of WCL38$^+$ gill cells could be considered as type 2 cells. Intermediate stages, with more cytoplasm but a lower number of granules, could also be found in gill suspensions. In any case, macrophages, granulocytes, erythrocytes, goblet cells, chloride cells and some residual epithelial cells were not recognized by WCL38. Immunoprecipitation of membrane lysates of intestinal leucocytes, whether or not reduced, revealed an immunoreactive band with a Mr of 40 kD (Fig. 5). In contrast, membrane lysates of thymocytes or PBL did not show these immunoreactive proteins.

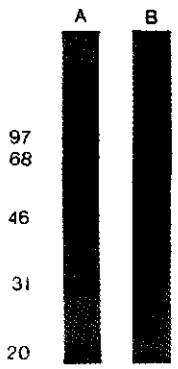


Figure 5. Immunoprecipitation of IEL membrane lysates (lane **B**) showing the presence of a WCL38$^+$ band of a Mr of 40 kD, and molecular weight markers (lane **A**). The immunoreactive 40 kD molecule was not detectable in PBL lysates.

Discussion

Immunisation of mice with membrane lysates of carp IEL resulted in a mAb (WCL38) specifically reacting with a sub-population (50-70%) of IEL and gill lymphoid cells and with a very limited number of lymphoid cells in other lymphoid organs of adult carp. The use of membrane lysates resulted in specific mAb against the major population of cells present in the original cell suspension, as was found in procedures using Ig^- PBL (WCL6 against thrombocytes; Rombout *et al.*, 1993b) and thymocytes lysates (WCL9 against early thymocytes; Rombout *et al.*, 1997). Although functional evidence is not available yet, $WCL38^+$ cells can be considered as T cells, because 1) they appear to be Ig^- lymphocyte-like cells, 2) they are predominantly present in the mucosal epithelia, which is comparable with the location of $CD8^+$ T cells in higher vertebrates ((Lefrançois & Puddington, 1995; McGhee *et al.*, 1992) and 3) they probably do not represent NCC cells because of the very low number of $5C6^+$ cells in IEL. In head kidney and spleen of carp considerable numbers of $5C6^+$ cells are found, however, the proportion is much lower than described for catfish, 40% and 30% respectively (Evans *et al.*, 1988). Like in channel catfish (Evans *et al.*, 1988; Evans & Jaso-Friedman, 1992) 5C6 is able to suppress non-specific cytotoxicity in carp against mouse myeloma cells (reduction of 20-30%; not published), which indicate that 5C6 is also reactive with a functional molecule on carp NCC.

Because of the distribution of $WCL38^+$ cells, which are abundantly present in mucosal epithelia, scarcely in thymus, spleen and head kidney and even more scarcely in blood, $WCL38^+$ cells may resemble the $CD8^+$ $\gamma\delta TCR^+$ cells of mammals (Lefrançois & Puddington, 1995; McGhee *et al.*, 1992; Itohara *et al.*, 1990). In mice 50-60% of IEL appeared to be $CD3^+$ $CD8^+$ $\gamma\delta TCR^+$ cells, similar to the proportion of $WCL38^+$ cells in carp IEL. $\gamma\delta$ T cells also seem to be abundantly present in the skin of mice (Allison et *al.*, 1991). Preliminary attempts to isolate lymphoid cells from skin of carp showed a large proportion of $WCL38^+$ lymphoid cells. At present it is difficult to prove whether $WCL38^+$ cells indeed represent the homologue of these $CD3^+$ $CD8^+$ $\gamma\delta TCR^+$ cells because of the absence of suitable tools (mAb). In addition, mammalian IEL appear to be unresponsive for T cell mitogens (Smart *et al.*, 1988; Sydora *et al.*, 1993), which is probably caused by their unique differentiation and activation state, possibly induced by the contact with the intestinal epithelial cells (Sydora *et al.*, 1993). Recently, the α (Partula *et al.*, 1996) and ß (Partula *et al.*, 1995) TCR chain of trout have been described and expression of γ/δ TCR genes is indicated in the clearnose skate (Rast *et al.*, 1997). In any case, it will be interesting to investigate whether cDNA of $WCL38^-$ Ig^- lymphoid cells of carp and not of $WCL38^+$ IEL hybridize with probes of the α and/or ß TCR chain of trout.

Mammalian $\gamma\delta TCR^+$ cells and natural killer (NK) cells have many characteristics in

common; they mediate "NK-like" cytotoxicity, secrete a similar panel of cytokines and express many of the same cell surface differentiation antigens (Lanier, 1995). In addition, γδTCR$^+$ cells recognize intact rather than processed polypeptides and consequently antigen recognition is not restricted by classical MHC molecules (Lanier, 1995; Raulet, 1994). It is suggested that the γδTCR acts more like an Ig molecule than αßTCR. Although, many functions are suggested for these γδ T cells, at present it is not clear whether certain functions measured are restricted to a subpopulation or to all IEL. At least a considerable proportion appear to have a cytolytic activity, especially when interleukin-2 is present (Sydora *et al.*, 1993; Moretta *et al.*, 1991). γδT cells are frequently described as large granular lymphocytes (Moretta *et al.*, 1991; Doherty *et al.*, 1992) which also fits very well in the ultrastructure of a considerable number of WCL38$^+$ cells found in gill leucocyte suspensions of carp. Although intermediate stages seem to be present in gill suspensions it is not yet proven that smaller WCL38$^+$ lymphoid cells and larger granular cells represent successive differentiation steps of one cell type. In this view, the absence of large granular WCL38$^+$ IEL is remarkable. Furthermore, γδ T cells are thought to have a role in preserving the integrity of the epithelial surfaces, since it was demonstrated that these cells recognize a ligand expressed by stressed autologous keratinocytes in skin or heat-shock protein (Allison & Havran, 1991; Raulet, 1994). An immunoregulatory role for γδ T cells is also suggested. Although peripheral tolerance can be transferred by γδ TCR$^+$ epithelial lymphocytes from airway epithelium (McMenamin *et al.*, 1991), transfer of intestinal γδ TCR$^+$ cells of naive mice to orally tolerised recipients have been shown to abrogate oral tolerance as measured by antibody production (Fujihashi *et al.*, 1992). A possible regulatory role is also supported by the panel of cytokines they have been shown to release: IL-2, IL-3, IL-6, IFN-γ, TGF-ß and IL-5 (Taguchi *et al.*, 1991; Barrett *et al.*, 1992).

The distribution of WCL38$^+$ cells also shows some similarity with RGL-1$^+$ cells in rat (Cerf-Bensussan *et al.*, 1986) and HML-1$^+$ cells in humans (Cerf-Bensussan *et al.*, 1987). The human mucosal lymphocyte antigen (HML-1) appears to be an integrin ($\alpha E\beta_7$) and was initially postulated to play a role in the mucosal localization or adherence of mucosal lymphocytes (Parker *et al.*, 1992). Interaction of this integrin with epithelial cells is demonstrated and is thought to be important for the retention and/or function of IEL within the epithelium (Cepek *et al.*, 1994). In addition, RGL-1 (Mr 100 and 125 kD) and HML-1 (Mr 105 and 150 kD) react with 2 large molecules, while WCL38 reacts with a molecule of a much lower molecular weight (Mr 40 kD), which possibly excludes homology with these mammalian molecules.

Mammalian mucosal lymphocytes express a specific mucosal homing receptor, the integrin $\alpha_4\beta_7$, which is the counterpart of MadCAM-1 on high endothelial venules (HEV; Springer, 1994). However, this receptor is present on both mucosal B and T cells, while

WCL 38 does not react with Ig^+ cells. Moreover, the Mr of integrin α_4 subunit (150 kD) and integrin $ß_7$ subunit (130 kD; Brines *et al.*, 1996) does not fit well with the Mr of the molecule recognized by WCL38 (40 kD). Consequently, it is not likely that WCL38 recognizes a similar mucosal homing receptor in carp. Nevertheless, the existence of a mucosal homing receptor in carp is supported by the presence of mucosal plasma cells in gut and gills and not in other lymphoid organs after oral immunisation (Joosten *et al.*, 1997). The Mr of the molecule recognized by WCL38 more resembles one of the two molecular forms of the mammalian γ chain (40 and 55 kD) and of the 43 kD δ chain (Porcelli *et al.*, 1991). However, comparison of $WCL38^+$ molecules with the $\gamma\delta$TCR is rather speculative at present.

In conclusion, a major part of fish mucosal T cells have membrane markers distinct from systemic lymphocytes. Whether the occurrence of $WCL38^+$ molecules is correlated with the $\gamma\delta$TCR, or play a role in the homing of mucosa associated T cells or are expressed or modified only in the epithelial microenvironment remains to be elucidated.

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Chapter 7

General discussion

Almost all available vaccines for fish are bacterins, which are based on inactivated whole bacterial cultures (Newman, 1993; Press & Lillehaug, 1995). Bacterins against vibriosis and Enteric Redmouth Disease (yersiniosis), administered by immersion or injection, resulted in protection against these diseases. Because of their efficacy, it seemed easy to develop a bacterial vaccine and it was not necessary to characterise the immunogenic component of the vaccines. However, several other bacterins tested were not effective by bath and gave limited protection by injection. For example, immunisation with *Aeromonas salmonicida* resulted in variable results. Vaccination appeared to be effective when the bacterin was administered in combination with an adjuvant and extracellular cell products turned out to be important for induction of protection (Newman, 1993). Knowledge of the immune response of fish and the pathogenic mechanisms and virulence factors of particular fish pathogens is required and has to be extended in order to find a better approach to vaccine development. In addition, the search for other routes of vaccine administration, which are less harmful to the fish and can be used at early ages, brings along different demands for a vaccine to be effective. Oral vaccination is a potential alternative for the existing vaccination routes and would meet these conditions.

Efficacy of vaccination

The entrance of the vaccine in the fish, i.e. uptake and processing of antigen in the gut, which needs to result in presentation to and activation of the immune system, is of prior importance for oral vaccination. The production of specific antibodies (Ab) can be an indication for effective vaccination. At least for *Vibrio anguillarum*, used for vaccination as described in this thesis (Chapter 2-4), protection and serum Ab titres are closely correlated (Chapter 2). However, the presence of Ab in the serum does not always correlate with protection. The ability to produce Ab to some of the antigenic components in a mixture does not necessarily guarantee immune protection (Vallejo *et al.*, 1992). Ab must be neutralizing to be effective in preventing disease and the part of the pathogen that induces these Ab is important for vaccination (Bøgwald *et al.*, 1990; 1992; Loghothetis *et al.*, 1994). Detection of the immune responses is not sufficient to evaluate a vaccine. The efficacy of a vaccine is only accepted after challenge experiments, in which the protection against the disease derived after vaccination is shown. The vaccine can be considered as effective, when mortality decreases significantly after vaccination. Strong Ab responses are reported in the absence of protection and protection can sometimes occur with only small amounts of Ab in the serum (Kusuda *et al.*, 1978). This indicates that the mucosal immune response can be an important factor in protection, because it may prevent the pathogen to enter the host (Kawai *et al.*, 1981). If protection is based upon Ab

responses in the mucosal surfaces of fish, mucosal delivery may be more efficacious than injection (Ellis, 1995). Consequently, a challenge via the natural route seems to be the best way to test protection against natural infection.

Although secondary serum Ab titres are a good indication for the development of immunological memory, the efficacy of oral vaccination using microparticles as described in Chapter 3, would eventually need to be tested by challenge experiments. However, in these studies with *V. anguillarum* this was not feasible. *V. anguillarum* is a strictly marine bacterium (Press & Lillehaug, 1995), whereas carp is a fresh water fish. Even an i.m. injection with high numbers of virulent bacteria did not result in disease in carp (unpublished results). Challenge tests in trout, using the effective microparticle formulation used in our study (Chapter 4), still have to be done.

After oral administration of viral peptides (KLG18) oral tolerance was demonstrated (Chapter 5), which was illustrated by lower Ab titres in orally vaccinated carp, compared to non-vaccinated controls. These data were supported by challenge experiments done by PHAROS with the protein in microparticles (M. Thiry; personal communication). Feeding of KLG18 to rainbow trout resulted in higher mortality than in unvaccinated controls, which increased when higher doses of protein were used for vaccination. This agrees with our observations of oral tolerance in carp. Neutralizing Abs are present in fish protected against Viral Haemorrhagic Septicemia (VHS), which are directed against the G protein and are the most important component of the protective immune response against VHS virus (Lorenzen *et al.*, 1990). However, Lecocq-Xhonneux *et al.* (1994) also mention that oral vaccination with recombinant VHS protein (produced in insect cells) failed to produce neutralizing Ab and protective responses, as was previously found to be the case for inactivated and attenuated VHS vaccines by this route. Purified proteins are usually poorly immunogenic *in vivo* (Mowat *et al.*, 1991) and soluble proteins normally induce oral tolerance (Mowat *et al.*, 1987). The development of subunit vaccines or peptide vaccines for oral vaccination needs to circumvent the induction of oral tolerance (Weiner et *al.*, 1994; Mowat, 1994).

The role of mucosal immunity in oral vaccination

The induction of mucosal immune responses as a defence against this invasion is important for vaccine development, since the majority of infectious microorganisms are encountered through mucosal surface areas (Michalek *et al.*, 1994; McGhee *et al.*, 1992). Vaccination by the mucosal route would be the best way to induce immune responses at mucosal sites (Manganaro *et al.*, 1994; Ruedl *et al.*, 1995). Several indications are available for the existence of a mucosal immune system in fish (Chapter 1) and it can play a major role in disease resistance in fish. Similarities as

well as differences with the mammalian mucosal immune system are found (Chapter 1). In mammals, protection at different mucosal sites has been observed and this indicates the ability of the mucosal immune system to communicate the immunogenic information arising at one mucosal site to other mucosal surfaces in the body (McGhee, 1992; Shalaby, 1995). Effector cells that are stimulated in the gut migrate to other mucosal sites via the lymphatic system (Manganaro *et al.*, 1994). In mammals mucosal Abs (IgA) have been shown to specifically inhibit microbial adherence and prevent absorption of antigens from mucosal surfaces (McGhee *et al.*, 1992).

The presence of Ab at different mucosal sites is also reported for fish (Rombout *et al.*, 1986; 1989; Jenkins *et al.*, 1994). It has been demonstrated in fish (ayu) that attachment of bacteria to the body surface mucus is decreased when specific Abs are present in skin mucus (Kawai *et al.*, 1981). However, the site of production of these Abs in secretions of fish was not specified in these studies. For oral vaccination more information concerning the local immune responses in the gut, skin and gills, induced by oral immunisation is needed to assess these potentials (Ellis, 1995). Many of its features still have to be elucidated in fish. In this thesis some features of the mucosal immune system in fish were studied. Mucosal Ig producing plasma cells have been found in gills and gut after oral immunisation of carp (Chapter 4). This indicates that also in carp mucosal Ig is produced in the mucosae. Furthermore, a distinct population of Ig^- (T) lymphocytes is present at the mucosal sites (Chapter 6). These observations indicate that mucosal homing of specific cell populations in fish occurs and can be related to a specialised function. Knowledge on the presence and function of cells in the intestine is important for evaluation of vaccination by this route. Memory formation or tolerance induction by oral administration seems to depend on the way of handling and presentation to certain cells present in the intestine. Oral tolerance induction in mammals has been described to be mediated by immunoregulatory T cells in the gut and to be dependent on the way of antigen presentation by APC (antigen presenting cells; Mowat, 1994; Wiener *et al.*, 1994, McDonald, 1994). The nature of the immune response that eventually occurs may reflect the outcome of the interactions between competing cytokines (Mowat, 1994).

Oral vaccination

Optimisation of antigen doses and duration of administration may increase the effect of oral vaccination (Ellis, 1995). In fish, the effective vaccination dose varies between species (Chapter 3 and 4). Differences in uptake of antigens in the intestine between different fish species were reported earlier (Vigneulle *et al.*, 1991; Doggett *et al.*, 1993). The uptake of *V. anguillarum* in the digestive tract of trout was slower, less efficient and only detected in a small portion of microfolds compared to sea bass and

turbot; this was observed for both anally and orally administered *V. anguillarum* (Vigneulle *et al.*, 1991). Usually, for oral vaccination much higher doses are needed than for injection vaccination (McGhee *et al.*, 1992). Protection of antigen from degradation in the anterior gut and enhancing uptake in the intestine can stimulate high immune responses, due to a better delivery of the antigen to the gut (Ellis, 1995). Besides the differences in intestinal uptake between fish, efficacy of oral vaccine is also dependent on the antigen, the gastrointestinal environment, the presence of adjuvants and the mode of delivery (Shalaby, 1995). For the development of oral vaccines it will be important to investigate if and how these factors interrelate.

In mammals particulate antigens are more effective oral immunogens than soluble antigens (Michalek *et al.*, 1994). The major portion of an ingested soluble protein antigen will cross the epithelial barrier of the gut as amino acids and low molecular weight peptides, which may induce systemic tolerance. Uptake of particulate material by M cells will result in processing and presentation of antigen by APCs to T cells in the same fashion as in a natural infection (Michalek *et al.*, 1994). Modern antigen delivery systems in mammals, e.g. encapsulation of vaccines in liposomes, microspheres, ISCOMS (Immune Stimulatory Complexes) etc. have the advantage of protection against degradation, the particulate antigen form, the immunopotentiating effects of these vehicles, their retarded antigen release and reduction of adverse effects. Particles may alter the processing of fed protein, either in the intestine or by APCs (Mowat *et al.*, 1991). Orally administered particles are taken up by mammalian M cells and transported to the Peyer's patches (PP). Traffic of microparticles containing macrophages seems dependent on the size of the microparticles; particles $\geq$ 5 μm stay in the PP for up to 35 days probably for induction of mucosal immune responses, while smaller particles are transported to the lymph nodes and spleen, probably inducing systemic immune responses (Eldridge *et al.*, 1991).

For fish the development of an oral delivery system may be different from those in mammals. The encapsulation of bacterial antigens in living food is a method suitable for fish (Chapter 2). However, most fish species are fed with live food only early in life, when vaccination has been demonstrated to be ineffective. At the age that they can be vaccinated they are mostly fed with pelleted food. Therefore, a delivery system which can be mixed with the fish food will be most practical for oral vaccination. In contrast to mammals, particles do not seem to be taken up in the intestine of fish. Dalmo *et al.* (1995) recently demonstrated that in salmon orally administered particles $\geq$ 45 nm were not taken up. In carp, gold particles of $\geq$ 10 nm, coated with goat IgG, could not be demonstrated in the intestinal tissue after anal administration (unpublished results). As described in Chapter 3 of this thesis, complete

bacteria are not taken up in carp or trout. It was concluded that, when using particles as delivery system, the soluble antigen has to be released from the particles in the lumen before uptake. On the other hand, O'Donnell (1996) recently described experiments on uptake of HGG from microparticles. The antigen stays present in the head kidney for a prolonged period (until 3 weeks), while soluble HGG disappeared after one week. This might indicate sustained release of HGG, and thus uptake of these microparticles. However, further details are not available yet on this mechanism.

Liposomes, in which the antigen is entrapped in micelles, may be an effective delivery system for fish. These liposomes act as immunoadjuvants, resulting in smaller amounts of antigen needed and soluble antigen are given in a particulate form. Liposomes may be a suitable carrier for oral vaccines in fish, because they eventually break down in the digestive system due to enzyme action (Chandrasekhar *et al.*, 1994).

Oral tolerance to synthetic soluble vaccines might be avoided by formulating particulate antigens, by protecting vaccines and by delivering antigen in recombinant bacteria (Friedman *et al.*, 1994). Production of particulate antigen to circumvent the induction of oral tolerance may not be effective in fish, because uptake of particulate antigens in the second gut segment seems to be impossible. Live bacteria are more effective in inducing an immune response than killed bacteria or viral proteins. When a viral protein can be expressed in avirulent bacteria, an immune response will be elicited against this viral immunogen. However, the live vaccine strain must be completely avirulent to the host and yet highly immunogenic for the induction of the desired immune responses (Michalek *et al.*, 1994). Recently, plasmid-encoded IHN and VHS epitopes have been inserted in *A. salmonicida*, which may result in duplicate vaccine for fish (Enzman *et al.*, 1996).

A relatively new way of vaccination has been developed by i.m. injection of plasmid DNA encoding for an antigen. The antigen is expressed by the host cells and an immune response is generated (Rabinovich *et al.*, 1994). Possibly this DNA vaccination can be applied via other routes. In mice, administration by mucosal route (intranasal and intraorbital) resulted in protective immune responses (Fynan *et al.*, 1993). In carp, DNA injection has been shown to result in increased protection, while bath also resulted in 20 % decrease in mortality (Alikin *et al.*, 1996). If the DNA can be protected in the digestive tract and the plasmid is taken up by the enterocytes, this would be a potential oral vaccination procedure.

Properties of alginate microparticles

Protection against degradation.

Alginate microparticles are an effective delivery system which results in effective oral vaccination, due to antigen protection and release in the posterior part of the digestive tract (Chapter 4). For vaccination against other diseases determination of the immunogenic part of each pathogen is important. The form of the encapsulated antigen must be suitable for uptake by the enterocytes after release from microparticles. Characteristics of the particles, important for release of the antigen at the right site, must be varied when delivered to different fish species, because of differences in the digestive tract. Also the encapsulation of adjuvants may be needed to obtain a better efficacy of vaccination, as demonstrated for furunculosis (Newman, 1993). Although oral vaccines can be applied easily, the degree to which different antigens can be combined in alginate microparticles must be determined (Morris, 1994). This would make the vaccine less expensive.

Adjuvant effect

The microparticle system used for oral vaccination effectively induces specific Ab titres to *V. anguillarum* (Chapter 4). However, this effect may not only be due to better delivery. Alginate microspheres were used for oral delivery of antigens to different animal species (mice, rabbits, cattle and chicken) and it was suggested that alginate microspheres act as adjuvant (Bowersock *et al.*, 1996). A strong stimulatory effect of alginates on cytokine production by human monocytes was reported (Otterlei *et al.*, 1991). Injection of sodium alginate in carp 3 or 6 days prior to challenge with *Edwardsiella tarda* resulted in increased resistance to disease (Fujiki *et al.*, 1994). Alginates in the food of marine fish larvae (turbot) were found to stimulate the non-specific system, resulting in a better protection against disease (Skjermo *et al.*, 1995). Therefore, an immunostimulatory effect of alginates can not be excluded and may increase the efficiency of the alginate microparticles used.

Concluding remarks and future prospects

Data presented in this thesis, clearly demonstrate that oral vaccination can be more effective when antigens are protected against degradation in the foregut. Alginate microparticles appear to be an effective delivery system that protects antigen and releases it in the antigen transporting endgut. However, for each fish species the formulation of the particles has to be adapted with respect to the characteristics of the digestive tract. In addition, the most immunogenic molecules, resulting in protective immunity, have to be incorporated in these microparticles. Consequently, for each

pathogen the immunogenic part suitable for encapsulation has to be established. Furthermore, the adjuvant effect of particles itself or encapsulation of adjuvants in the microparticles may enhance the effect of oral vaccination. Oral vaccination appears to evoke mucosal immune responses, which is important since most infectious microorganisms are encountered through mucosal surfaces. However, oral vaccination with soluble protein antigens can induce oral tolerance in fish. Procedures, like expression of the viral protein in an avirulent bacteria or oral vaccination with plasmid DNA, have to be investigated, to overcome this problem and to achieve an optimal stimulation of the immune system. Knowledge on the immune responses and gut physiology of fish, and the mechanisms and virulence factors of fish pathogens is required and has to be extended for development of optimal oral vaccines.

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With the increase and intensification of aquaculture, disease problems in fish are growing, causing considerable economic losses. Therefore, the control of fish diseases is of great concern in aquaculture. Prevention of diseases might be established by vaccination. Potentially the most useful method for fish vaccination will be oral vaccination, which can be applied stress free, does not require extra labour and can be applied on a large scale, even to small fish. However, oral vaccination has been less effective than other vaccination methods and large quantities of bacterin are required to be effective. Probably the antigen is degraded in the first part of the digestive tract, before it reaches the hindgut, which is the most important antigen transporting area in the digestive tract of fish. Macrophages and many lymphoid cells are present in the intestinal mucosa. Mucosal and systemic immune responses can be induced by anal administration of antigens. Consequently, for a more effective oral vaccination, antigens have to be protected against degradation in the first part of the digestive tract in such a way that it reaches the hindgut in sufficient quantities, to provoke a protective immune response. Two approaches to protect antigens are described in this thesis: Bioencapsulation, suitable for vaccination of young fish and microencapsulation, for older fish because microparticles can be mixed with pelleted food.

Artemia nauplii were used as an antigen delivery system for oral vaccination of young fish against *Vibrio anguillarum*. Juvenile carp (stomachless) of 2, 4 and 8 weeks old and gilthead seabream (stomach-containing) of 8 and 10 weeks old were fed with antigen-enriched *Artemia*. Antigen uptake and transport to intraepithelial macrophages in the hindgut of carp was demonstrated. However, in 2 weeks old carp these intraepithelial macrophages were not detectable yet. In gilthead seabream a different transport was observed and intraepithelial macrophages were not found. Ten weeks after oral vaccination fish received an intramuscular (i.m.) injection with bacterin and 3 weeks later serum antibodies (Abs) were measured to determine memory formation. Carp orally vaccinated at 2 or 4 weeks old showed a significantly lower response than non-vaccinated controls, indicating induction of tolerance at this young age. However, carp orally vaccinated at 8 weeks old tended to have a higher Ab response compared to the control. Orally vaccinated seabream showed significantly higher secondary responses compared to the control, which can be regarded as an indication for effective memory formation. Oral vaccination with bioencapsulated vaccines is very promising when applied at the right age. Application at too young fish seems to induce immunosuppression, instead of immunisation. However, since fish are mostly fed with pelleted food at the age that they can be vaccinated, this method does not seem practical for fish culture.

A delivery system which can be combined with pelleted food would be a better approach. Microencapsulation of antigens in alginate microparticles, which will protect the antigen against digestive enzymes and an acid environment and release the antigen in the hindgut, was studied. Release of antigen from the microparticles requires an antigen that can be taken up in the hindgut immediately after release. In order to determine which fraction of a bacterin results in optimal antigen transport and induces immunological memory following administration in the gut, rainbow trout (stomach-containing) and carp were anally intubated with different fractions of a *V. anguillarum* bacterin. Antigen uptake in the hindgut was demonstrated in fish intubated with complete vaccine, supernatant of the bacterin and LPS extracted from the bacterin, while whole bacteria were not taken up. Ab titres increased significantly in carp intubated with complete vaccine or supernatant of the bacterin. Fish received an i.m. injection with complete vaccine 10 weeks after anal intubation. Significant memory was induced in carp which were intubated with supernatant of the bacterin. Furthermore, Ab titres in carp intubated with complete vaccine or LPS tended to be increased compared to the non-vaccinated control. In contrast to carp, trout required 10 x more supernatant to obtain similar results. It is concluded that the supernatant contains the most immunogenic part of the bacterin, and consequently is the best candidate for encapsulation in microparticles.

Subsequently, this supernatant of the bacterin was encapsulated in alginate microparticles for oral vaccination, to protect the vaccine against degradation in the anterior part of the digestive tract. Microparticles show a pH-dependent release of the antigen and the resistibility can be varied for different fish species in accordance with differences in the digestive tract. Two types of microparticles, which differed in time of release and amount of encapsulated antigen, were tested in carp and trout. Alginate microparticles with or without antigen were added to food or food with non-encapsulated antigen was fed. Feeding with encapsulated antigen resulted in a better uptake compared to feeding with the same amount of non-encapsulated antigen. Memory formation after oral vaccination was tested and revealed differences in effectiveness of the different microparticle types between carp and trout: alginate microparticles suitable for induction of systemic memory in carp were not effective in trout and *vice versa*. Specific mucosal plasma cells appeared to be present mainly in gut and gills of carp after repeated oral vaccination with encapsulated antigen and absent after i.m. injection. These results indicate that oral vaccination with encapsulated antigens evokes systemic memory and induces mucosal immune responses in fish. Consequently, oral vaccination with microparticles appears to be a promising method to control bacterial diseases.

However, in mammals oral administration of protein antigens often results in oral tolerance. If oral tolerance also occurs in fish, it may have great impact on the development of oral vaccines against soluble protein antigens. Therefore, oral vaccination with different protein antigens (ferritin, recombinant surface glycoprotein of viral haemorrhagic septicemia virus (KLG18) and ovalbumin (OVA)) was studied in carp. Feeding of ferritin or KLG18 resulted in lower Ab titres compared to unprimed controls when animals were i.m. injected with protein 10 weeks later and sampled 3 weeks after injection. Responsiveness to OVA appeared to be carp strain dependent. OVA, administrated by different routes (oral, anal, i.m.), and i.m. injected 2 months later, resulted in very low Ab responses. An isogenic carp strain was selected for an optimal response to i.m. injection with OVA. In this strain, feeding high doses of OVA on 6 subsequent days resulted in immunological memory formation, instead of oral tolerance. Oral tolerance can be induced in carp, but differences in tolerance induction may depend on the protein used. Genetic factors may also play a role in the induction of oral tolerance in fish.

Since the majority of infectious microorganisms are encountered through mucosal surface areas, developing methods for the induction of mucosal immune responses as a defence against this invasion may be important for vaccine development. For oral vaccination more information concerning the local immune responses in the gut, skin and gills is needed to assess these potentials. Compared to mammals, only a small number of monoclonal Abs (mAbs) for identification of cell populations are available in fish. Production of mAbs specific for mucosal lymphocytes will give more information on the presence of these cells. In addition to the mAb specific for mucosal IgM (WCIM), a mAb against carp intestinal T cells (WCL38) was produced. WCL38 reacted with the majority of the lymphoid cells isolated from intestine or gills. In thymus, spleen and head kidney immunoreactive cells are scarce. In cryo-sections $WCL38^+$ lymphoid cells were abundantly found within the intestinal epithelium. In conclusion, like higher vertebrates, carp seem to have a distinct mucosal T and B cell population, which indicate that specific homing of distinct lymphocytes to mucosal tissues also occurs in fish. The mucosal T cells may have a specialised function, associated with their presence in the mucosae.

The results described in this thesis show that oral vaccination of fish can be more effective when antigens are encapsulated to protect them against degradation in the digestive tract. However, oral vaccination of too young fish is not effective. Encapsulation of antigens in alginate microparticles has to be adapted for each fish species and antigen, in a way that sufficient quantities can reach the hindgut in a

suitable form for uptake. Mucosal plasma cells are induced at mucosal sites after oral vaccination, indicating induction of a mucosal immune response. Next to mucosal B cells, distinct mucosal T cells could be demonstrated using a newly produced mAb. Furthermore, oral tolerance can be induced in fish after oral administration of protein antigens, which can be a problem for oral vaccination with (soluble) viral proteins.

Samenvatting

Met de groei en intensivering van de visteelt groeit ook het aantal ziektenuitbraken in vissen, die tot aanzienlijke economische schade kunnen leiden. Daarom is het onder controle houden van ziekten van groot belang voor de visteelt. Vaccineren zou ziektenuitbraken kunnen voorkomen en het gebruik van medicijnen (o.a. antibiotica) kunnen limiteren. Orale vaccinatie is in potentie de beste methode om vissen te vaccineren, omdat het stress-vrij kan worden toegediend, er geen extra arbeid voor nodig is en het op grote schaal kan worden toegepast, ook op kleine vissen. Tot nu toe is orale vaccinatie echter minder effectief geweest dan andere vaccinatiemethoden tegen bacteriële ziekten, zoals injectie of badvaccinatie en grote hoeveelheden van een gedood pathogeen zijn nodig om werkzaam te zijn. Waarschijnlijk wordt bij orale vaccinatie het antigeen (lichaamsvreemde stof die een afweerreactie kan veroorzaken) in het eerste deel van de darm afgebroken, voordat het de einddarm bereikt. De einddarm is nl. het belangrijkste deel voor de opname van antigenen in het spijsverteringskanaal van vissen. Grote aantallen macrofagen en lymfoïde cellen zijn aanwezig in de darm. Na anale toediening van antigenen kunnen zowel mucosale (locale) als systemische afweerreacties ontstaan. Om orale vaccinatie beter werkzaam te maken, zou het antigeen beschermd moeten worden tegen afbraak in het eerste deel van het spijsverteringskanaal, zodat het in voldoende hoeveelheden de einddarm bereikt en opgenomen kan worden, om zodoende tot beschermende afweerreacties te kunnen leiden. Twee methoden voor bescherming van het antigeen zijn in dit proefschrift beschreven: bioencapsulatie, het meest geschikt voor orale vaccinatie van jonge vissen, en microencapsulatie, geschikt voor oudere vissen waarbij micropartikels in het reguliere voer kunnen worden verwerkt.

Jonge vissen eten kleine kreeftjes en andere diertjes die in het water leven. Kleine voerkreeftjes werden gebruikt als antigeendragers voor orale vaccinatie van jonge vissen tegen een bacterie, *Vibrio anguillarum*. Jonge karpers van 2, 4 en 8 weken oud en zeebrasem van 8 en 10 weken oud werden gevoerd met antigeen-verrijkte kreeftjes. De kreeftjes kunnen verrijkt worden door ze bacteriën uit het water te laten filteren. Karpers en zeebrasems zijn gebruikt om te zien of zowel maagloze (karper) als maaghoudende (zeebrasem) vissen op deze manier kunnen worden gevaccineerd. Opname van het antigeen in de einddarm werd aangetoond na orale vaccinatie van zowel karpers als zeebrasems. Er bleken echter verschillen te zijn in opname en transport. Tien weken na orale vaccinatie werden de vissen in de spier (intramusculair, i.m.) ingespoten met het vaccin om vervolgens 3 weken later antilichamen in het bloed te meten. Het afweersysteem heeft een geheugen tegen een bepaald antigeen gevormd als na een tweede contact, in dit geval de i.m. injectie, meer en sneller antilichamen gevormd worden dan in dieren waarbij de i.m. injectie het eerste contact is. In karpers die op 2 of 4 weken oraal gevaccineerd werden, was

een lagere antilichaamproductie te meten dan in vissen die niet oraal gevaccineerd werden. Dit kan wijzen op tolerantie, wat betekent dat de vissen hun afweer hebben uitgeschakeld tegen deze specifieke ziekteverwekker. Omdat het antigeen zo vroeg in het leven al aanwezig was in hun lichaam wordt het later als 'eigen' herkend. In karpers die op een leeftijd van 8 weken oraal gevaccineerd werden, kon een verhoging in antilichaamproductie worden waargenomen. Oraal gevaccineerde zeebrasems hadden een significant verhoogde antilichaamproductie t.o.v. controle dieren. Orale vaccinatie van vissen met gebioencapsuleerde vaccins lijkt dus een veelbelovende methode, als het op de juiste leeftijd word toegediend. Toepassing op te jonge vissen lijkt de afweerreactie te onderdrukken, in plaats van te activeren. Omdat vissen vaak al met korrelvoer worden gevoerd op de leeftijd dat ze kunnen worden gevaccineerd lijkt deze methode minder practisch voor toepassing in de intensieve visteelt.

Een bescherming die gecombineerd kan worden met korrelvoer zou een betere benadering zijn. Alginaten zouden het antigeen kunnen beschermen tegen verteringsenzymen en de zure omgeving, en vervolgens het antigeen afgeven in de einddarm. Deze alginaten vormen een soort gel waarvan kleine partikels gemaakt kunnen worden, waarin antigenen kunnen worden verwerkt. Als het antigeen vrijkomt uit de partikels in de einddarm, moet het in een vorm zijn dat het meteen kan worden opgenomen door de darmcellen. Om vast te stellen welk deel van een vaccin het best wordt opgenomen en de beste afweerreacties veroorzaakt na toediening in de darm, zijn verschillende delen van een vaccin anaal toegediend aan forellen (maaghoudend) en karpers. Het vaccin komt zodoende meteen op de plaats waar het, onverteerd, kan worden opgenomen. Opname in de einddarm werd aangetoond met het hele vaccin, "oplosbare" deel (supernatant) van het vaccin en moleculen opgezuiverd uit de wand van de bacterie (LPS). Hele bacteriën werden niet opgenomen door de darmcellen. De hoeveelheid antilichamen nam significant toe in karpers die het complete vaccin of het supernatant ontvingen. Geheugen werd bewerkstelligd in karpers die geïntubeerd waren met het supernatant. Ook karpers die compleet vaccin of LPS ontvingen hadden een lichte verhoging van het antilichaamniveau, vergeleken met de niet-geïntubeerde controles. Bij de forel was, in tegenstelling tot de karper, 10 x zoveel supernatant nodig om vergelijkbare resultaten te bereiken. De conclusie uit deze experimenten is dat het supernatant het meest immunogene deel van een bacteriëel vaccin bevat, en daarom de beste kandidaat is voor encapsulatie in alginaat micropartikels.

Om het vaccin te beschermen tegen afbraak in het eerste deel van het spijsverteringskanaal werd vervolgens dit supernatant verwerkt in alginaat micropartikels voor oral vaccinatie. Alginaten gaan opzwellen in een minder zuur milieu, waardoor ze gaan kleven aan de darmwand en het antigeen afgeven. De

zuurgraad neemt toe aan het eind van het spijsverteringskanaal, waardoor de micropartikels het antigeen zullen afgeven in de einddarm. De resistentie van de micropartikels tegen vrijlaten van antigeen kan aangepast worden voor verschillende vissoorten, omdat er verschillen in het spijsverteringskanaal zijn tussen de vissoorten. Twee verschillende alginaten zijn getest in karpers en forellen, waarbij het ene type sneller het antigeen vrijliet en ook meer antigeen bevatte. Alginaat micropartikels met en zonder antigeen, of niet beschermd antigeen, zijn aan het voer toegevoegd. Het voeren met beschermd antigeen gaf een betere antigen opname, in vergelijking met dezelfde hoeveelheid niet beschermd antigeen. Verschillen in geheugenvorming werden gevonden tussen de twee typen micropartikels en vissoorten. Alginaten die in karpers geheugen opwekten, deden dat niet in forellen en omgekeerd. Na herhaalde orale vaccinatie van karpers met beschermd antigeen en niet na i.m. injectie, werden voornamelijk in de darm en de kieuwen specifieke antilichaamproducerende cellen aangetoond. Met andere woorden, orale vaccinatie met beschermde antigenen wekt een systemisch (algeheel) geheugen op van het afweersysteem en induceert ook locaal een afweerreactie. Daarom lijkt orale vaccinatie met micropartikels een veelbelovende methode voor de controle van bacteriële ziekten in vissen.

In zoogdieren wordt tegen oraal toegediende lichaamsvreemde eiwitten vaak tolerantie opgewekt, wat betekent dat het afweersysteem niet meer reageert op deze eiwitten en dus een afweerreactie achterwege blijft. In virussen is het deel dat het afweersysteem activeert een eiwit en dit zou dus problemen kunnen opleveren voor orale toediening van een eiwitvaccin. Als orale tolerantie ook in vissen optreedt, zal dit nadelige gevolgen kunnen hebben voor de ontwikkeling van orale vaccines tegen virale eiwitten. Daarom is in de karper orale vaccinatie met verschillende eiwitten bestudeerd; met ferritine, een eiwit uit de milt van paarden, KLG18, een eiwit dat een belangrijk deel is voor het ontstaan van antilichamen tegen een bepaald virus en ovalbumine (OVA), een eiwit opgezuiverd uit kippeneieren. Het voeren van KLG18 of ferritine resulteerde in lagere hoeveelheden antilichaam na een i.m. injectie met dit eiwit, vergeleken met controle dieren, hetgeen bewijst dat orale tolerantie in karpers geïnduceerd kan worden. De afweerreactie tegen OVA bleek sterk afhankelijk van de gebruikte karperlijn. Wanneer OVA werd toegediend via verschillende routes (oraal, anaal en i.m. injectie) resulteerde een tweede injectie 2 maanden later in erg lage hoeveelheden antilichamen in alle groepen. Na injectie van verschillende karperlijnen met OVA werd een lijn geselecteerd die wel op OVA reageerde. Echter in deze lijn resulteerde het voeren van hoge doses OVA op 6 opeenvolgende dagen in geheugen van het afweersysteem, in plaats van tolerantie. Orale tolerantie kan dus ontstaan in de karper, maar er zijn eiwitafhankelijke verschillen bij het ontstaan van deze tolerantie. Genetische factoren kunnen ook een rol spelen bij het ontwikkelen van

orale tolerantie in vissen, zoals dit eerder in muizen gevonden is.

Het merendeel van besmettelijke micro-organismen komt het lichaam binnen via de slijmvliezen (mucosae), zoals bijvoorbeeld spijsverteringskanaal en kieuwen. Daarom kan het ontwikkelen van methoden voor het opwekken en meten van locale afweerreacties, als een eerste verdedigingslijn, belangrijk zijn voor vaccinontwikkeling. Voor orale vaccinatie is meer informatie nodig over deze locale afweerreacties in de huid, darm en kieuwen van vissen. Vergeleken met zoogdieren is maar een klein aantal monoclonale antilichamen (mAl) beschikbaar voor de indentificatie van bepaalde celpopulaties in de vis. Productie van zulke mAl specifiek voor afweercellen in de mucosa zou meer informatie geven over de aanwezigheid van deze cellen. In aanvulling op een mAl dat specifiek reageert met locale antilichamen (WCIM) en B cellen, is een mAl (WCL38) ontwikkeld tegen afweercellen in de darm die geen antilichamen maken, maar wel een belangrijke rol spelen in de afweer (T cellen). WCL 38 reageert met de meerderheid van deze afweercellen in darm en kieuwen, terwijl in thymus, milt en kopnier maar enkele van deze cellen werden gevonden. Deze mucosale T cellen komen met name tussen de (darm)epitheelcellen voor en kunnen een speciale functie hebben die gerelateerd is aan hun plaats in de mucosa, zoals bij zoogdieren het geval is.

De resultaten, die in dit proefschrift zijn beschreven, laten zien dat orale vaccinatie van vissen mogelijk is en dat bescherming van het antigeen tegen afbraak in het spijsverteringskanaal effectief kan zijn. Orale vaccinatie is echter niet werkzaam in te jonge vissen. Verwerken van antigenen in alginaat micropartikels voor orale vaccinatie moet aangepast worden voor elke vissoort en elk antigeen, op een manier dat het in voldoende hoeveelheden, en in een geschikte vorm voor opname, de einddarm bereikt. Antilichaamproducerende cellen worden geactiveerd in de mucosae na orale vaccinatie, hetgeen wijst op de inductie van locale afweerreacties. Naast mucosale antilichaamproducerende cellen zijn er ook mucosale T cellen aangetoond door gebruik van een nieuw ontwikkeld mAl. Verder blijkt ook in vissen orale tolerantie te kunnen ontstaan na voeren met een eiwitantigeen, hetgeen problemen kan opleveren voor het ontwikkelen van een orale vaccinatie strategie met virale eiwitten.

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Ellen

Curriculum vitae

Petronella Henrica Maria (Ellen) Joosten werd geboren op 24 augustus 1967 in Son en Breugel. In 1986 deed zij eindexamen V.W.O. aan het Van der Puttlyceum te Eindhoven, en begon met haar studie Biologie aan de Landbouwuniversiteit te Wageningen. In januari 1992 studeerde zij af, met als afstudeervakken Celbiologie en Immunologie (vakgroep Experimentele Diermorfologie & Celbiologie), Dierfysiologie (vakgroep Fysiologie van mens en dier) en een stage Immunologie bij Mestrado de Imunologia, Instituto de Ciencias Biomedicas de Abel Salazar in Porto. Per 1 februari 1992 werd zij aangesteld als assistent-in-opleiding bij de vakgroep Experimentele Diermorfologie & Celbiologie van de Landbouwuniversiteit in Wageningen, op een deels door het FAR programma van de Europese Gemeenschap gefinancierd project. Het promotieonderzoek dat aldaar werd uitgevoerd resulteerde in dit proefschrift.

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