Characterisation of fish leucocytes

An immunocytochemical and functional study in carp (*Cyprinus carpio* L.)

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Promotor: dr. W.B. van Muiswinkel, hoogleraar zoölogische celbiologie

Co-promotor: dr. J.H.W.M. Rombout, universitair hoofddocent zoölogische celbiologie

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Johanna Catharina Elisabeth Koumans-van Diepen

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A panel of monoclonal antibodies (MAbs) against carp serum immunoglobulin (Ig), WCIs or carp thymocytes (T), WCTs were used for the characterisation of carp leucocytes. Unfortunately, all WCTs and some WCIs react with common carbohydrate determinants present on all leucocytes and Ig. Most WCIs react specific with protein determinants at the heavy chain of Ig. Consequently, B lymphocyte (sub) populations, plasma cells and Ig-binding cells could be studied. Ig molecules are found in clusters at the cell membrane of B cells and plasma cells, and in contrast to mammalian plasma cells, most carp plasma cells still have Ig at their surface membrane. Mainly the dull surface Ig-positive (sIg^+) cells were stimulated by the mammalian B cell mitogen LPS and not by PHA (T cell mitogen) *in vitro*, whereas the sIg-negative (sIg^-) cells were stimulated by PHA and not by LPS. The percentages of B cells and plasma cells showed an increase during ontogeny and reached a plateau at about 3 months and 8 months of age respectively. It is suggested that full development of the carp (humoral) immune system needs at least 8 months (at 21-22 °C). Three different subpopulations of B cells and plasma cells and at least two Ig isotypes can be distinguished based upon their reactivity with WCI 4 and WCI 12. The distribution of the three B cell subpopulations appeared to be organ and age dependent which indicates functional differences between the Ig isotypes. Fc-like receptors were mainly demonstrated on gut macrophages while pronephros macrophages and neutrophilic granulocytes did not show Ig binding. Consequently, other forms of antigen opsonisation (e.g. complement) may play a role in phagocytosis by these non Ig-binding cells. Several procedures were tested for obtaining MAbs specific for Ig^- lymphoid cells. It is concluded that the presence of immunodominant carbohydrate determinants is the major problem for obtaining specific MAbs. Tolerisation of mice against these determinants or the use of isolated membrane lysates from sIg^- PBL appeared promising but till now only specific thrombocyte markers have been obtained. The use of more purified antigen is recommended in further attempts. The data presented in this thesis can be used for fundamental studies on cell interactions in the immune response, but also for more applied investigations on fish health control.

PhD Thesis. Department of Experimental Animal Morphology and Cell Biology, Wageningen Agricultural University (WAU), P.O Box 338, 6700 AH Wageningen, The Netherlands

aan mijn ouders

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BIBLIOTHEEK
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STELLINGEN

1. Het verkrijgen van monoclonale antilichamen tegen afzonderlijke leucocytpopulaties van vissen wordt bemoeilijkt door de aanwezigheid van voor de muis immuundominante koolhydraatdeterminanten op deze cellen.
(dit proefschrift)

2. Er bestaan meerdere isotypen van het IgM molecuul bij de karper.
(dit proefschrift)

3. Een juiste negatieve controle is onontbeerlijk voor een positieve selectie van hybride cellen die de beoogde monoclonale antilichamen produceren.
(dit proefschrift)

4. Het voorkomen van een major histocompatibility complex (MHC) bij de karper vormt een indirect bewijs voor het bestaan van een T-cel receptor bij deze vis.
(Hashimoto *et al.*, Proc. Natl. Acad. Sci. USA 87:6863-6867, 1990; Stet *et al.*, Dev. Comp. Immunol. 17:141-156, 1993)

5. Het feit dat mannen tijdens vergaderingen gemiddeld meer dan twee keer zo lang en bovendien vaker aan het woord zijn dan vrouwen betekent meestal niet dat ze ook meer te vertellen hebben, maar wordt eerder veroorzaakt door een verschil in spreekstijl.
(D. Tannen, Je begrijpt me gewoon niet, Prometheus, Amsterdam, 1992)

6. Van de gehanteerde doelstellingen in de proefdierkunde samengevat als vervanging, vermindering en verfijning, is vermindering van het aantal proefdieren niet van direct belang voor een verbetering van het welzijn van het proefdier maar slechts van economisch en politiek belang.
(handleiding proefdierkunde, vakgroep Proefdierkunde Rijksuniversiteit Utrecht, 1988)

7. Vooral niet-wetenschappers dienen zich te realiseren dat onderzoeksresultaten vaak als 'waarheden' gepresenteerd worden terwijl ze zich op een schaal van wit tot zwart eerder in het gebied van lichtgrijs tot donkergrijs bevinden.

8. Het is een misverstand dat de aanschaf van een personal computer met de bijbehorende software de gebruiker specifieke kennis geeft over vakgebieden als bijvoorbeeld desktop-publishing en statistiek.

9. Het gebruik van een computer werkt pas tijdbesparend indien men **vooraf** de te investeren tijd voor het zich eigen maken van de werking van een programma afweegt tegen de tijdwinst die het gebruik ervan oplevert.

10. Ironisch genoeg zijn veel natuurgebieden niet of slechts lastig per openbaar vervoer bereikbaar.

11. De saamhorigheid binnen een groep mensen is af te lezen aan het percentage mensen dat gemeenschappelijk de pauzes doorbrengt.

12. Gezien de huidige werkgelegenheidsproblematiek onder academici bevalt het beter als doctorandus dan als doctor.

J.C.E. Koumans-van Diepen
Characterisation of fish leucocytes
An immunocytochemical and functional study in carp (*Cyprinus carpio* L.)
Wageningen, 15 oktober 1993

Contents

General introduction

This thesis deals with the immunocytochemical and functional characterisation of carp (*Cyprinus carpio* L.) leucocytes. Carp is a member of the cyprinid fish family (*Cyprinidae*) which belongs together with i.a. catfishes (*Ictaluridae*) and salmonids (*Salmonidae*) to the superorder *Teleostei* (modern bony fish) (Lagler *et al.*, 1962). These teleost fish belong to the class of the *Osteichthyes* (= bony fish). Next to bony fish, *Chondrichthyes* (= cartilaginous fish) and *Agnatha* (= jawless fish) constitute the primary aquatic vertebrates called *Pisces* (= fish) (Figure 1). The immune system of fish is of interest from a phylogenetic point of view because fish are the first group of animals showing the basic aspects of the immune system of higher vertebrates such as true lymphocytes, lymphoid tissues, antibody production, T cell cytotoxicity and long term memory (Rijkers, 1980; Lamers, 1985; Ratcliffe & Millar, 1988). Moreover, a thorough knowledge of the immune system of fish is indispensible for studies on fish diseases and vaccination which are important for fish health control in aquaculture. Leucocytes form an integral part of the defense system because they fulfil essential immune functions. Characterisation of leucocytes is mainly based on morphological, functional and ontogenetic criteria.

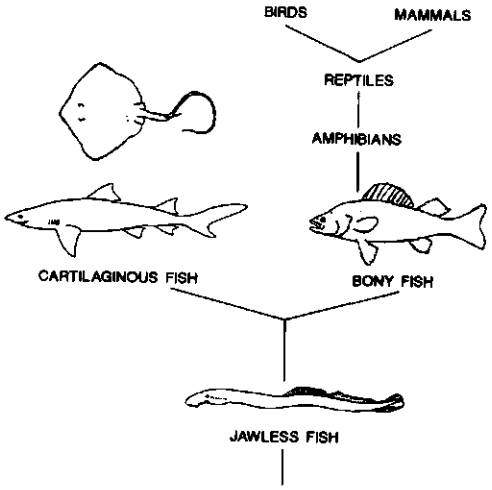

Figure 1. **Phylogenetic tree of the main groups of vertebrates showing the relationship between jawless fish (*Agnatha*), cartilaginous fish (*Chondrichthyes*) and bony fish (*Osteichthyes*) (from Cooper, 1982, with modifications).**

In this chapter the current knowledge on leucocyte characterisation of teleosts is described and compared with mammalian (man, mice) data. Special attention is paid to economic important fish species, e.g. catfish, trout, salmon and carp. Finally the aim and outline of this thesis is described.

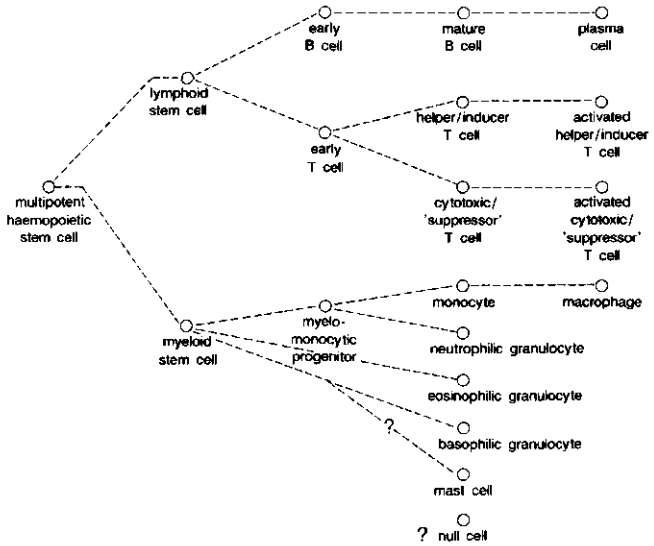

Figure 2. **Hypothetical scheme of human haemopoiesis (adapted from Adriaansen, 1992).**

Origin

Mammalian leucocytes originate from multipotent haemopoietic stem cells in the bone marrow (Figure 2). These stem cells (frequency: 1:10,000 bone marrow cells) give rise to two main cell lineages: myeloid cells and lymphoid cells. The myeloid stem cell can differentiate into several granulocyte types, mast cells, or mononuclear phagocytes in the bone marrow. The lymphoid progenitor cells stay in the bone marrow and differentiate into early B cells or migrate to the thymus and develop into several T cell types. The origin of a third population of cells, the null cells (e.g. natural killer (NK) cells), is still unknown.

The ontogeny of fish leucocytes is still rather unclear. Major lymphomyeloid organs in *Osteichthyes* are thymus, spleen, kidney and gut (Fänge, 1982; Rombout *et al.*, 1993a).

The anterior kidney (pronephros) and trunk kidney (mesonephros) are considered as the major haemopoietic organs. Most authors agree that this is the main site of erythrocyte, granulocyte, B lymphocyte and monocyte differentiation (Ellis, 1977a; Zapata, 1979; Bielek, 1981; Botham & Manning, 1981). Although the spleen is also involved in haemopoiesis its role is considered to be of less importance and mainly focussed on erythropoiesis in most fish species (Rowley *et al.*, 1988; Van Muiswinkel *et al.*, 1991). Haemopoietic cells were observed in kidney prior to the appearance of lymphocytes in any other lymphoid organ (Ellis, 1977a; Grace & Manning, 1980; Botham & Manning, 1981; Razquin *et al.*, 1990; Jósefsson & Tatner, 1993). The first lymphocytes were found in thymus and later in kidney and spleen (Ellis, 1977a; Grace & Manning, 1980; Botham & Manning, 1981; Secombes *et al.*, 1983a; Razquin *et al.*, 1990; Jósefsson & Tatner, 1993). Whether the lymphocyte precursors derive from other organs than thymus (e.g. kidney) is not known but Chilmonczyk (1992) concluded from these and other histological and functional studies that the thymus of fish can be regarded as a primary lymphoid organ for T cells. B cells in fish might develop in the anterior kidney because the first immunoglobulin-positive (Ig^+) cells were found in this organ in rainbow trout (*Oncorynchus mykiss*; Razquin *et al.*, 1990). Moreover affinity analysis of antibody forming cells in various lymphoid organs of coho salmon (*Oncorynchus kisutch*; Irwin & Kaattari, 1986) and morphological studies (Zapata, 1979) gave indications that anterior kidney might be the primary lymphoid organ for B cells (Kaattari, 1992).

Morphology

The characterisation of mammalian leucocytes was initially performed by morphological analysis in combination with (enzyme) cytochemistry (Parmley, 1988). However, the identification of leucocytes has been enormously improved during the last 15 years due to the availability of monoclonal antibodies (MAbs) to several cell surface markers. Since 1982 four international workshops on human leucocyte differentiation antigens have been held (Paris, 1982, Boston, 1984, Oxford, 1986, Vienna, 1989) and cluster of differentiation (CD) designations were assigned to the MAbs (Bernard *et al.*, 1984; Reinherz *et al.*, 1986; McMichael *et al.*, 1987; Knapp *et al.*, 1989). In this way a systematic

nomenclature for MAbs recognising the same surface molecules was developed. The same CD terminology was introduced for MAbs against homologous surface molecules in mouse. At present 89 different CD molecules can be identified in man and 16 different CD molecules in mouse, while a number of MAbs has not yet been classified. A recent summary of the CD system of human and mouse leucocyte surface molecules is given by Coligan *et al.* (1992). An overview of the human CD markers is given in Table I.

Early fish leucocyte characterisation studies were mainly based on morphological similarities with mammalian leucocytes and haematological staining (Rowley *et al.*, 1988). Ultrastructural similarities between lymphocytes and plasma cells from carp and mouse are obvious (Figures 3A+B and 4A+B, respectively). However, the nomenclature of fish granulocytes is still confusing e.g. neutrophilic granulocytes of carp (Figure 3C) show more resemblance with eosinophilic granulocytes than with neutrophilic granulocytes of mouse (Figure 4C+D). Carp basophilic granulocytes (Figure 3D) have an ultrastructure similar to mammalian mast cells (not shown). The production of conventional antisera and more recently MAbs to Ig and some other cell surface molecules allowed further identification of fish leucocytes. However, the panel of MAbs to fish leucocytes is still limited (Table II).

Function

The immune response can be separated in innate or aspecific immunity and adaptive or specific immunity (Golub and Green, 1991). The specific immune reponse has four main features: it is induced by antigen, displays self-non self discrimination, is antigen specific and shows immunologic memory. Aspecific immunity shows only the first two features. Cells from the myeloid lineage are usually considered as mediators of aspecific immunity, while lymphoid cells are the main mediators of specific immunity. However, monocytes/macrophages are involved in aspecific immunity as phagocytic cells and in the specific immune response as antigen-presenting and immunomodulating cells. Moreover, most phagocytes have a preference for the binding and uptake of antibody-antigen complexes. Therefore, it is difficult to make a distinction between cells involved in aspecific or specific immunity.

Table I. Monoclonal antibodies to human cell-surface antigens[a]

CD[b]	function	MW (kDa)	reactivity
1a	MHC class I-related protein	gp49	thy, DC, B sub, LHC
1b	MHC class I-related protein	gp45	thy, DC, B sub, LHC
1c	MHC class I-related protein	gp43	thy, DC, B sub, LHC
2	SRBC-receptor, signal transduction	gp50	T
2R	CD2 epitope unrelated to SRBC-receptor	gp50	T act
3	T3 antigen associated with TcR signal transduction	gp16-25	immature T (cy) mature T (m)
4	MHC II recognition HIV receptor	gp59	T_h
5	T proliferation	gp67	T, B sub
6	pan T antigen	gp100	T, B sub
7	FcμR	gp40	T
8	MHC I recognition	gp32	T_c/T_s
9	aggregation plate	p24	pre B, mo, throm
10	CALLA/neutral endopeptidase	gp100	pre B sub, GCB thy sub, gran, cALL
11a	adhesion molecule	gp180	leuc
11b	CR3	gp165	mo, gran, NK
11c	CR4	gp150	mo, gran, NK, B sub
w12	to be redefined		
13	pan myeloid antigen	gp150	mo, gran
14	PI-linked protein	gp55	mo, LHC, (gran)
15	X hapten, fucosyl-N-acetyllactosamine	several gp	gran, (mo) Reed-Ste rnberg
16	FcγRIII	gp50-65	NK, gran
w17	lactosyl ceramide	gl	gran, mo, plate
18	CD11 associated	gp95	leuc
19	pan B antigen	gp90	B
20	B activation	p35,37	B
21	CR2/EBV receptor	gp140	B
22	B activation	gp135	B (cy), B sub (m)
23	FcεRII	gp45-50	B sub, mo act, eo
24	PI-linked protein	gp38-41	B, gran
25	α chain IL-2 receptor	gp55	T act, B act, mo act
26	dipeptidyl peptidase IV	gp120	T act
27	homodimer	p110 p(55/55)	mature T, T act, B sub
28	homodimer	gp90 gp(44/44)	T sub
29	VLA-ß chain	gp130	broad
30	activation antigen	gp105	lymph act sub Reed-Ste rnberg
31	platelet GPIIa	gp130	gran, B, plate, mo, (T)
w32	FcγRII	gp39-48	gran, B, eo, mo,
33	pan myeloid antigen	gp67	pro, mo
34	progenitor antigen	gp105-120	pro
35	CR1	gp160-250	gran, B, mo
36	thrombospondin receptor	gp90	mo, plate, (B)
37	B antigen	gp40-52	B, (T), (mo), (gran)
38	GF receptor?	gp45	T act, lymph pro, PC
39	B antigen	gp70-100	mature B sub, (mo)
40	B activation	gp45-50	B sub
41	platelet GPIIb	gp135	plate
42a	platelet GPIX	gp23	plate
42b	platelet GPIb	gp170	plate
43	leucosialin, T proliferation	gp95	T, gran, mo, some B, brain

CD	function	MW (kDa)	reactivity
44	homing receptor	gp80-95	leuc
45	LCA, signal transduction	gp180-220	leuc
45RO		gp180	T sub, B, mo, ma, gran
45RA		gp220	T sub, B, gran, mo
45RB		gp190,205 220	T sub, B, gran, mo, ma
46	membrane co-factor protein	gp66/56	leuc
47	non-lineage antigen	gp47-52	broad
48	PI-linked protein	gp41	leuc
w49b	VLA-α2 chain/ collagen receptor	gp170	plate, T cult
w49d	VLA-α4 chain/ fibronectin receptor	gp150	T, B, (LHC) thy, mo
w49f	VLA-α6 chain/ laminin receptor	gp150 (120/30)	plate, (T)
w50	not well defined		leuc
51	VNR-α chain	gp140 (125/25)	(plate)
w52	Campath-1 antigen	gp21-28	leuc
53	CD37 homology	gp35	leuc
54	ICAM-1	gp90	broad
55	PI-linked protein	gp70	broad
56	NCAM	gp140	NK, T sub
57	human NK antigen	gp110	NK, T, B sub, brain
58	LFA-3	gp45-66	leuc, ery, epith
59	PI-linked protein	gp18-20	broad
w60	NeuAc-NeuAc-Gal	gl	T sub
61	GPIIIa/VNR-ß chain	gp33	plate
62	GMP-140	gp140	plate act
63	GP-53 antigen	gp53	plate act, mo, (gran, T, B)
64	FcγRI	gp75	mo, (gran act)
w65	fucoganglioside	gl	gran, (mo)
66	phosphoprotein	gp180-200	gran
67	PI-linked protein	p100	gran
68	macrophage antigen	gp110	ma
69	AIM	gp60 (28/34)	B act, T
w70	not fully defined		B act, T act Reed-Ste rnberg
71	transferrin receptor	gp 190 (95/95)	prolif cells, ma
72	B antigen	gp43/39	B
73	PI-linked protein	gp69	B sub, T sub
74	MHC II-associated invariant chain	gp41/35/ 33	B, mo
w75	B antigen	p53?	mature B, (T sub)
76	B antigen	p85, 67	mature B, T sub
77	B antigen	gl	B rest
w78	not fully defined		B, (mo)

[a]Adapted from Coligan *et al.*, 1992

[b]Abbreviations: **act**, activated; **AIM**, activation inducer molecule; **B**, B lymphocytes; **ba**, basophils; **cALL**, common acute lymphoblastic leukemia; **CALLA**, common acute lymphoblastic leukemia antigen; **CD**, cluster of differentiation; **CR**, complement receptor; **cult**, cultured; **cy**, cytoplasmic; **DC**, dendritic cells; **EBV**, Epstein-Barr virus; **eo**, eosinophils; **epith**, epithelial cells; **GCB**, germinal center B cell; **GF**, growth factor; **gl**, glycolipid; **GMP**, granulocyte membrane protein; **gp**, glycoprotein; **gran**, granulocytes; **HIV**, human immunodeficiency virus; **ICAM**, intercellular adhesion molecule; **IL**, interleukin; **LCA**, leucocyte common antigen; **LFA**, lymphocyte function-associated antigen; **LHC**, epidermal Langerhans cells; **leuc**, leucocytes; **lymph**, lymphocytes; **m**, membrane; **ma**, macrophage; **MHC**, major histocompatibility complex; **mo**, monocyte; **MW**, molecular weight; **NCAM**, neural cell adhesion molecule; **NK**, natural killer cells; **PI**, phosphoinositol; **plate**, platelets; **pro**, progenitor cells; **prolif**, proliferating; **R**, receptor; **rest**, resting cells; **SRBC**, sheep red blood cells; **sub**, subsets; **T**, T lymphocytes; **TcR**, T cell receptor; T_c, T cytotoxic, T_h, T helper/inducer; **thy**, thymocytes; T_s, T suppressor; **VLA**, very late activation antigen; **VNR**, vitronectin receptor

In general two aspects of specific immunity can be distinguished: 1. lymphoid cells can selectively kill other cells which express non-self molecules at their membrane by cell-cell contact (cellular immunity) or 2. lymphoid cells can produce specific antibodies to non-self molecules (humoral immunity). A lot of attention has been payed to memory formation, resulting in faster and stronger immune responses to antigens entering the animal for a second time. As will be addressed in the following section aspecific and (cellular and humoral) specific immune functions are described in fish. It obvious that the availability of MAbs is not only important for the identification of leucocyte types, but also plays an important role in the functional characterisation. MAbs can be used for isolation of distinct cell populations for *in vitro* studies or cell functions may be activated or inhibited by MAbs against receptor molecules.

Fish leucocyte types

Granulocytes

Recently two reviews have been published on the granulocytes of fish (Ainsworth, 1992; Hine, 1992). The identification of fish granulocytes is mainly based on haematological stainings or their ultrastructure and similarity with mammalian granulocytes but also on their (enzyme) cytochemical reactions (Ainsworth, 1992), e.g. peroxidase activity (Bielek, 1981) or Sudan black B staining (Ellsaesser *et al.*, 1985). MAbs are only available for the identification of neutrophilic granulocytes from channel catfish (*Ictalurus punctatus*; Ainsworth *et al.*, 1990; Bly *et al.*, 1990). Like in man, three main granulocyte types can be distinguished: neutrophils, eosinophils and basophils. There is a considerable diversity in granulocyte morphology in different fish species. Moreover, not every granulocyte type is present in every species. For instance in channel catfish only neutrophilic granulocytes are found (Ellsaesser *et al.*, 1985). However, all three granulocyte types are present in carp (Cenini, 1984; Temmink & Bayne, 1987) but basophils and eosinophils are considered to be of the same lineage (Temmink & Bayne, 1987; Rombout *et al.*, 1989a). This idea is based upon: 1. the existence of intermediate cells with both basophilic and eosinophilic granules, 2. the same distribution of these cell types in kidney and gut.

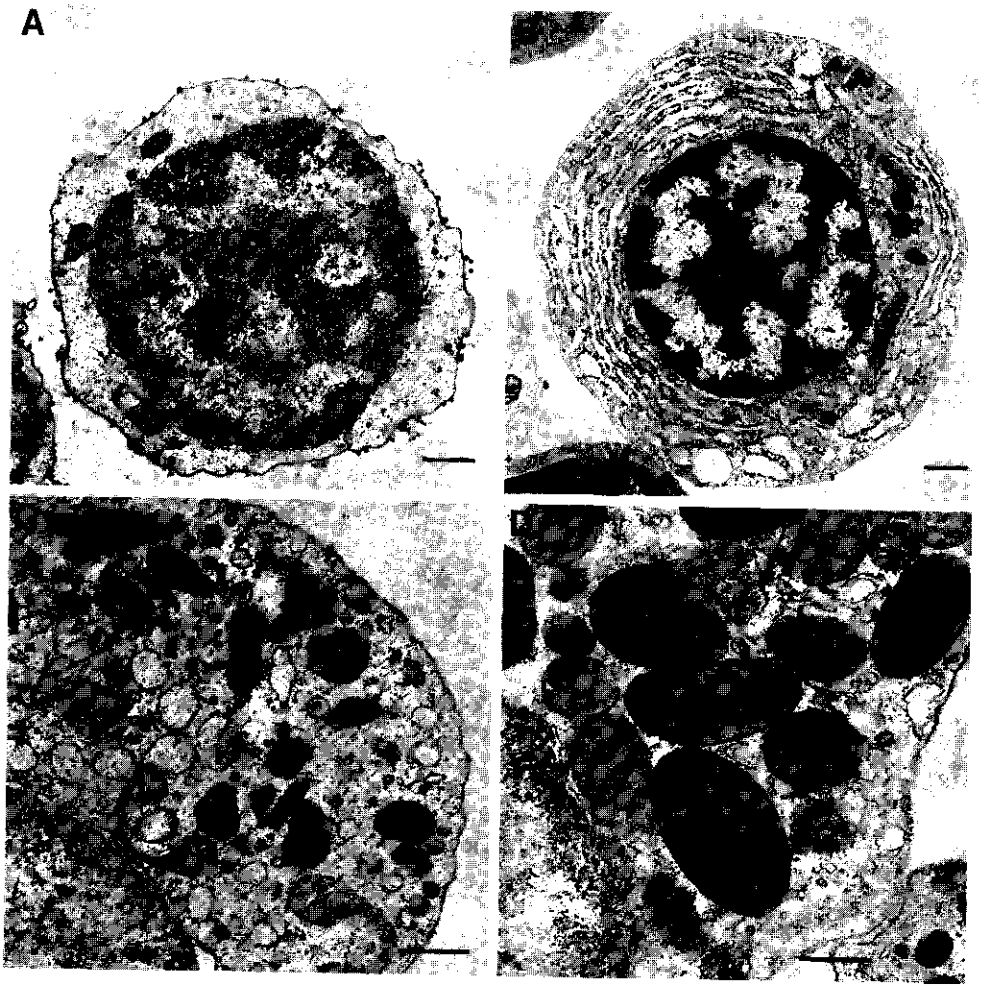

Figure 3. Electron micrographs of carp leucocytes. bar = 0.5 μm **A.** lymphocyte, **B.** plasma cell, **C.** neutrophilic granulocyte, **D.** basophilic granulocyte

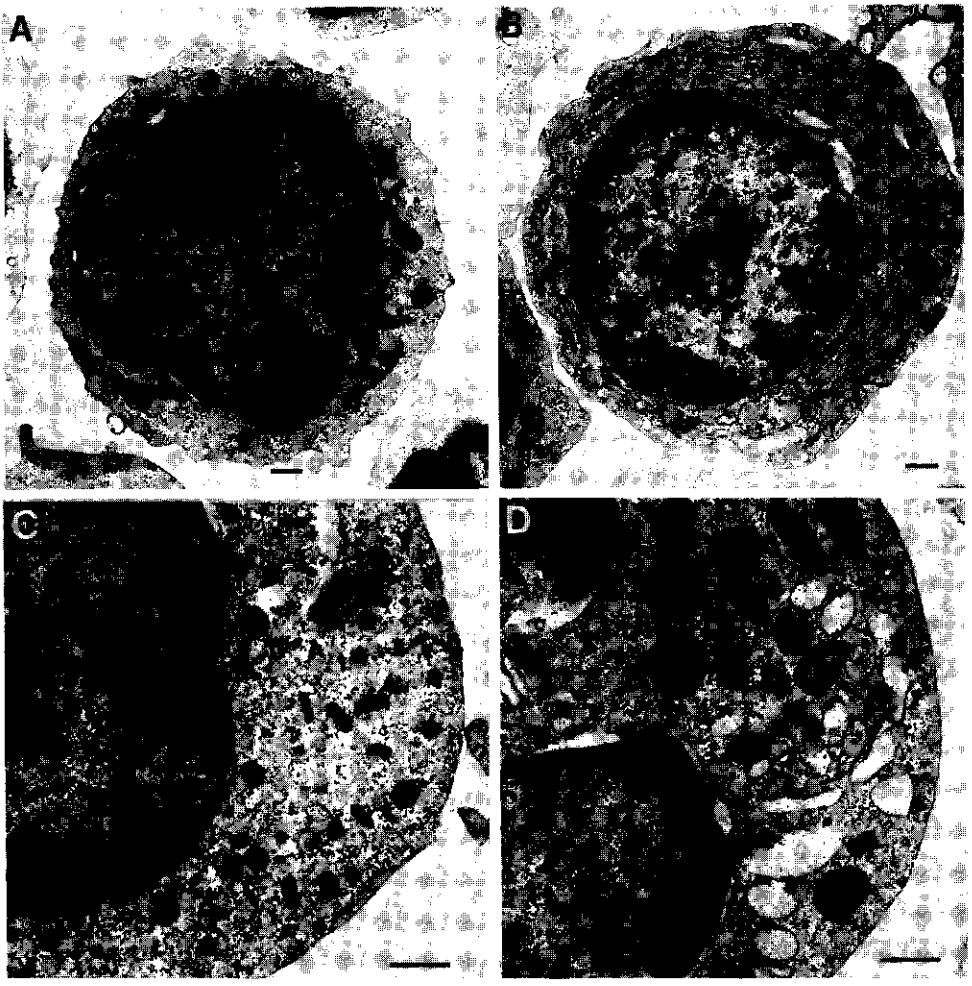

Figure 4. Electron micrographs of leucocytes from mouse. bar = 0.5 μm **A.** lymphocyte, **B.** plasma cell, **C.** neutrophilic granulocyte, **D.** eosinophilic granulocyte (courtesy J.J. Taverne-Thiele)

Table II. Monoclonal antibodies against cell-surface molecules of teleost fish species

fish species	MAb	reactivity	molecule (kDa)	references
Atlantic cod	1F1	N.D.	H chain Ig (81)	Pilström and Petersson, 1991
Atlantic cod	2A2	N.D.	L chain Ig (27.5)	Pilström and Petersson, 1991
Atlantic cod	several	N.D.	H chain Ig	Israelsson *et al.*, 1991
Atlantic salmon	several	B sub	H chain Ig	Killie *et al.*, 1991
Atlantic salmon	several	N.D.	Ig	Falk *et al.*, 1991
carp	WCI 4	B sub	H chain Ig (70)	Secombes *et al.*, 1983b; this thesis, chapter 6
carp	WCI 12	B sub	H chain Ig (70)	Secombes *et al.*, 1983b; this thesis, chapter 6
carp	WCI M	B sub	H chain mIg (70)	Rombout *et al.*, 1993b
carp	WCT	leuc	several	Secombes *et al.*, 1983b; Rombout *et al.*, 1990; this thesis, chapter 2
carp	WCL 2	leuc sub	several	this thesis, chapter 7
carp	WCL 6	throm	(40)	this thesis, chapter 7
channel catfish	3E11	B sub	H chain Ig (70)	Lobb and Clem, 1982; Lobb and Olson, 1988
channel catfish	3D11	B sub	H chain Ig (70)	Lobb and Clem, 1982; Lobb and Olson, 1988
channel catfish	1H6	B sub	H chain Ig (70)	Lobb and Clem, 1982; Lobb and Olson, 1988
channel catfish	9E1	B	H chain Ig (70)	Sizemore *et al.*, 1984
channel catfish	1G7	B sub	L chain Ig (26)	Lobb *et al.*, 1984
channel catfish	3F12	B sub	L chain Ig (22/24)	Lobb *et al.*, 1984
channel catfish	13C10	T,thy,N,throm	(150)	Miller *et al.*, 1987
channel catfish	13C5	N	N.D.	Bly *et al.*, 1990
channel catfish	C2-3a/ C2 4a	T*	N.D.	Ainsworth *et al.*, 1990
channel catfish	C3-1/ 51A	N	N.D.	Ainsworth *et al.*, 1990
channel catfish	5C6	NCC	vimentin-like (40)	Evans *et al.*, 1988; Evans and Jaso-Friedmann, 1992
rainbow trout	1.14	B	Ig	DeLuca *et al.*, 1983
rainbow trout	1A6	N.D	Ig	Sánchez *et al.*, 1989
rainbow trout	2H9/ 2A1	N.D.	L chain Ig	Sánchez and Domínguez, 1991
rainbow trout	3B10/ 4D11	N.D.	H chain Ig	Sánchez *et al.*, 1993a; Sánchez *et al.*, 1993b
rainbow trout	several	B	H chain Ig	Thuvander *et al.*, 1990
sea bream	WSI 5	B	L chain Ig	Navarro *et al.*, 1993

Abbreviations: **B**, B lymphocytes; **H chain**, heavy chain; **leuc**, leucocytes; **L chain**, light chain; **mIg**, mucus Ig; **N**, neutrophilic granulocytes; **NCC**, non-specific cytotoxic cells; **N.D.**, not determined; **sub**, subsets; **T**, T lymphocytes; **throm**, thrombocytes; **thy**, thymocytes

* not fully evidenced

Recently a fourth type is described in carp: the eosinophilic granular cell (Cross & Matthews, 1991). It is only present in the skin and not in the blood or lymphoid organs. A similar cell type is described for salmonids and it is believed to represent a primitive mast cell (Ezeasor & Stokoe, 1980; Vallejo & Ellis, 1989; Powell *et al.*, 1990). Like in mammals, neutrophils of teleosts mediate the accute inflammatory response (Hine, 1992), they migrate to the site of inflammation by chemotaxis and show phagocytic activity (Ainsworth, 1992). Phagocytosis by carp and plaice (*Pleuronectes platessa*) neutrophils could be enhanced by complement opsonisation while antibody showed little or no effect (Nash *et al.*, 1987; Matsuyama *et al.*, 1992). This indicates the presence of complement receptors but, in contrast to mammals, the absence of Fc receptors. Carp neutrophils show respiratory burst and microbicidal activity (Verburg-van Kemenade *et al.*, 1989). Basophilic and eosinophilic granulocytes from carp are not phagocytic according to Temmink & Bayne (1987) but Verburg-van Kemenade *et al.* (1989) reported phagocytosis by carp basophilic and eosinophilic granulocytes. Peroxidase activity is described for neutrophils and eosinophils of carp, while basophils were peroxidase negative (Bielek, 1981). There is some evidence that goldfish (*Carassius auratus*) eosinophils are involved in parasitic infections (Huizinga, 1980). Although the carp basophil shows similarities with mammalian mast cells it is still unknown whether this cell type possess mast cell-like functions.

Monocytes/macrophages

Like in mammals, the name monocyte is used for circulating mononuclear phagocytes, while macrophages are fixed or wandering cells in tissues. The cytochemical features of these cells in different fish species are reviewed by Rowley *et al.* (1988). Monocytes are acid phosphatase- and PAS-positive, but they are variably positive for peroxidase and alkaline phosphatase (dependent on investigated species) . Two types of macrophages can be distinguished: 1. melanomacrophages containing melanin and other pigments like lipofuscin and haemosiderin (Agius, 1985), 2. other macrophages differing from monocytes in size and lysosome numbers. It is expected that subpopulations are present in this group. For monocytes/macrophages in fish the same functions as in mammals have

been demonstrated: phagocytosis (rev. Secombes & Fletcher, 1992), antigen presentation (rev. Vallejo *et al.*, 1992) and the production of cytokines (Secombes, 1991) and other immunomodulating factors like leukotrienes and lipoxins (rev. Secombes & Fletcher, 1992). Like in mammals several receptor types are demonstrated on fish macrophages which are able to enhance phagocytosis. Lectin receptors were demonstrated in salmonid fish (Ozaki *et al.*, 1983). Opsonisation by complement was described for rainbow trout (*Oncorhynchus mykiss*; Honda *et al.*, 1985, 1986; Michel *et al.*, 1990) which indicates the presence of complement receptors. Opsonising activity was still found in anti-*Yersinia ruckeri* immune serum after inactivation of complement (Griffin, 1983), which indicates the presence of Fc receptors. Melanomacrophages (Lamers, 1986) and gut macrophages (Rombout *et al.*, 1989a) from carp were immunoreactive with MAbs to carp serum Ig and the presence of Fc receptors binding immune complexes was suggested. Fc receptors are most evidently demonstrated in nurse shark (*Ginglymostoma cirratum*) by an erythrocyte-antibody (EA) rosette method (Haynes *et al.*, 1988). Evidence for an antigen presenting function of carp macrophages comes from the detection of antigenic determinants on the surface of melanomacrophages in spleen and kidney after injection with *Aeromonas hydrophila* (Lamers & De Haas, 1985) and on the surface of gut macrophages after anal intubation with ferritin or *Vibrio anguillarum* (Rombout and Van de Berg, 1989). Antigen processing and presentation has been frequently studied in channel catfish by *in vitro* antigen pulsing of *in vivo* primed antigen-presenting cells (e.g. monocytes). Leucocyte proliferation and antibody production was measured and a MHC (major histocompatibility complex) class II-type pathway for exogeneous antigen processing and presentation was suggested (Vallejo *et al.*, 1992). Till now the production of an IL (interleukin)-1-like factor by fish macrophages has been demonstrated in channel catfish (Clem *et al.*, 1991) and there is some evidence for this activity in carp (Verburg-van Kemenade *et al.*, 1991).

Non-specific cytotoxic cells (NCC)

Fish NCC are an equivalent of mammalian natural killer (NK) cells and they play an important role in the defense against microbial and protozoan infections. Both NCC and NK

cells need a direct contact with the target cell in order to kill by apoptic and necrotic mechanisms (Geenlee *et al.*, 1991). NCC and NK differ in killing kinetics, target cell specificity and morphology (Evans & Jaso-Friedmann, 1992). MAbs prepared against purified catfish NCC (Evans *et al.*, 1988), were also reactive with NK cells from rat or man (Avecedo *et al.*, 1988). This antibody appeared to inhibit the cytotoxic activity of these cells (Evans *et al.*, 1988). Purification and sequencing of the putative rat NK receptor using this MAb revealed that this molecule was a vimentin-like protein. It was therefore suggested that the receptor on fish NCC is also a vimentin-like protein (Evans & Jaso-Friedmann, 1992).

Lymphocytes

Lymphocytes form the majority of the blood leucocytes and are probably the best studied leucocyte population. The morphology of fish lymphocytes is similar to mammalian lymphocytes: small cells (5-8 μm) with a high nucleus:cytoplasm ratio (Figure 3A, 4A). Lymphocytes identified by light microscopy can easily be confused with some morphological different types of thrombocytes (Ellis, 1977b). Carp thrombocytes cut in transverse section were similar to lymphocytes at the ultrastructural level (Cenini, 1984). Heterogeneity among fish lymphocytes has been estimated indirectly by functional studies and directly by immunocytochemical characterisation with MAbs against Ig. The functional studies include an estimation of cellular immunity, humoral immunity, mitogen stimulation or production of immunomodulating factors. Specific cellular immune responses in which T cells are the effector cells, can be demonstrated by the mixed leucocyte reaction or MLR (Ellis, 1977a; Caspi & Avtalion, 1984a; Miller *et al.*, 1986; Kaastrup *et al.*, 1988; rev. Kaattari & Holland, 1990), delayed type hypersensitivity (Bartos & Sommer, 1981; Pauley & Heartwell, 1983; rev. Stevenson & Raymond, 1990) or transplantation immunity (Hildemann, 1958; Rijkers & Van Muiswinkel, 1977; Botham & Manning, 1981). Humoral immune responses in which B cells and plasma cells are the effector cells, could be demonstrated by the detection of specific antibodies after immunisation, e.g. by agglutination assays (Sailendri & Muthukkaruppan, 1975; Lamers *et al.*, 1985), enzyme-liked immunosorbent assay (ELISA; Thuvander *et al.*, 1990; Killie *et al.*, 1991; rev. Arkoosh and Kaattari, 1990; Rombout *et*

al., 1986, 1989b), ELISPOT (Secombes *et al.*, 1991; Davidson *et al.*, 1992; rev. Davidson & Secombes, 1992) or plaque forming cell assay (Anderson *et al.*, 1979; Rijkers *et al.*, 1980a,b; Miller & Clem, 1984; rev. Anderson, 1990). Mitogen stimulation assays comprise *in vitro* stimulation of leucocytes by mammalian B cell (lipopolysaccharide, LPS) or T cell (concanavalin A, ConA; phytohaemagglutinin, PHA) mitogens. In this way indications for lymphocyte heterogeneity could be obtained (Etlinger *et al.*, 1976; Chuchens & Clem, 1977; Warr & Simon, 1983; Caspi *et al.*, 1984; Reitan & Thuvander, 1991). Cooperation between T helper and B cells could be demonstrated by hapten-carrier assays (Yocum *et al.*, 1975). More direct evidence for lymphocyte heterogeneity has been obtained by the production of MAbs against specific surface molecules. The development of MAbs against Ig from several fish species (Table II) revealed that thymocytes and a part of other lymphoid cells were surface Ig-negative (sIg^-). These MAbs have been used for the separation of sIg^+ and sIg^- cells which were subsequently tested in mitogen stimulation assays (DeLuca *et al.*, 1983; Sizemore *et al.*, 1984; Ainsworth *et al.*, 1990) and hapten-carrier assays (Miller *et al.*, 1985). However, not all sIg^- lymphoid cells have to be T cells because they could also be null cells. MAbs immunoreactive with sIg^- lymphocytes showing helper T cell activity in an antibody response against a thymus dependent antigen have only been described for channel catfish (Miller *et al.*, 1987). However, this MAb reacted also with most thymocytes, neutrophils, thrombocytes, a few hepatocytes and some brain cells and hence cannot be considered as a specific T cell marker. Ainsworth *et al.* (1990) also described a MAb reacting with sIg^- catfish PBL. Panned immuno-positive cells showed a better mitogen response to Con A than to LPS but further characterisation was not performed.

Indications have been found for the production of cytokines by lymphocytes (rev. Secombes, 1991). In carp, an IL-2-like growth promoting activity was demonstrated in culture supernatant from mitogen (PHA)- or alloantigen (MLR)-stimulated leucocyte cultures (Caspi & Avtalion, 1984b; Grondel & Harmsen, 1984). Panned sIg^- lymphocytes from rainbow trout were able to secrete a macrophage activating factor (MAF) following stimulation with Con A/phorbol myristate acetate (PMA), while panned sIg^+ lymphocytes did not (Graham & Secombes, 1990a). Separation of the MAF-containing supernatants by

high performance liquid chromatography (HPLC) showed MAF activity and anti-viral activity in the same fractions and the presence of an IFN-γ-like molecule was suggested (Graham & Secombes, 1990b). MAF activity in carp has also been observed (Verburg-van Kemenade *et al.*, 1991).

It can be concluded that B cell functions are present in fish and that B cells can be identified in many fish species by specific MAbs against Ig. T cell functions are also described and can be divided in cytotoxic (e.g. graft rejection, MLR) and helper (hapten-carrier assay, MAF production) functions. Unfortunately, T cell (sub)populations can still not be well identified by specific MAbs.

Aim and outline of the thesis

Although many leucocyte types can be distinguished and specific and aspecific immune functions are described, the correlation between cell type and function is still difficult in fish. The main reason for this is an apparent lack of the right tools for the identification, separation and functional characterisation of distinct cell populations. As mentioned earlier, MAbs have become a major tool for the characterisation of leucocytes in mammalian immunology (Table II). Unfortunately a panel of these mammalian MAbs tested on catfish leucocyes did not cross-react (Miller *et al.*, 1987). Also MAbs against CD 4 and CD 8 antigens of mouse or MAbs against catfish sIg^- lymphocytes (Miller *et al.*, 1987) were not reactive with carp cells (unpublished results). It is concluded that species-specific MAbs have to be developed for most fish species.

In this study methods for identification, separation and functional characterisation were adapted for carp leucocytes. MAbs produced earlier against carp Ig or thymocytes (Secombes *et al.*, 1983b) were further characterised. Most promising MAbs appeared to be specific for carp Ig and hence could be used for investigations on B cell (sub)populations, plasma cells and Ig-binding leucocytes. These MAbs were also useful for isolation of sIg^+ and sIg^- leucocytes, which allowed a much better *in vitro* study of these cell types. In addition, isolated sIg^- lymphoid cells were used for immunisation of mice in order to produce new MAbs.

The following topics, in order of their appearance in this thesis, were investigated:

- The immunocytochemical detection of membrane antigens using light and electron microscopic techniques (Chapter 2).
- The ontogeny of B cells and plasma cells in carp from 2 weeks till 16 months of age, studied by flow cytometry and fluorescence microscopy (Chapter 3).
- Ig binding by carp leucocytes using an EA rosette method and immuno (electron) microscopy (Chapter 4).
- Mitogen (LPS or PHA) stimulation of sIg^+ and sIg^- peripheral blood leucocytes. The cell populations were enriched by magnetic cell sorting (MACS) or analysed by simultaneous detection of sIg and 5-bromo-2'-deoxyuridine with fluorescence microscopy or flow cytometry (Chapter 5).
- B cell and Ig heterogeneity, estimated by a variety of immuno(cyto)chemical methods (Chapter 6).
- The production of new MAbs against Ig^- lymphoid cells of carp, including a characterisation of the reaction pattern of promising MAbs (Chapter 7).

Finally, a summary of the results and main conclusions is given in English and Dutch.

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Immunocytochemical detection of membrane antigens of carp leucocytes using light and electron microscopy

J.C.E. van Diepen, G.T.M. Wagenaar & J.H.W.M. Rombout

Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH, Wageningen, The Netherlands.

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Abstract

Monoclonal antibodies against carp serum immunoglobulin (Ig; WCI 12 of IgG class) and thymocytes (WCT 23 of IgM class) were characterised at the ultrastructural level for their reactivity with leucocytes from carp pronephros, and for the corresponding distribution of the cell membrane molecule(s) recognised by these antibodies. To this end, the immunogold (double) labelling technique was applied to cells in suspension, as well as to a monolayer of cells. Immunofluorescence labelling was also performed on fixed cytocentrifuge preparations. With WCI 12, 20 - 30 % of the lymphocytes, probably B cells, and the majority of the plasma cells appeared to be Ig-immunoreactive. It was found that membrane Ig mainly occurred in clusters at the B cell membrane in pre- as well as in post-fixed preparations. WCT 23 reacted with all leucocytes present and the molecules recognised by WCT 23 appeared to be regularly distributed at the cell membrane.

Introduction

In contrast to leucocytes of higher vertebrates, those of fish are not well-characterised. Based on their ultrastructural resemblance with mammalian leucocytes, a more or less similar distinction in lymphocytes, plasma cells, monocytes, macrophages and granulocytes can be made among the leucocytes of fish (Ellis, 1977; Cenini, 1984; Temmink & Bayne, 1987). Most of these cells however, cannot be unambiguously identified with light-microscopic techniques, and even at the ultrastuctural level the distinction between B and T lymphocytes will be difficult. Thus, leucocyte characterisation in fish would benefit very much from the availability of monoclonal antibodies recognising specific membrane markers. Monoclonal antibodies against serum immunoglobulin (Ig) of catfish (*Ictaluras punctatus*) (Lobb & Clem, 1982), trout (*Oncorhynchus mykiss*) (DeLuca *et al.*, 1983), and carp (*Cyprinus carpio*) (Secombes *et al.*, 1983) have been shown to be able to differentiate between fish lymphocytes. These data indicate the presence of Ig-immunopositive B lymphocytes and Ig-

negative lymphoid cells (which can be T or null cells). In addition, monoclonal antibodies against carp thymocytes (WCTs), produced in our laboratory, have been shown to react with a larger proportion of leucocytes than the monoclonal antibodies against serum Ig (WCIs) (Secombes *et al.*, 1983). However, due to the indiscriminatory character of the light-microscopic techniques used (cryostat sections, cytocentrifuge slides), the immunoreactive cells could not be precisely identified.

In this study, the further characterisation of two monoclonal antibodies, made against carp immunoglobulin (WCI 12 of IgG class) and carp thymocytes (WCT 23 of IgM class), respectively, is described. These antibodies are chosen because of their high affinity, and their difference in Ig class which allows double labelling. In order to identify the immunoreactive cells, and to analyse the membrane distribution of the molecules recognised by the antibodies, an immunogold labelling technique has been applied to living cells from the pronephros after which the cells were processed for scanning and transmission electron microscopy. Immunofluorescence labelling was also performed on cytocentrifuge slides of pronephros cells, in order to compare both labelling methods.

Materials and methods

Fish

Fifteen carp, *Cyprinus carpio* L., bred in our laboratory and kept at 23 $\pm$ 0.5 °C in an UV-filtered recirculating system, were used. Before dissection, fish were anesthetised in MS 222 (Sandoz, Basel, Switzerland) and bled by syringe from the caudal vein in order to reduce the blood volume in the pronephros.

Cell suspensions

Cell suspensions of pronephros were prepared in a TBS^+ buffer (18.18 mM Tris, 0.82 % NaCl, 0.73 mM $MgCl_2$, 0.18 mM $CaCl_2$, 1 % BSA, 0.1 % NaN_3) by squeezing tissue pieces from pronephros through a nylon gauze filter. Since non-siliconised glassware was

used, macrophages and monocytes were hardly present in the final cell suspensions, probably due to their adherence to the test-tubes.

Antibodies

The preparation of monoclonal antibodies WCI 12 and WCT 23, raised against carp serum Ig and carp thymocytes, respectively, has been previously described (Secombes *et al.*, 1983). Both antibodies were generally used as 1:100 dilutions of ascitic fluid. Gold probe conjugates of different sizes were tested: goat anti-mouse IgG coupled to 5, 15, 30 or 40 nm gold particles (GAM-IgG G5, G15, G30 and G40) for detection of WCI 12, and GAM-IgM G10 and G30 for detection of WCT 23. In the case of scanning electron microscopy, only a 30 nm gold probe conjugate was used. All gold probe conjugates were used at a dilution of 1:5, and were purchased from Janssen Pharmaceutica (Beerse, Belgium), except GAM-IgG G15 which was purchased from Peninsula Laboratories Europe Ltd. (St. Helens, U.K.). For fluorescence microscopy, a 1:100 dilution of FITC-labelled rabbit anti-mouse antibody (RAM-FITC; 1:100; Dakopatts, Glostrup, Denmark) was used.

Immunogold labelling of cells in suspension

Cells (2.5×10^6) were washed by means of centrifugation for 5 min at 350 x g and 4 °C, and then resuspended in 1 ml TBS^+ buffer. After incubation for 1 h at 0 °C with 1 ml WCI 12 or WCT 23 the cell suspensions were washed three times, followed by an incubation for 1 h at 0 °C with 100 μl of the corresponding gold probe conjugates. During the incubations, cells suspensions were swirled every ten minutes.

Immunogold labelling of a monolayer of cells

Pronephros cells (1.2×10^6/ml) in TBS^+ buffer without BSA were centrifuged at 1 ml per well in 24-well tissue culture plates (Costar, Cambridge, U.K.) for 5 min at 350 x g and 4 °C. A slip of filter paper was fixed to the wall of the wells to prevent accumulation of cells in the corner. After centrifugation, the filter paper was removed, and the resulting monolayer of cells was incubated for 45 min at 0 °C with 1 ml BSA containing TBS^+ buffer

in order to prevent non-specific binding of antibodies to the bottom of the wells. The monolayer was then incubated for 1 h at 0 °C with 1 ml WCI 12 or WCT 23, gently washed three times, and incubated with 200 μl of the corresponding gold-conjugated antibodies for 1 h at 0 °C. After another three washes with TBS^+ buffer, the cells were finally resuspended by vigorously pipetting up and down in the wells. Resuspended cells were collected in tubes, and centrifuged into a pellet. During the incubations, plates were swirled every 10 min.

Immunogold double labelling

A four step double labelling procedure was carried out. Cells in suspension were incubated with WCI 12 and an anti-IgG labelled gold probe. Subsequently a monolayer of these cells was made which was incubated with WCT 23 and an anti-IgM labelled gold probe. The labelling of the cells in suspension and of the monolayer of cells was carried out in the same way as described above.

Electron microscopy

For transmission electron microscopy, cell pellets were fixed in 1 % (w/v) $K_2Cr_2O_7$, 2 % (v/v) glutaraldehyde and 1 % (w/v) OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 0 °C, and subsequently washed in double-distilled water, dehydrated in alcohol and propylene oxide, and embedded in Epon 812. Ultra-thin sections were cut on a Reichert Ultracut E, and after routine staining with uranyl acetate and lead citrate, examined in a Philips 201 electron microscope. Characterisation of different leucocyte types was based on morphologic criteria described by Temmink & Bayne (1987). For scanning electron microscopy, cells were centrifuged for 5 min at 350 x g and 4 °C to a gold-palladium-coated nucleopore filter (Agar Scientific Ltd., Stansted, U.K.) which was placed at the bottom of a 24-well tissue culture plate. They were subsequently fixed for 30 min at 0 °C in 2 % (v/v) glutaraldehyde, dehydrated in ethanol, critical-point dried, and examined in a Philips 400 T scanning transmission electron microscope using the backscattered electron imaging (BEI) mode.

Immunofluorescence

Cell suspensions in TBS^+, or PBS with 5 % BSA and 0.1 % EDTA were centrifuged on slides previously coated with poly-L-lysine (PLL) at 0.01 % (w/v) or BSA at 3 % (w/v), respectively. These cytocentrifuge slides were dried for 30 min with cold air. PLL-coated slides were fixed for 2 min at 0 °C in acetone, and BSA-coated slides were fixed for 12 min at 0 °C in a solution of acetic acid:ethanol 1:19 (v/v). After fixation the slides were washed three times in PBS at 0 °C. Slides were then incubated for 1 h at room temperature with 30 μl WCI 12 or WCT 23, followed by three washes in PBS. After incubation for 1 h at room temperature with RAM-FITC the non-bound antibody conjugate was removed by three washes in PBS, and the slides were studied under a fluorescence microscope (Nikon Microphot FXA).

Controls

The following controls were carried out:

1. Gold probes and fluorescence conjugates were applied with the monoclonal antibodies (WCI 12 and WCT 23) omitted.
2. GAM IgG and GAM IgM were tested for cross-reactivity with non-corresponding antibodies (WCT 23 and WCI 12, respectively).
3. Both WCI 12 and WCT 23 were preabsorbed with carp Ig and thymocytes.

Results

WCI 12 (anti-Ig)

WCI 12, applied to cells in suspension as well as to a monolayer of cells, was immunoreactive with only a subset of the lymphocytes (about 20-30 %). After preabsorption of WCI 12 with carp Ig this reaction did not occur, whereas the reaction was still present after absorption with thymocytes. Gold particles were mainly found in clusters at the cell membrane with transmission and scanning electron microscopy (Figures 1 and 7).

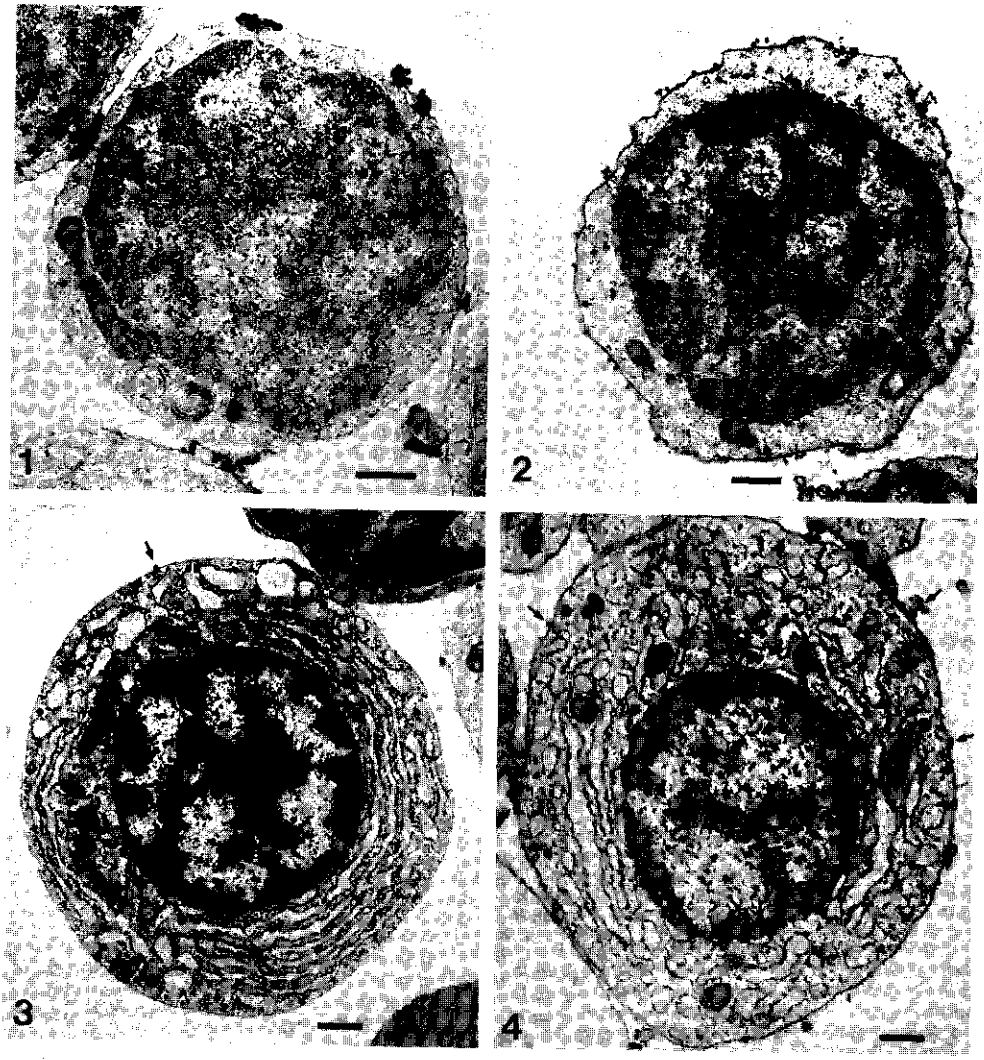

Figure 1. Electron micrograph of a WCI 12-immunoreactive lymphocyte (GAM-IgG G40). bar = 0.5 μm. **Figure 2.** Electron micrograph of a WCT 23-immunoreactive lymphocyte (GAM-IgM G30). bar = 0.5 μm. **Figure 3.** Electron micrograph of a plasma cell incubated with WCI 12 showing only one GAM-IgG G30 particle at the cell membrane (arrow). bar = 0.5 μm. **Figure 4.** Electron micrograph of a WCI 12- and WCT 23-immunoreactive plasma cell (GAM-IgG G40 and GAM-IgM G10, respectively). Many G40 particles are present at the cell membrane (arrows) next to many G10 particles which are less readily visible due to the low magnification. bar = 0.5 μm.

Fluorescence microscopy also revealed a clustered labelling independent of the fixation used (Figure 8). Morfologic differences between WCI 12-positive and WCI 12-negative lymphocytes were not found. Cells with the ultrastructural feature of plasma cells (lymphoid nucleus, rough endoplasmic reticulum whether or not with dilated cisternae), showed a labelling varying from no goldparticles present, to about the same amount as found on lymphocytes (Figures 3 and 4). Granulocytes and erythrocytes did not react with WCI 12. Differences in the number of cells labelled with WCI 12 were not found when gold probes of different sizes were used. However, smaller gold probes did result in increased numbers of gold particles per cell.

WCT 23 (anti-thymocyte)

WCT 23 caused clumping of cells when used on cell suspensions. This clumping frequently gave rise to a reduced ultrastructure, and absence of labelling at places where cell membranes adhered to each other. A monolayer of cells was used to overcome this problem, and resulted in a clear ultrastructure and optimal labelling of the cells. WCT 23 appeared to be strongly immunoreactive with all leucocytes present, but not with erythrocytes. The reaction was absent after preabsorption with carp Ig or thymocytes. Gold particles were regularly distributed along the cell membrane. Again, the use of smaller gold probes resulted in a higher density of gold particles at the cell membrane (Figures 2 and 5). Labelling of fixed cytocentrifuge preparations with WCT 23 also showed a regular distribution of fluorescence label (Figure 9).

Double labelling with WCI 12 and WCT 23

Immunoreaction of WCI 12 on cell suspensions, followed by the reaction of WCT 23 on the same cells in a monolayer, yielded an optimal double labelling. Only WCI 12-immunoreactive cells appeared to be double-labelled; determinants recognised by WCI 12 (immunoglobulin) again occurred in clusters whereas determinants recognised by WCT 23 were regularly distributed on the surface of all leucocytes (Figures 4 and 6). Some WCT 23 labelling was present within the WCI 12-labelled Ig clusters.

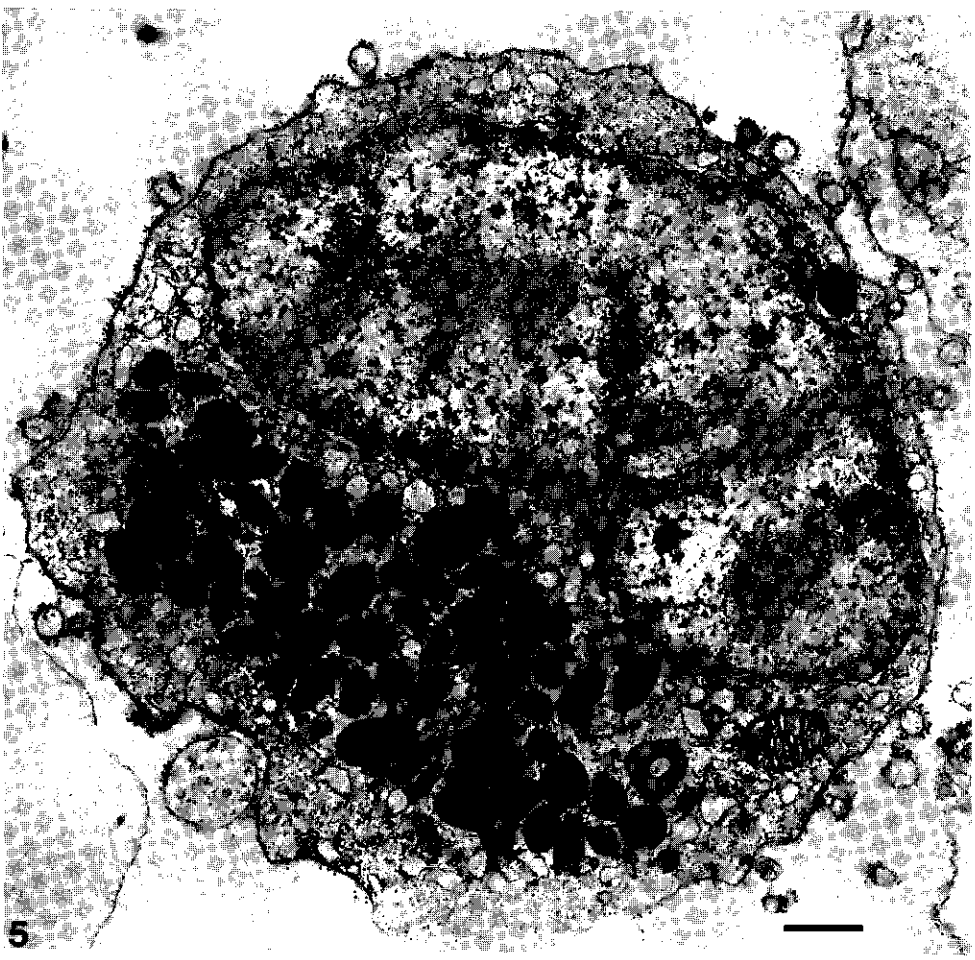

Figure 5. Electron micrograph of a WCT 23-immunoreactive neutrophilic granulocyte (GAM-IgM G10). bar = 0.5 μm

Figure 6. Electron micrograph of a WC1 12 and WCT 23 double-stained lymphocyte (GAM-IgG G40 and GAM-IgM G10, respectively) and a part of a WCT 23-immunoreactive basophilic granulocyte (GAM-IgM G10). bar = 0.5 μm

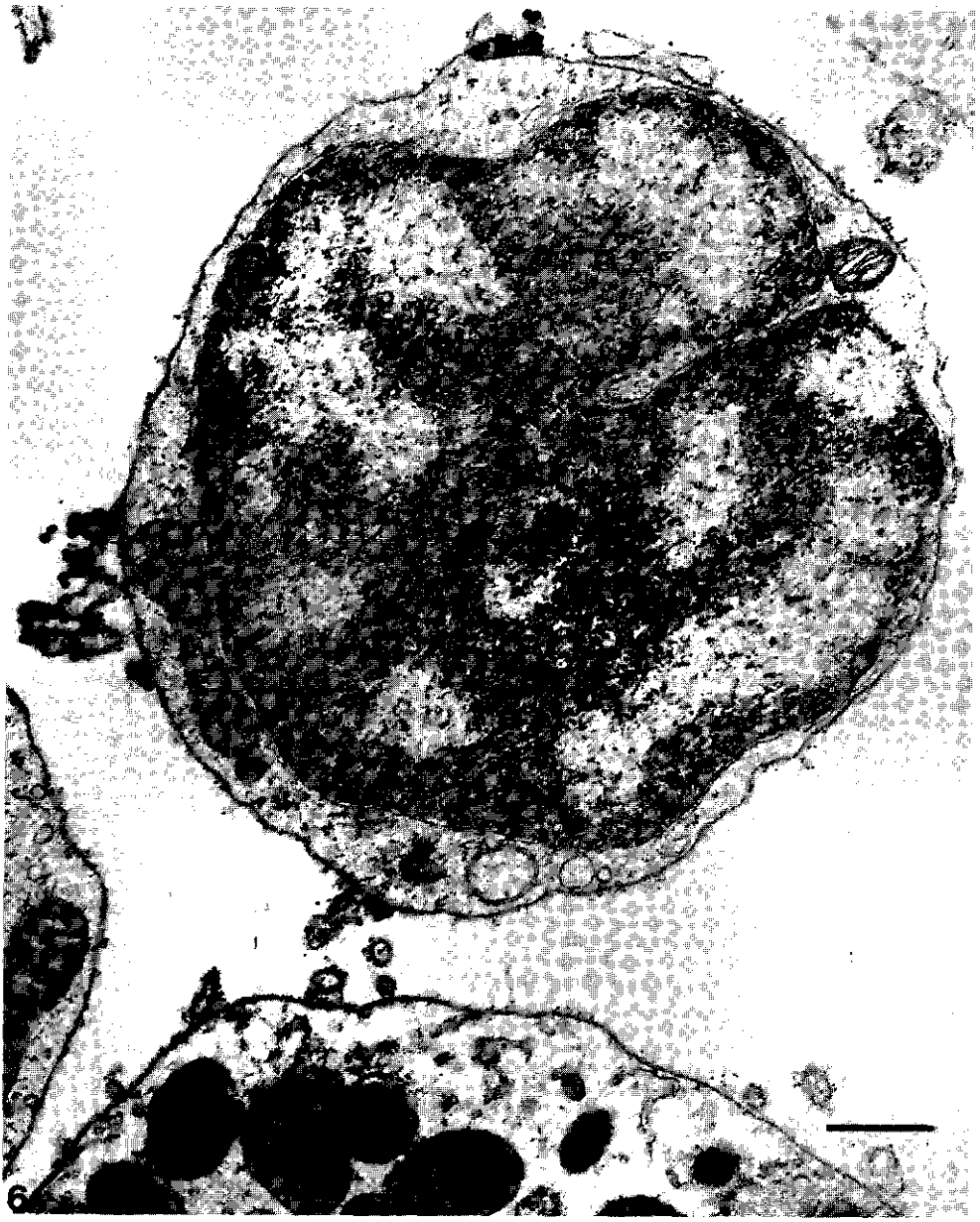
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Figure 7. Scanning electron micrograph in BEI mode of a WCI 12-immunoreactive lymphocyte (GAM-IgG G30). bar = 0.5 μm

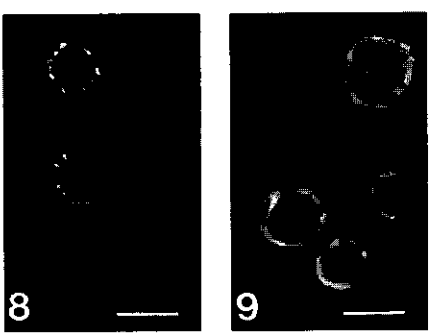

Figure 8. Fluorescence micrograph of lymphocytes labelled with WCI 12 and GAM-FITC after fixation in acetone. The fluorescence label appeared to be present in clusters. bar = 3 μm

Figure 9. Fluorescence micrograph of lymphocytes (L) and a granulocyte (G) labelled with WCT 23 and GAM-FITC after fixation in acetone. The fluorescence label appeared to be regularly distributed along the cell membrane. bar = 3 μm

Discussion

This study describes the identification of carp pronephric leucocytes immunoreactive with monoclonal antibodies WCI 12 (anti-Ig) and/or WCT 23 (anti-thymocyte), and the distribution of the corresponding membrane molecule(s) at the plasma membrane of these cells. Immunoreactions with WCI 12 (of IgG class) were successfully carried out on cell suspensions, resulting in a proper surface labelling and an optimal ultrastructure after subsequent fixation. However, the use of WCT 23 (of IgM class) on cell suspensions resulted in clumping of the leucocytes and, consequently, in irregular labelling and a moderate ultrastructure. Therefore, WCT 23 had to be used on a monolayer of cells. Hoogeveen *et al.* (1988), who recently described a similar immunogold labelling method on cell suspensions of human lymphocytes did not report cell clumping when using IgM antibodies. However, in contrast to our studies the frequencies of labelled cells and the number of antibodies per cell in their experiments were low. The combination of a high frequency of determinants at the cell membrane, the presence of many immunoreactive cells, and the use of IgM antibodies (a pentamer with ten binding sites) may account for the cell clumping observed under our conditions. Smaller gold probes produced a higher labelling density than larger probes, a well-known feature that can be explained by the occupation of more binding sites (Kehle & Herzog, 1987) and an increase in steric hinderance (De Waele, 1984) by the larger gold probe conjugates. In conclusion, the technique described here appears to be very suitable for detailed morphologic characterisation of immunoreactive leucocytes, and for detailed localisation of the corresponding antigenic determinants.

WCI 12 appears to be immunoreactive with the surface of most, but not all, plasma cells found, and with a part of the lymphocytes, probably belonging to the B cell line. B and T cell functions (Etlinger *et al.*, 1976; Caspi *et al.*, 1984; Sizemore *et al.*, 1984), and distinct Ig-positive and Ig-negative lymphocytes (Lobb & Clem, 1982; DeLuca *et al.*, 1983; Secombes *et al.*, 1983; Miller *et al.*, 1987), are generally accepted for fish. This study for the first time attempts to characterise these cells at the ultrastructural level. In contrast to mammals (Roitt *et al.*, 1989) morphologic differences between Ig-positive lymphocytes and

Ig-negative lymphocytes were not found in this study. Contrary to mammalian B cells (Schreiner & Unanue, 1976; De Groot & Wormmeester, 1981), Ig molecules were found to be present in clusters at the cell surface membrane. This observation could probably not be ascribed to patching, because clustering of label is still found in cytocentrifuge preparations pre-fixed in acetone or ethanol/acetic-acid (De Petris, 1978) and in addition WCT 23 did not show clustering under the same conditions although it is of IgM class and reacts with many more antigens. Additional evidence can be obtained by the use of directly labelled monovalent WCI 12.

In mammals, plasma cells lose their surface Ig during differentiation (Hämmerling *et al.*, 1976). Carp pronephric plasma cells, distinguished by their ultrastructure, appeared to have a variable amount of Ig molecules at their surface. Although the numbers of plasma cells found in this electron-microscopic study were low, the amount of surface Ig seems not to be correlated with the maturation of these cells. This premise, however, has to be studied in more detail with light-microscopic techniques in which internal and external Ig can be discriminated and will be the subject of our next study.

Since all leucocytes analysed appear to be positive for WCT 23, this antibody cannot be considered as defining a T cell marker. Secombes *et al.* (1983) have characterised this antibody in an ELISA as positive for thymocytes and Ig. In their cytocentrifuge preparations, the vast majority of lymphocytes from carp pronephros showed surface staining, and plasma cells could be identified by cytoplasmic staining. In the present study it is shown that not only lymphocytes react with WCT 23, but all granulocytes as well. As WCT 23 does not react with erythrocytes, it might be considered as a monoclonal antibody defining a leucocyte common marker. However, from our absorption tests (WCT 23 absorbed with carp Ig) and from previous ELISA data (Secombes *et al.*, 1983) it can be concluded that WCT 23 cross-reacts with Ig, which is unusual for a leucocyte common marker. Preliminary results from Western blots of serum Ig or lymphocyte membrane preparations showed that both WCT 23 and WCI 12 recognised the 70 kDa Ig heavy chain. In contrast to WCI 12, WCT 23 recognised many other molecules. Probably WCT 23 recognises a common antigenic determinant present on several membrane and serum molecules, including immunoglobulin.

In conclusion, this study has demonstrated a difference in distribution of molecules on the membrane of leucocytes; clustered Ig molecules present at B and plasma cells versus randomly distributed more common molecules present on all leucocytes.

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Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L.); an ontogenetic study

J.C.E. Koumans-van Diepen, J.J. Taverne-Thiele, B.T.T.M. van Rens & J.H.W.M. Rombout

Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Key words: fish; ontogeny; B cell; plasma cell; immunoglobulin; immunofluorescence; flow cytometry

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Abstract

The ontogeny of B cells and plasma cells in carp (2 weeks-16 months) was studied at 21.5 °C. Percentages of surface immunoglobulin-positive (sIg^+) lymphoid cells in pro- and mesonephros, spleen, thymus and blood were determined by flow cytometry. Plasma cells were studied with a dual-color immunofluorescence technique in order to discriminate between sIg and cytoplasmic Ig (cIg). Although a limited number of B cells (< 1 % of lymphoid cells) were found in 2 week old carp, plasma cells could not be detected at this age. From 1 month onwards (sIg^+) plasma cells could be detected in pro- and mesonephros but not or only occasionally in spleen, thymus or blood. The percentage of plasma cells which were sIg^+ decreased with age from 100 % sIg^+ plasma cells at 1 month of age to the level found in adults (80 %). Percentages of B cells and (sIg^+ + sIg^-) plasma cells increased during the development of the carp reaching a plateau at about 3 months of age for plasma cells and around 8 months of age for B cells. The ontogeny of the carp (humoral) immune system is discussed with respect to these data.

Introduction

The histogenesis of teleost lymphoid organs has been studied in salmon (*Salmo salar*; Ellis, 1977), rainbow trout (*Oncorhynchus mykiss*; Grace & Manning, 1980) and carp (*Cyprinus carpio*; Botham & Manning, 1981; Van Loon *et al.*, 1981; Van Loon *et al.*, 1982). In these studies the morphologic maturation of the lymphoid organs has been described, but the morphologic maturity does not necessarily coincide with maturity of immune functions (rev. Kaattari, 1992). For example, it has been shown in salmon that although mature lymphocytes are present in the thymus and kidney at 14 days pre-hatch, these cells do not have the capacity for mixed lymphocyte reactions until 45 days after hatching (Ellis, 1977).

The production of monoclonal antibodies against lymphocyte surface antigens allowed the study of the ontogeny of specific lymphocyte populations in carp (Van Loon *et al.*, 1982; Secombes *et al.*, 1983a) and rainbow trout (Razquin *et al.*, 1990). Most attention in carp has been paid to the development of the T cell compartment and data on the maturation of B cells are scarce.

In this study a monoclonal antibody (WCI 12) reactive with the heavy chain of carp Ig (Secombes *et al.*, 1983b; Rombout *et al.*, 1990) was used to examine the development of B cells and plasma cells in carp. The percentages of surface Ig^+ (sIg^+) lymphoid cells in the different lymphoid organs of carp from 2 weeks-16 months old were determined by flow cytometric analysis. Immunogold labelling was used for ultrastructural identification of sIg^+ cells. A quantitative investigation was performed on the presence of sIg on plasma cells (Van Diepen *et al.*, 1991) in individual carp. The (sIg^+) plasma cells were studied in the lymphoid organs by dual-colour immunofluorescence microscopy in order to discriminate between sIg and cytoplasmic Ig (cIg).

Materials and methods

Fish

Wild-type carp, *C. carpio* , aged 2 weeks and 1, 3, 5, 8, and 16 months were used. The animals were bred in our laboratory and kept at 21.5 $\pm$ 0.5 °C in a recirculating system. The filtered water was disinfected by UV light. The carp were fed *Artemia salina nauplii* for a period of 2 weeks followed by pelleted food (Trouvit/K30; Trouw and Co., Putten, The Netherlands) at a daily, age-dependent ration of 10 % (2 weeks old), decreasing to 2 % (6 months old) of the body weight. Before dissection of the lymphoid organs (pro- and mesonephros, spleen, and thymus) fish were anaesthetised in a solution of tricaine methane sulphonate (TMS; 3 g / 10 l water; Crescent Research Chemicals, Phoenix, USA) and bled by syringe filled with 0.5 ml heparin solution (50 IU/ml cRPMI (RPMI + 10 % double-distilled water)) per 2 ml blood. The tail of 2 week old fish was cut off and blood was taken

into a capillary tube part filled with heparin solution. Blood and organs from 30 individuals of 2 weeks old were pooled in order to obtain enough cells for analysis (2 x 10^6).

Cell suspensions

Cell suspensions of pronephros, mesonephros, and spleen were prepared in cRPMI by squeezing the tissue pieces through a nylon gauze filter. The thymus was squeezed between two slides. Peripheral blood leucocyte (PBL) suspensions were prepared as follows: heparinized blood was diluted 1:1 in cRPMI, allowed to settle for 1 hour at 0 °C, and centrifuged for 15 min at 100 x g and 4 °C. The supernatant containing white blood cells and a very small part of the erythrocyte pellet was pipetted off. Blood from 2 week old carp was used undiluted for Percoll separation.

All cell suspensions were layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in cRPMI and DMEM (10 x concentrated; Life Technologies Ltd, Paisley, U.K.) to densities of 1.020 and 1.070 g/ml. After centrifugation for 30 min at 840 x g and 4 °C, cells at the 1.02-1.07 interface were harvested with a Pasteur pipette. Cells were washed twice for 10 min at 680 x g and 4 °C and resuspended in a TBS^+ buffer (18.18 mM Tris, 0.82 % NaCl, 0.73 mM $MgCl_2$, 0.18 nM $CaCl_2$, 1 % BSA, 0.1 % NaN_3) to 2.5 x 10^6 cells per ml.

Flow cytometry

Purified lymphocytes (6 x 10^5) were incubated in 250 μl of a 1:100 diluted WCI 12 monoclonal antibody anti-carp Ig H chain containing culture supernatant (Secombes *et al.*, 1983b; Rombout *et al.*, 1990) for 30 min at 0 °C, washed by centrifuging for 5 min at 680 x g and 4 °C and resuspended in TBS^+ buffer. Subsequently, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (RAM) antibody (1:100; Dakopatts, Glostrup, Denmark) containing 1 % pooled carp serum for 15 min at 0 °C. Cells were washed again and analysed on a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, USA) using the Consort 30 data analysis package. For analysis 10,000 cells were counted and only the cells with low side scatter (SSC) were used to

determine the percentage of WCI 12-positive lymphoid cells (Figure 1). Cells with a high SSC were excluded from calculation of the percentage of WCI 12-positive lymphoid cells because these were mainly macrophages and granulocytes as was proven by fluorescence activated cell sorting and electron microscopic examination of the sorted cells (unpublished).

Electron microscopy

Immunogold labelling of the cell suspensions was performed using WCI 12 followed by a gold-conjugated goat anti-mouse antibody. A detailed procedure is described previously (Van Diepen *et al.*, 1991).

Dual-colour immunofluorescence

Cells were labelled in suspension for sIg following the same procedure as described for FACS analysis. Instead of RAM-FITC, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated RAM (1:100; Dakopatts , Glostrup, Denmark) antibody was used. After the labelling, cells were incubated in 250 μl normal mouse serum (1:25) during 1 h at 0 °C in order to block the free binding sites on the RAM-TRITC. The cells were washed twice in TBS^+ and resuspended in TBS^+ buffer without BSA. Cytocentrifuge preparations were made on poly-L-lysine (0.01 %) coated slides as described previously (Van Diepen *et al.*, 1991). The preparations were stained for cIg. Subsequently, the fixed cells were incubated with biotin-conjugated WCI 12 for 45 min at room temperature (RT), rinsed three times in PBS, incubated with avidin-FITC (1:25; Becton Dickinson, Mountain View, USA) for 30 min at RT, rinsed in PBS thrice, and embedded in 0.1 % (w/v) paraphenylenediamine containing PBS/glycerine 1:9 (v/v) solution. The preparations were studied under a fluorescence microscope (Nikon Microphot FXA). The numbers of cIg^+ cells were counted and expressed as percentage of sIg^+ cells. Each cIg^+ cell was checked upon sIg-positivity and the percentage of sIg^+ plasma (cIg^+) cells was determined. The percentage of lymphoid cells which were cIg^+, was calculated using the percentage of sIg^+ cells determined by flow cytometry.

Statistical analysis

F-tests followed by paired comparisons were used in order to estimate significant differences between groups. Values obtained from 2 week old fish were excluded from statistical analysis due to pooling of cells from different individuals.

Results

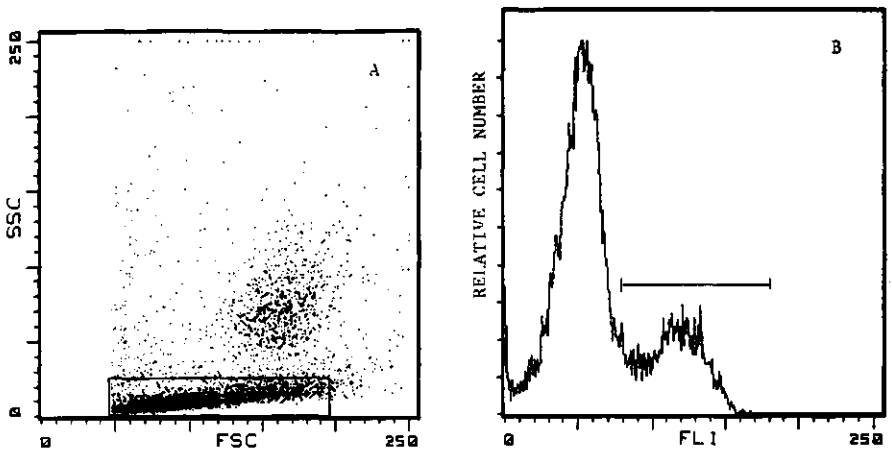


Figure 1. A. Dot plot of WCI 12-labelled pronephros cells showing the gate used to determine the percentage of sIg^+ lymphoid cells (B). FSC = forward scatter, SSC = side scatter

B. Fluorescence histogram of gated (A) WCI 12-labelled pronephros cells. The horizontal bar indicates the region of sIg^+ lymphoid cells (21.6 %). FL 1 = fluorescence intensity

sIg^+ lymphoid cells

Representative flow cytometric profiles of WCI 12-labelled pronephros cell suspensions from 16 month old fish are shown in Figure 1. Cells within the gate were regarded as lymphoid cells, mainly composed of lymphocytes: smooth cells with a small amount of cytoplasm. The percentages of sIg^+ lymphoid cells are given in Table I; the

Table I. Percentages of lymphoid cells in blood and lymphoid organs from carp at different ages which are surface Ig-positive determined by flow cytometry

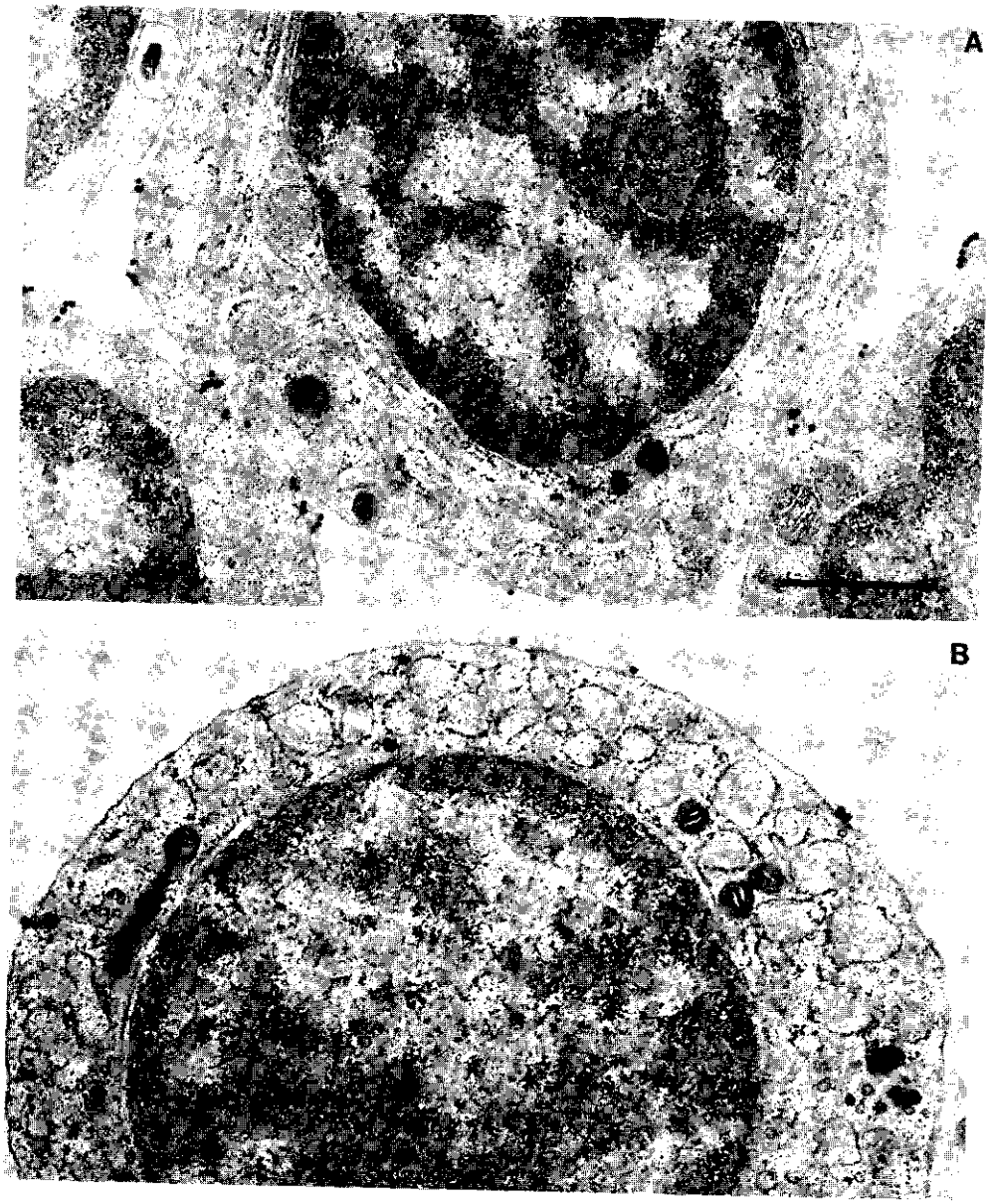
age	blood	pronephros	mesonephros	spleen	thymus	
2 weeks*	0.0	0.6	0.9	0.8	ND**	
1 month	ND**	7.2 ± 2.1 ^{a}	3.7 ± 1.6 ^{a}	5.7 ± 3.7 ^{a}	0.5 ± 0.4 ^{a}	n = 6
3 months	15.8 ± 9.8 ^{a}	9.7 ± 1.7 ^{ab}	7.2 ± 3.3 ^{a}	5.9 ± 2.0 ^{a}	1.5 ± 0.6 ^{a}	n = 6
5 months	20.6 ± 9.4 ^{a}	10.9 ± 3.3 ^{ab}	6.6 ± 2.5 ^{a}	5.0 ± 2.3 ^{a}	1.0 ± 0.8 ^{a}	n = 6
8 months	33.5 ± 5.8 ^{ab}	14.5 ± 1.9 ^{bc}	9.9 ± 1.0 ^{ab}	14.2 ± 2.2 ^{ab}	1.8 ± 0.6 ^{ab}	n = 3
16 months	48.1 ± 7.8 ^{b}	21.6 ± 2.4 ^{c}	16.8 ± 3.7 ^{b}	21.6 ± 6.0 ^{b}	3.7 ± 0.6 ^{b}	n = 6

The percentages represent the mean value ± SD. Values with a different superscript letter (a, b, c) are significantly different (within the same organ) at $P < 0.01$. * Values of pooled cell suspensions from 30 animals, ** not done

highest percentages were found in the blood followed by the pronephros, the spleen, the mesonephros with only a few percent of sIg^+ lymphoid cells in the thymus. The age of the fish and the percentage of sIg^+ lymphoid cells in the lymphoid organs were positively correlated; the lowest percentage was found in 2 week old fish while 16 month old fish revealed the highest percentage of sIg^+ lymphoid cells (Table I). The differences between the percentage sIg^+ lymphoid cells in 16 month old fish and in 1, 3, and 5 month old fish were statistically significant at $P < 0.01$. The percentage sIg^+ lymphoid cells in 16 month old fish and in 8 month old fish were not however, significantly different.

The majority of sIg^+ cells had the ultrastructural features of lymphocytes (lymphoid nucleus, small rim of cytoplasm, no granules). However, most plasma cells found (lymphoid nucleus, rough endoplasmic reticulum with or without dilated cisternae) were also sIg^+ (Figure 2).

Figure 2. Electron micrographs of immunogold stained sIg^+ plasma cells. bar = 1 μm A. sIg^+ plasma cell (30 nm gold particles) containing narrow rough endoplasmic reticulum (rER). B. sIg^+ plasma cell (40 nm gold particles) showing dilated rER.


A
B

cIg$^+$ lymphoid cells

Cytocentrifuge preparations of purified pro- and mesonephros lymphoid cells contained cIg$^+$ cells, but these cells were rare in spleen suspensions and absent or only occasionaly found in thymus and PBL preparations. The percentage of cIg$^+$ lymphoid cells in the kidney from different ages of carp are given in Table II. In preparations from 2 week old carp, cIg$^+$ lymphoid cells were not found. The percentage of cIg$^+$ cells in 1 month old fish was significantly ($P < 0.05$) lower than the percentage of cIg$^+$ cells in 3 month old fish. From 3 months onwards, no significant increase in the percentage of cIg$^+$ cells could be observed. The percentage of cIg$^+$ cells which were sIg$^+$ (Figure 3) in kidney from different ages of carp are shown in Table III. Striking was that in 8 month old carp about 80 % of the cIg$^+$ cells were sIg$^+$ which was significantly ($P < 0.05$) lower than 98 % in 3 and 5 month, and 100 % in 1 month old carp. Microscopic examination revealed that the amount of sIg and cIg varied, but did not show any correlation with each other.

Table II. Percentages of lymphoid cells in pro- and mesonephros from carp at different ages which are cytoplasmic Ig-positive determined by immunocytochemical staining of cytocentrifuge preparations

age	pronephros	mesonephros	
2 weeks*	0	0	
1 month	0.17 ± 0.16^{a}	0.27 ± 0.29^{a}	n = 6
3 months	1.46 ± 0.38^{b}	0.96 ± 0.48^{b}	n = 6
5 months	0.70 ± 0.19^{c}	0.66 ± 0.50^{ab}	n = 6
8 months	0.93 ± 0.14^{bc}	1.02 ± 0.20^{ab}	n = 3/2

The percentages represent the mean value $\pm$ SD. Values with a different superscript letter (a, b, c,) are significantly different (within an organ) at $P < 0.05$. * Values of pooled cell suspensions from 30 animals

Figure 3. **Fluorescence micrographs of a cytocentrifuge preparation of pronephros cells double-stained for sIg and cIg. bar = 10 μm A. Micrograph taken with the FITC filter combination showing a cIg^+ plasma cell. B. Micrograph of the same preparation taken with the TRITC filter combination. The plasma cell shows a comparable amount of sIg^+ as the (cIg^-) B lymphocytes.**

Table III. **Percentages of cytoplasmic Ig-positive cells which are surface Ig-positive in pro- and mesonephros from carp at different ages determined by immunocytochemical staining of cytocentrifuge preparations**

age	pronephros	mesonephros	
1 month	100 ± 0 [a]	100 ± 0 [a]	n = 6
3 months	98.4 ± 2.2 [a]	97.7 ± 2.9 [a]	n = 6
5 months	98.2 ± 3.0 [a]	98.0 ± 1.9 [a]	n = 6
8 months	85.3 ± 11.5 [b]	74.8 ± 11.5 [b]	n = 3/2

The percentages represent the mean value ± SD. Values with a different superscript letter (a, b) are significantly different (within an organ) at $P < 0.05$

Discussion

Monoclonal antibody WCI 12, reactive with the heavy chain of carp Ig, was used for a flow cytometric determination of the percentages of sIg^+ lymphoid cells in the different lymphoid organs (pro- and mesonephros, spleen, thymus, and blood) from carp of 2 weeks and 1, 3, 5, 8 and 16 months old. The percentages of sIg^+ cells in adult carp found in this study are slightly different from those described by others (Schneider & Ambrosius, 1989); 21.6 vs. 33 % in pronephros, 21.6 vs. 34 % in spleen, 48.1 vs. 37 % in blood, and 3.7 vs. 0 % in thymus. These differences may be explained by genetic and environmental differences between the fish and in addition by the different methods used (immunoperoxidase and polyclonal antibody). Flow cytometric analysis of sIg^+ cells from adult channel catfish revealed similar percentages in blood (40 %) and thymus (3.8 %) (Sizemore *et al.*, 1984; Miller *et al.*, 1987) to those found in this study.

The electron microscopic study showed that the majority of the sIg^+ lymphoid cells were lymphocytes, but in addition, most cells with the ultrastructural feature of plasma cells were also sIg^+. Two types of plasma cells could be distinguished; one type with narrow rough endoplasmic reticulum (rER) and another type with more extended rER, which has also been reported by others (Imagawa *et al.*, 1991). In this study both types appeared to be sIg^+.

Dual colour immunofluorescence microscopy was used for a quantitative study on (sIg^+) plasma cells. Cells with a considerable amount of Ig^+ cytoplasm and an eccentric nucleus were considered to be plasma cells; this morphology has recently been confirmed for carp by others (Imagawa *et al.*, 1991). Most plasma cells were found in the pro- and mesonephros, and occasionally a plasma cell could be detected in preparations from the spleen and thymus. Preparations of blood did not contain plasma cells. These results are in agreement with earlier studies of plaque forming cell (PFC) responses measured after immunisation with sheep red blood cells (SRBC) in that the pronephros and mesonephros accounted for the major part of the total PFC activity, 53 and 40 % respectively, while the spleen accounted for only 5 %, with the PFC activity in the blood and thymus being minimal

(Rijkers *et al.*, 1980). The dual color immunofluorescence method revealed excellent discrimination between sIg and cIg. In 1 month old fish, 100 % of the plasma cells were sIg^+. This percentage decreased in 3 and 5 month old fish to 98 % and in 8 month old fish to about 80 %. For mammals it is well accepted that plasma cells lose sIg during their differentiation (Hämmerling *et al.*, 1976; Roitt *et al.*, 1989); only 50 % or less of human plasma cells apppear to be sIg^+ (Grossi & Greaves, 1981). This difference in sIg^+ plasma cells between human and carp may be explained by a lower turn-over time of sIg on carp plasma cells compared to mammalian plasma cells, possibly due to lower body temperatures. However, it is not a likely explanation for the difference in sIg expression between the plasma cells of different aged carp. The amount of sIg may be related to the functional capability of the plasma cells as in mammals. However, in this study the amount of cIg and sIg associated with a plasma cell could not be correlated.

In this study, B cells could already be demonstrated by flow cytometric analysis in lymphoid organs from 2 week old carp. This is in concordance with earlier light-microscopic studies which showed that Ig^+ cells first appear in the pronephros about 2 weeks after hatching (Secombes *et al.*, 1983a). In rainbow trout Ig^+ cells first appear in kidney 4-5 days after hatching (Razquin *et al.*, 1990) while in salmon Ig^+ lymphoid cells were not found until 48 days after hatching (Ellis, 1977). Plasma cells were not detected in 2 week old carp but could be found in 1 month old carp. Probably plasma cells appear between 2 weeks and 1 month of age, which is also supported by a steady increase in serum Ig levels from 3 weeks onwards (Van Loon *et al.*, 1981). Although the presence of both B cells and plasma cells is found in 1 month old carp, immunisation of carp (raised at 21 ^{0}C) at this age induced tolerance when SRBC was used as the antigen (Van Loon *et al.*, 1981). Adequate T helper functions may not yet have been developed at this stage. Immunisation experiments with T-dependent and T-independent antigens in rainbow trout revealed that the response to T-independent antigens preceded the response to T-dependent antigens during the ontogeny of the immune system (Tatner, 1986). Whether the plasma cells present in 1 month old carp are the result of a T-independent reponse remains to be investigated.

The percentage of plasma cells increased during development and reached a plateau at about 3 months of age. This might indicate maturation of at least a part of the humoral immune system at this time. Carp (raised at 21 °C) which were given a primary injection at 4 months of age showed a normal primary immune response (Van Loon *et al.*, 1981). In contrast to plasma cells, the percentage of B cells seem to reach a plateau around 8 months of age. It can be suggested that some aspects of the humoral immune system probably mature during the first 3 months of age, while development of the (humoral) immune system as a whole seems to be completed around 8 months. This is supported by the fact that adult serum Ig levels are reached at 5-8 months of age (carp raised at 21 ° C; Van Loon *et al.*, 1981).

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Characterisation of immunoglobulin-binding leucocytes in carp (*Cyprinus carpio* L.)

J.C.E. Koumans-van Diepen, M.H.M van de Lisdonk, J.J. Taverne-Thiele, B.M.L. Verburg-van Kemenade & J.H.W.M. Rombout

Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Key words: fish, immunoglobulin binding, macrophages, neutrophilic granulocytes, immunofluorescence, immuno-electron microscopy, erythrocyte-antibody rosette assay

submitted

Abstract

This study shows the immunoglobulin (Ig)-binding capacity of Ig-positive macrophages for the first time in a teleost fish. Ig binding by Ig-positive carp leucocytes was demonstrated using an immunofluorescence method. Moreover, the Ig-binding cells were identified at the ultrastructural level with a similar immunogold technique. After internalisation of fluorescent- or gold-labelled Ig (1 h at room temperature), most macrophages from the hindgut were able to bind added purified carp Ig, which could be demonstrated clearly with a second fluorescent or gold label. In pronephros, an important haemopoietic organ in fish, only a few monocyte-like cells showed Ig binding, macrophages and neutrophilic granulocytes appeared to be Ig-negative.

The use of goat-anti-mouse Ig gold probes coated with carp anti-goat antibodies revealed that, besides hindgut macrophages and monocyte-like cells from pronephros, also some lymphoid cells in both hindgut and pronephros cell suspensions were able to bind Ig.

The Ig binding was quantified using the erythrocyte-antibody (EA) rosette method. Percentages of Ig-binding leucocytes from the hindgut of carp were higher than those derived from the pronephros.

Introduction

In general, macrophages are involved in the aspecific immune response by phagocytosis and killing of micro-organisms but also in the specific immune response in which they play a role in antigen-presentation or in the clearance of immune complexes (Unanue, 1984; Male *et al.*, 1987). In order to perform these functions, macrophages possess several molecules and receptors at their cell membrane. One of these is the Fc receptor (FcR), which binds the Fc part of the immunoglobulin molecule. In this way, antibody-antigen complexes can be bound by macrophages and phagocytosed for further

processing.

In mammals a number of FcR types are found for different immunoglobulin isotypes (Unkeless *et al.*, 1981, 1988; Vojtíšková & Franěk, 1989; Van de Winkel & Anderson, 1991). FcR are present on macrophages and certain FcR types are also found on blood platelets, lymphocytes (T and B), granulocytes and other leucocytes. In addition to mammals, FcR have been described for birds (Darby & Van Alten., 1982), amphibians (Coosemans & Hadji-Azimi, 1988) and cartilaginous fish (Haynes *et al.*, 1988). The existence of Ig-binding receptors in bony fish has not yet been proven, although Ig-positive macrophages were described in carp spleen and kidney (Lamers, 1986) and more recently in carp hindgut (Rombout *et al.*, 1989).

In this study the Ig positivity of carp macrophages is investigated in more detail. A fluorescence microscopic method was developed in order to estimate Ig binding by Ig-positive macrophages. A similar immunogold method was performed at electron microscopic level, which allowed the identification of Ig-binding cells. Electron microscopic characterisation is a necessity in fish immunology because monoclonal antibodies recognising different fish leucocyte populations are not or scarcely available. Various methods have been described for studying FcR expression (rev. Kerbel & Elliott, 1983). The erythrocyte-antibody (EA) rosette assay is the most established technique for the demonstration of Fc receptors (Coombs & Wilson, 1982), but this method reveals no information on the nature of Ig-binding cells. Therefore, an electron microscopic variant of the EA rosette test was developed using carp Ig-coated gold probes. The conventional EA rosette assay has been used for the quantification of Ig-binding cells in pronephros and hindgut cell suspensions.

Materials and methods

Animals

Carp, *Cyprinus carpio* L., of 6-8 months were bred and kept in our laboratory as described earlier (Van Diepen *et al.*, 1991). Before dissection of pronephros and hindgut, fish were killed in a solution of tricaine methane sulphonate (TMS; Crescent Research Chemicals, Phoenix, USA; 3 g/10 l water) and bled by syringe in order to reduce the final blood volume in the organs.

Cell isolation

All materials (tubes e.g.) used were siliconised with Sigmacote (Sigma chemical co., St. Louis, USA) in order to avoid adherence of cells. The hindgut was dissected out and the mucosa was mechanically stripped off. Both pronephros and the mucosa were sqeezed through a nylon gauze while adding medium (cRPMI (RPMI + 10 % double-distilled water) + 0.1 % NaN_3 + 10 IU heparin/ml). Cell suspensions were washed twice in medium by centrifuging for 10 min at 680 x g and 4 °C. A continueous Percoll gradient was prepared by centrifuging 10 ml 60 % Percoll (Pharmacia AB, Uppsala, Sweden) in medium for 45 min at 25,000 x g and 4 °C. Cells were layered over this gradient, centrifuged for 30 min at 840 x g and 4 °C, pipetted off at a density of 1.07 and washed twice in medium + 1 % BSA. Pronephros cells at this density are mainly macrophages, lymphocytes and granulocytes (each population $\pm$ 30 %). Isolated hindgut cells are mainly lymphocytes, only $\pm$ 5 % are macrophages (Rombout *et al.*, 1993).

Fluorescence microscopy

Cells (2 x 10^6) from pronephros and hindgut were incubated in 1 ml of a 1: 100 diluted culture supernatant containing WCI 12, a monoclonal antibody reactive with the heavy chain of carp Ig (Secombes *et al.*, 1983; Rombout *et al.*, 1990) for 30 min on ice. The suspensions were washed twice in medium + 1% BSA by centrifuging for 10 min at 680 x g and 4 °C. Then, suspensions were incubated with tetramethylrhodamine

isothiocyanate conjugated rabbit anti-mouse Ig antibodies (RAM-TRITC; 1:100; Dakopatts, Glostrup, Denmark) for 30 min on ice, washed as mentioned above and resuspended in medium + 1% BSA without NaN_3. The cells were kept for 30 min at room temperature to allow internalisation of the label and after centrifugation the pellet was resuspended in purified carp Ig (8 μg/ml) containing medium + 1% BSA for 30 min at 4 °C. Control suspensions were kept in medium. Subsequently, suspensions were washed and a second labelling with WCI 12 followed by fluorescein isothiocyanate (FITC) conjugated RAM (1:100; Dakopatts, Glostrup, Denmark) was performed as described above. After labelling, cytocentrifuge preparations were made and studied with a fluorescence microscope (Nikon Microphot FXA).

Electron microscopy

The ultrastructural characterisation of Ig-binding leucocytes was performed using two different methods.

The first method was similar to that described for fluorescence microscopy, but in this case goat anti-mouse (GAM) Ig conjugated to 10 or 30 nm gold probes (1:5; E-Y Laboratories, Inc., San Mateo, CA, USA) were used.

The second method can be considered as an electron microscopic variant of the EA rosette assay. GAM conjugated to 30 nm gold probes were incubated in a subagglutinating dilution of carp anti-goat (CAG) serum (1:200). CAG serum was prepared by intramuscular immunisation of carp with 160 μg goat Ig (Dakopatts, Glostrup, Denmark) in Freunds' complete adjuvant. Pronephros and hindgut cell suspensions were first kept at room temperature in NaN_3-free medium for 1 h and then incubated with coated and uncoated gold probes in medium for 30 min on ice. The binding of carp Ig-coated gold probes was controlled by studying cytocentrifuge preparations of the cell suspensions using the epipolarisation mode on a Zeiss fluorescence microscope. Then, cells were prepared for electron microscopy as earlier described (Van Diepen *et al.*, 1991) and examined in a Philips 201 electron microscope.

Erythrocyte-antibody (EA) rosette assay

Sheep red blood cells (SRBC) in RPMI (5 x 10^7/ml) were incubated in a subagglutinating dilution of carp anti-SRBC serum during 30 min at room temperature. Cells were washed twice in RPMI by centrifuging for 7 minutes at 490 x g and room temperature. Coated SRBC (SRBC-carp Ig) or uncoated SRBC (20 x 10^6/ml) were mixed 1:1 with cell suspensions of pronephros or gut (4 x 10^6/ml) and incubated for 30 min on ice. As control, carp cell suspensions were incubated with purified carp Ig (8 μg/ml) for 30 min on ice before addition of SRBC. Finally cells were centrifuged for 7 min at 490 x g and 4 °C, resuspended carefully and examined in a Bürker-counting chamber. A carp leucocyte with at least one SRBC was considered as a positive rosette. For quantification 3 carp were used and at least 10^3 carp cells per specimen were studied for SRBC binding.

Results

Fluorescence microscopy

In cell suspensions of hindgut and occasionally in pronephros suspensions double positive cells were found showing red fluorescent phagosome-like structures in their cytoplasm and green fluorescence on their surface (Figure 1). These cells were regarded as macrophages because of the phagosome-like structures and their cell size; predominantly large ($\pm$ 20 μm) in hindgut but smaller in pronephros. Some Ig-positive macrophages in hindgut suspensions did not have the green fluorescence label at their cell membrane. Smaller positive lymphoid cells were also found showing both labels on the same spots at the cell membrane. In control preparations (without addition of carp Ig) the macrophages showed only red fluorescence in their phagosome-like structures while the lymphoid cells were still double-positive at their cell membrane.

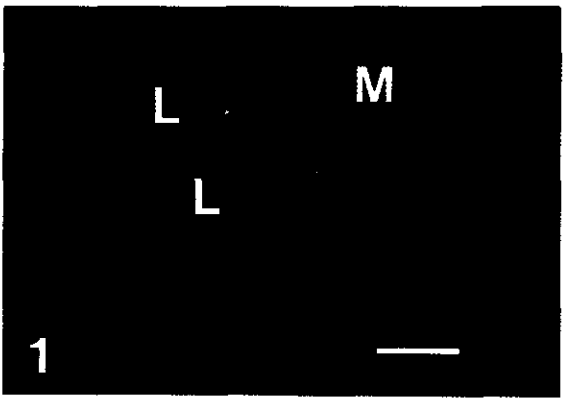

Figure 1. **Fluorescence micrograph of a cytocentrifuge preparation of hindgut leucocytes stained in suspension with a monoclonal antibody against Ig (WCI 12). Double staining showing TRITC-labelled internalised Ig (red) and, after incubation with carp Ig, FITC-labelled newly bound Ig (green). M = double-stained macrophage, L = Ig-negative lymphocyte, bar = 10 μm**

Electron microscopy

Results obtained with fluorescence microscopy could be confirmed with electron microscopy using two distinct gold probes instead of fluorescent labels. Again different labelling patterns could be observed and the positive cells were identified as: 1. Macrophages in gut suspensions (Figures 2,3) and monocyte-like cells from pronephros (Figure 4) showing 30 nm gold probes in endosome- and/or phagosome-like structures and 10 nm, but no 30 nm, gold probes on their surface. 2. Macrophages from hindgut with only 30 nm gold probes in endosome- and/or phagosome-like structures and no 10 nm particles at their cell membrane. 3. Lymphocytes from both organs having both small and large gold particles at the cell membrane (Figure 5).

Most of the intestinal macrophages were large (10-30 μm) double-positive cells (Figure 2), but smaller double-positive macrophages could be found as well (Figure 3). The single positive intestinal macrophages showing only 30 nm gold probes in their cytoplasm were less frequently found. The number of double-positive lymphocytes was very low in intestinal cell suspensions. In pronephros cell suspensions the double-

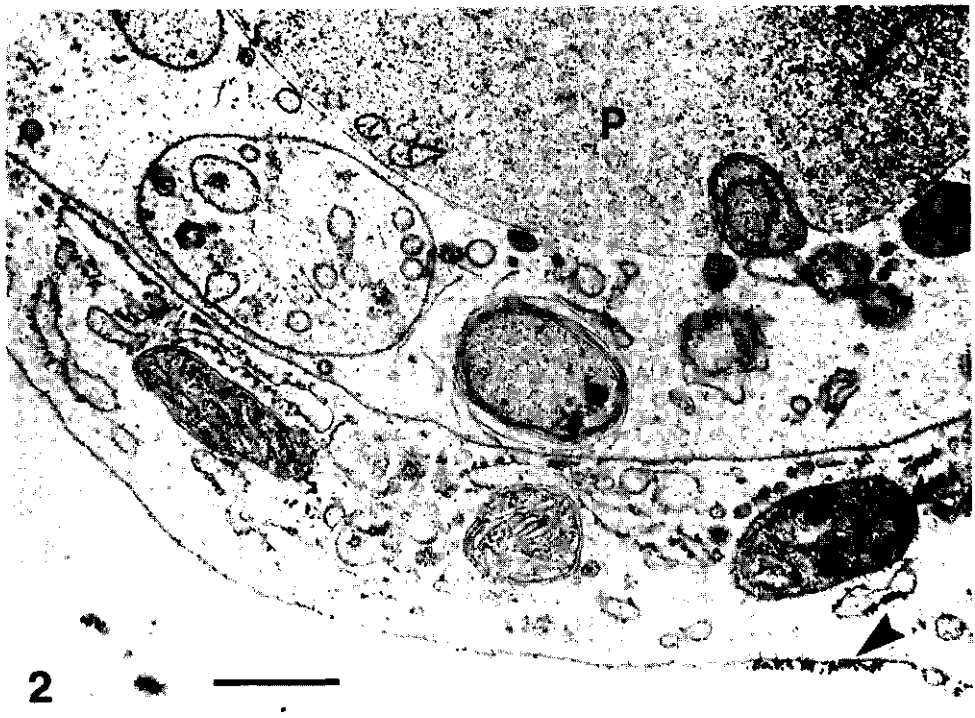

Figure 2. **Electron micrograph of a large hindgut macrophage labelled with a monoclonal antibody against Ig (WCI 12). Large gold particles (30 nm) represent internalised Ig within endosomal structures (arrow) and small gold particles (10 nm) represent newly bound carp Ig at the cell membrane (arrowhead). P = phagosome, bar = 0.5 μm**

positive monocyte-like cells (Figure 4) formed a minor population. These monocyte-like cells were about the same size as lymphocytes, but they showed lysomes and endosomes in their cytoplasm. However, sometimes it was difficult to discriminate between a lymphoid cell which had endocytosed a few gold particles and a monocyte-like cell. A reasonable part (about 20 %) of the lymphocytes was double positive (Figure 5). The macrophages in pronephros were smaller than intestinal macrophages and moreover completely Ig-negative (Figure 6). Neutrophilic granulocytes were Ig-negative and also did not show Ig binding.

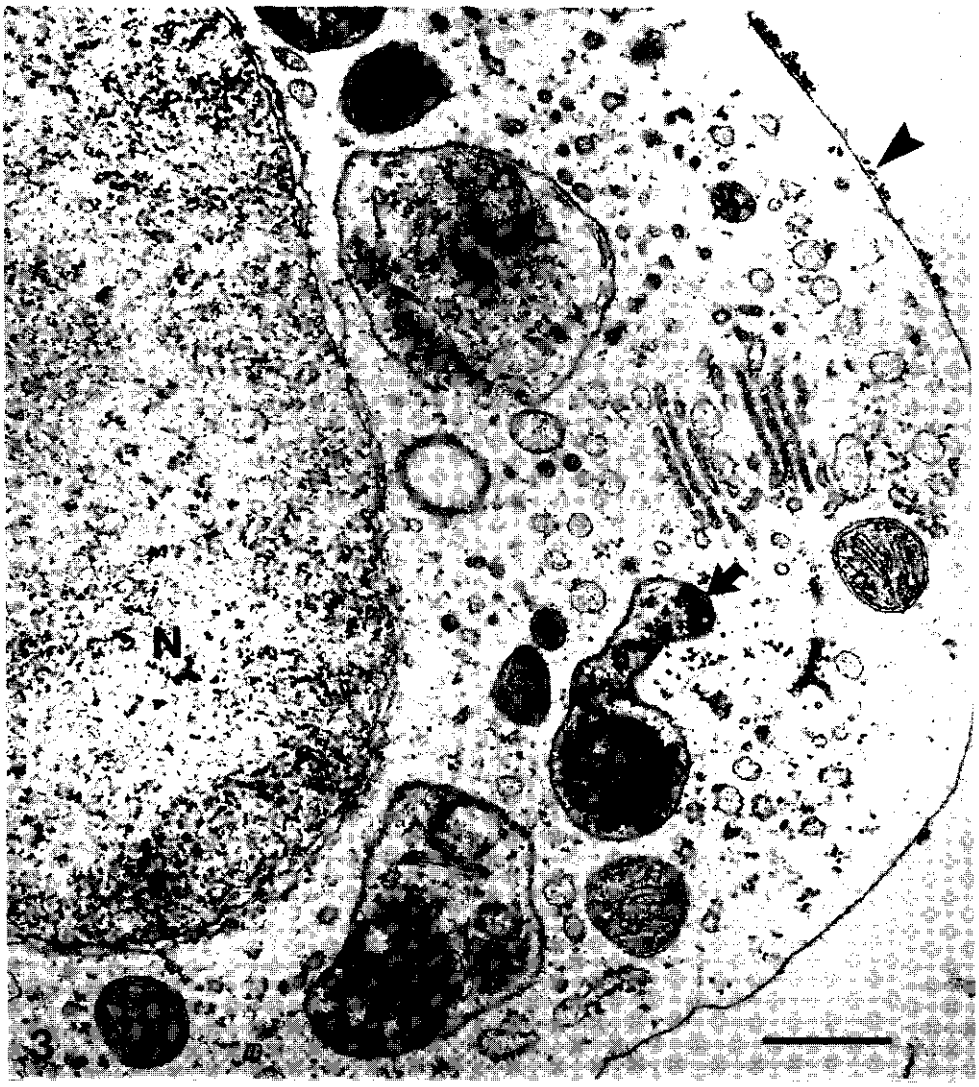

Figure 3. Electron micrograph of a small hindgut macrophage labelled with a monoclonal antibody against Ig (WCI 12). Large gold particles (30 nm) represent internalised Ig within endosomal structures (arrows) and small gold particles (10 nm) represent newly bound carp Ig at the cell membrane (arrowhead). N = nucleus, bar = 0.5 μm

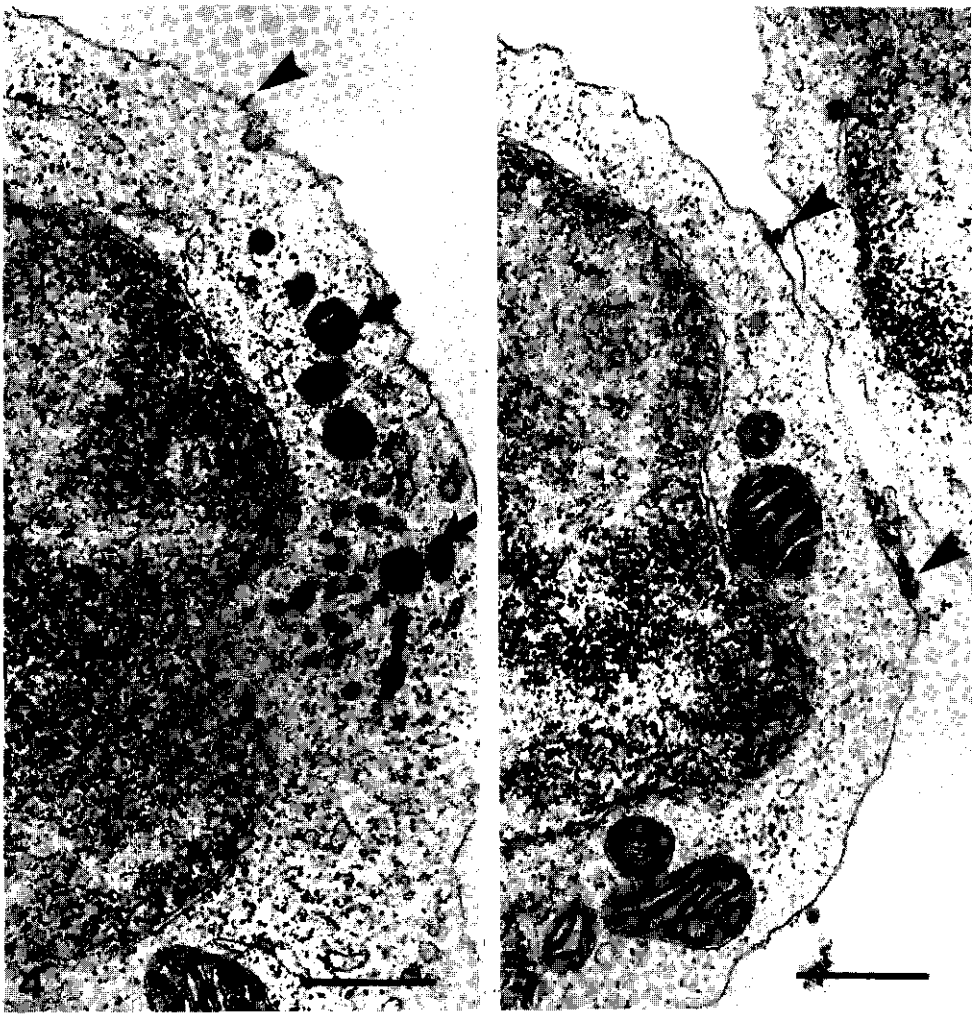

Figure 4. Electron micrograph of a pronephros monocyte-like cell labelled with a monoclonal antibody against Ig (WCI 12). Large gold particles (30 nm) represent internalised Ig (arrows) and small gold particles (10 nm) represent newly bound carp Ig at the cell membrane (arrowhead). bar = 0.5 μm

Figure 5. Electron micrograph showing a pronephros lymphocyte labelled with a monoclonal antibody against Ig (WCI 12) which is represented by large (30 nm) and small (10 nm) gold particles at the cell membrane (arrowheads). bar = 0.5 μm

Incubation with the carp Ig-coated gold probes revealed binding of these probes to macrophages in hindgut (Figure 7) but also to lymphoid cells (Figure 8) in hindgut and pronephros. Control labelling with uncoated gold probes did not show this reaction pattern.

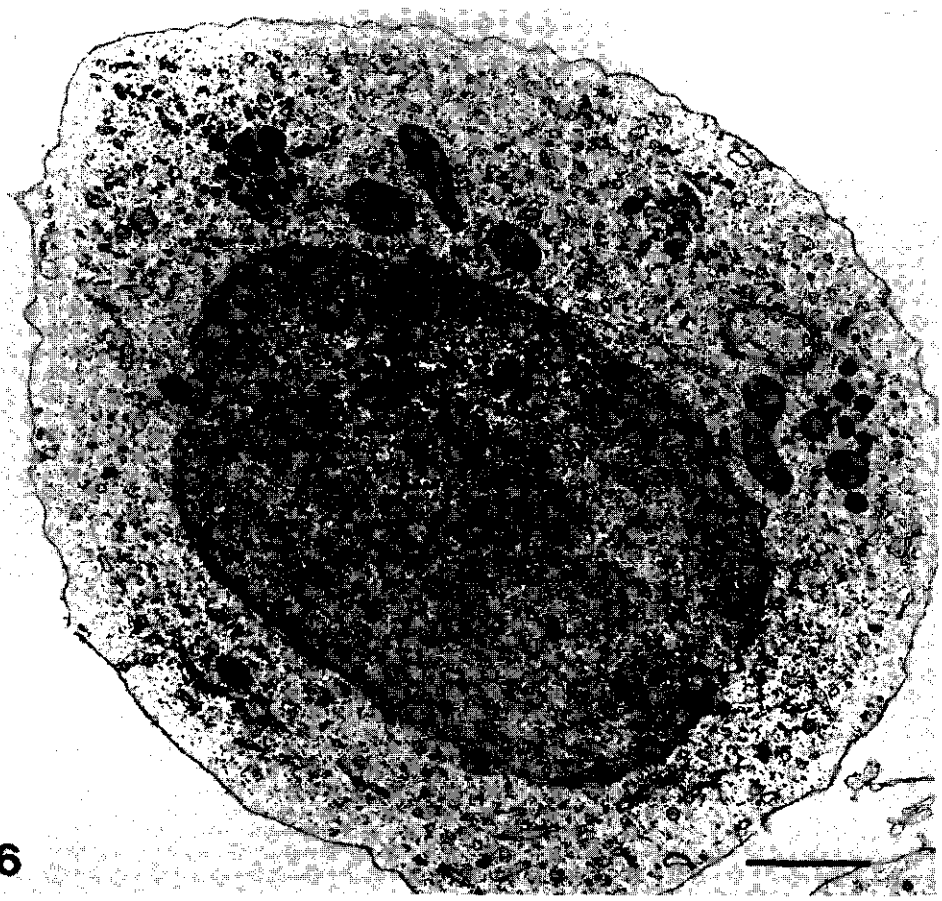

Figure 6. Electron micrograph showing a pronephros macrophage which is negative after labelling with a monoclonal antibody against Ig (WCI 12). bar = 1 μm

Rosette assay

Percentages of rosette-forming leucocytes in pronephros and hindgut suspensions are given in Table I. Background rosette-forming (with uncoated SRBC) appeared to be around 2 % in both organs. However, the percentage of Ig-specific rosette-forming leucocytes in hindgut (4.8 %) is higher than in pronephros (1.8 %). Preincubation with carp Ig reduced the percentage of Ig-specific rosette-forming cells to background level in hindgut.

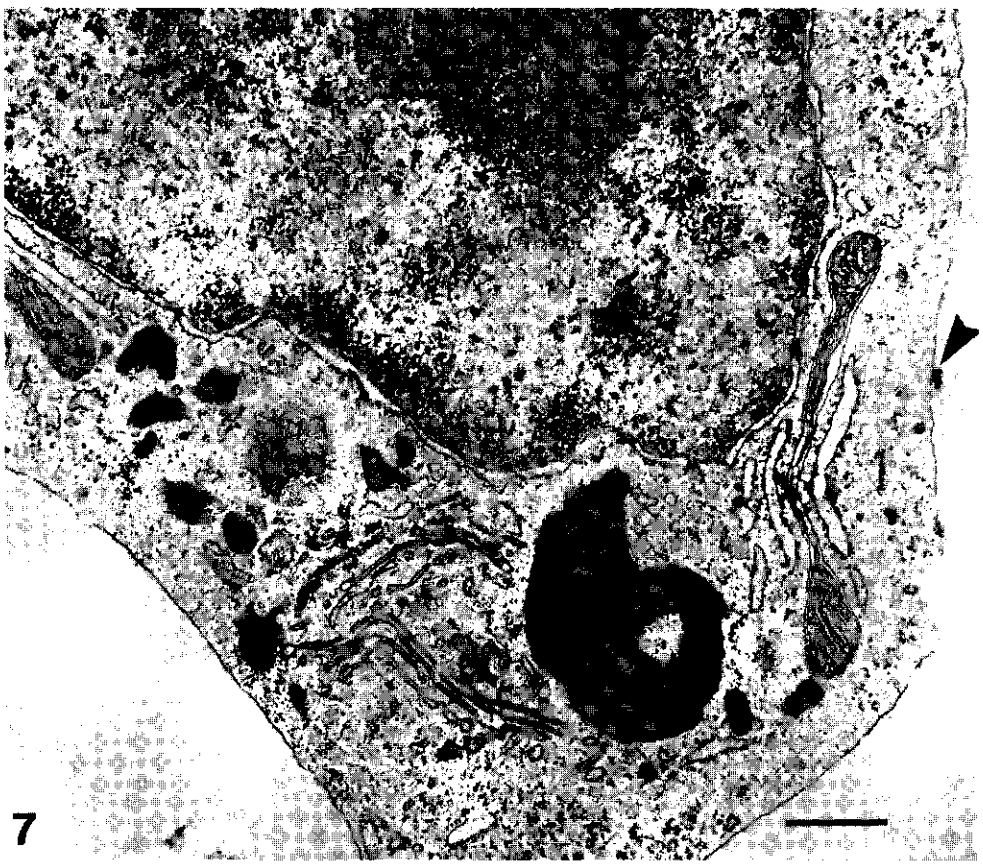

Figure 7. **Electron micrograph showing a hindgut macrophage which has bound carp Ig-coated 30 nm gold probes (arrowhead), bar = 0.5 μm**

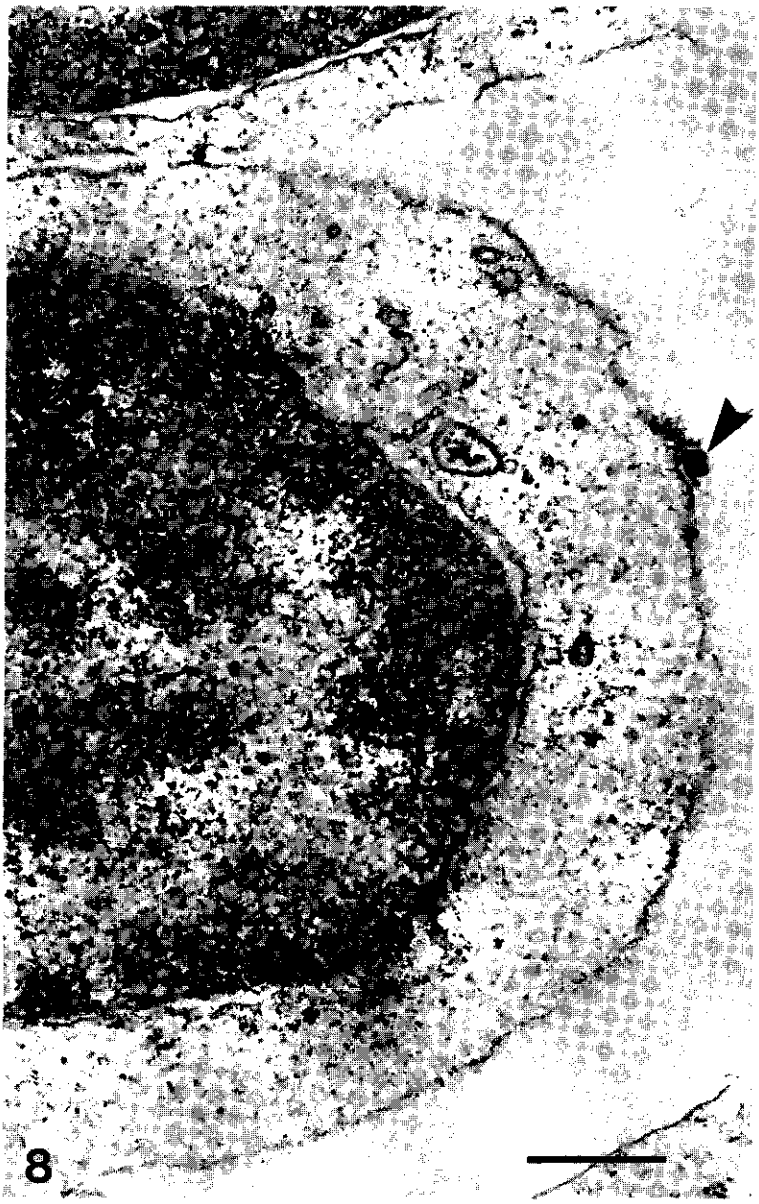

Figure 8. Electron micrograph showing a pronephros lymphocyte which has bound carp Ig-coated 30 nm gold particles (arrowhead), bar = 0.5 μm

Table I. **Percentages of rosette-forming cells in cell suspensions from pronephros and hindgut of carp after incubation with carp Ig-coated SRBC and uncoated SRBC**

organ	SRBC-carp Ig	SRBC
hindgut	6.8 + 3.5	2.0 $\pm$ 0.6
pronephros	3.6 $\pm$ 0.6	1.8 $\pm$ 0.6

The percentages represent the mean value $\pm$ SD. n = 3

Discussion

In this study the Ig-binding capacity of leucocytes from carp is investigated using light and electron microscopy.

A fluorescence microscopic assay was developed for demonstration of Ig binding by Ig-positive leucocytes. Cells were labelled with a monoclonal antibody against carp Ig and allowed to internalise their bound Ig. Ig binding was demonstrated after subsequent incubation with purified carp Ig and a second labelling against Ig, while binding was absent without the addition of Ig. The same procedure was followed for electron microscopic study of Ig binding, with small and large gold particles instead of immunofluorescence labels. Ig-positive lymphocytes showed both labels at their cell membrane and not in their cytoplasm; apparently they do not endocytose the fluorescent or gold labels. The light and electron microscopic observations showed that most if not all of the intestinal macrophages have an Ig-binding capacity. It is suggested that the Ig binding is mediated by Fc-like receptors (Fc-likeR), analogous to Ig binding by FcR in mammals and other vertebrates. Whether the Fc part of carp Ig binds to the Fc-likeR remains to be investigated. Therefore Fc and/or $F(ab')_2$ fragments of carp Ig, which are not available yet, have to be prepared. The number of rosette-forming cells determined in

hindgut cell suspensions is in agreement with the number of macrophages present (Rombout *et al.*, 1993). It is concluded that Fc-likeR are expressed on a great majority of the macrophages isolated from the hindgut. Some Ig-positive macrophages did not bind added Ig; they had only fluorescent or gold label in their endosomes and not at the cell membrane. Several explanations for the absence of Ig binding can be suggested: 1) Studies on internalisation and fate of macrophage FcR during receptor-mediated endocytosis in mice revealed that phagocytosis of multivalent Ig complexes which cause clustering of the FcR, leads to a selective loss of FcR at the cell membrane due to degradation of both receptor and Ig complexes in lysosomes (Mellman & Ukkonen, 1985). The amount of surface FcR can be decreased by > 50 % for several hours. 2) The existence of more than one FcR type like in mammals which posses FcR for several isotypes of Ig (Unkeless *et al.*, 1981; Verspaget & Beeken, 1985). In teleost fish different Ig heavy chains are reported for channel catfish (Lobb & Olson, 1988) and rainbow trout (Sánchez *et al.*, 1989). In carp indications are present for differences between mucus Ig and serum Ig, but both molecules are recognised by monoclonal antibody WCI 12 (Rombout *et al.*, 1990) used here. In this study binding of Ig from serum is investigated, while only receptors for mucus Ig may be present on some of the macrophages and monocytes.

In pronephros cell suspensions the Ig-binding cells are monocyte-like cells which form a great minority in contrast with the high number of pronephros macrophages. This corresponds with the very low number of Ig-binding cells in pronephros cell suspensions estimated with the EA rosette assay. The pronephros macrophages are different from those in hindgut; they were smaller and completely Ig-negative. Neutrophilic granulocytes from pronephros were also Ig-negative. Although pronephros macrophages and neutrophilic granulocytes seem to lack FcR, they are functionally active as demonstrated by their high phagocytic, respiratory burst and microbicidal activity (Temmink & Bayne, 1987; Verburg-van Kemenade *et al.*, 1989). Possibly opsonisation of the antigen by complement instead of antibodies can enhance phagocytosis by these cells. Recent data on neutrophilic granulocytes of carp have shown that normal serum is highly effective in

opsonisation and that this effect can be blocked by anti-C3, while opsonisation by antiserum is absent (Matsuyama *et al.*, 1992). Although opsonising effects of antiserum have been demonstrated for rainbow trout (*Oncorhynchus mykiss*; Griffin, 1983; Sakai, 1984), coho salmon (*O. kisutch*) and masu salmon (*O. masou*; Sakai, 1984), channel catfish (*Ictalurus punctatus*; Scott *et al.*, 1985) and Japanese eel (*Anguilla japonica*; Kaige *et al.*, 1990), a dominant role of complement as opsonising factor has been reported for rainbow trout (Sakai, 1984; Michel *et al.*, 1990), coho salmon and masu salmon (Sakai, 1984), channel catfish (Scott *et al.*, 1985) and plaice (*Pleuronectes platessa*; Nash, *et al.*, 1987). In carp pronephros effective binding of immune complexes could be performed by melanomacrophages which are reported to be Ig-positive in cryo-sections of pronephros (Lamers, 1986). These cells are situated in the connective tissue around blood vessels and apparently are not isolated from pronephros by the procedure used here.

Although FcR were already found on leucocytes of mammals (Unkeless *et al.*, 1981, 1988; Vojtíšková & Franěk, 1989; Van de Winkel & Anderson, 1991), birds (Darby & Van Alten, 1982) and amphibian (Coosemans & Hadji-Azimi, 1988), demonstration of FcR on fish leucocytes turned out to be rather difficult. Wrathmell & Parish (1980) could not demonstrate FcR on leucocytes of dogfish (*Scyliorhinus canicula*), ray (*Raja clavata*) and plaice, whereas Haynes *et al.* (1984), using an adapted rosette assay, revealed that shark leucocytes actually do posses FcR. As mentioned earlier, studies on the presence of FcR on fish cells based on enhanced phagocytic activity after opsonisation of antigen by antiserum are contradictory. Moreover, Ig-positive macrophages are present in spleen, kidney (Lamers, 1986) and hindgut (Rombout, 1989) of carp. This study for the first time demonstrates Ig binding by hindgut macrophages, monocyte-like cells in pronephros and lymphoid cells in both organs of carp. The Ig-binding capacity of hindgut macrophages and some lymphoid cells is similar to the mammalian situation. However, the finding that neutrophilic granulocytes from pronephros do not have active Fc-likeR is in contrast with the presence of FcR on all mammalian neutrophilic granulocytes (Anderson & Looney, 1986; Male *et al.*, 1987). Although the expression of FcR on human macrophages is complex (Van de Winkel &

Anderson, 1991), the absence of active FcR on a distinct macrophage population, as described here for carp pronephros macrophages, has not been reported.

It is concluded that a great majority of carp intestinal macrophages possess active Fc-likeR in contrast to pronephros macrophages and neutrophilic granulocytes. This suggests a functional difference between hindgut and pronephros macrophages: opsonisation of antigen by complement probably plays a major role in phagocytosis by pronephros macrophages and neutrophilic granulocytes while Fc-likeR are likely more important for effective phagocytosis by intestinal macrophages.

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Immunocytochemical analysis of mitogen responses of carp (*Cyprinus carpio* L.) peripheral blood leucocytes

J.C.E. Koumans-van Diepen, E.G.M. Harmsen & J.H.W.M. Rombout

Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands.

Key words: fish; mitogen response; fluorescence microscopy; flow cytometry; magnetic cell sorting; B lymphocyte; T lymphocyte

submitted

Abstract

PHA and LPS responses of surface immunoglobulin-positive (sIg^+) and sIg-negative (sIg^-) carp peripheral blood leucocytes (PBL) were studied. sIg^+ cell-enriched and sIg^+ cell-depleted carp PBL populations were obtained by magnetic cell sorting (MACS) and mitogenic stimulation *in vitro* was measured by ^{3}H-thymidine incorporation. The mitogen responses of sIg^+ and sIg^- cells in non-separated carp PBL cultures were analysed by simultaneous detection of incorporated 5-bromo-2'-deoxyuridine (BrdU) and sIg with the fluorescence microscope and flow cytometer. Flow cytometric determination of the % sIg^+ cells in combination with absolute cell counting revealed an increase of sIg^+ cells but not of sIg^- cells after LPS stimulation while the number of sIg^- cells and not of sIg^+ cells was enhanced after PHA stimulation. LPS stimulation showed an increased ^{3}H-thymidine incorporation in the sIg^+ cell-depleted population compared with non-separated cells and BrdU incorporation was observed in sIg^- cells from LPS-stimulated cultures by fluorescence microscopy. However, flow cytometric analysis showed that mainly dull sIg^+ cells and not sIg^- cells are stimulated by LPS. These dull sIg^+ cells were not sorted from sIg^- cells with MACS and could apparently not be distinguished from sIg^- cells by light microscopy. PHA stimulates sIg^- cells and not sIg^+ cells as was estimated by all techniques used.

Introduction

Phytohaemagglutinin (PHA) and concanavalin A (Con A) stimulate the proliferation of mammalian T cells while lipopolysaccharide (LPS) is considered as a B cell mitogen (reviewed by Greaves & Janossy, 1972). These mitogens have been applied to study lymphocyte heterogeneity of several teleost fish species (reviewed by Rowley *et al.*, 1988). Leucocytes from several lymphoid organs such as pronephros, spleen and peripheral blood, were able to respond to both T and B cell mitogens. Thymocytes from rainbow trout

(*Oncorynchus mykiss*; Etlinger *et al.*, 1976) and Atlantic salmon (*Salmo salar*; Reitan & Thuvander, 1991) responded to Con A or PHA and not LPS. However, other authors reported that both Con A and LPS could stimulate thymocytes from rainbow trout (Warr & Simon, 1983) and blue gill (*Lepomis macrochirus*; Chuchens & Clem, 1977). After panning of leucocytes with monoclonal antibodies specific to immunoglobulin (Ig), surface Ig-positive (sIg^+) cell-enriched and sIg^+ cell-depleted populations of rainbow trout or channel catfish (*Ictalurus punctatus*) were stimulated with LPS or PHA; although sIg^+ cell-depleted lymphocytes showed a better response to Con A, they also responded to LPS (DeLuca *et al.*, 1983; Sizemore *et al.*, 1984). Kaattari (1992) even suggested that sIg^+ and sIg^- lymphocytes of fish may share one function, i.e. LPS reactivity. Till now mitogen stimulation of carp cells was only performed on non-separated leucocytes (Grondel & Boesten, 1982; Caspi *et al.*, 1984; Pourreau *et al.*, 1987). However, co-stimulation experiments with Con A, PHA and LPS and the electron microscopic characterisation of PHA- and LPS-stimulated peripheral blood leucocytes (PBL) indicated that PHA/Con A responsive cells were distinct from LPS responsive cells (Caspi *et al.*, 1984).

In this study, new techniques were applied to analyse mitogen responses of carp PBL. Firstly, sIg^+ cell-enriched and sIg^+ cell-depleted PBL, obtained by magnetic cell sorting (MACS), were stimulated with PHA or LPS and the responses were measured by incorporation of ^{3}H-thymidine. Secondly, LPS or PHA-stimulated PBL were analysed individually by simultaneous detection of sIg and incorporated 5-bromo-2'-deoxyuridine (BrdU) with fluorescence microscopy or flow cytometry. Special attention was payed to the responsiveness of sIg^- cells to LPS.

Materials and methods

Carp

Adult carp (*Cyprinus carpio* L.) bred at the central fish facilities of our university

were used. The animals were kept at 21.5 $\pm$ 0.5 °C in a recirculating system with filtered water disinfected by UV-light. The carp were fed pelleted food (K30; Trouw & Co., Putten, The Netherlands) at a ration of 2 % of the body weight.

Lymphocyte isolation and culturing

Siliconised (Sigmacoate; Sigma chemical co., St. Louis, MO, USA) materials were used in order to avoid adherence of the cells. PBL were isolated from heparinised blood by centrifugation over Lymphoprep (Nycomed, Oslo, Norway) for 30 min at 1500 x g as described previously (Koumans-van Diepen *et al.*, 1993). Isolated cells were suspended at 5 x 10^6/ml in carp culture medium (cRPMI (RPMI + 10 % double-distilled water) + 100 U/ml penicillin + 100 μg/ml streptomycin + 2 mM L-glutamine + 0.02 mM 2-mercaptoethanol + 1 % heat-inactivated pooled carp serum). One hundred μl, 500 μl or 5 ml cell suspension was added to each well of flat-bottemed 96-well tissue culture plates, 24-well tissue culture plates (Costar, Cambridge, MA, USA) or 10 ml tissue culture flasks (Costar, Cambridge, MA, USA) respectively. PHA (2.5 μg/ml, Sigma chemical co., St. Louis, MO, USA) or LPS (31.25 μg/ml; *Salmonella thyphimurium*; Difco Laboratories, Detroit, Mi, USA) was added (for the 96-well plates in triplicate) and the cells were cultured for 4 days at 27 °C in a 5 % CO_2 incubator. At 16 h before harvesting, ^{3}H-thymidine (1 μCi/96-well; Amersham International plc, Buckinghamshire, U.K.) or BrdU (10 μM; Sigma chemical co., St. Louis, MO, USA) were added to the 96-well plates or the 24-well plates or culture flasks respectively.

The cells from the 96-well plates were harvested onto filter paper using a semi-automatic cell harvester (Skatron; Lier, Norway) and the radioactivity was measured in a liquid scintillation counter (Beckman LC 1701) after drying of the paper. The cells in the 24-well plates or culture flasks were resuspended by vigorous pipetting up and down and subsequently collected in tubes. These cells were washed in flow cytometry-medium (cRPMI + 10 U/ml heparin + 0.1 % NaN_3 + 1 % BSA) by centrifuging for 10 min at 680 x g and 4 °C and counted in a Bürker counting chamber.

Flow cytometric analysis

The percentage of sIg^+ cells was determined by flow cytometry before and after cell culturing in 24-well plates (Koumans-van Diepen *et al.*, 1993). Briefly, cells were labelled with mouse anti-carp Ig (1:100; WCI 12) followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig antibodies (RAM-FITC; 1:100; Dakopatts, Glostrup, Denmark). Lymphoid cells were gated and analysed for labelling using the Consort 30 data analysis package (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA).

Magnetic cell sorting (MACS)

During the labelling procedure TBS^{++} buffer (18.18 mM Tris, 0.82 % NaCl, 0.73 mM $MgCl_2$, 0.18 mM $CaCl_2$, 1 % BSA) was used instead of cRPMI because normal culture media contain biotin which may disturb the labelling. Cells (4 x 10^7/ml) were incubated with biotin-conjugated mouse anti-carp Ig (1:250; WCI 12; Secombes *et al.*, 1983) for 30 min at 0 °C and washed once in TBS^{++} and a second time in TBS^{++} without BSA (which may contain biotin as well). Resuspension in TBS^{++} without BSA followed: 10^7 cells per 90 μl TBS^{++} and 10 μl streptavidin conjugated superparamagnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added. After 15 min at 0 °C, FITC-conjugated avidin (1:4; Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) was added and incubated for 5 min at 0 °C. The cells were washed in TBS^{++}, resuspended to 10^8 cells/ml and separated using MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as described by Miltenyi *et al.* (1990). The columms filled with ferromagnetic stainless steelwool were sterilised in an autoclave at 120 °C. For separation a 21 G 2 needle was used while for washing the flow was increased by the use of a 20 G $1^1/_2$ needle. Separated cells were washed twice in carp culture medium and cultured in 96-well plates in presence of PHA or LPS as described above.

Simultaneous detection of incorporated BrdU and sIg

A. Fluorescence microscopy: Cultured cells from 24-well plates were pulsed with BrdU and subsequently labelled with anti-carp Ig (WCI 12) as described for flow cytometric analysis. Tetramethyl rodamine isothiocyanate (TRITC)-conjugated RAM (1:100; Dakopatts; Glostrup, Denmark) was used as second antibody. Cytocentrifuge preparations were made using poly-L-lysine coated slides. These slides were fixed in ice cold methanol for 10 min after drying for 10 min. Subsequently, the preparations were rinsed twice in TBS (0.02 M Tris-HCl pH 7.4, 0.9 % NaCl), incubated with heat-inactivated normal mouse serum (NMS; 1:5) for 15 min at room temperature (RT), rinsed twice in TBS, incubated with 0.1 N HCl for 10 min at RT, incubated in digestion buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol) for 10 min at RT and incubated with exonuclease III (2600 U/ml digestion buffer; BRL, Gaithersburg, MD, USA) for 45 min at 37 °C. After digestion, preparations were rinsed once in TBS + 10 % heat-inactivated Newborn calf serum and incubated with FITC-conjugated anti-BrdU (1:10; Boehringer Mannheim GmbH, Germany, Mannheim) for 30 min at RT. Subsequently, they were rinsed twice in TBS + 0.5 % Tween 20 and embedded in 0.1 % (w/v) paraphenylenediamine containing PBS/glycerine 1:9 (w/v) solution. The preparations were studied under a fluorescence microscope (Nikon Microphot FXA). Percentages of $BrdU^+$ cells and percentages of $BrdU^+$ cells which were sIg^+ were determined by analysing 700-2500 cells for BrdU incorporation and/or sIg.

B. Flow cytometry: A modification of the method described by Bayer *et al.* (1990) was performed. Harvested BrdU-pulsed PBL from culture flasks were labelled as described for flow cytometric analysis, as second antibody phycoerythrin-conjugated RAM (RAM-PE; 1:100; Dakopatts, Glostrup, Denmark) was used. Then cells were incubated with NMS (1:100) for 30 min on ice and fixed in 1 % phormaldehyde + 4.8 % acetic acid in flow cytometry-medium for 15 min on ice. Subsequently, the cells were washed twice in flow cytometry-medium and twice in digestion buffer and then incubated with exonuclease III (250 U/100 μl) for 45 min at 37 °C. After washing, cells were incubated in FITC-conjugated anti-BrdU (1:20) for 30 min, washed twice again in flow cytometry-medium + 0.5 % Tween 20

and resuspended in flow cytometry-medium. Analysis by flow cytometry was performed as described above.

Results

Table I. Relative numbers of sIg^+ and sIg^- carp PBL after PHA and LPS stimulation expressed as the percentage of the number of cells before stimulation (day 0)*

cell types	day 0	+ PHA (day 4)	+ LPS (day 4)
sIg^+ cells	100 / 100	55 / 49	249 / 243
sIg^- cells	100 / 100	123 / 105	79 / 63

* values for two fish

Numbers of sIg^+ and sIg^- cells after stimulation

Relative numbers of sIg^+ and sIg^- cells before and after cell culture with the mitogens were calculated from the percentage of sIg^+ cells determined by flow cytometric analysis and absolute cell countings (Table I). The number of sIg^+ cells increased after stimulation with LPS but decreased after stimulation with PHA. The numbers of sIg^- cells were slightly enhanced after PHA stimulation but decreased after stimulation with LPS.

Stimulation of MACS-separated cells

Fluorescence histograms of sIg^+ cell-depleted and sIg^+ cell-enriched populations are shown in Figure 1. The percentage of sIg^+ cells before and after MACS separation are given. The contamination of sIg^+ cells (15 %) in the sIg^+ cell-depleted fraction mainly consisted of dull sIg^+ cells. The responses to PHA and LPS of the sIg^+ cell-depleted and sIg^+ cell-enriched fractions were compared with responses of non-separated cells (whether or not

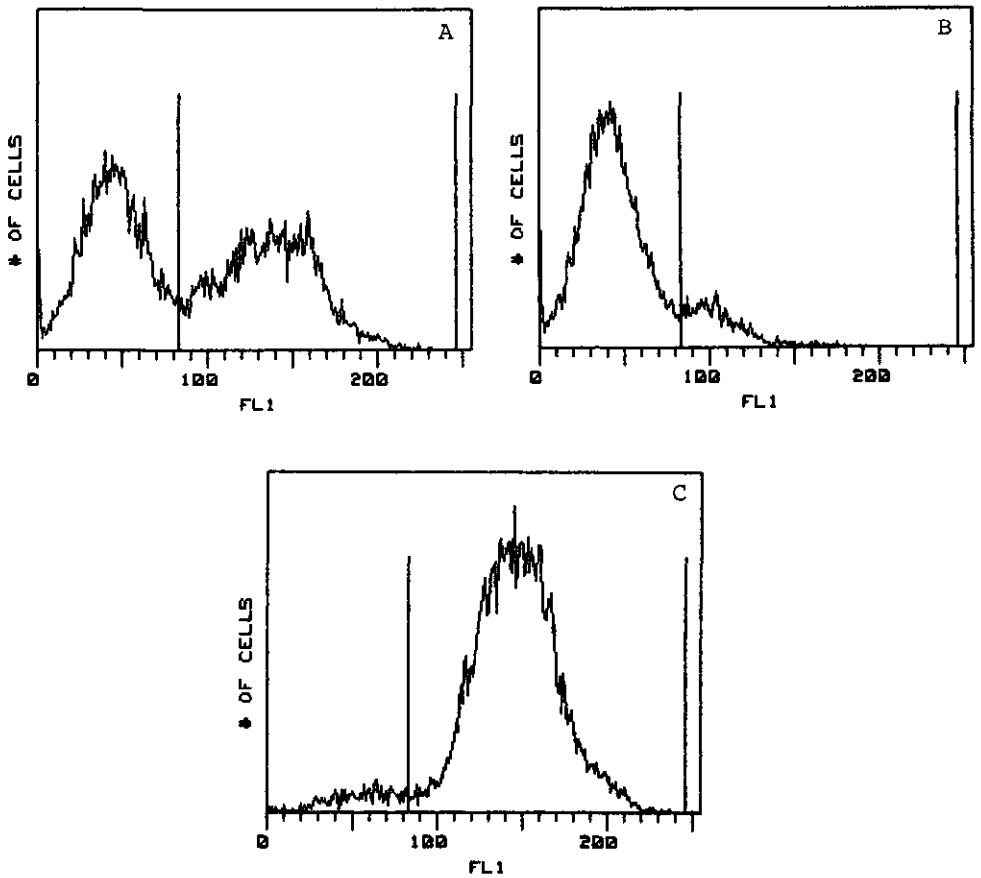

Figure 1. Representative fluorescence histograms of anti-Ig labelled carp PBL before and after MACS separation. **A.** non-separated fraction (50.2 and 42.9 % sIg^+ cells in fish 1 and 2, respectively) **B.** negative fraction (15.0 and 15.2 % dull sIg^+ cells), **C.** positive fraction (94.2 and 92.2 % sIg^+ cells)

labelled) or remixed sIg^+ and sIg^- cells after separation (Table II). Although labelling of the cells with biodegradable superparamagnetic microbeads slightly increased ^{3}H-thymidine incorporation in mitogen stimulated and control groups, the labelling did not influence the mitogen response. Mitogen responses to PHA and LPS were increased in the sIg^- lymphocyte fraction compared to both the non-separated and remixed sIg^+ and sIg^- populations. The sIg^+ cell-enriched fraction showed a similar response (fish 1) or a lower response (fish 2) to both PHA and LPS compared with the non-separated and remixed populations.

Table II. ***In vitro*** **mitogen responses* of MACS-separated subpopulations of carp PBL measured by ^{3}H-thymidine incorporation**

	fish 1			fish 2		
cell fraction	control	+ PHA	+ LPS	control	+ PHA	+ LPS
non-separated unlabelled	123	643	633	115	771	667
non-separated labelled	168	888	765	167	933	1295
sIg^+ cell-depleted	376	5458	3799	202	5176	5097
sIg^+ cell-enriched	137	746	752	99	269	313
remixed	102	539	548	112	688	780

*** Mean counts per minute for triplicate cultures**

Analysis of stimulated cells by simultaneous detection of BrdU and sIg

BrdU incorporation was detected in the cytocentrifuge slides as green fluorescent spots in the cell nuclei and sIg was simultaneously identified by a red membrane fluorescence (Figure 2). Table III shows the percentages of $BrdU^+$ PBL found in non-stimulated and PHA- or LPS-stimulated cultures and percentages of $BrdU^+$ cells which were sIg^+ or sIg^- in the same cultures. In PHA-stimulated cultures much more ($\pm$ 62.7 %) cells had incorporated

BrdU compared with LPS-stimulated cultures (± 18.4 %) or controls (± 2.6 %). In PHA-stimulated cultures > 99 % of the $BrdU^+$ cells were sIg^-, while in the LPS-stimulated cultures around 25 % of the $BrdU^+$ cells were sIg^+.

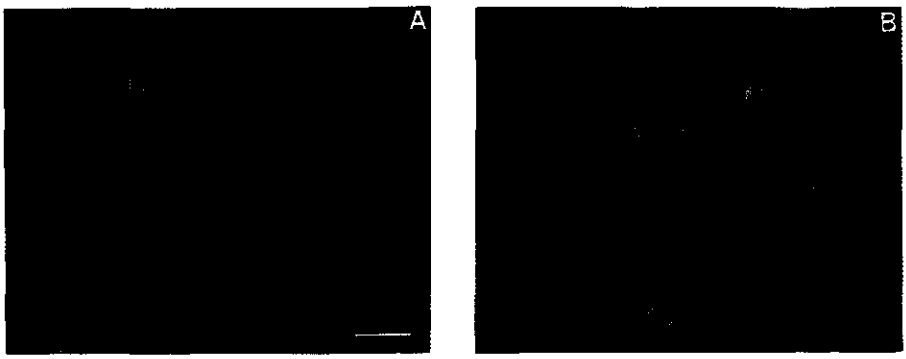

Figure 2. Fluorescence micrographs of anti-BrdU (green fluorescence) and anti-carp Ig (red fluorescence) double-stained carp PBL after mitogen stimulation. **A.** LPS-stimulated culture: $sIg^+/BrdU^+$, $sIg^+/BrdU^-$, $sIg^-/BrdU^+$ and $sIg^-/BrdU^-$ cells. **B.** PHA-stimulated culture: $sIg^+/BrdU^-$, $sIg^-/BrdU^+$ and $sIg^-/BrdU^-$ cells.

Table III. Percentages of $BrdU^+$ carp PBL, whether or not stimulated by PHA or LPS, and percentages of $BrdU^+$ cells which were sIg^+ or sIg^-

	fish 1			fish 2		
cells	control	+ PHA	+ LPS	control	+ PHA	+ LPS
PBL	3.1	62.5	19.3	2.2	62.9	17.5
sIg^+	ND*	0.6	26.2	ND*	0.6	21.6
sIg^-	ND*	99.4	73.8	ND*	99.4	78.4

* Not determined because the number of $BrdU^+$ cells was too low to calculate a reliable percentage

Representative dot plot profiles of anti-carp Ig and anti-BrdU double-stained LPS- or PHA-stimulated PBL are shown in Figure 3. In the LPS-stimulated cultures the majority of the $BrdU^+$ cells were dull sIg^+. In the PHA-stimulated cultures the $BrdU^+$ cells were mainly sIg^-.

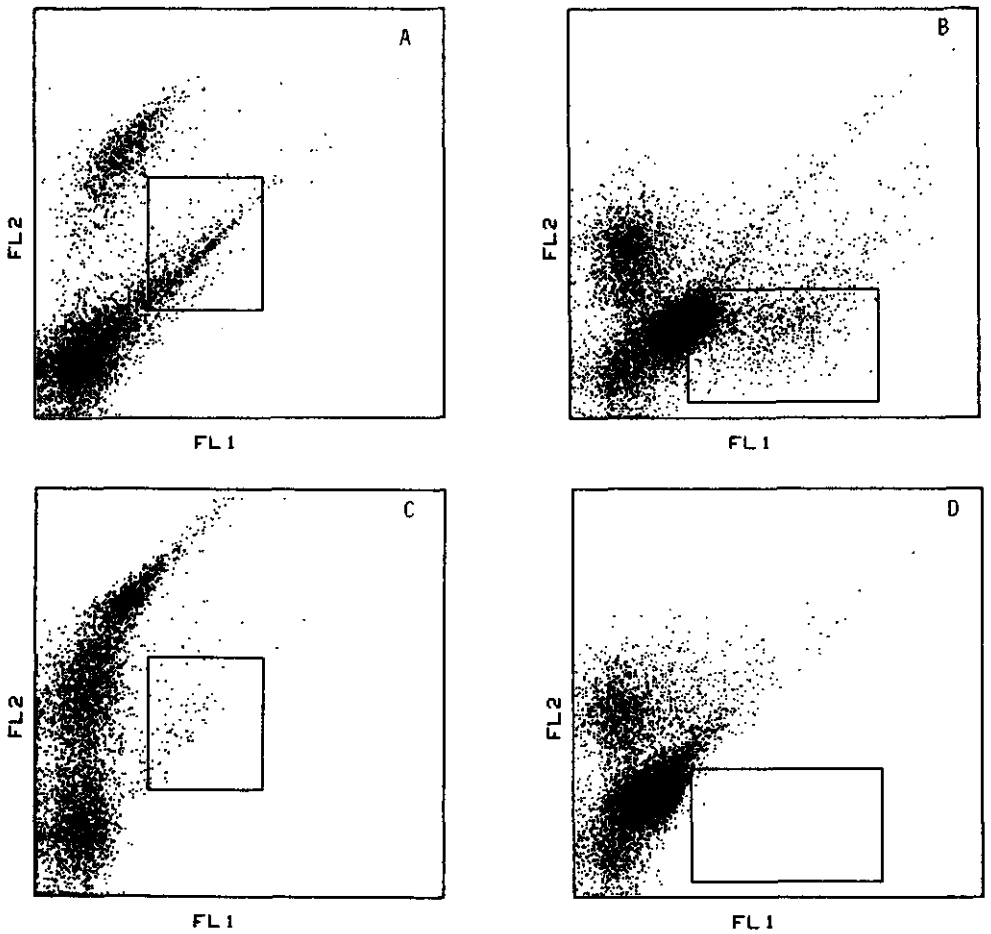

Figure 3. **Dot plots of anti-carp Ig (FL 2) and anti-BrdU (FL 1) double-stained mitogen-stimulated carp PBL. $BrdU^+$ cells are boxed. A. LPS-stimulated PBL, $BrdU^+$ cells are mainly dull sIg^+. B. PHA-stimulated PBL, $BrdU^+$ cells are mainly sIg^-. C. Control, LPS-stimulated cells without anti-BrdU staining. D. Control, PHA-stimulated cells without anti-BrdU staining.**

Discussion

In this study mitogen responses of carp PBL to LPS and PHA were studied using new techniques for fish. Several authors (Grondel & Boesten, 1982; Caspi *et al.*, 1984; Pourreau *et al.*, 1987) demonstrated the response of carp PBL to these mitogens but no distinction was made between responding B and T cells.

A monoclonal antibody specific for carp Ig (WCI 12; Secombes *et al.*, 1983; Van Diepen *et al.*, 1991) was used for physical separation of sIg^+ and sIg^- cells. As panning of carp lymphocytes did not work sufficiently (unpublished results), magnetic cell sorting was used and revealed a suitable separation of sIg^+ ($\pm$ 93 % pure) and sIg^- cells ($\pm$ 85 % pure). A great benefit of this method is the absence of sIg^+ cell loss or damage due to adherence. Moreover, labelling with biodegradable superparamagnetic beads and avidin-FITC did not clearly influence the responsiveness of the cells to PHA or LPS. Therefore, MACS seems to be a promising method for cell separation especially in cases where panning does not work well enough.

Mitogen stimulation of sIg^+ and sIg^- carp lymphocytes was also studied on non-separated cells by the simultaneous detection of sIg and incorporated BrdU. The advantage of this technique is the presence of all accessory cells necessary, which had to be added to panned catfish lymphocytes after separation (Sizemore *et al.*, 1984). The double-stained cells were studied and quantified under a fluorescence microscope or analysed with a flow cytometer. Quantitative studies by fluorescence microscopy showed a small variation in results from different fish but the method is rather laborious. Flow cytometric analysis does not have this drawback but for this method cells have to be fixed in suspension which influenced FSC-SSC profiles.

PHA, an established T cell mitogen in mammals, stimulates the proliferation of sIg^- cells and not of sIg^+ cells as can be concluded from the following observations: 1. The absolute number of sIg^- cells was enhanced after PHA-stimulation while the number of sIg^+

cells was decreased. 2. The sIg^+ cell-depleted fraction, but not the sIg^+ cell-enriched fraction, showed an increase of ^{3}H-thymidine incorporation by PHA compared with non-separated cells. 3. More than 99 % of the $BrdU^+$ cells in PHA-stimulated cultures were sIg^- as can be concluded from fluorescence microscopy and flow cytometry. Stimulation of sIg^- cells by a related T cell mitogen, ConA, has been reported for two other teleost fish species; rainbow trout (DeLuca *et al.*, 1983) and catfish (Sizemore *et al.*, 1984). Con A applied for carp revealed the same results as with PHA but the mitogen responses were lower (unpublished results).

LPS stimulates sIg^+ cells as can be concluded from: 1. The absolute number of sIg^+ cells showed a strong increase in the LPS-stimulated cultures. 2. The sIg^+ cell-enriched fraction showed an increased ^{3}H-thymidine incorporation after LPS stimulation compared with control cultures. 3. After LPS stimulation around 25 % of the cells incorporating BrdU were sIg^+. However, LPS seems to have a stronger stimulating effect on sIg^- cells because the LPS response in the sIg^+ cell-depleted fraction was much higher than in the non-separated cell population or the sIg^+ cell-enriched fraction. Moreover, around 75 % of the $BrdU^+$ cells in the LPS-stimulated cultures were sIg^-. On the other hand, the absolute number of sIg^- cells as calculated from cell counting and flow cytometric analysis decreased in the cultures with LPS. These contradictory results can be explained by the fact that the sIg^+ cell-depleted fraction still contained around 15 % dull sIg^+ cells and apparently these cells can be well stimulated by LPS. The absence of dull sIg^+ cells in the sIg^+ cell-enriched fraction also explains that the LPS response in this fraction was not enhanced compared with the response in the non-separated and remixed cell populations. Probably the LPS-responding dull sIg^+ cells cannot be detected with the fluorescence microscope. This supposition is strongly supported by the simultaneous flow cytometric detection of sIg and BrdU after LPS stimulation, showing that mainly dull sIg^+ cells incorporated BrdU. Consequently, these results indicate that sIg^+ cells and especially dull sIg^+ cells are stimulated by LPS. In addition, this conclusion is sustained by results of Mond *et al.* (1980) showing that B cells of mouse, present in the non-adherent sIg^- cell fraction after panning, had up to a 12-fold

increased responsiveness to T-independent antigens in an *in vitro* assay of antibody formation compared with B cells in the adherent sIg^+ fraction.

LPS stimulation of the sIg^+ cell-depleted fraction (obtained by panning) has also been described for rainbow trout (DeLuca *et al.*, 1983) and channel catfish (Sizemore *et al.*, 1984). At present it can not be excluded that dull sIg^+ cells in the sIg^+ cell-depleted fraction are also responsible for the LPS responsiveness in these species. On the other hand Sizemore *et al.* (1984) argued that LPS-responsive cells in the sIg^+ cell-depleted fraction and in the sIg^+ cell-enriched fraction belong to distinct cell populations because accesory cells were required for the LPS response in the sIg^+ cell-depleted fraction, while LPS responsiveness in the sIg^+ cell-enriched fraction was independent on the presence of accessory cells.

In conclusion, PHA stimulates sIg^- cells and not sIg^+ cells, while LPS appears to stimulate sIg^+ cells, especially the dull sIg^+ cells in carp. The results described here for carp contradict the conclusion made by Kaattari (1992) that two different fish lymphocyte populations share LPS reactivity. The reported LPS responsiveness of panned sIg^- lymphocytes from trout (DeLuca *et al.*, 1983) and channel catfish (Sizemore *et al.*, 1984) may also be due to the presence of dull sIg^+ cells in these fractions which can be examined with the techniques described.

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B cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.); an immuno(cyto)chemical study

J.C.E. Koumans-van Diepen, E. Egberts, B.R. Peixoto, N.Taverne & J.H.W.M. Rombout

Department of Experimental Animal Morphology and Cell Biology, Agricultural University, P.O. Box 338, 6700 AH Wageningen, The Netherlands

Key words: fish; immunocytochemistry; B cells; immunoglobulin; isotypes; ontogeny

submitted

Abstract

B cell and immunoglobulin (Ig) heterogeneity was demonstrated in carp, *Cyprinus carpio* L., using two monoclonal antibodies (MAbs; WCI 4, WCI 12) produced against carp serum Ig.

Immunochemical results showed that both WCI 4 and WCI 12 reacted with a protein determinant on a heavy chain of Ig (relative molecular mass ~70,000). Immunofluorescence microscopic and flow cytometric analysis of lymphoid cells showed three distinct subpopulations of B cells and plasma cells: WCI 4^+ 12^- cells, WCI 4^- 12^+ cells and WCI 4^+ 12^+ cells. WCI 4^- 12^+ and WCI 4^+ 12^+ anti-DNP antibody-secreting cells were demonstrated with ELISPOT in pronephros and spleen suspensions from primary immunised carp. Affinity chromatography of carp serum and sequential immunoprecipitation of ^{125}I-labelled peripheral blood leucocytes (PBL) membrane molecules indicated the presence of at least two antigenically different Ig (iso) types namely WCI 4^- 12^+ and WCI 4^+ 12^+ molecules. WCI 4^+ 12^- molecules could not be detected by immunoprecipitation. During ontogeny a shift in percentages of WCI 4^+ 12^- cells and WCI 4^- 12^+ cells was found in spleen and pronephros. WCI 4^+ 12^- cells formed the majority of B cells at 2 weeks of age but the percentages decreased during ontogeny. On the other hand, the percentages WCI 4^- 12^+ cells increased and these cells became the major population of B cells from 13 weeks onwards. The proportion of WCI 4^+ 12^+ cells remained stable during ontogeny. The distribution of B cell subpopulations in blood was more or less stable at all ages.

Introduction

From a phylogenetic point of view, fish are the first group of animals in which immunoglobulins (Ig) can be demonstrated (Litman *et al.*, 1991; Ratcliffe & Millar, 1988). Early studies on the presence of Ig on lymphocyte populations of several teleost fish species,

using polyclonal antisera against serum Ig, revealed that high percentages of lymphocytes including thymocytes were Ig-immunoreactive (Warr *et al.*, 1979; Clem *et al.*, 1977; Emmerich *et al.*, 1975). In later work with carp, it was argued that the immunoreactive molecule present on thymocytes was different from serum Ig because it contained two heavy (H) chains but lacked light (L) chains (Fiebig *et al.*, 1980). Other authors explained the initial observations by a cross-reaction between carbohydrate moieties on the Ig H chain and the thymocytes (Yamaga *et al.*, 1978a; 1978b). The development of monoclonal antibodies (MAbs) against serum Ig provided evidence for distinct Ig-positive and Ig-negative lymphocyte populations, and mainly Ig-negative thymocytes in carp (*Cyprinus carpio*; Secombes *et al.*, 1983), channel catfish (*Ictalurus puntatus*; Lobb & Clem, 1982; Ainsworth *et al.*, 1990), rainbow trout (*Oncorynchus mykiss*; DeLuca *et al.*, 1983; Thuvander *et al.*, 1990), Atlantic salmon (*Salmo salar*; Killie *et al.*, 1991; Falk *et al.*, 1991), Atlantic cod (*Gadus morhua*; Pilström & Petersson, 1991; Israelsson *et al.*, 1991) and sea bream (*Sparus aurata*; Navarro *et al.*, 1993).

The bony fish serum Ig is a tetrameric molecule with eight L and eight H chains (Wilson & Warr, 1992). However, monomeric and/or dimeric molecules have also been found in carp (Rombout *et al.*, 1993) and some other teleost fish species (Wilson & Warr, 1992). Within the tetrameric configuration minor differences in antigenicity are found suggesting Ig heterogeneity in channel catfish (Lobb & Olson, 1988), rainbow trout (Sanchez *et al.*, 1989), Atlantic salmon (Killie *et al.*, 1991; Falk *et al.*, 1991) and brown trout (*Salmo trutta*; Falk *et al.*, 1991). However, till now no reports are available on heterogeneity of B cells or plasma cells.

This study deals with B cell and Ig heterogeneity in carp using a variety of immunocytochemical and immunochemical techniques. A selected panel of MAbs produced against carp serum Ig (Secombes *et al.*, 1983) was used.

Materials and methods

Fish

Outbred carp, *Cyprinus carpio* L., of 2 weeks - 4 years old were raised at the central fish facilities 'De Haar vissen' at 21.5 $\pm$ 0.5 °C in a recirculating system with filtered and UV-sterilized water. Hatched carp were fed *Artemia salina nauplii* for a period of 2 weeks followed by pelleted food (Trouvit/K30; Trouw and Co., Putten, The Netherlands) at a daily, age-dependent ration of 10 % (at 2 weeks old) decreasing till 2 % (at 24 weeks old) of the body weight. Fish were anaesthetised in 0.03 % tricaine methane sulphonate (TMS; Crescent Research Chemicals, Phoenix, AR, USA) and bled from the dorsal aorta by syringe filled with 0.5 ml heparin solution (50 IE/ml cRPMI (RPMI 1640 + 10 % double-distilled water)) per 2 ml blood. The tail of 2 and 4 weeks old fish was cut off and blood was taken into a capillary tube filled with heparin solution.

Leucocyte isolation

All materials used were coated with Sigmacoate (Sigma chemical co., St. Louis, MO, USA) in order to avoid adherence of cells. Heparinized blood was diluted 1:1 in cRPMI + 0.1 % NaN_3, allowed to settle for 1 hour at 0 °C and centrifuged for 15 min at 100 x g and 4 °C. Supernatant containing white blood cells and a very small part of the erythrocyte pellet was pipetted off and layered over Lymphoprep (Nycomed, Oslo, Norway) centrifuged for 30 min at 1500 x g and 4 °C. Peripheral blood leucocytes (PBL) harvested from the interface with a Pasteur pipette were washed twice for 10 min at 680 x g and 4 °C, and resupended in cRPMI + 0.1 % NaN_3 + 1 % BSA + 10 IU heparin/ml. Dissected pronephros and spleen were sqeezed through a nylon gauze while adding cRPMI + 0.1 % NaN_3 + 1 % BSA + 10 IU heparin/ml.

For flow cytometric analysis lymphocytes from pronephros and spleen were enriched: cell suspensions were prepared in medium without BSA and layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in cRPMI and Dulbecco's modified Eagle medium (DMEM, 10 x concentrated; Life Technologies Ltd., Paisley, U.K.)

to densities of 1.020 and 1.070 g/ml and centrifuged for 30 min at 840 x g and 4 °C. Organs from 2 respectively 4 weeks old carp were pooled before Percoll separation. Cells at the 1.02-1.07 interface were harvested with a Pasteur pipette, washed twice for 10 min at 680 x g and 4 °C and resuspended in cRPMI + 0.1 % NaN_3 + 1 % BSA + 10 IU heparin/ml.

Monoclonal antibodies and conjugates

WCI 4, WCI 12 and WCI 14 were raised against carp serum Ig as described by Secombes *et al.*(1983). The antibodies were generally used as 1:50 - 1:200 dilutions of culture supernatant. Biotinylated WCI 12 (WCI 12-biotin) was prepared for the application of double staining. Fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig antibodies (RAM-FITC; 1:50-1:100; Dakopatts, Glostrup, Denmark) was used as second antibody for flow cytometry and fluorescence microscopy. In case of double staining, phycoerythrin-conjugated streptavin (SA-PE; Southern Biotechnology Associates Inc., Birmingham, AL, USA) was used for flow cytometry and tetramethyl rodamine isothiocyanate-conjugated RAM (RAM-TRITC; 1:100; Dakopatts, Glostrup, Denmark) for fluorescence microscopy. Goat anti-mouse Ig antibodies coupled to 30 nm gold particles (GAM-Ig G30; 1:5; E-Y Laboratories, Inc., San Mateo, CA, USA) were applied as second antibody for electron microscopy. Alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) antiserum (GAM-AP; 1:3000; Bio-rad Laboratories, Richmond, CA, USA) was used for Western blots and ELISPOT. FITC conjugated concanavalin A (Con A-FITC; 1:100; Calbiochem Co., San Diego, CA, USA) was used for the detection of carbohydrates.

Flow cytometry

Cells (6 x 10^5) were incubated in 250 μl of diluted MAb or a combination of two MAbs (additional staining) for 30 min at 0 °C and washed by centrifuging for 5 min at 680 x g and 4 °C, and resuspension in cRPMI + 0.1 % NaN_3 + 1 % BSA + 10 IU heparin/ml. Subsequently the cells were incubated with RAM-FITC for 15 min at 0 °C. For double staining, single labelled cells were incubated with normal mouse serum (1:25) for 30 min at 0 °C in order to occupy the free binding sites on the RAM-FITC. Then, a second labelling

with a biotinylated monoclonal antibody followed by SA-PE was performed similar to the first labelling. The labelled cells were washed and 10,000 cells were analysed on a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) tuned at 488 nm using the Consort 30 data analysis package. Cells with a low forward scatter (FSC) and side scatter (SSC), representing small and smooth cells with a low cytoplasm/nucleus ratio, were gated in order to determine the percentages of sIg-positive lymphoid cells.

Fluorescence and electron microcopy

Cell suspensions were labelled with a MAb and RAM-FITC as described for flow cytometry. Cytocentrifuge preparations of these labelled cell suspensions and untreated cell suspensions were prepared as reported previously (Van Diepen *et al.*, 1991). In order to investigate whether carbohydrate or protein determinants are recognised, cytocentrifuge slides of spleen and pronephros leucocytes were treated for 5 min with cold (4 °C) acetone, followed by an incubation for 1 h at 4 °C in 50 mM periodate in 50 mM acetate buffer (pH 4.5), and for 30 min at room temperature (RT) in 10 mM sodium borohydride in PBS (pH 7.2) and finally washed with PBS; this method has been reported to remove carbohydrates very effectively (Mattes & Steiner, 1978; Basbaum *et al.*, 1986). As a control for the removal of carbohydrates, labelling of the cells with Con A-FITC was performed. The periodate treated and control slides were stained with MAb as described earlier (Van Diepen *et al.*, 1991). Double staining of fixed cell preparations with WCI 4 and WCI 12 was performed by labelling first with WCI 4 and RAM TRITC, followed by WCI 12 and RAM FITC and reversed. The preparations were studied with a Nikon Microphot FXA fluorescence microscope. Cells with a considerable amount of Ig^+ cytoplasm and an eccentric nucleus were considered to be plasma cells (cf. Imagawa *et al.*, 1991). Electron microscopic preparations were made using immunogold labelling (Van Diepen *et al.*, 1991).

ELISPOT

A modification of the ELISPOT described by Secombes *et al.* (1991) was followed. Fish were immunised (intramuscularly) with 25 μg DNP_{494}-KLH in Freunds' incomplete

adjuvant. At day 14 after primary immunisation pronephros and spleen were dissected and the lymphoid cells were isolated by Percoll separation. A sheet of nitrocellulose (NC; BA85; Schleicher & Schull, Dassel, Germany) was incubated in a 96-well dotblot apparatus (minifold SRC-96; Schleicher & Schuell, Dassel, Germany) with 100 μl of a solution of DNP-BSA (ratio 3600:1; 500 μg protein/ml) in PBS per well for 15 min at RT. After replacing the DNP-BSA by PBS, the incubation was continued for another 15 min at RT. The NC sheet was then taken out, blocked in PBS + 1 % BSA for 1 h at RT and returned to the dotblot apparatus with a sheet of parafilm beneath it in order to avoid leaking. Cell suspensions in cRPMI (100 μl) were added to the wells in triplicate at four different concentrations: from 5 x 10^5 to 6.25 x 10^4 per well. The cell suspensions were poured off after incubation for 3 h at 25 °C. Subsequently, the wells were washed trice with PBS. For the detection of the bound DNP-specific antibodies, 150 μl of WCI 4, WCI 12 or a mixture of both were used overnight at 4 °C. After washing trice in PBS, the sheet was incubated with GAM-AP in TBS (0.02 M Tris, 0.05 M NaCl pH 7.4-7.6) for 1 h at RT. Following three other washings in TBS + Tween 20, incubation in substrate buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$) was performed for 10 min at RT. The spots were developed in substrate buffer with 0.1 % nitro blue tetrazolium (NBT) and 0.04 % 5-bromo-4-cloro-3-indolyl-phosphate (BCIP). The NC sheet was rinsed in aqua bidest, dried at RT and stored in the dark. Spots were counted under low magnification.

Western blotting

Samples of normal carp serum (0.8 μl) were analysed by SDS-PAGE and transferred onto nitrocellulose (BA85; Schleicher & Schuell, Dassel, Germany) at 4 °C. Transfers were cut and one of the lanes with sample was stained in 0.1 % Amido Black. (Prestained) SDS-PAGE Molecular Weight Standards (Bio-rad Laboratories, Richmond, CA, USA) were used. Transfers were saturated in TTBS (0.02 M Tris-HCl pH 7.4-7.6, 0.05 M NaCl, 0.05 % Tween 20) for 1 h at RT, washed twice in TTBS and incubated with one of the MAb for 1 h at RT. Subsequently, transfers were washed trice in TTBS, incubated with GAM-AP for 1 h at RT, washed trice in TTBS, washed in substrate buffer and incubated with substrate

buffer containing 0.1 % NBT and 0.04 % BCIP.

Immunoprecipitation

PBL (5 x 10^8) in 0.5 ml PBS were radioiodinated with 0.5 mCi $Na^{125}I$ (Amersham International plc, Buckinghamshire, UK) using single reaction enzymobead radioiodination reagent (Bio-Rad Laboratories, Richmond, CA, USA). Cells and enzymobeads were resuspended in 1 ml lysisbuffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 % (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate), 0.1 mM PMSF (phenyl methyl sulphonyl fluoride), 0.1 mM ethylmaleïnide) and incubated for 1 h on ice. The mixture was centrifuged for 10 min at 15,000 x g and the supernatant was collected and stored at -20 °C. Incorporation of ^{125}I was assessed by counting radioactivity of a small sample in scintillation cocktail (Beckman readysafe) in a Beckman LC 1701 counter (^{35}S-channel).

Culture supernatants containing WCI 4 or WCI 12 were concentrated over a Sepharose 4B-protein A column and dialysed against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.8). The antibodies were coupled to swollen CNBr-activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden). Two to 3 mg protein was bound per ml of gel.

Sequential immunoprecipitation of ^{125}I-labelled PBL membrane proteins was performed as follows: 200 μl ^{125}I-labelled PBL lysate (2 x 10^7 cpm) was precleared with 20 μl normal rabbit serum and 200 μl control (uncoupled) Sepharose gel end-over-end for 1 h at 4 °C. The beads were spun down by centrifugation for 5 min at 15,000 x g and the supernatant was taken off. After another preclearing with 200 μl control Sepharose beads for 1 h at 4 °C, the supernatant was depleted thrice with 150-300 μl WCI 4- or WCI 12-coupled Sepharose for 1-2 h at 4 °C, and finally precipitated with 300 μl WCI 12- or WCI 4-coupled Sepharose for 2 h at 4 °C. MAb-coupled beads were washed twice in 1 ml NET-NO and twice in 1 ml NET-N (NET-NO without BSA). Then MAb-coupled beads were mixed with 100 μl sample buffer and boiled for 5 min. The beads were spun down by centrifugation for 10 min at 15,000 x g, and the supernatant samples were analysed by SDS-PAGE.

Gels were stained in Vesterberg stain (0.7 M trichloroacetic acid (TCA), 0.1 % (w/v)

Coomassie Brilliant Blue (R-250), 0.13 M 5-sulfosalicylic acid (SSA), 30 % (v/v) methanol) for 30 min at RT, destained in 7.5 % (v/v) acetic acid, 22.5 % (v/v) ethanol) over night at RT. After incubation in 10 % (v/v) acetic acid, 1 % (v/v) glycerol for 30 min at RT, they were dried under vacuum at 70 °C for 2 h. Autoradiography was performed on Kodak XAR film with Kodak intensifying screens at -80 °C.

Affinity chromatography

A column was prepared of WCI 4 coupled to swollen CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Three mg protein was bound per ml gel. Five ml of pooled carp serum diluted to 100 ml in 20 mM phosphate buffer pH 8.5 were applied onto the column. The column was washed with 5 volumes of washing buffer (20 mM phosphate pH 8.5) and eluted with 5 volumes of elution buffer (20 mM phosphate + 0.5 M NaCl pH 8.5). Fractions of 2.5 ml were collected and samples were investigated for WCI 4 or WCI 12 immunoreactivity using a dotblot assay (Rombout *et al.*, 1993). Immunoreactivity was quantified by analysing a photograph (negative) of the dotblot with an Ultroscan XL gel scanner (Pharmacia, Uppsala, Sweden). Absorption (A) was expressed as percentage of the highest A (= 100 %) measured. Samples were also applied for Western blotting (see above) in order to check the relative molecular mass (Mr) of the immunopositive molecule(s).

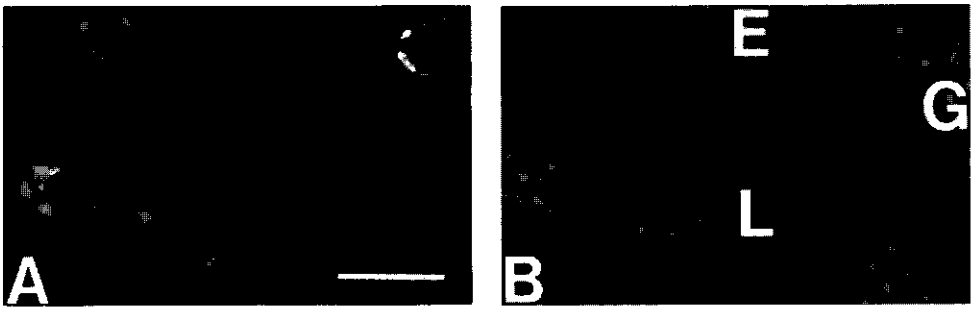

Figure 1. **Fluorescence micrograph (A) and a phasecontrast photograph (B) of WCI 14-labelled cytocentrifuge preparation of pronephros cells. Granulocytes (G) and lymphocytes (L) are positive, erythrocytes (E) are negative. bar = 20 μm**

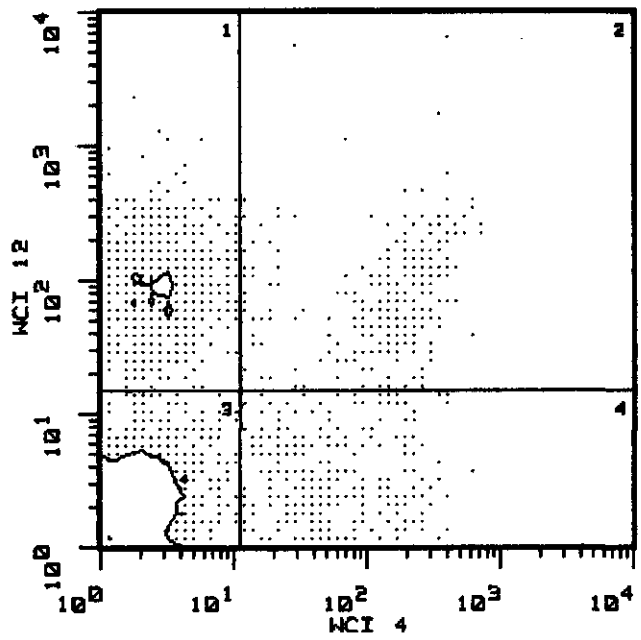

Figure 2. Flow cytometry profile of WCI 4 - WCI 12 double-stained lymphoid cells from blood.

Results

Immunocytochemistry

WCI 4 and WCI 12 both appeared to react with a subpopulation of lymphocytes and with plasma cells as estimated by labelling of pronephros cell suspensions for fluorescence and electron microscopy. On the contrary, WCI 14 stained all leucocytes but not erythrocytes (Figure 1). Labelling with WCI 4 and WCI 12 on fixed cytocentrifuge preparations showed plasma cells with a highly positive cytoplasm. Microscopic indications that WCI 4 reacted with a smaller percentage of lymphocytes compared to WCI 12 could be confirmed by flow cytometry.

Double staining of blood lymphoid cells from 6 months old carp revealed that 9 % was WCI 4^+ 12^-, 7 % was WCI 4^+ 12^+ and 17 % was WCI 4^- 12^+ (Figure 2). WCI 4^+ 12^- cells showed an average lower fluorescence intensity than WCI 4^+ 12^+ cells and WCI 4^- 12^+ cells. Double staining of plasma cells on fixed cytocentrifuge preparations of pronephros (Figures 3, 4) revealed 70-80 % WCI 4^- 12^+, 20-30 % WCI 4^+ 12^+ and only a low number (< 1 %) WCI 4^+ 12^- plasma cells.

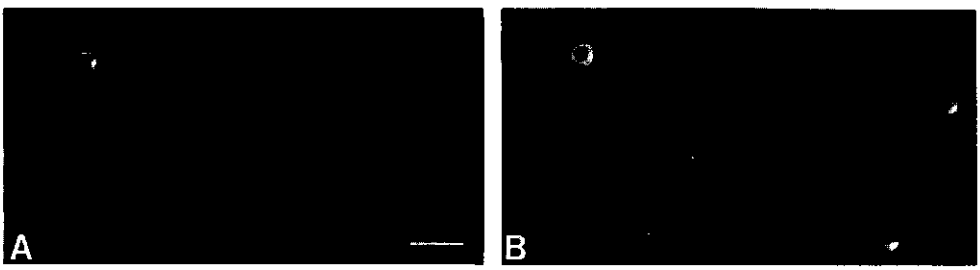

Figure 3. **Fluorescence micrographs of additional stained plasma cells from pronephros.**
bar = 20 μm A. WCI 4-labelled cells visualised with TRITC filter B. WCI 4 and WCI 12-labelled cells visualised with FITC filter.

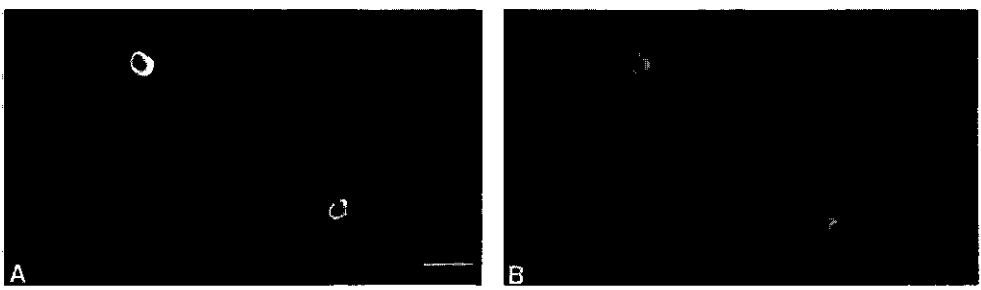

Figure 4. **Fluorescence micrographs of additional stained plasma cells from pronephros.**
bar = 20 μm A. WCI 12-labelled cells visualised with FITC filter B. WCI 12 and WCI 4-labelled cells visualised with TRITC filter.

Immunofluorescence reactions of WCI 14 but not of WCI 4 or WCI 12 were periodate-sensitive. WCI 4, applied in an ELISPOT assay for the detection of anti-DNP antibody-secreting plasma cells, stained 16-40 % of the WCI 12^+ spots in pronephros and 21-65 % of these spots in spleen (Table I). Additional staining of the spots with WCI 4 did not reveal more spots than a single labelling with WCI 12 in most cases. The number of positive spots were lower in spleen compared to pronephros.

Table I. Number of DNP-specific spot-forming cells per 10^6 pronephros or spleen cells from individual carp detected with WCI 4, WCI 12 or a combination of WCI 4 and WCI 12 after primary immunisation with DNP-KLH. The ratio WCI 4^+ / WCI 12^+ spots x 100 % is given

Primary response, day 14

organ	WCI 4	WCI 12	WCI 4 + WCI 12	% WCI 4/ WCI 12
pronephros 1	98	256	220	38
pronephros 2	156	984	968	16
pronephros 3	35	97	103	36
pronephros 4	68	170	160	40
spleen 1	13	20	27	65
spleen 2	86	208	246	41
spleen 3	5	24	18	21
spleen 4	22	54	54	41

Immunochemistry

Western blotting of serum proteins with WCI 4 or WCI 12 revealed in both cases a band with a relative molecular mass (Mr) of ~70,000 but the reaction with WCI 12 was stronger (Figure 5). WCI 14 also revealed a strong positive Mr ~70,000 band but next to it many other bands (Figure 5). Mr ~65,000 bands were found in all blots due to aspecific staining of carp serum albumin.

Immunoprecipitation of ^{125}I-labelled membrane proteins of PBL with WCI 4 and WCI 12 showed a Mr ~70,000 band and a Mr ~25,000 band (Figure 6). Positive bands could not be detected with WCI 4 after initial precipitation with WCI 12. After depletion with WCI 4, clear Mr ~70,000 and Mr ~25,000 bands were found after subsequent precipitation with WCI 12 (Figure 6).

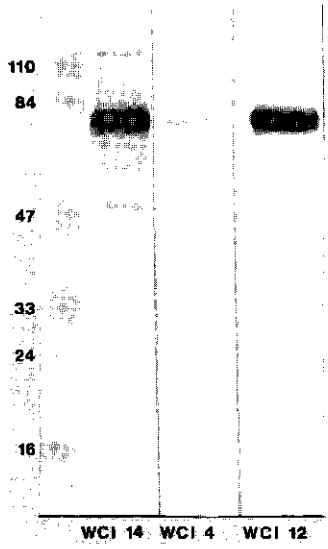

Figure 5. **Western blotting of serum proteins. WCI 4 and WCI 12 show both Mr ~70,000 bands while WCI 14 is positive with a Mr ~70,000 band and many other bands. The Mr ~65,000 bands are due to aspecific staining of carp serum albumin.**

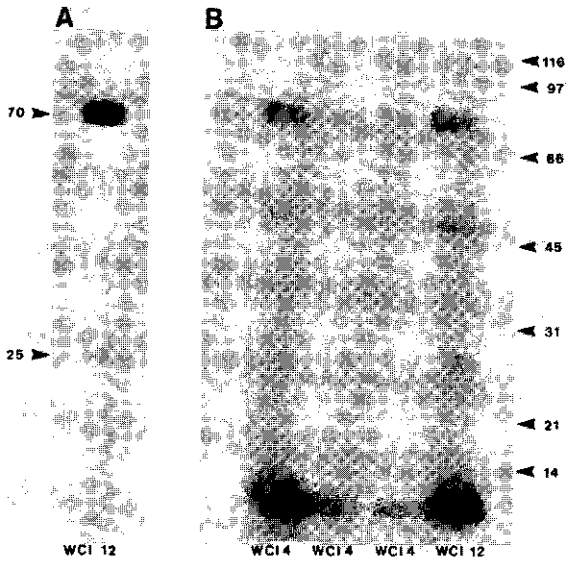

Figure 6. **Immunoprecipitation of ^{125}I-labelled membrane proteins of PBL with WCI 4 or WCI 12. Both MAbs show positive bands with Mr ~70,000 and ~25,000 (A). Depletion with WCI 4 still reveals WCI 12$^+$ bands (B).**

Results from the affinity chromatography using a WCI 4-coupled Sepharose column are shown in Figure 7. Dotblots of the eluted fraction were strongly stained with both WCI 4 (A = 92) and WCI 12 (A = 100) while blots of the flow-through fraction were WCI 4$^-$ (A = 2) and WCI 12$^+$ (A = 8). Western blotting of samples from flow-through and eluted fractions showed that WCI 4- and/or WCI 12-positive molecules had a Mr ~70,000.

Ontogeny

Single and additional stainings with WCI 4 and WCI 12 for flow cytometric analysis were used to determine the percentages of WCI 4$^+$ 12$^-$, WCI 4$^-$ 12$^+$ and WCI 4$^+$ 12$^+$ B lymphocytes in spleen, pronephros and blood from 2 weeks - 56 weeks old carp (Figure 8). The distribution of the WCI 4$^+$ 12$^-$ and WCI 4$^-$ 12$^+$ B cells changed during ontogeny in

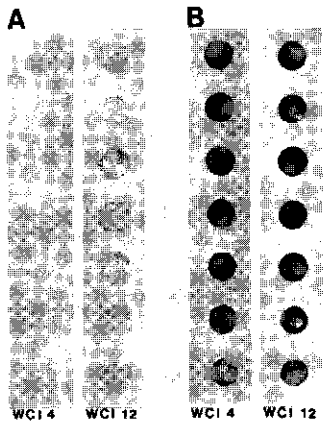

Figure 7. Dotblots stained with WCI 4 or WCI 12, from flow-through (A) and eluted (B) fractions obtained after affinity chromatography of serum using a WCI 4-conjugated Sepharose column.

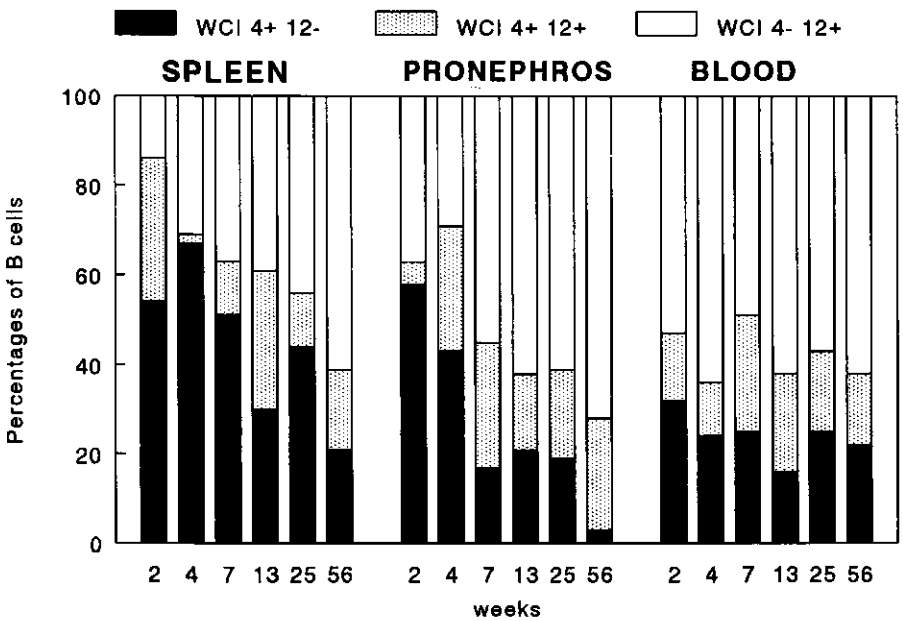

Figure 8. Distribution of B cell subpopulations of carp in spleen, pronephros and blood during ontogeny (age in weeks). n = 6-8

spleen and pronephros while in blood the distribution remained almost the same. In spleen and pronephros from 2 weeks old carp the majority of the B lymphocytes were WCI 4^+ 12^- cells while WCI 4^- 12^+ cells formed a minority. The percentages of WCI 4^+ 12^- cells decreased during ontogeny and formed a minor B cell population in 56 weeks old carp, while the WCI 4^- 12^+ cells became the most common B lymphocytes in these organs. The percentages of WCI 4^+ 12^+ cells did not show a clear shift during ontogeny. In blood the distribution of the three B cell subpopulations was more or less stable during ontogeny.

Discussion

The Mabs produced against carp serum Ig used in this study could be divided into two groups by ELISA: Ig^+ thymocyte $(T)^+$ and Ig^+ T^- (Secombes *et al.*, 1983). The WCI 14 MAb (Ig^+ T^+) is not Ig-specific but reacts with a common carbohydrate determinant present at the H chain of Ig but also at many other molecules present on all leucocyte types. Therefore, this MAb was considered as a control for the specificity of the WCI 4 (Ig^+ T^-) and WCI 12 (Ig^+ T^-) reactions.

WCI 4 and WCI 12 both seem to be specific for a protein antigenic determinant at the H chain of an Ig molecule. This can be concluded from the absence of any effects of periodate treatment on their reactivity, Western blotting and immunoprecipitation. Double or additional staining with WCI 4 and WCI 12 of membrane Ig (flow cytometry) or cytoplasmic Ig (cytocentrifuge slides) of lymphoid cells from pronephros showed three types of labelled B cells and plasma cells: WCI 4^+ 12^-, WCI 4^- 12^+, and WCI 4^+ 12^+. ELISPOT assays revealed WCI 4^- 12^+ and WCI 4^+ 12^+ anti-DNP antibody-secreting cells from immunised carp, while only occasionally WCI 4^+ 12^- anti-DNP antibody-secreting cells were detected. In cytocentrifuge preparations of pronephros < 1 % of the plasma cells appeared to be WCI 4^+ 12^-, which may explain the absence of clear WCI 4^+ 12^- spots. The observed

B cell and plasma cell heterogeneity can be explained by the presence of antigenically different Ig types. Although co-expression of two or even three Ig isotypes is found at the membrane of mammalian B cells, plasma cells produce only one type of Ig (Burrows & Cooper, 1984). Consequently WCI 4^+ 12^+ plasma cells probably produce an Ig type reactive with both MAbs and hence B cells expressing this Ig type may exist as well. Therefore, three different Ig types can be suggested: 1. with a determinant recognised by WCI 4, 2. with a determinant recognised by WCI 12 and 3. with both determinants and hence recognised by WCI 4 and WCI 12. The fact that the WCI 4 and WCI 12 antibodies both react with two different Ig types is not suprising. In human up to 80 % identity is found between the individual C domains of the γ isotypes and some antibodies against Ig react with determinants expressed on different isotypes (Male *et al.*, 1987). The sequential immunoprecipitations of ^{125}I-labelled PBL membrane molecules provide indications for the presence of at least two antigenically different immunoglobulin molecules: WCI 12^+ Mr ~70,000 and Mr ~25,000 bands could be found after depletion with WCI 4. Reverse immunoprecipitation (WCI 12 first) did not reveal WCI 4^+ bands. These results can be explained by the presence of WCI 4^+ 12^+ and WCI 4^- 12^+ Ig types. The absence of WCI 4^+ 12^- molecules after depletion with WCI 12 can be due to the relative low number of WCI 4^+ 12^- B cells (22 %) compared to the WCI 4^- 12^+ B cells (62 %) in blood. Moreover, WCI 4^+ 12^- cells showed an average lower fluorescence intensity than the WCI 4^+ 12^+ cells, which might be caused by a lower number WCI 4^+ 12^- Ig molecules per cell or a lower affinity of WCI 4 for WCI 4^+ 12^- molecules. Two Ig types were also detected in serum by affinity chromatography. Pooled carp serum passed through a WCI 4-coupled Sepharose column revealed a WCI 4^- 12^+ flow-through fraction while the eluted fraction was WCI 4^+ 12^+.

Immunoglobulin heterogeneity has been described for other teleost fish using MAbs: catfish (Lobb & Olson, 1988), rainbow trout (Sanchez *et al.*, 1989), Atlantic salmon (Killie *et al.*, 1991, Falk *et al.*, 1991) and brown trout (Falk *et al.*, 1991). However, none of these studies have reported on Ig heterogeneity at the B cell level. In catfish at least four heavy chains isotypes can be distinguished with three MAb each reacting with $\pm$ 20 % of the total

serum Ig. The isotypes had the same Mr of ~70,000. In rainbow trout at least two Ig isotypes are present; a MAb against Ig reacted with about 30 % of the total serum Ig in an ELISA (Sanchez *et al.*, 1989). Also for rainbow trout the Ig isotypes were not different in mass. Using MAbs to Atlantic salmon Ig (Killie *et al.*, 1991, Falk *et al.*, 1991) and rainbow trout Ig (Falk *et al.*, 1991) it was concluded that also Atlantic salmon and brown trout may have more than one Ig isotype. Also, molecular analysis of the Ig gene complex in channel catfish and Atlantic salmon indicates the presence of more than one CH gene which give possibilities for C-region isotypic variation (Ghaffari & Lobb, 1989a; Hordvik *et al.*, 1992).

The present study shows that the distribution of the three B cell subpopulations is different in spleen, pronephros and blood and moreover, changes during ontogeny. This rules out the possibility of a variation in the glycosylation of the heavy chain, which might alter the antigenic properties of the Ig molecule as discussed by others (Killie *et al.*, 1991; Ghaffari & Lobb, 1989b), because it seems unlikely that the glycosylation of immunoglobulin will differ between young and old fish.

At the moment it is still unknown whether the WCI 4 and WCI 12 recognise epitopes in the V-region or C-region of the Ig molecule. Also for the other fish species mentioned above the antigenic differences between the Ig isotypes are not yet localised at certain regions of the molecule. Killie *et al.* (1991) rules out the possibility of idiotypic variation detected by their MAb because of the relative high percentages (5-20 %) of positive lymphocytes. However, in human the V-regions can be divided in groups and subgroups with specific antigenic determinants and also for channel catfish six VH gene families are described (Ghaffari & Lobb, 1991; Warr *et al.*, 1991). If MAbs will recognise such a constant part of the V-region the reaction could be confused with isotypic variation of the C-region. In this study the percentages of WCI 4^+ and WCI 12^+ B cells are extremely high (sometimes > 60 % of the B cells) and moreover show a shift during ontogeny. Therefore, the immunoglobulin heterogeneity described here probably can not be explained by V-region differences but seems to be isotypic variation of Ig.

In mammals Ig types (IgM, IgD, IgG, IgE and IgA) have their own biological functions (Male *et al.*, 1987). However, differences in functions between IgG isotypes are

not very well established yet (Male *et al.*, 1987). In carp evidence for structural and functional differences between skin mucus Ig (mIg) and serum Ig was described (Rombout *et al.*, 1993). Additional immunofluorescence staining for flow cytometry showed that B cells expressing mIg were a subpopulation of the WCI 4^- 12^+ B cells and not of the WCI 4^+ 12^+ or WCI 4^+ 12^- B cell subpopulations (unpublished results). Thus, two or three different Ig isotypes present in serum and one isotype mainly present in mucus can be distinguished in carp. Although the function of the mIg type is obvious, functional differences between the serum Ig isotypes have still to be determined. The fact that the three B cell subpopulations described in this study show changes during ontogeny and are not equally distributed in different organs, might indicate functional differences. Functional significance of more than one isotype for teleost fish has been shown for catfish (Lobb & Olson, 1988), rainbow trout (Sanchez *et al.*, 1989) and Atlantic salmon (Killie *et al.*, 1991); a shift in Ig isotype proportion has been described during humoral immune responses in these fish species.

It can be concluded that carp show B cell and Ig heterogeneity, which can be demonstrated with MAbs. The presence of Ig isotypes in carp might be confirmed in the future by molecular analysis of the immunoglobulin gene complex.

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The production of monoclonal antibodies against immunoglobulin-negative lymphoid cells of carp (*Cyprinus carpio* L.)

J.C.E. Koumans-van Diepen, P.M. Emmer, A.G.P Oomens, N. Taverne,
J.J. Taverne-Thiele & J.H.W.M. Rombout

Department of Experimental Animal Morphology and Cell Biology, Agricultural University,
P.O. Box 338, 6700 AH Wageningen, The Netherlands.

Key words: fish; monoclonal antibody; T cell; cyclophosphamide; thrombocytes

Abstract

Several immunisation and selection methods were used to produce monoclonal antibodies (MAbs) against immunoglobulin-negative (Ig^-) lymphocytes. Immunisation of mice with peripheral blood leucocytes (PBL) and selection of hybridoma culture supernatants with fluorescence microscopy revealed only MAbs reactive with all leucocytes. Earlier immunisations with thymocytes and selection with ELISA had revealed comparable MAbs which recognised common carbohydrate determinants present on many molecules and all leucocytes. After immunisation with enriched sIg^- pronephros lymphoid cells, still 98 % of the MAbs reacted with all leucocytes and 1.5 % reacted with B cells and plasma cells. Two MAbs (WCL 1 and 2) were immunoreactive with around 17 % of the sIg^- pronephros lymphoid cells, 0.5 % of the sIg^- PBL and 30 % of the thymocytes. However, 17 % of pronephros B cells and neutrophilic granulocytes and 55 % of blood B cells were also stained. The recognised antigenic determinant again appeared to be of carbohydrate nature and the proportion of cells expressing the carbohydrate determinants varied strongly dependent on the carp strain used. Moreover, LPS-stimulated B cells and PHA-stimulated sIg^- PBL showed an increased proportion of cells having these determinants. In a third MAb procedure, mice were tolerised against the immunodominant carbohydrate determinants by means of cyclophosphamide. Although tolerance was not complete, immunisation with carp thymocytes resulted in 6 % of hybridomas producing MAbs positive with 45 % of the pronephros lymphoid cells; unfortunately the reactivity of all MAbs was lost. Finally, mice were immunised with membrane lysates of sIg^- PBL. Two MAbs (WCL 6 and 8) recognised thrombocytes in blood and spleen but not in pronephros, thymus and hindgut. Only 13 % of the hybridomas produced MAbs reactive with all leucocytes and 85 % showed no reaction.

Introduction

The immune system of teleost fish shows an obvious morphological and functional resemblance with the mammalian immune system. A humoral and cellular systemic immune response can be distinguished. The humoral immune response has been demonstrated by the appearance of antibody-secreting cells and the production of antibodies (Lamers *et al.*, 1985; Wilson & Warr, 1992). The cellular immune response has been demonstrated by the mixed leucocyte reaction (Caspi & Avtalion, 1984; Kaastrup *et al.*, 1988) or allograft rejection (Rijkers & Van Muiswinkel, 1977; Tatner & Manning, 1983). The presence of specific antibodies in mucus and bile and not in serum after intestinal immunisation (Rombout *et al.*, 1986) indicate the presence of a mucosal immune system. These different aspects of the immune system implies the presence of leucocytes which are involved in these immune functions. Lymphoid cells, different types of granulocytes and mononuclear phagocytes can be identified by electron microscopic examination (Bielek, 1981; Cenini, 1984; Temmink & Bayne, 1987; Rowley *et al.*, 1988). However, a clear distinction in B and T cells can not be made upon morphological criteria. Therefore, monoclonal antibodies (MAbs) have been produced against serum immunoglobulin (Ig) of several teleost fish species: catfish (*Ictalurus punctatus*, Lobb & Clem, 1982; Ainsworth *et al.*, 1990), carp (*Cyprinus carpio* L., Secombes *et al.*, 1983), rainbow trout (*Oncorynchus mykiss*, DeLuca *et al.*, 1983; Thuvander *et al.*, 1990), Atlantic salmon (*Salmo salar*, Killie *et al.*, 1991; Falk *et al.*, 1991), Atlantic cod (*Gadus morhua*, Pilström & Petersson, 1991; Israelsson *et al.*, 1991) and sea bream (*Sparus aurata*, Navarro *et al.*, 1993). By means of these MAbs, a distinction between Ig-positive (Ig^+) and Ig-negative (Ig^-) lymphoid cells can be made. Surface Ig^+ (sIg^+) lymphocytes are accepted as B cells but Ig^- lymphoid cells may not only represent T cells but also null cells. Efforts have been undertaken to develop T cell specific MAbs for carp and catfish. The MAbs produced against carp thymocytes (Secombes *et al.*, 1983) appeared to be reactive with determinants present at all leucocytes (Rombout *et al.*, 1990; Van Diepen *et al.*, 1991ab). MAbs to channel catfish sIg^- lymphocytes were obtained by Miller *et al.*

(1987) and Ainsworth *et al.* (1990). The immunopositive cells showed helper activity during *in vitro* antibody reponses against the T-dependent antigen TNP-KLH (Miller *et al.*, 1987). Unfortunately, the MAb produced by Miller *et al.* (1987) reacted not only with sIg^- lymphocytes but also with most neutrophilic granulocytes and thrombocytes. The MAbs produced by Ainsworth *et al.* (1990) did not react with granulocytes but were not fully characterised. It can be concluded that research on fish immune system functions would benefit a lot from the availability of T cell specific MAbs.

In this study several strategies were followed in order to produce such antibodies for carp. It has been suggested that common carbohydrate moieties present at the leucocyte membrane probably play a disturbing role because of their strong antigenicity in mouse (Rombout *et al.*, 1990). Therefore, one of the procedures described here include efforts to induce tolerance to these common determinants by cyclophosphamide treatment. Mice were immunised with peripheral blood leucocytes (PBL), enriched sIg^- pronephros lymphoid cells or thymocytes. The use of isolated molecules was shown to be successful for obtaining anti-Ig MAbs (Secombes *et al.*, 1983) and the production of a polyclonal anti-serum reactive with T cells of the Mexican Axolotl (Kerfourn *et al.*, 1992). Therefore, mice were also immunised with membrane lysates of sIg^- PBL.

Materials and methods

Fish

Two different strains of carp bred at the central fish facilities 'De Haar vissen' were used: outbred carp and the F1 of homozygous cloned carp (E20 x E6; Komen *et al.*, 1990). The adult animals were kept at 21.5 °C $\pm$ 0.5 °C in a recirculating system. The filtered water was disinfected by UV light. The carp were fed pelleted food (K30; Trouw & Co., Putten, The Netherlands) with a ration of 2 % of the body weight per day.

Antigen preparation

Surface Ig^- carp pronephros lymphoid cells were prepared as follows: pronephros lymphoid cells were isolated by Percoll separation (Koumans-van Diepen *et al.*, 1993a) and labelled with anti-carp Ig monoclonal antibody (WCI 12; Secombes *et al.*, 1983; Koumans-van Diepen *et al.*, 1993b) and fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig antibodies (RAM-FITC; 1:100; Dakopatts, Glostrup, Denmark) and kept in cRPMI (RPMI + 10 % double-distilled water) + 5 % foetal calf serum. A > 95 % pure cell suspension of sIg^- carp lymphoid cells was obtained by fluorescence activated cell sorting with a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). Small, smooth and fluorescence-negative cells were gated, isolated, washed twice in PBS and stored at -20 °C.

Peripheral blood leucocytes (PBL) were isolated as described (Koumans-van Diepen *et al.*, 1993c) washed twice in PBS and stored at -20 °C.

Surface Ig^+ PBL were obtained by magnetic cell sorting (MACS; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) of isolated PBL. The cells were prepared for MACS-separation as follows: isolated PBL in TBS^+ (18.18 mM Tris/HCl pH 7.4, 0.82 % NaCl, 0.73 mM $MgCl_2$, 0.18 mM $CaCl_2$, 1 % BSA, 0.1 % NaN_3) were incubated with biotinylated WCI 12 for 30 min at 0 °C. Subsequently the cells were washed twice, incubated in FITC-conjugated avidin (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) in TBS^+ without BSA (may contain biotin) for 15 min at 0 °C, washed in TBS^+ without BSA, incubated in TBS^+ without BSA + 1 % biotinylated superparamagnetic microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) for 5 min and washed in TBS^+. The MACS column (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) was cooled by passing through some volumes of ice-cold TBS^+. Subsequently, the cell suspensions were pipetted onto the column and separated as described by Miltenyi *et al.* (1990). The separated cells were analysed by flow cytometry and the sIg^+ cells were > 90 % pure. The cells were washed twice in PBS and stored at -20 °C.

Thymocytes were obtained by sqeezing dissected thymi through a nylon gauze while

adding PBS. Cells were washed twice in PBS and stored at -20 °C.

Membrane lysates of sIg^- PBL were obtained from MACS-separated sIg^- PBL (see above) as follows. The sIg^- PBL (1×10^8) were centrifuged for 10 min at 680 x g and 4 °C and resuspended in 1 ml TBS^+ without BSA + 0.1 mM PMSF (phenyl methyl sulphonyl fluoride) + 1 mM ethylmaleïnide. The cells were fragmented in a small Potter tube for 4 min on ice. Nuclei were separated by centrifuging for 5 min at 160 x g and 4 °C. The supernatant was centrifuged for 1 h at 100,000 x g at 4 °C. The membranes (pellet) were washed in TBS^+ without BSA and centrifuged again for 1 h at 100,000 x g at 4 °C. Membranes were incubated with TBS^+ without BSA + 1 % CHAPS (3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulphonate) for 1 h at 4 °C and centrifuged for 1 h at 100,000 x g and 4 °C. The protein concentration in the supernatant was measured at 280 nm and the supernatant was stored at - 20 °C until use.

Tolerisation and immunisation procedures of Balb/c mice

A. Mice were intraperitoneally (i.p.) immunised with high numbers of PBL (Table I).

Table I. Immunisation schedule

day[a] mouse #	0	31	70	73
1-2	PBL[b]	PBL[b]	PBL[b]	fusion # 1

[a] at day 9 and 41 mice were bled and anti-sera were prepared

[b] i.p. immunisation with 5×10^7 carp PBL in 100 µl PBS

B. Mice were i.p. immunised with sIg^- pronephros lymphoid cells (Table II).

Table II. Immunisation schedule

day[a] mouse #	0	28	101	104
3-5	100 µl sIg^- [b]	300 µl sIg^- [b]	300 µl sIg^- [c]	fusion # 3

[a] at day 13 and 39 mice were bled and anti-sera were prepared

[b] i.p. immunisation with carp sIg^- pronephros lymphoid cells: 1 x 10^7/ml PBS

[c] 15 x 10^6 sIg^- pronephros lymphoid cells/300 µl PBS

C. Cyclophosphamide treatment was used in order to destroy lymphocytes of mice stimulated by Ig and other common (carbohydrate) antigens present on carp B cells. The method described by Matthew & Sandrock (1987) was used. Mice were i.p. injected with sIg^+ PBL followed by cyclophosphamide and subsequently immunised with thymocytes (Table III).

D. Membrane lysates of sIg^- PBL were used for i.p. immunisation of mice (Table IV).

Fusion

Spleen cells from the hyperimmunised Balb/c mice were fused with SP2/0-Ag-14 myeloma cells (Schullman *et al.*, 1978) according the hybridoma technique described by Köhler & Milstein (1975). A detailed description of the followed procedure is given by Schots *et al.* (1992). The supernatant of the hybridoma cultures was screened for specific antibodies after 10-14 days.

Table III. Tolerance induction by cyclophosphamide treatment and immunisation schedule

day[a] mouse #	0 (birth)	14	28	42	64	67
6-7	200 µl B[b]	200 µl B[b]	200 µl B[b]	500 µl Th[c]	500 µl Th[c]	fusion
8-9 control 1	200 µl B[d]	200 µl B[d]	200 µl B[d]	200 µl B[d]		
10 control 2				500 µl Th[c]		

[a] at day 10, 24, 38, and 52 mice were bled and (anti-) sera were prepared

[b] i.p. immunisation with B cells = sIg^+ PBL (5 x 10^7/ml PBS) followed after 10 min, 24 h and 48 h by i.p. injections with cyclophosphamide in PBS (100 mg/kg)

[c] i.p. immunisation with thymocytes (7 x 10^7/ml PBS)

Table IV. Immunisation of mice with membrane lysates of sIg^- PBL

day mouse #	0	56	91	94
11-12	200 µl[a]	150 µl[b]	100 µl[b]	fusion # 11

[a] in Freund's incomplete adjuvant (1:1), 0.5 mg protein/ml

[b] 1 mg protein/ml lysate

Screening of MAbs

In all cases a fluorescence microscopic screening method was used. Preparations of lymphocytes and granulocytes from pronephros or of enriched sIg^+ and sIg^- PBL were made onto 12-well microprint stock slides (Nutacon b.v., Hoofddorp, The Netherlands).

Pronephros lymphoid cells and neutrophilic granulocytes were enriched in one fraction by Percoll separation (Koumans-van Diepen *et al.*, 1993a) and diluted in TBS^+ without BSA to 1.5 x 10^6 cells/ml. Suspensions of sIg^+ or sIg^- PBL were prepared by MACS separation as described above. Multi-well slides were coated with 0.075 % poly-L-lysine (PLL) by incubation of each well with 30 μl PLL-solution for 1 h at room temperature (RT). Slides were rinsed thrice in double-distilled water and dried. Then, 35 μl of cell suspension was added to each well and allowed to adhere for 1 h at 4 °C. The liquid was removed by flicking and a layer of filter paper with 12 holes was attached onto the slide. The slides were centrifuged in a cytocentrifuge (Nordic Immunological Laboratories b.v., Tilburg, The Netherlands), dried quick with cold air and fixed for 12 min in ice-cold ethanol-acetic acid (19:1). After fixation slides were rinsed trice in TBS^+ (without BSA), once in aqua bidest and finally dried quick with cold air. The slides were stored over night in an exicator at 4 °C. Antibody-containing hybridoma culture supernatants (50 μl) were applied onto the slides for 45 min at RT. Subsequently, the slides were rinsed in TBS^+ and incubated with FITC- or tetramethyl rodamine isothiocyanate (TRITC)-conjugated RAM (1:50; Dakopatts, Glostrup, Denmark) for 30 min at RT. The preparations were rinsed four times in TBS^+ and embedded in 0.1 % (w/v) paraphenylenediamine containing PBS/glycerine 1:9 (v/v) solution and studied under a fluorescence microscope (Nikon Microphot FXA).

Isotyping of the MAbs

Determination of the MAb isotypes was performed by ELISA as described by Schots *et al.* (1992).

Characterisation of MAbs

Several techniques were applied in order to characterise the reaction pattern of interesting MAbs.

Leucocytes from blood, spleen, thymus, pronephros and hindgut (Koumans-van Diepen *et al.*, 1990d) labelled with MAb and a second fluorescence conjugated antibody were analysed by fluorescence microscopy and/or flow cytometry (Van Diepen *et al.*, 1991a; Koumans-van Diepen *et al.*, 1993a). Double-labelling or additional staining with WCI 12 was performed for flow cytometric analysis (Koumans-van Diepen *et al.*, 1993b) before and after PHA or LPS stimulation (Koumans-van Diepen *et al.*. 1993c). Electron microscopic identification of reactive cells was performed by immunogold labelling (Van Diepen *et al.*, 1991) of PBL and spleen suspensions. Periodate treatment of cytocentrifuge preparations of carp leucocytes and cryo-sections of lymphoid organs gave information about a possible carbohydrate nature of the antigenic determinant (Koumans-van Diepen *et al.*, 1993b). Information about the relative molecular mass (Mr) of the reactive molecule(s) was obtained by Western blotting of PBL lysates or immunoprecipitation of ^{125}I-labelled PBL membrane proteins (Koumans-van Diepen *et al.*, 1993b).

Results

Tolerisation, immunisation and selection procedures

The results from the four performed tolerisation and/or immunisation procedures are summarised in Table V.

Screening of clones obtained after immunisation of mouse with PBL (procedure **A**) revealed that all obtained MAbs were reactive with all leucocytes. Immunisation with sIg^- lymphoid cells from pronephros (procedure **B**) showed 98 % of the clones reactive with all leucocytes.

Table V. Results of tolerisation and/or immunisation procedures

procedure / mouse #	tolerance induction	tolerogen	reactivity preimmune sera	antigen	percentages of clones reactive with: part of leucocytes	all leucocytes	none	no. of clones
A # 1	-	-	N.D.	PBL	0	100	0	1472
B # 3	-	-	*negative*	sIg^-	2*	98	0	680
C # 6-7	Cp	B cells	neglectable	Th	6	70	24	700
D # 11	-	-	N.D.	M.L. sIg^- PBL	2	13	85	280

*** 1.5 % was only reactive with B cells and plasma cells**

abbreviations: B cells, sIg^+ PBL; Cp, cyclophosphamide; M.L., membrane lysates; N.D., not done; PBL, peripheral blood leucocytes; sIg^-, surface Ig-negative pronephros lymphoid cells; Th, thymocytes

Only a minor part (2 %) was reactive with a limited number of cells which reaction pattern could be divided in two groups:

1. immunoreactive with B cells and plasma cells (1.5 %)
2. immunoreactive with other leucocytes (0.5 %)

Sera obtained from cyclophosphamide-treated mice (procedure C) showed a neglectable reaction with leucocytes compared to sera from control mice (# 8-9; Figure 1). On the other hand, after first immunisation with thymocytes no difference was found between the reaction

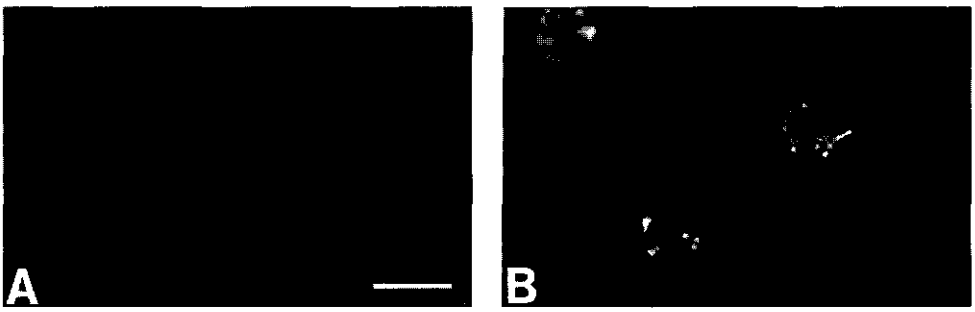

Figure 1. Fluorescence micrographs of cytocentrifuge preparations of pronephros leucocytes stained with sera from **A.** cyclophosphamide-treated mice (# 6-7) and **B.** control mice (# 8-9). bar = 20 μm

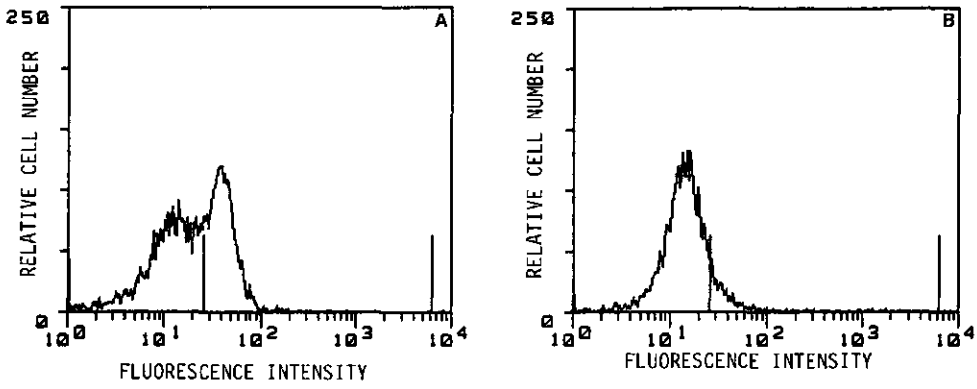

Figure 2. **Fluorescence histogram of A. gated pronephros lymphoid cells and B. gated pronephros granulocytes, labelled with culture supernatant from one of the interesting hybridomas obtained by procedure C. The region between the two markers indicate the immunopositive cells.**

pattern of sera from cyclophosphamide-treated mice (# 6-7) and control mouse (# 10); all pronephros leucocytes and thymocytes in cytocentrifuge preparations were reactive. However, compared to procedures **A** and **B** a higher percentage of clones (6 %) reacted with a part of the leucocytes and a much higher proportion of clones (24 %) did not show any reaction. Flow cytometric analysis demonstrated that the promising MAbs were reactive with 45 % of the gated lymphoid pronephros cells, while gated granulocytes (large, rough cells with a high cytoplasm-nucleus ratio) only showed a background reaction (Figure 2). Unfortunately the reactivity of these MAbs was lost. Immunisation of mouse with membrane lysates of sIg^- PBL (procedure **D**) revealed a limited percentage (13 %) of clones which produced MAbs reactive with all leucocytes. Only 2 % reacted with a subpopulation of sIg^- PBL and not with sIg^+ PBL.

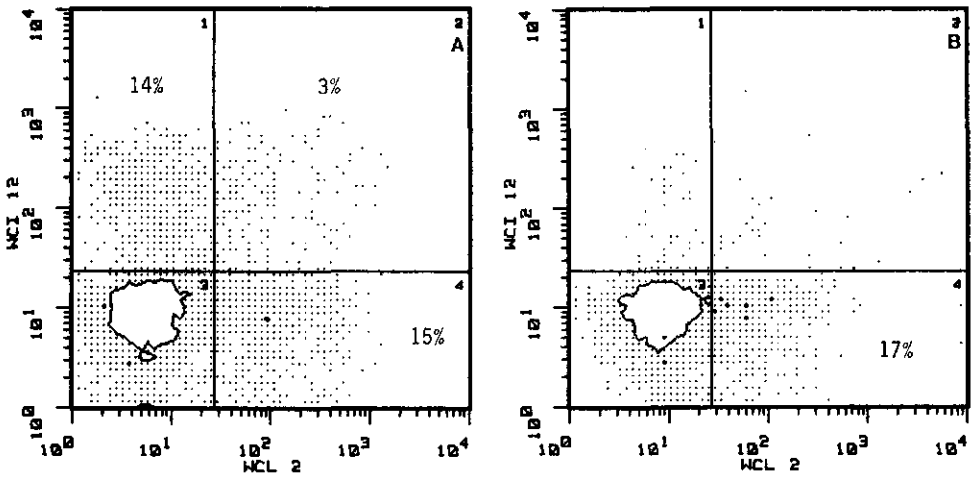

Figure 3. Contour graphs of WCL 2- and WCI 12-double-stained pronephros cells. **A.** Gated lymphoid cells. **B.** Gated neutrophilic granulocytes.

Characterisation of the reaction pattern of MAbs

Two interesting MAbs obtained by procedure **B**, WCL 1 of the IgM class and WCL 2 of the IgG3 class recognised the same cell population as estimated by flow cytometry. Therefore, only the reaction pattern of WCL 2 was further characterised. WCL 2 appeared to be reactive with around 17 % of sIg^- lymphoid cells, of **B** cells and of neutrophilic granulocytes from pronephros (Figure 3) and with 30 % of the thymocytes as estimated by flow cytometry and electron microscopy.

Table VI. Percentages of sIg^+ or sIg^- carp PBL (n=2) which are WCL 2^+ before (day 0) and after PHA or LPS stimulation (day 4)

	day 0	+ PHA (day 4)	+ LPS (day 4)
sIg^+ cells	62 / 48	40 / 55	74 / 67
sIg^- cells	0 / 1	25 / 29	0 / 0

For PBL other percentages were obtained: about 0.5 % of sIg^- and 55 % of sIg^+ PBL were WCL 2^+ (Table VI). It was estimated that only a part of the individuals from the outbred carp showed this limited reaction, in other individuals WCL 2 was positive with all leucocytes. However, this variation in reaction pattern was not found within the F1 of homozygous cloned carp; all individuals showed the same specific reaction. Therefore, these carp were used for further characterisation of the Mab. The antigenic determinant recognised by WCL 2 was periodate sensitive because no labelling was found after periodate treatment of cytocentrifuge preparations or cryo-sections of pronephros. Western blotting of PBL lysates and immunoprecipitation of ^{125}I-labelled PBL membrane proteins revealed many WCL 2 positive bands (Figure 4) independent the carp strain used. Double staining of mitogen-stimulated PBL with WCL 2 and WCI 12 for flow cytometry showed that the percentage of sIg^+ cells immunoreactive with WCL 2 was increased in LPS-stimulated cultures, while the percentage of sIg^- cells immunoreactive with WCL 2 was increased in PHA-stimulated cultures (Table VI).

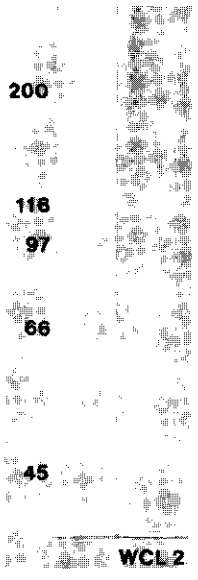

Figure 4. **Western blot of PBL lysates showing many WCL 2^+ bands.**

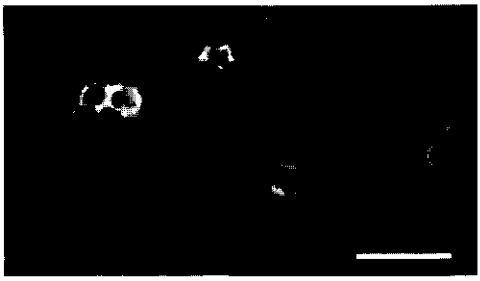

Figure 5. **Fluorescence micrograph of MACS-enriched sIg^- PBL labelled with WCL 6. Note spindle-shaped fluorescent cells. No reaction was found with sIg^+ cells. bar = 40 μm**

Five MAbs obtained by procedure **D**, WCL 6, 7, 8, 9 and 10 were reactive with a part of the sIg^- leucocytes, which were frequently spindle-shaped (Figure 5). Western blotting of PBL lysates with WCL 7, 9 or 10 revealed many positive bands. The reactivity of WCL 7, 9 or 10 was abolished by periodate treatment of cytocentrifuge preparations. Consequently, these Mabs were not investigated in more detail. Western blotting of PBL lysates with WCL 6 or 8 yielded a slight positive band with a Mr of ~40,000. The reaction with WCL 6 or 8 on cytocentrifuge preparations was not periodate sensitive. Additional staining of PBL with both WCL 6 and 8 showed that these MAbs reacted with the same cells. Flow cytometric analysis of PBL only showed a positive reaction with WCL 6 (IgG1 class) but not with WCL 8. The percentages of WCL 6^+ cells in isolated PBL suspensions were 5 - 50 % (n = 8). Percoll gradient (density 1.07) enriched spleen leucocyte suspensions contained 5-17 % WCL 6^+ cells (n = 3). Double staining of PBL or spleen leucocytes with WCI 12 revealed three separate cell populations: WCL 6^+ WCI 12^-, WCL 6^- WCI 12^+ and WCL 6^- 12^-, double positive cells were absent or rare (Figure 6). Suspensions from thymus, pronephros and hindgut hardly contained WCL 6^+ cells (n = 3). Both WCL 6^+ 12^- and WCL 6^- WCI 12^+ cells had a low forward scatter (FSC) and side scatter (SSC) but the WCL 6^+ WCI 12^- cells tended to have a slightly higher FSC. Immunogold labelling of PBL and spleen leucocytes was performed. WCL 6^+ PBL could be

characterised as cells with a more or less pycnotic irregular nucleus and electron dense chromatin. A variable number of small granula was present in the cytoplasm of these cells (Figure 7). In contrast to PBL, WCL 6^+ spleen cells frequently had a normal lymphoid nucleus and less granular inclusions (Figure 8), but cells resembling WCL 6^+ PBL were found as well.

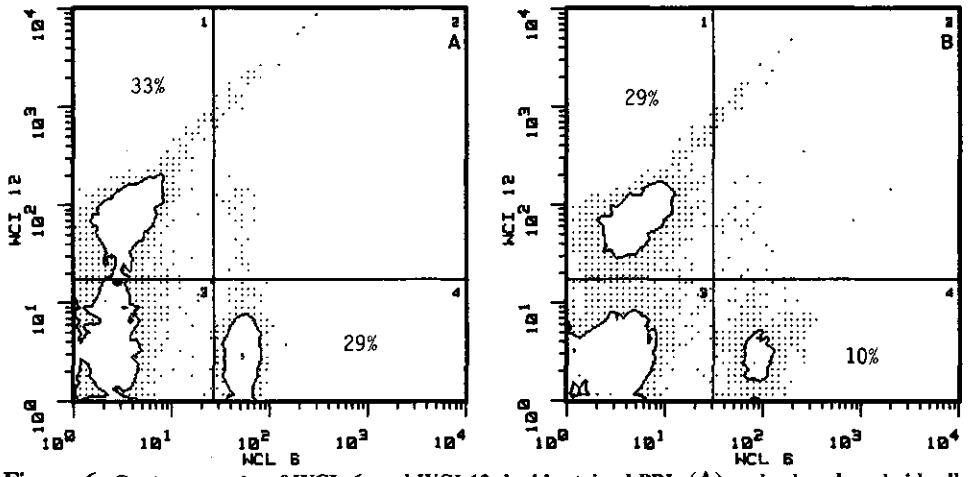

Figure 6. Contour graphs of WCL 6- and WCI 12-double-stained PBL (A) and spleen lymphoid cells (B).

Discussion

In this study efforts were undertaken to produce MAbs specific for Ig^- carp lymphocytes. It was known from an earlier attempt (Secombes et al., 1983) that immunisation of mice with carp thymocytes revealed only MAbs (WCTs) against common leucocyte determinants (Rombout *et al.*, 1990; Van Diepen *et al.*, 1991ab). The carbohydrate nature of these common determinants was confirmed by periodate

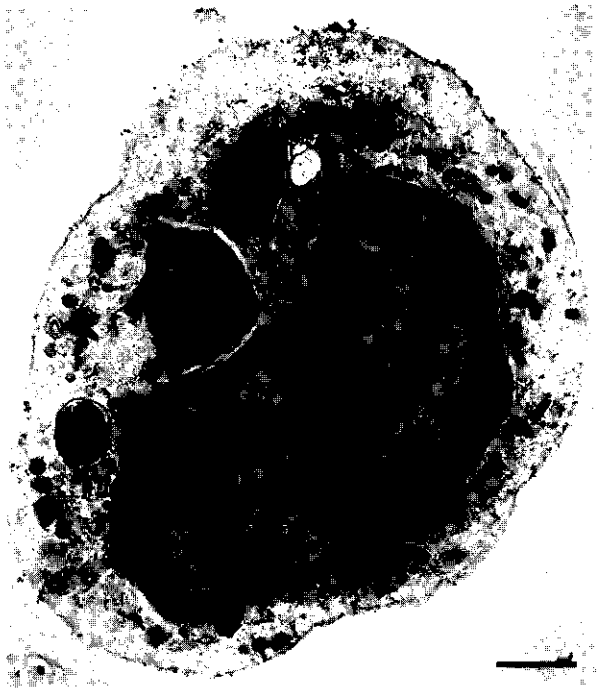

Figure 7. Electron micrograph of immunogold-stained (G15) WCL 6^+ PBL. Note indented pycnotic nucleus, electron dense chromatin and granular inclusions. bar = 0.5 μm

sensitivity of the antigen and by the fact that WCTs stained many bands in Western blots of PBL lysates (unpublished results). In this study, tolerance induction in mice to those common determinants was investigated by cyclophosphamide treatment. Moreover, immunisation procedures with PBL and more purified antigen (isolated sIg^- pronephros lymphoid cells or membrane lysates of sIg^- PBL) were performed. In contrast to earlier procedures (Secombes *et al.*, 1983), in which MAbs were selected using an ELISA, screening of hybridoma culture supernatants was done on cytocentrifuge preparations of lymphocyte- and granulocyte-containing suspensions from pronephros or of MACS-separated sIg^+ and sIg^- PBL. These screening methods comprise an internal control of cells which should be negative

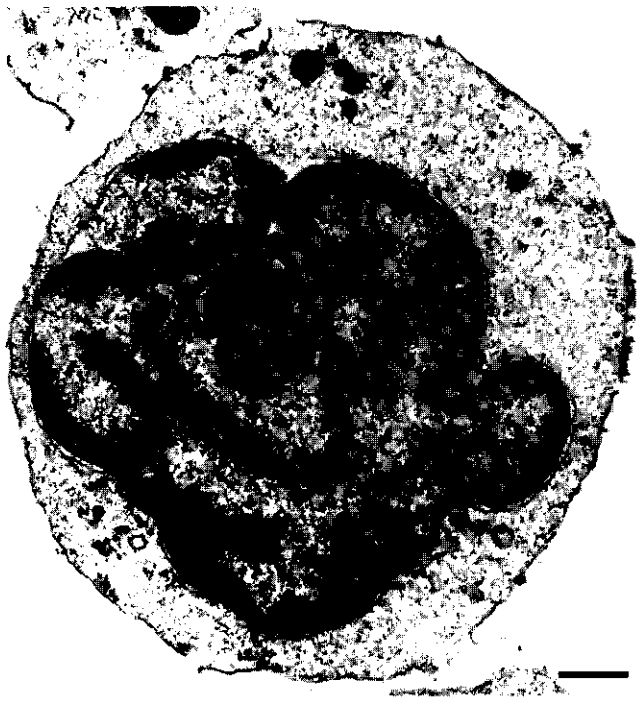

Figure 8. *Electron micrograph of immunogold-stained (G15) WCL 6⁺ spleen cell with a lymphoid nucleus and less granular inclusions.* bar = 0.5 μm

(granulocytes or sIg^+ PBL, respectively) in order to exclude MAbs against common determinants present on all cell types. Granulocytes were distinguished from lymphocytes by their size and shape, while MACS-separated sIg^+ PBL showed green (FITC) fluorescence.

None of the monoclonal antibody procedures described in this study revealed specific MAbs to carp Ig^- lymphocytes. All MAbs derived from mouse immunised with PBL and 98 % of the MAbs obtained from the mouse immunised with sIg^- pronephros lymphoid cells were reactive with carbohydrate determinants present on all carp leucocytes. These results sustain the idea that the common (carbohydrate) determinants on carp leucocytes are immunodominant as suggested earlier by Rombout *et al.* (1990).

The tolerisation of mice to common leucocyte determinants seems to be rather successful by cyclophosphamide treatment of mice after injection with the tolerogen. Neonatal and high dose tolerance induction have also been tried, but immunisation instead of tolerisation was obtained (unpublished results). Cyclophosphamide treatment of mice strongly reduced the immune response; antisera from repeatedly cyclophosphamide treated mice showed only a very weak reaction while antisera from control mice were strongly positive with all leucocytes. Although the response is strongly reduced by cyclophosphamide treatment, it is not completely abolished as described by Matthew & Sandrock (1987). Probably as a consequence, the sera of the tolerised mice were reactive with all leucocytes after first immunisation with thymocytes. On the other hand, a different panel of MAbs was produced compared to non-tolerised mice. Only 70 % was reactive with all leucocytes while 24 % did not react at all. A minority of the hybridoma culture supernatants (6 %) showed a reaction with 45 % of the pronephros lymphoid cells, which is clearly more than the 2 % of specific MAbs obtained by convential immunisation with sIg^- pronephros cells. Unfortunately, because of till now unresolved reasons, the reactivity of these MAbs was lost during the procedure. If this instability was not caused by the cyclophosphamide treatment, it is worth while to repeat this procedure, as it revealed completely different antibodies. However, the immunisation of mouse with isolated membrane lysates from sIg^- PBL also appeared to be promising. In this procedure a minority of 13 % of hybridoma culture supernatants was reactive with all leucocytes, a high percentage (85 %) did not show any reaction, while 2 % of the MAbs gave an interesting reaction pattern.

A few MAbs were further characterised. The antigenic determinant recognised by WCL 2 (procedure **B**) has probably a carbohydrate nature, because it is periodate sensitive and shows many positive bands in Western blotting and immunoprecipitation of PBL lysates. The expression of the antigenic determinant recognised by WCL 2 seems to be dependent on the degree of activation of the carp leucocytes: sIg^+ PBL stimulated with LPS and sIg^- PBL stimulated with PHA showed a higher proportion of WCL 2^+ cells compared with unstimulated cultures, sIg^+ cells cultured with PHA or sIg^- cells cultured with LPS. LPS and PHA have been shown to be mitogens for sIg^+ and sIg^- cells of carp, respectively (Koumans-

van Diepen *et al.*, 1993c). Expression of the WCL 2-immunoreactive determinant on lymphoid cells appears also to be organ-dependent. In pronephros (F1 of homozygous cloned carp) 17 % of the B lymphocytes and sIg^- lymphoid cells were WCL 2^+ while in blood 48-62 % of the B cells and 0-1 % of the sIg^- cells were WCL 2^+. However, besides indications that the expression of the antigenic determinant recognised by WCL 2 is enhanced on activated cells, a functional significance of the WCL 2^+ molecules is not clear at the moment. The fact that the expression of the WCL 2^+ determinant is also dependent on the carp strain used, makes it even more difficult to give suggestions for the function of these molecules.

WCL 6 and 8 (procedure D) recognise the same cell population as was estimated by additional staining of cytocentrifuge preparations. Moreover, they seem to recognise the same molecule with a Mr of ~40,000. In contrast to WCL 6, WCL 8 was not reactive with living cells as observed by flow cytometry. Probably, the antigenic determinant reactive with WCL 8 is located at an intracellular or intramembraneous part of the molecule, whereas WCL 6 seems to recognise an extracellular determinant on the same molecule. Cells recognised by WCL 6 and 8 most likely represent thrombocytes because of:

1. their abundancy in blood (Ellis, 1977),
2. their spindle shape in cytocentrifuge slides (Ellis, 1977),
3. their pycnotic nuclei and granular inclusions as observed at the ultrastructural level (Rowley *et al.*, 1988).

Their variable percentage found in PBL suspensions (5 - 50 %) might be explained by lability of the thrombocytes in the procedures used. The WCL 6^+ cells with a normal lymphoid nucleus found in spleen may be considered as progenitor cells of the thrombocytes and hence carp spleen seems to be involved in thrombopoiesis.

It can be concluded that following conventional procedures MAbs reactive with carbohydrate determinants present at all carp leucocytes are produced. Apparently the glycocalyx of carp leucocytes is very immunodominant for mice. Tolerance induction in mice to these common determinants turned out to be rather difficult. Nevertheless the cyclophosphamide method revealed the highest percentage of hybridomas specific for a

limited number of leucocytes. The use of membrane lysates from sIg^- PBL resulted in a specific thrombocyte marker. This marker can be used in the future to deplete the suspension of sIg^- PBL from thrombocytes, in order to repeat the MAb procedure with membrane lysates from better purified sIg^- PBL.

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Summary and conclusions

Several leucocyte populations and different immune functions have been described for fish. However, a correlation between cell type and cell function is often hard to proof. This is mainly due to an apparent lack of suitable methods and tools for the identification, separation and functional characterisation of fish leucocytes. In mammals, monoclonal antibodies (MAbs) are the major tools for leucocyte characterisation. MAbs against surface molecules of fish leucocytes are scarce and most of them appeared to be species-specific. A panel of MAbs against carp serum immunoglobulin (Ig), WCIs, or carp thymocytes (T), WCTs, has been developed at this department. In earlier studies, three groups of MAbs could be distinguished based upon their reactivity in enzyme-linked immunosorbent assays: Ig^+ T^-, Ig^+ T^+ and Ig^- T^+.

In this study, the reaction patterns of WCIs and WCTs were analysed in more detail and promising MAbs were used for the characterisation of carp leucocytes. For this aim, a variety of techniques was adapted for the use in carp. Several procedures were tested for obtaining new MAbs specific for Ig^- lymphoid cells, because none of the existing MAbs appeared to be specific for T cells.

Unfortunately, all WCTs and Ig^+ T^+ WCIs react with common carbohydrate determinants present on all leucocytes and Ig (**Chapters 2, 6, 7**) and hence these MAbs could not be used for further functional investigations. The Ig^+ T^- WCIs recognise protein determinants at the heavy chain of Ig. Consequently, B lymphocytes, plasma cells and Ig-binding cells could be identified using these MAbs (**Chapters 2, 4, 6**). Ig molecules are found in clusters at the cell membrane of B cells and plasma cells (**Chapter 2**). In contrast to mammalian plasma cells, most plasma cells in carp still have Ig at their surface (**Chapter 3**).

In general, the percentage of B cells and plasma cells showed an increase during ontogeny, as studied by fluorescence microscopy and flow cytometry (**Chapter 3**). The percentage of plasma cells reached a plateau at about 3 months of age which might indicate

maturation of the humoral immune system by that time. However, a significant increase in the percentage of B cells was observed till 8 months of age. Therefore, it can be suggested that a full development of the (humoral) immune system of carp needs at least 8 months (21-22 °C).

Ig-binding by leucocytes from pronephros and hindgut was demonstrated with an immunofluorescence method and erythrocyte-antibody (EA) rosette method (**Chapter 4**). The Ig-binding cells were identified with similar immunogold methods. In pronephros only a few monocyte-like cells and some lymphoid cells showed Ig-binding while macrophages and neutrophilic granulocytes did not. On the contrary, most macrophages and some lymphoid cells from hindgut were able to bind Ig. These results suggest that Fc-like receptors are probably important for effective phagocytosis by gut macrophages. Other forms of antigen opsonisation (e.g. complement) may play a role in phagocytosis by pronephros macrophages and neutrophilic granulocytes.

In vitro stimulation of surface Ig^+ (sIg^+) and sIg^- peripheral blood leucocytes (PBL) with phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) was studied by the following methods (**Chapter 5**):

1. Incorporation of ^{3}H-thymidine in mitogen-stimulated cultures of sIg^+ cell-enriched and sIg^+ cell-depleted PBL obtained by magnetic cell sorting (MACS).
2. Analysis of mitogen responses of sIg^+ and sIg^- cells in non-separated PBL cultures by simultaneous detection of incorporated 5-bromo-2'-deoxyuridine (BrdU) and sIg using fluorescence microscopy or flow cytometry.
3. Flow cytometric analysis of sIg^+ and sIg^- cell numbers before and after mitogen stimulation.

It were mainly the dull sIg^+ cells which were stimulated by LPS and not by PHA, whereas the sIg^- cells were stimulated by PHA and not by LPS. These results are in agreement with data from mammals showing that PHA and LPS are T and B cell mitogens, respectively.

Three different subpopulations of B cells and plasma cells can be distinguished based upon their reactivity with WCI 4 and WCI 12: WCI 4^+ 12^-, WCI 4^+ 12^+ and WCI 4^- 12^+

(**Chapter 6**). Evidence for at least two antigenically different Ig isotypes was obtained by sequential immunoprecipitation and affinity chromatography. The distribution of the three B cell subpopulations appeared to be organ and age dependent which indicates functional differences between the isotypes. The WCI 4^- 12^+ B cell subpopulation formed the majority in blood at all ages studied (2-56 weeks). In spleen and pronephros, the majority of the B cells at 2 and 4 weeks of age were WCI 4^+ 12^- but these cells decreased in number during ontogeny. The WCI 4^- 12^+ subpopulation increased and formed the majority from 13 weeks of age onwards. The percentage of WCI 4^+ 12^+ B cells was relatively stable during ontogeny in blood, spleen and pronephros.

Procedures for the production of MAbs against Ig^- lymphoid cells were described (**Chapter 7**). Immunisation of mice with PBL or enriched sIg^- pronephros lymphoid cells revealed that all or nearly all produced MAbs were reactive with all leucocytes. The presence of immunodominant carbohydrate determinants, present on several molecules found at all leucocytes, appears to be the major problem for obtaining specific MAbs. Mice were tolerised against these common determinants by immunisation with carp B cells followed by cyclophosphamide treatment. The immune response against carp leucocytes was strongly reduced although not completely abolished by this treatment. As a consequence, a different panel of MAbs was obtained after subsequent immunisation with carp thymocytes. About 70 % of the hybridoma culture supernatants was reactive with all leucocytes, 24 % did not react at all and 6 % showed a reaction with 45 % of the pronephros lymphoid cells, but not with granulocytes. Unfortunately, the reactivity of the MAbs was lost for unknown reasons. Immunisation with isolated membrane lysates from sIg^- PBL appeared to be another promising approach. In this case only 13 % of the hybridoma culture supernatants was reactive with all leucocytes, but not more than 2 % was reactive with a subset of the sIg^- leucocytes. Two of these MAbs were characterised and appeared to be thrombocyte markers. It can be concluded that the use of purified antigen (membrane molecules of better enriched cell populations) is recommended in further attempts for the development of specific MAbs against sIg^- lymphoid cells.

This thesis provides detailed information on a variety of methods which are required for optimal identification, separation and functional characterisation of carp leucocytes. The use of cell-specific MAbs appeared to be essential in these studies. The available MAbs against carp Ig allowed a successful characterisation of the B cell compartment. On the other hand, there is still a need for T cell-specific MAbs. Efforts to produce such antibodies have not been successful, probably due to fish-specific problems, but revealed new technical possibilities for further attempts. The data presented in this thesis can be used for fundamental studies on cell interactions in the immune response, but also for more applied investigations on fish vaccination or the interaction between environmental factors (e.g. stress, pollution) and the immune system.

Samenvatting en conclusies

Er zijn bij vissen verschillende populaties van leucocyten (witte bloedcellen) en verscheidene immunologische functies beschreven. De correlatie tussen het celtype en de celfunctie is echter vaak lastig aan te tonen. Dit wordt veroorzaakt door een klaarblijkelijk gebrek aan geschikte methoden en materialen voor de identificatie, scheiding en functionele karakterisering van visleucocyten. Het gebruik van monoclonale antilichamen (MA) is bij zoogdieren het belangrijkste middel voor de karakterisering van leucocyten. MA die gericht zijn tegen oppervlaktemoleculen van leucocyten zijn schaars voor vissen en bovendien zijn de meeste MA vissoort-specifiek. Op onze vakgroep werd door Dr. E. Egberts een set MA ontwikkeld tegen serum-immuunglobuline (Ig) van de karper, WCIs en tegen thymocyten (T) van de karper, WCTs. Op grond van hun reactie in een 'enzyme-linked immunosorbent assay' (ELISA) konden de volgende drie groepen MA onderscheiden worden: Ig^+ T^-, Ig^+ T^+ en Ig^- T^+.

In het hier beschreven onderzoek werden de reactiepatronen van de WCIs en de WCTs nader geanalyseerd en veelbelovende MA werden gebruikt voor de karakterisering van karperleucocyten. Een verscheidenheid aan technieken werd hiertoe aangepast voor het gebruik bij de karper. Verschillende procedures werden uitgevoerd om nieuwe MA te verkrijgen die specifiek zijn voor Ig^- lymfoïde cellen omdat geen van de bestaande MA specifiek tegen T-cellen gericht bleek te zijn.

Alle WCTs en de Ig^+ T^+ WCIs reageren jammer genoeg met algemene koolhydraatdeterminanten op diverse moleculen die aanwezig zijn op alle typen van leucocyten en op het Ig-molecuul (**Hoofdstuk 2, 6, 7**). Deze MA zijn alleen geschikt voor het herkennen van leucocyten in bloed of andere organen maar kunnen niet gebruikt worden voor onderzoek naar celfuncties. De Ig^+ T^- WCIs herkennen eiwitdeterminanten op de zware keten van het Ig-molecuul. Met behulp van deze MA konden B-lymfocyten, plasmacellen en Ig-bindende cellen geïndentificeerd worden (**Hoofdstuk 2, 4, 6**). De Ig-moleculen bevinden zich in clusters op de celmembraan van B-cellen en plasmacellen

(**Hoofdstuk 2**). De meeste plasmacellen bezitten Ig op hun celmembraan in tegenstelling tot plasmacellen van zoogdieren (**Hoofdstuk 3**).

Met behulp van fluorescentie-microscopie en flowcytometrie werd vastgesteld dat het percentage B-cellen en plasmacellen toeneemt gedurende de ontogenie. Het percentage plasmacellen bereikt een plateau bij een leeftijd van 3 maanden. Dit wijst op een mogelijke rijping van het humorale immuunsysteem rond deze tijd. Een significante toename in het percentage B cellen werd echter waargenomen tot een leeftijd van 8 maanden. Een complete ontwikkeling van het (humorale) immuunsysteem van de karper duurt dus mogelijk tenminste 8 maanden (bij 21-22 ^{0}C).

Binding van Ig door leucocyten uit de pronephros en het tweede segment van de darm werd vastgesteld met behulp van een immuunfluorescentiemethode en een erythrocyt-antilichaam rozetmethode (**Hoofdstuk 4**). De Ig-bindende cellen werden tevens geïdentificeerd met vergelijkbare immuungoudmethoden. In de pronephros werd Ig alleen gebonden door een beperkt aantal monocyt-achtige cellen en enkele lymfoïde cellen, maar niet door macrofagen en neutrofiele granulocyten. De meeste macrofagen en enkele lymfoïde cellen uit de darm bonden Ig. Hieruit wordt geconcludeerd dat Fc-achtige receptoren waarschijnlijk belangrijk zijn voor een effectieve fagocytose door darmmacrofagen terwijl andere vormen van opsonisatie van het antigeen (b.v. complement) mogelijk een rol spelen bij fagocytose door macrofagen en neutrofiele granulocyten uit de pronephros.

Stimulatie van membraan-Ig^{+} (mIg^{+}) en mIg^{-} perifere bloed leucocyten (PBL) met phytohaemagglutinine (PHA) en lipopolysaccharide (LPS) werd in vitro bestudeerd door middel van de volgende methoden (**Hoofdstuk 5**):

1. Bepaling van de ^{3}H-thymidine incorporatie in met mitogeen gestimuleerde kweken van mIg^{+} cel-verrijkte en mIg^{+} cel-gedepleteerde PBL. De celpopulaties werden verkregen door middel van magnetische celscheiding (MACS).
2. Analyse van mitogeenresponsen van mIg^{+} en mIg^{-} cellen in kweken van niet-gescheiden PBL door middel van simultane detectie van geïncorporeerd 5-bromo-

2'deoxyuridine (BrdU) en sIg met behulp van fluorescentiemicroscopie en flowcytometrie.
3. Bepaling van het aantal mIg^+ en mIg^- cellen voor en na mitogeenstimulatie met behulp van flowcytometrie.
Vooral de zwak Ig^+ cellen werden gestimuleerd door LPS maar niet door PHA, terwijl de mIg^- cellen werden gestimuleerd door PHA maar niet door LPS. Deze resultaten zijn in overeenstemming met die verkregen uit onderzoek bij zoogdieren waaruit bleek dat PHA en LPS respectievelijk T- en B-celmitogenen zijn.

Op grond van hun reactiviteit met WCI 4 en WCI 12 kunnen drie subpopulaties van B-cellen en plasmacellen onderscheiden worden: WCI 4^+ 12^-, WCI 4^+ 12^+ en WCI 4^- 12^+ (**Hoofstuk 6**). Aanwijzingen voor tenminste twee verschillende Ig-isotypen werden verkregen door middel van sequentiële immuunprecipitatie en affiniteitschromatografie. Er bestaan mogelijk functionele verschillen tussen de isotypen omdat de verhouding tussen de subpopulaties van B-cellen afhankelijk is van het type orgaan en de leeftijd van de vis. De WCI 4^- 12^+ B-celpopulatie vormde de grootste populatie in het bloed op alle bestudeerde leeftijden (2-56 weken). Het grootste deel van de B-cellen in de milt en de pronephros was WCI 4^+ 12^- op een leeftijd van 2 en 4 weken maar het percentage van deze B-cellen nam af in de loop van de verdere ontwikkeling. Het aantal WCI 4^- 12^+ cellen nam toe en vormde een meerderheid vanaf 13 weken. Het percentage WCI 4^+ 12^+ B-cellen in bloed, milt en pronephros bleef ongeveer gelijk gedurende de ontogenie.

Verschillende procedures voor de produktie van MAbs tegen Ig^- lymfoïde cellen zijn uitgevoerd (**Hoofdstuk 7**). Nagenoeg alle geproduceerde MAb waren reactief met alle leucocyten indien muizen geïmmuniseerd werden met PBL of verrijkte mIg^- lymfoïde cellen uit de pronephros. Het grootste probleem bij de produktie van specifieke MAb is waarschijnlijk de aanwezigheid van immuundominante koolhydraatdeterminanten op diverse membraanmoleculen van alle leucocyten. Getracht werd om muizen tolerant te maken voor deze algemene determinanten door middel van immunisatie met B-cellen van de karper gevolgd door een behandeling met cyclofosfamide. De immuunrespons tegen de

karperleucocyten bleek sterk verminderd in deze muizen maar was helaas niet compleet verdwenen. Toch werd een andere set van MA verkregen na immunisatie van deze 'tolerante' muizen met karperthymocyten. Ongeveer 27 % van de kweeksupernatanten van de hybridomas reageerde met alle leucocyten, 24 % reageerde niet en 6 % reageerde met 45 % van de lymfoïde cellen uit de pronephros maar niet met granulocyten. De reactiviteit van deze MA ging jammer genoeg verloren om onbekende redenen. Een andere veelbelovende benadering was immunisatie met geïsoleerde membraanlysaten van mIg^- PBL. Hierbij was slechts 13 % van de kweeksupernatanten reactief met alle leucocyten maar slechts 2 % reageerde met een deel van de mIg^- leucocyten. Twee van deze MAbs bleken specifiek voor thrombocyten. Geconcludeerd kan worden dat het gebruik van opgezuiverd antigeen (membraanmoleculen van verrijkte celpopulaties) aan te bevelen is bij verdere pogingen tot het verkrijgen van specifieke MA tegen mIg^- lymfoïde cellen.

Dit proefschrift verschaft gedetailleerde informatie over een verscheidenheid aan methoden die onmisbaar zijn voor optimale identificatie, scheiding en functionele karakterisering van karperleucocyten. Het gebruik van celspecifieke MA is hierbij essentieel. De beschikbaarheid van MA tegen Ig van de karper leidde tot een succesvolle karakterisering van het B-celcompartiment. Er is echter nog steeds behoefte aan T-celspecifieke MA. Pogingen om deze MA te verkrijgen zijn tot nu toe niet succesvol wat mogelijk veroorzaakt wordt door voor de muis immuundominante koolhydraat-determinanten op visleucocyten. Wel werden nieuwe methoden ontwikkeld die gebruikt kunnen worden bij verdere pogingen. De resultaten die in dit proefschrift worden beschreven kunnen gebruikt worden voor fundamenteel onderzoek naar de interactie van cellen tijdens de immuunrespons maar ook voor meer toegepast onderzoek naar vaccinatie van vissen of naar de interactie tussen milieufactoren (b.v. stress, vervuiling) en het immuunsysteem.

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Curriculum vitae

Johanna Catharina Elisabeth (José) van Diepen (e.v. Koumans) werd op 19 juli 1965 geboren te Krommenie (Noord-Holland). In 1983 behaalde zij het diploma Atheneum B, aan Scholengemeenschap "Bertrand Russell" in die plaats. Dat zelfde jaar werd gestart met een studie Biologie aan de Vrije Universiteit te Amsterdam. Deze studie werd in 1988 afgesloten, met als doctoraalonderwerpen Endocrinologie en Histologie uitgevoerd bij de vakgroep Organismale Dierkunde (VU). Kort hierna werd zij aangesteld als assistent in opleiding (AIO) bij de vakgroep Experimentele Diermorfologie en Celbiologie, sectie Celbiologie aan de Landbouwuniversiteit te Wageningen. De resultaten van het AIO-onderzoek zijn beschreven in dit proefschrift. Het AIO-onderwijs heeft de volgende cursussen omvat: "Methoden en technieken in de celbiologie" en "Immunologie voor gevorderden" (PAOG, Amsterdam), proefdierkunde (KUN), "Communicatievaardigheden I+II", "Mondeling presenteren" en "Organisatie en begeleiding van een afstudeervak" (LUW), Engels (Quintrix, Wageningen). Als onderwijstaak werd een achttal studenten tijdens hun doctoraalonderzoek begeleid.