Ontogeny of the immune system of fish using specific markers

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Nicla Romano

Stellingen

1) When the thymus of fish, like in other vertebrates, is important for the selection of T cells, the absence of thymus involution in immunologically mature carp is possibly related to the absence of distinct T cell regions in fish lymphoid organs.
   This thesis.

2) Although B cells seem to originate from head kidney, indications for B cell selection or expression of recombination activating genes (Rag) are not available in the first month of cyprinid development.
   This thesis; Trede and Zon (1998), Developmental and Comparative Immunology, 22, 253-263.

3) Because of the very low number of blood thrombocytes in the first weeks of development, blood clotting after an injury is not possible or must be based on other mechanisms in young fish.
   This thesis.

4) Monoclonal antibodies (mAbs) against fish leucocyte subpopulations are without doubt a big step forward in fish immunology, although no CD classification can be used till now. This problem may be solved when mAbs can be made against recombinant proteins of fish CD antigens, of which some are already sequenced.

5) The immune system of Antarctic teleosts must be adapted to low environmental temperatures. Preliminary observations have shown that lymphomyeloid tissue develops slowly and has extensive large vascularisation and peculiar erythrocytes, but other physiological adaptations related to the immune response can also be expected.

6) A PhD degree has to reflect the scientific capacities of a candidate and should not, like in Italy, be restricted to the working position of the candidate.

7) One of the major threads of science is the strong development of bureaucracy, which probably does not only occur in Italy.

8) Unfortunately some scientific referees have a prejudice against papers coming from southern European countries; in general they don't look at the message of a paper but more to the prestige of the author or linguistic failures.

9) A relationship with horses allows people to understand more about the nature of our human being.

10) The difference between Dutch and Italian tomatoes is clearly a matter of taste, probably merely related to a difference in production methods (industrial versus natural).

Stellingen belonging to the thesis: "Ontogeny of the immune system of fish using specific markers"
from Nicla Romano, Wageningen, December 14, 1998

Man monotonous Universe,
Thinks to increase good,
But from his fevered hands
fall only limits without end.

Giuseppe Ungaretti

To my parents, Giuseppe and Rie

Abstract

A panel of monoclonal antibodies (mAbs) was used for the characterisation of leucocyte subpopulations during the ontogeny of common carp (*Cyprinus carpio*, L.) and sea bass (*Dicentrarchus labrax*, L.). In carp the leucocytes were monitored in different lymphoid organs by immunofluorescence and flow cytometry using specific mAbs for early T cells (WCL9), B cells (WCI12), monocytes/macrophages (WCL15) and thrombocytes (WCL6). Early T cells were very numerous (~77 %) in thymus during the first weeks post fertilisation (p.f.), but also present in other organs, especially head kidney. Subsequently, these cells disappeared from all organs, except the thymus (~40%). B cells appeared in the head kidney from the second week p.f., and later on in the spleen and blood, but their number remained low in the thymus and gut. Thrombocytes were detected in cell suspensions of spleen from the first week p.f. and their percentage increased until the 4th week (~30%) and then decreased in spleen (~10%), but increased in blood (~30%). Monocyte/macrophage-like cells were present in all organs from the first week p.f. and their percentage gradually increased until the 8th week p.f.. By using mAb WCL15 on fixed tissue the *in situ* distribution of monocytes/macrophages was studied in thymus, head kidney, spleen and gut from 2 days until 60 weeks p.f.. Macrophages were found from day 2 p.f. in the head kidney and in the dorsal part of the yolk sac epithelium. From 1 week onwards, macrophages were also found scattered in the thymus and gut and during the second week also in spleen. The number of macrophages increased in all lymphoid tissues until the 6th-8th week p.f., then decreased except in the thymus, where they became localised mainly at the cortical-medullary boundary. By using mAb WCL38 increasing numbers of mucosal T cells were observed in cell suspensions of gills and intestine from the first week p.f. onwards. With immuno-histochemistry the early appearance of mucosal T cells could be confirmed in the gills and intestine but was also detected in skin. With mAb WCL9 the ontogeny of the carp thymus was studied and special attention was paid to the development of cortex and medulla. The differentiation between cortex and medulla started in the 4th week p.f.. Ultrastructural study of the developing thymus confirmed these data and permitted the analysis of the distribution and morphological differences of the epithelial cells. In addition, numerous apoptotic cells appeared in the cortex from the 4th week p.f. onwards, while lower numbers were observed in medulla. In sea bass, mAbs DLT15 and DLIg3 specific for T and B cells, respectively, were employed to describe the morphology and distribution of these cells. Enriched leucocyte fractions from different tissues (thymus, spleen, head kidney, intestine and blood) were used. High numbers of T cells were detected in thymus and intestine, whereas B cells were more numerous in blood, head kidney and spleen. Furthermore, an ontogenetic study with DLT15 revealed that the thymus was the first organ with T cells, followed by head kidney and spleen. Although some differences were observed between the two commercially important fish species studied, the data presented in this thesis are of phylogenetic interest and can be used for the development of vaccination strategies in young fish.

Contents

Chapter 1

General Introduction

The environment contains a large number of microbial agents that could cause pathologies in vertebrates. However, in most cases the adverse effects of invading micro-organisms can be limited by an active immune system. In fact, a distinction can be made between an innate and adaptive immune system. The innate immune system can be regarded as the first line of defence against infections, acting in an unspecific manner. This system could also activate adaptive immune responses, which produce specific reactions to antigens. Adaptive immunity is characterised by specificity and memory, and can be divided into humoral and cell mediated immunity. In the innate and adaptive immune system a variety of molecules and cells (mainly leucocytes) are involved, distributed throughout the body and each having its specific function (Roitt _et al._, 1993).

From the phylogenetic point of view fish, especially teleosts, share both features in the immune system with other vertebrates. In addition, a thorough knowledge of the teleost immune system is necessary to develop health-protective strategies to prevent disease and immunotolerance under intensive culture conditions. Fish farming at high densities can easily result in the spreading of infective diseases at all stages of the production cycle (Ellis, 1995). Therefore, a thorough knowledge of the immune system and its development can be of high value for the protection of fish in aquaculture.

In this chapter, the current knowledge on leucocytes, lymphoid organs and their development in bony fish is summarised. Finally the aim and outline of this thesis will be described.

I.1. Fish leucocytes

Within the immune system of teleost fish leucocyte subpopulations including B cells (Irwin & Kaattari, 1986; Miller _et al._, 1994; Koumans-Van Diepen _et al._, 1994a), T cells (Lin _et al._, 1992; Miller _et al._, 1994), granulocytes (Ainsworth, 1992; Hine, 1992; Lamar & Ellis, 1994), thrombocytes (Esteban _et al._, 1989; Rombout _et al._, 1996), macrophages (Meseguer _et al._, 1991; Hardie _et al._, 1994; Sveinbjornsson & Seljelid, 1994), and natural killer cells (Evans _et al._, 1992; Hogan _et al._, 1996) play an important role. These cells are present in lymphoid and non-lymphoid organs or can be associated with epithelia to form a mucosa-associated lymphoid tissue (MALT) (van Muiswinkel, 1995). The production and use of monoclonal antibodies (mAbs) directed against distinct cells have facilitated the characterisation of leucocytes. From the early eighties, numerous mAbs have been developed against leucocytes and their

subpopulations in various fish species. Tables 1 and 2 show the panel of specific antibodies produced in the last 20 years.

I.1.1.Lymphocytes

Humoral and cellular reactions in vertebrates are mainly driven by macrophages, B cells and T cells (Roitt *et al.*, 1993). In teleosts humoral responses that have been shown to take place both *in vivo* and *in vitro*, include secretion of IgM-like immunoglobulins by B cells and plasma cells (Miller *et al.*, 1985; Castillo *et al.*, 1993; Koumans-Van Diepen *et al.*, 1994a; Killie & Jorgensen, 1995). Cellular activities, by putative T cells (and macrophages), were demonstrated both *in vivo* and *in vitro* by the presence of antigen-enhanced cell proliferation (Marsden *et al.*, 1996), antigen processing and presentation (Vallejo *et al.*, 1992), macrophage-activating factor (Francis & Ellis, 1994; Hardie *et al.*, 1994), mitogen responses (Sizemore *et al.*,1984), mixed leucocyte reactions (Miller *et al.*, 1985) and allograft rejections (Botham & Manning, 1981).

A boost for the research on the biology of fish B and T cells has been given by the use of mAbs directed against subpopulations of lymphocytes. In attempts to prepare mAb against B cells mice were immunised with purified Ig from PBL or mucus (see Table 1) and numerous mAbs are available. In an attempt to obtain mAbs against T cells (Secombes *et al.*, 1983) the preparation revealed several difficulties (see Table 2) until a few years ago. Many hybridomas obtained secreted antibodies recognising a broad spectrum of antigens, because of their reaction with the glycosylated epitopes common to various leucocyte populations (Rombout *et al*, 1990). Thus, only few mAbs were developed as specific for thymocytes and T cells (Scapigliati *et al.*, 1995; Passer *et al.*, 1996; Rombout *et al.*, 1997; 1998). The successful T cell-specific mAbs were obtained by immunising mice with thymocyte or intestinal intraepithelial lymphocyte (IEL) plasma membrane preparations (Rombout *et al.*, 1997; 1998), or whole cells such as thymocytes (Scapigliati *et al.*, 1995), and thymocytes plus Ig-negative PBL (Passer *et al.*, 1996).

I.1.1.1. B cells

B cell heterogeneity was demonstrated in several fish species as channel catfish (Lobb & Olson, 1988), rainbow trout (Sanchez *et al.*, 1995), carp (Koumans-van

Diepen *et al.*, 1995), Atlantic salmon (Pettersen *et al.*, 1995; Magnadottir *et al.*, 1996), and sea bass (dos Santos *et al.*, 1997) by using different Ig-specific mAbs. Moreover, immuno-electronmicroscopical studies have shown morphological differences among B cells and plasma cells in carp (Koumans-van Diepen *et al.*, 1994a).

B cell functions in fish were demonstrated after agglutination in carp, (Lamers *et al.*,1986) and in a species of tilapia (Sailendri & Muthukkaruppan, 1975). Van Ginkel *et al.* (1994) provided in channel catfish evidence for the activation of B cells by membrane immunoglobulin cross-linking. Moreover, catfish B cells were purified by magnetic cell sorting from peripheral blood leucocytes (PBL) and then stimulated by membrane immunoglobulin cross-linking. The stimulated cellular fraction showed a B cell receptor-like complex composed of two non-covalently associated molecules (Rycyzyn *et al.*, 1996). In carp, the mAb WCI12 specific for serum Ig and Ig-bearing cells has been used to immunopurify by magnetic sorting fractions of Ig^+ and Ig^- PBL (Koumans-van Diepen *et al.*, 1994b). Immuno-purified cell fractions were stimulated with phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) mitogens. The Ig^- cells were hardly stimulated with LPS, whereas both fractions showed mitogen responses by PHA. In the sea bass, the mAb DLIg3 specific for Ig and Ig-bearing cells (Scapigliati *et al.*, 1996), has also been used to purify by magnetic sorting cross-reacting cells from PBL. The B cell identity of these purified cells has been proven by their proliferation with LPS, but not with concanavalin A (in some species know as a T cell mitogen), confirming the *in vitro* activity of B cells purified from PBL. In agreement with the heterogeneity of B cells, the immunoglobulin molecules also show differences. Diversity in MW, structure (Havarstein *et al.*, 1988; Wilson & Warr, 1992; Warr, 1995) and/or antigenicity in IgM are reported in many teleost (Lobb *et al.*, 1984; Lobb & Olson, 1988; Koumans-van Diepen *et al.*, 1995; dos Santos *et al.*, 1997). Furthermore in carp, differences between serum and mucus immunoglobulins were demonstrated using the mAbs WCI12 and WCI-M indicating the existence of a different mucosal IgM isotype (Rombout *et al.*, 1993a). In addition an another IgD-like molecule was recently described in channel catfish (Wilson *et al.*,1997). The occurrence of IgD in fish suggests the ancient character of this Ig isotype (Wilson *et al.*,1997).

Table I. Monoclonal antibodies (mAbs) against Ig molecules and Ig-positive cells in bony fish. The asterisks indicate the proven reactivity of mAb with B cells or plasmacells.

Fish species	Name	Reactivity	Specificity	References
Common carp	WCI12	serum Ig	Ig heavy-chain *	Secombes et al., 1983a
Cyprinus carpio	WCI4	"	"	Koumans-Van Diepen et al., 1995
	WCI-M	mucus	"	Rombout et al., 1993a
Channel catfish	3E11	serum Ig	Ig heavy chain *	Lobb & Olson., 1988
Ictalurus punctatus	3D11	serum Ig	"	
	1H6	serum Ig	"	
Atlantic cod	several	serum Ig	Ig heavy chain	Israelsson et al., 1991
Gadus morhua		serum Ig	Ig light chain	
European eel	WEI1	serum Ig	Ig heavy chain *	van der Heijden et al., 1995
Anguilla anguilla	WEI2	serum Ig	Ig light chain *	
Japanese flounder	several	serum Ig	Ig heavy chain	Bang et al., 1996
Paralichthys olivaceus				
Red drum	RDG013	serum Ig	Ig heavy chain *	MacDougal et al., 1995
Sciaenops ocellatus				
Atlantic salmon	several	serum Ig	Ig heavy chain *	Killie et al., 1991
Salmo salar	several	serum Ig	Ig heavy chain*	Pettersen et al.,1995
	Type-I	serum Ig	Ig heavy chain	Magnadottir et al., 1996
	Type-II	serum Ig	Ig heavy vhain	"
Gilthead sea bream	WSI-5	serum Ig	Ig light chain *	Navarro et al., 1993
Sparus aurata				
Sea bass	3B5	serum Ig	Ig heavy chain *	Romestand et al., 1995
Dicentrarchus	6E11	serum Ig	Ig heavy chain *	Breuil et al., 1997
labrax	DLIg14	serum Ig	Ig heavy chain *	Scapigliati et al., 1996
	DLIg3	serum Ig	Ig light chain *	"
	WDI1	serum Ig	Ig heavy chain *	dos Santos et al., 1997
	WDI2/3	serum Ig	Ig light chain *	"
White sturgeon	I-10/13	serum Ig	Ig heavy chain	Adkinson et al., 1996
Acipenser	several		Ig light chain	"
transmontanus				
Rainbow trout	1.14	serum Ig	n.d.	DeLuca et al., 1983
Oncorhynchus	DCI14	serum Ig	Ig heavy chain	Thuvander et al., 1990
mykiss	2A1	serum Ig	Ig light chain	Sanchez & Dominguez, 1991
	2H9	serum Ig	Ig heavy chains*	"
	3B10	serum Ig		Sanchez et al., 1993, 1995
	4D11	serum Ig		
Turbot	URI-7	serum Ig	Ig heavy chain	Estévez et al., 1994
Scophtalmus				
maximus				

I.1.1.2. T cells

Evidence for cell-mediated immune reactions in fish has been provided by studies on T cell responses using functional criteria such as proliferation induced by T cell mitogens (Sizemore *et al.*, 1984), mixed-leucocyte reactions (MLR) (Miller *et al.*, 1985), T helper cell functions in antibody production against thymus-dependent

Table 2. A list of mAbs against Ig-negative leucocytes in bony fish. The symbol MΦ indicate macrophages reactivity.

Fish species	Name	Immunization antigen	MW (kDa)	Specificity	Reference
Common carp	WCL6	Ig⁻ leucocyte	90	thrombocytes	Rombout *et al.*,1996
Cyprinus carpio	WCL9	thymocytes	150/200	early T cells	Rombout *et al.*,1997, this thesis
	WCL38	Intestinal Lymphoid cells	38	mucosal T cells	Rombout *et al.*,1998, this thesis
	WCL15	adherent Ig⁻ HK cells	several	MΦ/monocytes thrombocytes	Weyts *et al.*,1996, this thesis
	TCL/BE8	PBL	122	monocytes neutrophils	Nakayasu *et al.*,1998
Channel catfish	13C10	thymocytes Ig-negative PBL	150	T cells, neutrophils thrombocytes	Miller *et al.*, 1987
Ictalurus punctatus	C3-1	Ig-negative PBL	n.d.	neutrophils	Ainsworth et al., 1990
	5C6	Ig-negative PBL	40	NCC	Evans & Jaso-Friedmann,1992
	CfT1	thymocytes and Ig-negative PBL	35	peripheral T cells	Passer *et al.*, 1996
	4-20/7-2	Ig-negative PBL	150/32	thrombocytes	Passer *et al.*, 1997
Sea bass	DLT15	thymocytes	40	thymocytes and peripheral T cells	Scapigliati *et al.*, 1995, this thesis
Dicentrarchus labrax					
Yellowtail	YeT-2	thymocytes	n.d.	Ig⁻ leucocytes	Nishimura *et al.*, 1997
Seriola quinqueradiata					
Rainbow trout	Hyb106-9	serum Ig	n.d.	granulocytes and thrombocytes	Slierendrecht *et al.*, 1995
Oncorhynchus					
mykiss	21G6/ 21F11	head kidney leucocytes	n.d.	MΦ, lymphocytes, granulocytes	Bowden *et al.*,1997

antigens (Miller *et al.*,1987; Clem *et al.*, 1985), secretion of lymphokines (Secombes *et al.*, 1996; Graham & Secombes, 1990) and allograft rejection (Botham & Manning, 1981).Unfortunately, in these studies T cell responses have been monitored indirectly, due to the lack of specific markers. The generation of permanent T cell lines has been achieved in catfish (Miller *et al*,1994), and these cells permitted elegant studies on *in vitro* T cell responses. However, it is also important to monitor T cell response after *in vivo* challenge with antigens and, in this respect, the possibility of purifying and/or labelling T cells with the aid of a specific mAb offer new possibilities for fish immunology. In attempts to obtain mAbs against T cells many research groups immunised mice with thymocytes or PBL. Unfortunately, in most cases a cross-reaction with other leucocyte subpopulations was revealed. Table 2 shows an updated list of the mAbs developed against Ig-negative leucocytes.

In order to minimise the cross-reaction of anti-thymocyte mAb with other leucocytes; mice were immunised with isolated membrane molecules from carp thymocytes excluding many intracellular antigens (Rombout *et al.*,1997). The resulting mAb WCL9 displayed a peculiar reaction with a subpopulation of thymocytes and reacted with two polypeptides (155 and 200 kDa). This mAb is considered as a marker for early thymocytes. A similar immunisation strategy was employed by using membrane lysates of intestinal leucocytes of carp (Rombout *et al.*, 1998), which has been previously shown to be rich in Ig-negative lymphoid cells (Rombout *et al.*, 1993b). The resulting mAb WCL38 cross-reacted with 50-60% of gut, skin and gill lymphoid cells and with approximately 5% of the thymocytes and a negligible percentage of lymphoid cells in other organs and blood, suggesting being a marker of mucosal T cells. WCL38 appeared to react with a dimeric molecule of 76 kDa, consisting of two subunits of 38 kDa (Rombout *et al.*,1998). In channel catfish, the mAb CfT1 has been obtained using a mixture of thymocytes and Ig-negative PBL (Passer *et al.*, 1996). This mAb, recognised a 35 kDa non-glycosylated antigen, and displayed the characteristics of a good marker for thymocytes and peripheral T cells, since it reacted with a high percentage of thymocytes, and a lower percentage of cells from PBL, spleen and head kidney in immunofluorescence and flow cytometry. These data agree with those found with mAb DLT15 in sea bass thymocytes and peripheral T cells in spleen (Scapigliati *et al.*, 1995). Moreover, DLT15 can also be used in immunohistochemistry on paraffin sections from lymphoid and non-lymphoid organs showing the *in situ* distribution of T cells (Scapigliati *et al.*, 1995). In juvenile sea bass, the immunostaining with DLT15 revealed a high concentration of positive cells

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in the outer region of thymic lobuli and scattered DLT15^{+} cells were found in distinct lymphoid areas of the head kidney and spleen (Scapigliati _et al._, 1995).

I.1.2 Non-specific cytotoxic cells

The non-specific cytotoxic cell (NCC) is another type of fish leucocyte that can induce the destruction of foreign cells. Granular lymphoid cells were found in the peripheral blood, spleen and head kidney of teleost (rev. Manning, 1994). The NCC could be the teleost equivalent of mammalian natural killer cells (NK) (Evans & Jaso-Friedmann, 1992; Evans, 1997). It has been suggested that the NCC may represent a progenitor of the NK, but with more diversity in function (Manning, 1994). They may provide the first barrier of defence against tumour growth and infectious agents, as it occurs in mammals by NK cells. Both NCC and NK cells need a direct contact with the target cell in order to kill by apoptotic or necrotic mechanisms (Greenlee _et al._, 1991). A mAb (5C6) developed against channel catfish NCC (Evans _et al._, 1988) seems to be a non-species specific NCC marker. Recently, 5C6 has been characterised as reacting with a vimentin-like protein present on the NCC (NCCRP-1, Jaso-Friedmann & Evans, 1997). In mammals, lymphokine-activated killer (LAK) cells exist in addition to NK cells, these cells need IL-2 to mediate cytotoxicity (Lanier, 1995). In contrast to the NK cells, they expose the γδTCR (Raulet, 1994). The lack of specific markers against γδTCR in fish dues not permits the existence of LAK cells in teleosts.

I.1.3. Thrombocytes

Fish thrombocytes are very abundant in blood and the majority shows a spindle shape, with irregular nucleus and many vacuolar and granular inclusions (Ellis, 1977, Rowley _et al._,1988). Thrombocytes showed the same reaction upon collagen treatment as mammalian platelets (Uchida _et al._,1992). In this respect, despite their different cellular identity, they were considered as the equivalent of the mammalian platelets (Parmley, 1988) since fish thrombocytes are responsible of blood clotting when an injury occurs (Uchida, 1992). A mAb against rainbow trout granulocytes and thrombocytes was produced (Slierendrecht _et al._, 1995), but the reaction with thrombocytes was weaker to compare with granulocytes. An interesting mAb was developed against carp thrombocytes and precursors (WCL6, Rombout _et al._,1996)

which appeared to be a suitable tool to study the appearance of thrombocytes during ontogeny. More recently, a thrombocyte-specific antigen CD41/ CD61-like was identified in catfish by using mAbs 4-20 and 7-2 (Passer *et al.*,1997)

I.1.4. Monocytes/macrophages

Macrophages and neutrophilic granulocytes in fish are the principal phagocytic cells, which phagocytose inert or antigenic material, exert cytotoxic activity and stimulate lymphocytes by secreting interleukin-1-like factors (Secombes & Fletcher, 1992; Verburg-van Kemenade *et al.*, 1995). In fish, macrophages can be distinguished on the basis of presence or absence of melanin. Melanomacrophages, contain melanin or other pigments and lysosomes and are often organised in groups so called melanomacrophage centres (Agius, 1995). Despite in carp "melanomacrophages" are similar to those in other species they do not contain melanin (Agius, 1995).

The other macrophages, are localised more solitary in tissues, lack pigments and contain numerous lysosomes or phagosomes (rev. Secombes & Fletcher, 1992). Although macrophage-like cells were observed in numerous tissues of fish their classification on the basis of their heterogeneity was not performed, probably due to the lack of specific markers. A direct lineage relationship between monocytes and macrophages was supposed, but never proven, and the characterisation of both cells in adult or larval fish was performed by functional (Faisal & Ahne, 1990; Sveinbjornsson & Seljelid, 1994; Verburg-van Kemenade *et al.*, 1994) or (ultra) structural studies (Zapata & Cooper, 1990; Koumans-van Diepen *et al.*, 1994c). In general, macrophages are involved in phagocytosis (rev. Secombes & Fletcher, 1992), antigen processing and presentation (Vallejo *et al.*, 1992), production of cytokines (Secombes, 1991) and other modulating factors (Secombes & Fletcher, 1992) and immune-complex binding (Koumans-van Diepen *et al.*, 1994c). *In vitro* assays of prolipherative and antibody response to various thymus-dependent antigens provided evidence that the major histocompatibility complex (MHC) molecules govern the antigen presentation (Vallejo *et al.*, 1992), and that MHC seem to be expressed early during the development (Rodrigues *et al.*, 1996).

A marker for monocytes and macrophages, WCL15, was recently reported by Weyts *et al.* (1997). A permanent cell line originating from carp peripheral blood leucocytes (Faisal & Ahne, 1990) having morphological and functional characteristics of macrophages appeared to be reactive with this mAb. However, with live peripheral

blood leucocytes WCL15 recognised also thrombocytes and showed a weak reaction with basophils (Rombout *et al.*, unpublished).

I.1.5. Granulocytes

Fish granulocytes can be classified into three subtypes: basophils, neutrophils and eosinophils (Ellis, 1977). However, based on their staining properties, a clear identification of the subtypes was not easy in each species. Moreover, the respective roles of the different populations is not clear and vary from species to species (rev. Manning, 1994). Anyway, numerous ultrastructural studies were published in the last ten years, indicating different granulocyte cytology in each species. Generally, they are involved in early non-specific immunity since the first reaction during inflammation is an influx of granulocytes. Their presence at the site of inflammation reaches a peak after 12-24 h and is followed by an invasion of monocytes and lymphocytes (Griffin, 1984). Neutrophilic granulocytes, are characterised by cytoplasmic round-oval granules often containing of a crystalline structure (Rowley *et al.*, 1988). Neutrophils are involved in fish inflammatory responses and show chemotactic (Hine, 1992) and phagocytotic reactions (Ainsworth, 1992). Carp neutrophils showed respiratory burst and microbicidal activity and interleukin 1-like secretion (Verburg van Kemenade *et al.*, 1989, 1995).

In contrast to mammals, the presence of basophils and mast cells is still a matter of debate in fish. In carp, cells with a similar ultrastructural feature were described (Rombout *et al.*, 1989; Verburg-van Kemenade *et al.*, 1994). Basophilic-like cells in fish could differentiate into mast cells when activated and they may function in inflammatory reactions as occurs in mammals (Ellis, 1977, Reite, 1998). However, it is not proven yet whether histamine-like molecules are produced in fish (Ellis, 1977). The literature on fish eosinophils is contradictory, they have been reported to contain large cytoplasmic "granules" stained with eosin (Kelényi & Némèth, 1969) but their function is still unknown. In carp, basophils and eosinophils are considered to belong to the same lineage (Temmink & Bayne, 1987), because the existence of intermediate cells with both "basophilic" and "eosinophilic" granules was described (Rombout *et al.*, 1989). However, a real eosinophilic cell may exist in carp, since a fourth granular cell type was described with eosinophilic granules, however, its presence seems to be restricted to the skin (Cross & Matthews, 1991). Thus, the controversial classification of granulocytes in fish also suffers of the lack of specific markers for each

subpopulation. Again, the use of granulocyte specific mAb against (channel catfish, Ainsworth, 1990; carp, Nakayasu *et al.*, 1998) can be important in this respect.

## I.2. Ontogeny of the immune system of fish

I.2.1. Histogenesis of the lymphoid organs in fish

The fish immune system involves lymphoid organs as thymus, (head and trunk) kidney, spleen and mucosal associated lymphoid tissues in skin (SALT), gills (GiALT) and gut (GALT), as shown in Figure1.

Complete histological description of the ontogenetic development of the teleost lymphoid organs are reported in rainbow trout (Grace & Manning, 1980), carp (Botham & Manning, 1981), yellowtail, red sea bream and Japanese flounder (Chantanachookhin *et al.*, 1991), gilthead sea bream (Josefsson & Tatner, 1993), rosy barb (Grace *et al*, 1981), Atlantic salmon (Ellis, 1977), Mozambique mouth brooder (tilapia) (Doggett & Harris, 1987), an other tilapia species (Fishelson, 1995), and in antarctic species, as *Harpagifer antarcticus* (O'Neill, 1989).

Like in all vertebrates, the thymus of fish probably plays an important role in the

Figure 1. The localisation of lymphoid organs and tissues in teleost fish (TH=thymus, HK=head kidney, M=mesonephros, SP=spleen, GiALT=gill-associated lymphoid tissue, GALT= gut-associated lymphoid tissue, SALT=skin-associated lymphoid tissue).
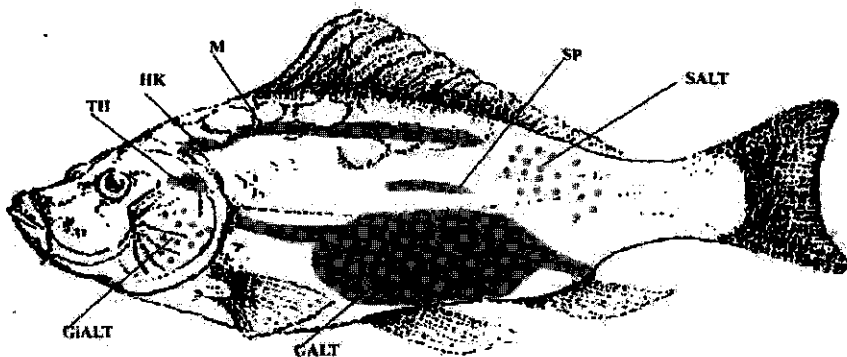
development of the functional immune system, as demonstrated with early thymectomy (Saliendri, 1973; Nakanishi, 1986). Fish thymus is mainly composed by Ig-negative lymphoid cells (Scapigliati _et al._,1995) within a network of reticular epithelial cells, and generally with a cortex and medulla organisation (rev. Manning, 1994). Although the anlage of head kidney appears earlier than thymus (Zapata & Cooper, 1990) the first appearance of lymphoid cells was generally observed in thymus (Botham & Manning, 1981; Grace _et al._, 1981; O'Neill, 1989). Migration of thymocytes to the head kidney has been demonstrated during development of sea bream (Josefsson & Tatner, 1993) and suggested for carp (Secombes _et al._, 1983) and sea bass (Abelli _et al._, 1996, this thesis).

The kidney is an important lymphopoietic organ in teleost. It appears as a sheet of tissue, located dorsally in the peritoneal cavity. Lymphopoietic areas were distinguished in head and trunk kidney. The structure of lymphohaemopoietic tissue is the same in both portions, however, in trunk kidney lymphoid areas are restricted among renal tubules that lacked in the head kidney (Zapata & Cooper, 1990).

The kidney seems to be a multi-functional organ in fish. It has endocrine functions (Abelli _et al._, 1995), myelopoietic functions (rev. Zapata, 1996) and immune functions. Most authors agree that the head kidney together with haematopoietic areas of mesonephros (trunk kidney) are the main sites of granulocyte, B lymphocyte, monocyte differentiation and partially, the site of erythropoiesis, (Ellis, 1977; Zapata, 1979; Bielek, 1981; Botham & Manning, 1981), although the presence of macrophages before appearance of the kidney suggest the existence of other lymphopoietic foci in the fish embryo (Zapata _et al._, 1996). Haematopoietic blast cells were observed in the head kidney prior to the appearance of lymphocytes in any other lymphoid organ (Ellis, 1977; Grace & Manning, 1980; Botham & Manning, 1981; Razquin _et al._, 1990; Zapata & Cooper, 1990; Josefsson & Tatner, 1993) suggesting the possibility that the head kidney could be the first haematopoietic organ.

The spleen develops later and remains predominantly erythroid in most teleost species (Rowley _et al._, 1988; van Muiswinkel _et al._, 1991), however, in some species it is possible to distinguish red and white pulp areas (i.e. sea bass, Abelli _et al._, 1996). Only a few studies were published on the ontogeny of the spleen (Quesada _et al._, 1995). It seems to be a central organ for thrombopoiesis in juvenile carp (Rombout _et al._, 1996) which was shown by using a mAb specific for thrombocytes and their precursors (WCL6).

The epithelial surfaces in contact with the external environment, i.e. skin, gills and gut, representing the first barriers for pathogens, seem to be protected by a mucosal immune system (Rombout *et al.*, 1989, 1993a; van Muiswinkel, 1995, Joosten *et al.*, 1997, Abelli *et al.*, 1997). In the intestine of fish numerous leucocytes were found (Rombout *et al.*, 1989) with prevalence of T lymphocytes and large macrophages among epithelial cells and B cells in the lamina propria (Abelli *et al.*, 1997; Rombout *et al.*, 1998). Although not much is known about antigen processing in gills and skin, indications are available for antigen uptake by these epithelia and for the presence of leucocytes at these locations (Lobb, 1987; Rombout *et al.*, 1993a; Iger & Wendelaar Bonga, 1994). Furthermore, the mucus itself is constantly being secreted and shed, and used to prevent mechanical and pathogenic diseases (Manning, 1994). Only very few studies are conducted to the ontogeny of these tissues (Botham & Manning, 1981; Picchietti *et al.*, 1997). In carp the first histological localisation of lymphoid-like cells in gut of larvae was reported around 9 days p.f. (Botham & Manning, 1981) and in sea bass around the 30 days post hatch (p.h.) (Picchietti *et al.*, 1997).

I.2.2. Ontogeny of specific immunity

I.2.2.1. Immunoglobulin presence in early development

The presence of Ig-like molecules was detected in fish embryos before any organs and tissue are formed. This suggests their role as the first barrier against pathogens. Ig-like molecules were also found in the eggs and/or new-born fries of pike *Exos lucius* (Clerx, 1978), carp (Van Loon *et al.*, 1981), plaice *Pleurocetes platessa* (Bly *et al.*, 1986), guppy *Poecilia reticulata* (Takahashi & Kawahara, 1987), rainbow trout (Shors & Winston, 1989; Castillo *et al.*, 1993; Oshima *et al.*, 1996), tilapia (Mor & Avtalion, 1990), chum salmon *Oncorhyncus keta* (Fuda *et al.*, 1992), channel catfish (Hayman & Lobb, 1993), red sea bream *Pagrus major* (Kanlis *et al.*, 1995), coho salmon *Oncorhyncus kisutch* (Yousif *et al.*, 1995), Atlantic salmon *Salmo salar* (Lillehaug *et al.*, 1996; Olsen & Press, 1997) and sea bass (Breuil *et al.*, 1997). Using ^{125}I-labelled homologous Ig, Bly (1984) demonstrated the uptake of Ig in the eggs of plaice (oviparous fish) and in the young of viviparous blenny (ovarian gestation) and swordtail *Xiphophorus hellery* (follicular gestation). In the channel catfish, IgM was found both in the yolk and in the egg membranes, suggesting that it provides an

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immune barrier at the surface of the egg, as well as protection for the developing fry (Hayman & Lobb, 1993).

Transfer of specific antibody from an immunised female to her eggs has been reported in many oviparous species (Bly *et al.*, 1986; Mor & Avtalion, 1990; Kawakara *et al.*, 1993; Kanlis *et al.*, 1995) while in an ovoviviparous guppy immunity was transferred to the new-born fry by the mucous feeding (Takahashi & Kawahara, 1987). In the red sea bream, the concentration of Ig in the oocytes was found to increase during vitellogenesis. Interestingly, the oocytes as well as the fertilised eggs of the *Vibrio*-immunised females had a much higher specific antibody titre than the same stages from control mothers (Kanlis *et al.*, 1995). Sin *et al.* (1994) have demonstrated passive transfer of protective immunity against *Ichthyophthirius* in tilapia, as well as a beneficial effect of mouthbreeding. The protective immunity was correlated with the anti-*Ichthyophthirius* titres in soluble extracts of fry tissues and maternal plasma. Hence the protective Ig was from two sources: the eggs, but also mother's mouth cavity. These studies indicate that a transfer of maternal antibody takes place in many fish species and suggest that female fish can vaccinated to provide their offspring with protection against specific pathogens.

In rainbow trout, IgM was detected in unfertilised eggs. Subsequently, in embryos, Ig levels increased slowly to reach a peak at the time of hatching, before slowly declining to initial values (11 µg/g egg weight) by 2 months p.h. (Castillo *et al.*, 1993). In tilapia, Ig levels declined during the prelarval stages and reached the lowest value 12 days p.h. (Takemura, 1993). In sea bass, Ig was also found in the eggs (3 µg/g egg weight) with an Ig level decrease in prelarvae and a gradual increase from day 18 p.h. onwards (Breuil *et al.*, 1997). These studies indicated that the maternal supply of IgM was almost depleted by the time of yolk exhaustion at the start of feeding, after which the larvae apparently begin to synthesise endogenous Ig.

I.2.2.2. Lymphoid cells

Specific immunity (in *sensu stricto*) includes the reactions of lymphoid cells in addition to other cellular and humoral components that are related to the immune response of these cells. This type of defence is characterised by antigen specificity and memory formation. In the last case the immune system reacts faster and stronger after repeated contact with the same antigen. This process includes antigen processing and cell cooperation between distinct leucocyte subpopulations (i.e. macrophages, B and T

cells). In fish, functional tests such as hapten-carrier assays suggested a cooperation between T helper and B cells (Yocum *et al.*,1975). In recent years, the availability of specific mAbs has improved the knowledge on the leucocyte subpopulations, but only few studies were related to the ontogeny of the immune system. The central point now is to study the development of immunological structures in relation with the first appearance of leucocyte subpopulations and their functional capacity. The proposed age-equivalence model of lymphoid development, which suggested that different fish species attain the same level of immunological maturity at equivalent age/temperature (Solomon, 1978), was mainly documented by histological observations (Ellis, 1977; Grace & Manning, 1980; Grace, 1981; Botham & Manning, 1981; Schneider, 1983; Josefsson & Tatner, 1993). Unfortunately, functional studies supporting this model are scarce.

The first indications for humoral immunity were found in carp (Secombes *et al.*, 1983b; Koumans-van Diepen *et al.*, 1994a). The appearance of membrane Ig$^+$ cells was detected in the head kidney by day 14 post fertilisation (p.f.), although head kidney lymphoid tissue was already observed 10 days earlier (Botham & Manning, 1981). During carp ontogeny the presence of different B cell subpopulations was suggested and their percentages varied during the larval development (Koumans-van Diepen *et al.*, 1994a). In 14 days-old carp the main population of Ig-bearing cells is recognised by the mAb WCI 4, while at older stages another mAb (WCI12) against Ig reacts with the major B cell population (Koumans-van Diepen *et al.*, 1995). A comprehensive analysis using immunohistochemistry indicates that the kidney of many teleost species could the first organ containing Ig$^+$ cells (rev. Tatner, 1996). These findings together with functional studies support the idea, that the fish kidney may be a primary lymphoid organ for B cell maturation (Kaattari & Irwin, 1985; Irwin & Kaattari, 1986; Schroder *et al.*, 1997). The early detection of Ig$^+$ cells in rainbow trout embryos (Castillo *et al.*, 1993) 8 days before the first identification of renal lympho-haematopoietic tissue (Grace & Manning, 1980; Razquin *et al.*, 1990) suggests that only a limited number of cells is derived from other sources. The *in situ* localisation of such cells has not been established yet. Thus, ontogeny of B cell precursors in fish apparently requires further investigation.

Numerous morphological studies were performed aiming to define the ontogeny of teleost cell-mediated immunity, but general pattern of development in different teleost species has not been firmly established (Manning, 1994; Tatner, 1996; Zapata *et al.*, 1996 for reviews). This is mainly due to the lack of specific markers to detect

early haematopoietic cells. Allograft rejection has been studied in fish to assess the ontogenetic development of cellular immunity. Studies in rosy barb (Rijkers & van Muiswinkel, 1977), carp (Botham & Manning, 1981) and rainbow trout (Tatner & Manning, 1983) have demonstrated the early functional maturation of cytotoxic T cells, few days after their morphological detection during development (Tatner, 1996). Studies to clear up whether antigens encountered early in life can prime the immune system to yield positive or negative (immunological tolerance) memory responses upon subsequent challenge with the same antigen were conducted in carp and sea bream (van Muiswinkel *et al.*, 1985; Mughal *et al.*,1986; Joosten *et al.*,1995). Carp vaccinated by immersion or by antigen injection with *Aeromonas salmonicida* bacterin (thymus independent antigen) 4 week p.f. showed an enhancement of the serum Ig levels. However, when the carp were vaccinated with thymus-dependent antigens, such as human gamma globulin (HGG), the secondary challenge showed development of the immunological tolerance (Mughal *et al.*, 1986). Carp orally vaccinated with *Vibrio anguillarum* bacterin at 2 or 4 weeks p.f. showed antigenic tolerance, whereas an oral vaccination at 8 weeks p.f. appeared to develop immunological memory (Joosten *et al.*, 1995) suggesting that the maturation of immune system in carp takes place in the first two months p.f.

I.2.3.Ontogeny of non-specific immunity

In addition to any maternally derived protection, larvae and fry possess a variety of non-specific defence mechanisms that provide them with protection prior to the maturation of the specific immune system. In addition to non-specific lectins, haemagglutinins and maternal IgM, fries also possess an efficient phagocytic system (rev. Tatner, 1996). In trout at 4 days p.h. phagocytosis of carbon particles was observed by mobile macrophages, which accumulated in connective tissues and epidermis of skin, gut and gills. The trapping of carbon particles in the gills of young fry was interpreted as a specialised mechanism to protect the developing thymus from undesirable and possibly tolerogenic antigen exposure (Tatner & Manning, 1985). The hypothesis that non-specific mechanisms are developed earlier than specific responses is further supported by the observation that carp macrophages were already localised in the yolk sac (Botham & Manning, 1981).

I.3.Aim and outline of this thesis

Although the teleost immune system has not been studied as extensively as that of mammals or birds, it can be stated that a number of structural and functional features related to humoral, cell-mediated, and non-specific immunity are shared by all classes of vertebrates. The immune system, of teleost fish is composed of similar leucocyte subpopulations compared with other vertebrates and these cells are localised in lymphoid organs and can be associated to epithelia to form a mucosa-associated lymphoid tissue (MALT). However, the exact correlation with the mammalian immune system appears to be difficult in fish, due to the lack of a set of specific identification markers.

This thesis aims to contribute to the knowledge of the developing immune system in fish. Specific mAbs are used to detect the distribution and percentages of leucocytes subpopulations during ontogeny. The freshwater teleost carp (*Cyprinus carpio*, L.) and the sea bass (*Dicentrarchus labrax*, L.) were used because of the availability of specific T cell mAbs in these species.

In **chapter 2**, the early presence of leucocytes subpopulations were detected by immunofluorescence/flow cytometric analysis using specific carp mAbs for early T cells, B cells, monocytes/macrophages and thrombocytes. The relative percentages of immunocompetent cells in the important lymphoid tissues (thymus, head kidney, spleen, blood and intestine), from 1 week to 30 weeks p.f., provides information on the quantitative distribution of leucocyte subpopulations during development, and on the origin of these leucocyte types.

In **chapter 3**, a mAb (WCL15) only reactive with monocytes/macrophages in fixed tissue was used to describe the distribution *in situ* of these cells in lymphoid organs of carp (thymus, head kidney, spleen and gut) from 2 days p.f. until 60 weeks p.f. The positive cells were detected by avidin-biotinylated peroxidase immunostaining with nickel-enhancement on paraffin section, indirect immunofluorescence and by immuno-gold electron microscopy.

In **chapter 4**, the ontogeny of the carp thymus is defined by using the mAb WCL9 specific for early T cells. Special attention was paid to the development of cortex and medulla by using immunohistochemical, confocal-laser-scanning analyses. The ultrastructural study of the developing thymus permitted the analysis of the

distribution and morphological differences of the epithelial cells. In addition, the *in situ* localisation of apoptotic cells were studied in paraffin embedded thymuses, trying to connect the distribution of thymocytes and epithelial cells with the proposed selection process in the thymus.

In **chapter 5**, mAb WCL38, was used during the ontogeny of carp to detect mucosal T cells in cell suspensions from systemic and mucosal lymphoid organs by flow cytometry. The appearance and distribution of lymphocytes in some developing epithelia such as intestine, gills and skin has also been included.

In **chapter 6**, the mAbs DLT15 and DLIg3 specific for T and B cells of sea bass, were employed to describe the morphology of these cells. Enriched leucocyte fractions from different tissues (thymus, spleen, head kidney, intestine and blood) were used.

In **chapter 7**, the mAb DLT15 was employed to analyse the ontogenetic development of lymphoid cells within thymus, head kidney and spleen of sea bass, with the aim of obtaining new information on the differentiation of T cells in this marine species.

In **chapter 8**, the observations described in this thesis are discussed in relation to their possible implications for phylogeny and aquaculture.

Chapter 2

Leucocyte subpopulations in developing carp (*Cyprinus carpio* L.): immunocytochemical studies.

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Abstract

The distribution of leucocyte subpopulations was studied in thymus, head kidney, spleen, gut and blood of developing carp by using monoclonal antibodies against early T-cells, B-cells, thrombocytes and macrophage-like cells. In the first week post-fertilisation (p.f.), early T-cells are very numerous (approximately 77 %) in thymus, but also present in other organs, especially the head kidney; subsequently these cells gradually disappear in all organs, except thymus (approximately 40%). B-cells appear in head kidney from the second week p.f., and later in spleen and blood, but their number remains low in thymus and gut. Thrombocytes first appear in spleen during the first week p.f. and their percentage increases until the fourth week (approximately 30%). Monocyte/macrophage-like cells are present in all organs from the first week p.f., and their percentage gradually increases until eighth week p.f. This study indicates that: (1) thymus may be the primary lymphoid organ for T lymphocytes; (2) head kidney could be the primary lymphoid organ for B lymphocytes; (3) spleen is a primary site for thrombopoiesis; and (4) monocyte/macrophage-like cells are already present in early age. These findings provide new information on development of fish immune system.

Introduction

The teleost immune system has not been studied as extensively as that of mammals, although a number of structural and functional features related to humoral, cell-mediated, and non-specific immunity are shared by both classes of vertebrates (Ellis, 1977; Esteban _et al._, 1989). At present, there is increasing interest for the immune system and immune responses of fish, both to establish comparative relationships with other vertebrates and to control fish health in aquaculture.

Previous studies of leucocytes distribution during fish development were performed by light and electron microscopic observation in marine species (Ellis, 1977; Esteban _et al._, 1989; Jósefsson & Tatner, 1993) and freshwater species (Cannon _et al._, 1980; Grace & Manning, 1980; Fishelson, 1990). In carp, microscopical observations revealed macrophages presence before the appearance of lymphoid tissues (Manning, 1994) and first lymphocyte-like cells around the first week post-fertilisation (p.f.)

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(Botham & Manning, 1981). Obvious progress derived from functional tests and the production of antibodies recognising fish leucocytes subpopulations such as granulocytes (Ainsworth, 1992; Hine, 1992), monocytes/macrophages (Hardie _et al._, 1994, Sveinbjornsson & Seljelid, 1994; Koumans-van Diepen _et al._, 1994_a_), thrombocytes (Rombout _et al._, 1996), B-lymphocytes (Irwin & Kaattari, 1996; Miller _et al._, 1994; Koumans-van Diepen _et al._, 1995; Scapigliati _et al._, 1996), and T-cells (Scapigliati _et al._, 1996; Miller _et al._, 1987; Koumans-van Diepen _et al._, 1994a) in a variety of fish species. Particularly for carp, monoclonal antibodies specific for immunoglobulins and Ig-producing cells (Koumans-van Diepen _et al._, 1995; Koumans-van Diepen _et al._, 1994_b_), early T-cells (Koumans-van Diepen _et al._, 1994b), thrombocytes (Rombout _et al._, 1996) macrophages and thrombocytes (Weyts _et al._, 1996) are available.

In this study we used these monoclonal antibodies (WCL9, early T-cells, WCI12, Ig-producing cells, WCL6, thrombocytes, WCL15, monocyte/macrophages and thrombocytes) to analyse the presence of immunocompetent cells in the most important lymphatic tissues (thymus, head kidney, spleen, blood and intestine) during carp development (from 1 week to 30 weeks post-fertilisation). Although the ontogeny of B-cells has been described from 2 weeks (except intestine and thymus) (Koumans-van Diepen _et al._, 1994_b_), an earlier presence of B cells before this date were suggested. For this reason, this study included the analysis of B-cells from 1 week for confirmation and comparison with other leucocyte subpopulations. The immunoreactive cells were detected by immunofluorescence/flow cytometric analysis to assess the ontogenetic development (timing and percentage) of leucocytes subpopulations.

Materials and Methods

Animals

Wild-type carp, _Cyprinus carpio_ (L.) 1 to 30 weeks old and adult (80 weeks), were bred and kept in the laboratories of the Department of Experimental Animal Morphology and Cell Biology (now, Dept. of Animal Sciences) of the Agricultural University of Wageningen (The Netherlands). They were reared in recirculating, filtered, UV-

sterilised water at 23°C and fed with _Artemia salina_ nauplii for 2 weeks followed by Trouvit K 30 pellets (Trouw & Co., Putten, The Netherlands) at a daily age-dependent ration of 10% (2 week-old) decreasing to 2% at 30 weeks onwards.

Before the dissection of head kidney, thymus, whole gut and spleen, fish were killed by an overdose of 0.03% tricaine methane sulfonate (Crescent Research Chemicals, Phoenix, AZ) in water (w/v). From 6 weeks onwards, blood was sampled from the caudal vein using a syringe containing 0.5 ml heparin solution (50 IU/ml) in c-RPMI (RPMI+10% double-distilled water). From animals of 1-to 5-weeks of age, blood was collected upon severing the tail. Leucocytes from blood and organs from 6-30 individuals (number decreasing from 30 to 6 with age) were pooled in order to obtain enough cells ($>2X10^6$) for analysis.

Cell suspension

Cell suspensions from head kidney, thymus, spleen and gut were prepared in c-RPMI containing 0.1% sodium azide by teasing the tissues through a nylon gauze filter (50 μm mesh). Peripheral blood leucocytes (PBL) were prepared from heparinised blood diluted 1:1 in c-RPMI and centrifuged (15 min at 100 g) at 4°C, subsequently the buffy coat was collected. All cell suspensions were washed ones (from 1 to 6 weeks) or twice for 10 min at 680g and 4°C in c-RPMI and resuspended in 2 ml of c-RPMI. These cell suspensions are layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in c-RPMI to yield densities of 1.020 and 1.070 g ml^{-1} (leucocytes lacking basophils collect at the interface, as previously demonstrate by Koumans-van Diepen _et al._, 1994_a_). After centrifugation (30 min at 840 _g_) at 4°C, cells layered between the densities mentioned above were collected and washed twice (10 min at 680g) at 4°C. The pellet was resuspended in c-RPMI with 1% BSA and 0.1% sodium azide. Peripheral blood leucocytes (PBL) were prepared from heparinised blood (5 ml) diluted 1:1 in RPMI and centrifuged (15 min at 100 g) at 4°C. The buffy coat was collected, resuspended in RPMI and layered as described above.

Monoclonal antibodies

WCI12 is specific for carp immunoglobulins and Ig-producing cells was previously described (Koumans-van Diepen *et al.*, 1994c), as a murine monoclonal antibody reactive with the heavy chain of carp Ig. WCL9 reacts with a membrane antigen on a thymocyte subpopulation (early T-cells, Rombout *et al.*, 1997). The anti-thrombocyte monoclonal antibody, WCL6 reacts with living (Rombout *et al.*, 1996) and fixed cells (unpublished data) and WCL15 is reactive with monocytes/macrophages and thrombocytes (Weyts *et al.*, 1997). All monoclonal antibodies are of IgG_1 class except WCL15, which is IgM.

Immunofluorescence and flow cytometry

Leucocytes were incubated suspensions of 250 µl in different tubes with WCI12 (1:100), WCL9 (1:20), WCL15 (1:100) and WCL6 (1:100). Labelled cells were then washed (10 min at 680g) at 4°C and incubated for 30 min with fluorescent-conjugated rabbit anti-mouse Ig (RAM-FITC, Dako, Glostrup, Denmark) diluted 1:100 in c-RPMI. After washing, 10^4 cells were measured with a flow cytometer (FACStar, Becton Dickinson Immunocytometry System, Mountain View, CA, USA), gated specifically for lymphoid cells (Fig.1) and analysed using the Consort 30 data analysis package The percentage of antibody-positive were calculated from pools of different individuals (N=30 in 1 and 2 week old carp; N =11 from 3 to 5 week old and N =5 from 6 week onwards). Controls for non-specific reactions were performed and in each experiment cell suspensions from 60 week old fish were used as positive controls. The percentage of monoclonal antibody positive cells was calculated by subtracting the percentage of cells labelled with only the secondary antibody (RAM-FITC).

Results

Figure 1 shows the forward scatter (FSC) and 90° side scatter (SSC) profile of all organs used from 2 and 30 week old carp, including the gate used to calculate the percentage of immuno-reactive leucocytes. Events with a low FSC (non-cellular particles) or cells with a high SSC were excluded from the gate; these cells mainly represent granulocytes as was proven by fluorescence activating cell sorting and
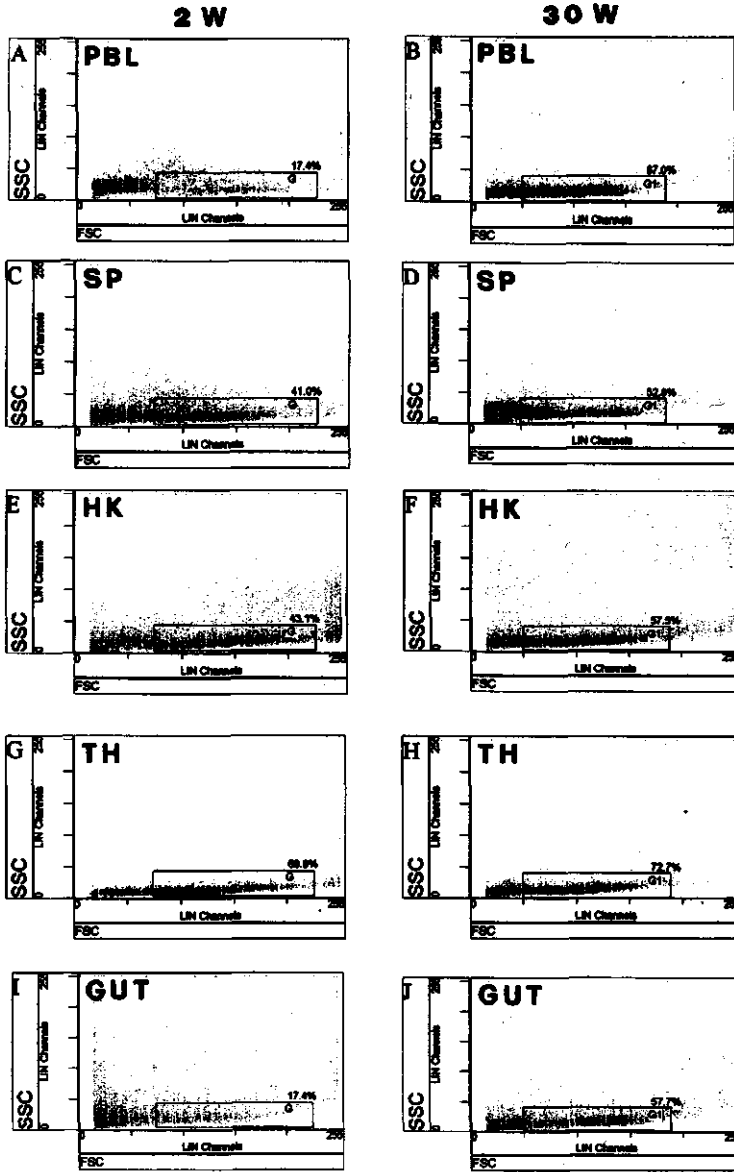
Fig.1 FCS/SSC profiles of each organ used of 2 and 30 weeks p.f. old carp showing the gate used for calculating the percentage of lymphoid cells with a low side scatter. The percentage of gated cells is given in each profile (a-j): a, b) PBL suspension of 2 and 30 week-old carp, respectively; c, d) splenocytes of 2 and 30 week-old carp, respectively; e, f) head kidney cells of 2 and 30 week-old carp, respectively; g, h) T-cells of 2 and 30 week-old carp, respectively; i, j) gut leucocyte suspensions of 2 and 30 week-old carp, respectively.

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WEEKS

		1	2	3	4	6	8	12	16	80
WCII12	TH	0.1±0.2	0	0	0.1±0.1	0.2±0.0	0	0.7±1.0	0.2±0.2	0.9±0.9
	HK	0	1.5±1.5	2.5±1.5	6.8±3	13.5±6.7	17.4±3.5	19.2±2.7	20.4±0.9	24.5±4.7
	SP	0	0.5±0.9	2±1.4	3.4±13	5.9±1.7	8.5±3.2	10.3±0.2	14.8±0.3	17.9±2.0
	GUT	0.3±0.1	0	0.8±0.8	0.8±0.2	2.3±0.5	2.8±0.9	3.6±3.7	4.4±01	6.9±2.7
	PBL	0.3±0.1	0.5±0.5	0.3±0.0	1.3±1.8	5±4.4	6.7±5	19.9±6	11.9±4.5	33±2.7
WCL9	TH	77.4±1.6	58.3±7.3	57.0±19.2	57.4±16.7	67.6±9.2	50±16.5	41.5±5.6	39.7±8.3	51.2±5.3
	HK	23.3±12.3	10.6±0.6	5.5±4	5.4±3	2±1.2	1.7±0.7	0.8±1.1	0.3±0.2	0.8±0.21
	SP	4.9±7	2.5±1.6	1.5±1.6	1.4±1.2	1.1±1.5	1.6±1.0	1.3±0.6	0.4±0.6	0.3±0.5
	GUT	5.8±6	2.5±2.1	3±2.7	5.1±5	2±1	0.2±0.3	0.7±0.0	0.1±0.2	1.5±0.6
	PBL	10.6±13.3	1.2±0.6	1.5±0.5	2.7±3.9	3.5±0.7	0.1±0.1	2.2±1.7	0.8±0.4	0.6±0.4
WCL6	TH	0.2±0.2	0	0.1±0.1	0.1±0.1	0.35±0.0	0	0	0	6.6±0.0
	HK	1.3±1.3	0.5±0.9	0.8±0.7	0.7±0.5	1.3±0.5	0.8±0.7	0.7±0.3	0.7±0.4	0.9±0.0
	SP	3.6±0.6	10.7±2	20.8±7.8	19.3±8.6	13.0±0.5	9.2±0.6	11.9±13	14.7±93	9.7±0.3
	GUT	0.3±0.0	0	0	0.17±0.0	0.5±0.3	0	0	0.4±0.5	0.9±0.1
	PBL	1±1.8	2.1±1	4.1±5.9	1.2±1.2	21.4±14.8	9.2±5.3	25.5±10.3	11.3±2	35.1±6.1
WCL15 (+)/ WCL6 (-)	TH	0.8±0.5	6.5±0.7	21.6±5	44±12.8	46.8±8.4	46.2±8.4	41.0±10.3	31.8±7.1	6.09±0.7
	HK	3.8±1.5	17.8±3.7	19.9±8.6	25.6±8.9	36.0±1.9	36.7±1.9	30.9±5	21.5±7.3	20.2±4.0
	SP	0.9±0.6	0.3±0.3	3.7±3	17.5±10.7	38.3±10	41.5±4	29.9±1.9	25.4±10.2	35.6±0.3
	GUT	5.5±1.3	8.4±2.3	14.4±7	19.4±9	30.2±7.8	26.4±10.4	38.3±4.3	26.5±2.2	33.7±8.0
	PBL	0.9±0.3	3.2±3.9	5.7±2.4	32.4±17.5	31.7±22.5	34.6±17.9	38.8±15.2	39.9±16.7	31.1±19.4

Table 1 Percentages of WCII12, WCL9, WCL6 and WCL15 positive/WCL6 negative cells in leucocyte fractions of thymus (TH), head kidney (HK), spleen (SP), gut and peripheral blood leucocytes (PBL) of larval and juvenile carp, calculated by flow cytometry. Data are expressed as mean ± standard deviation of three experiments made on different offsprings of the same carp strain.

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electron microscopic examination of sorted cells (Verburg-van Kemenade *et al.*, 1994). In each organ the percentage of gated cells increased with age, especially during the first 8 weeks. From 8 to 30 weeks there was a slight increase. Small change was found in thymus (from 70 to 73%) and the biggest in PBL (from 17 to 67%). The majority of immunoreactive cells were present in the gate, with the exception of WCL15-immunoreactive cells, with a minor population with SSC higher of that used for the gate. Figs. 1-5 represent the results obtained in one offspring. The Table 1 show the mean ± standard deviation of results obtained in three different offsprings (with the same strain).

The percentage of Ig-bearing cells (WCI12$^+$cells) in thymus, head kidney, spleen, gut and blood of 1 to 30 week-old is depicted in Fig. 2. The percentage of Ig-bearing cells was very limited (<1%) in all organs of 1 week-old larvae and became detectable in head kidney (3%), spleen (1%) and blood (1%) in 2 week-old carp. From the second week to the thirtieth week these percentages increased gradually in head kidney and spleen (for both, up to 20%). From the sixth week the percentage also increased in blood (up to 35% after 30 weeks) but it remained very low in thymus and gut.
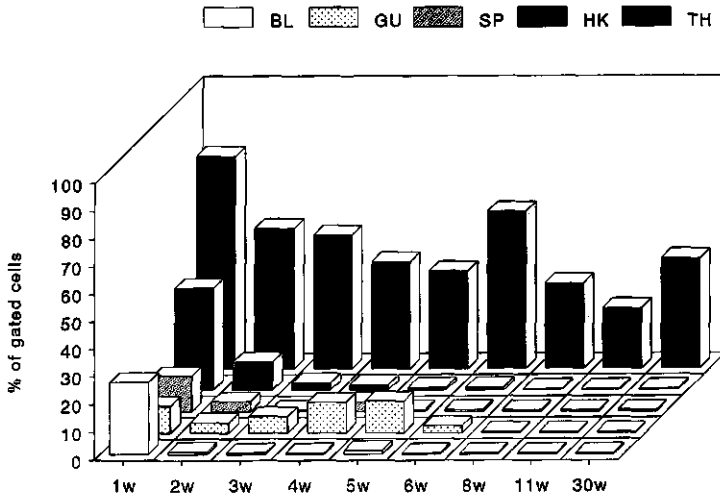
The percentage of early T-cells (WCL9$^+$cells) in thymus, head kidney, spleen, gut and blood of 1 to 30 week-old is depicted in Fig.3. Early T-cells were very numerous (77%) in thymus and clearly detectable in cell suspensions from head kidney (37%), spleen (13%), blood (26%) and gut (10%) of one week old animals. Throughout development, the percentage of early T-cells sharply decreased in all organs (<1%), remaining high only in the thymus (around 40%).

The percentage of thrombocytes (WCL6$^+$ cells) in thymus, head kidney, spleen, gut and blood of 1 to 30 week-old is depicted in Fig.4. Thrombocytes first appeared in the spleen from 2 weeks post-fertilisation (4%) and their percentage increased up to 30% in the third week. In the other organs the percentage remained very low (< 1%). In the fourth week WCL6$^+$ cells became detectable (5%) in blood. From the fifth week onwards, the number of thrombocytes quickly reached the adult levels of approximately 10% in spleen and 30-40% in blood.

The percentage of monocyte/macrophages (WCL15$^+$/WCL6$^-$ cells) in thymus, head kidney, spleen, gut and blood of 1 to 30 week-old is depicted in Fig.5. The
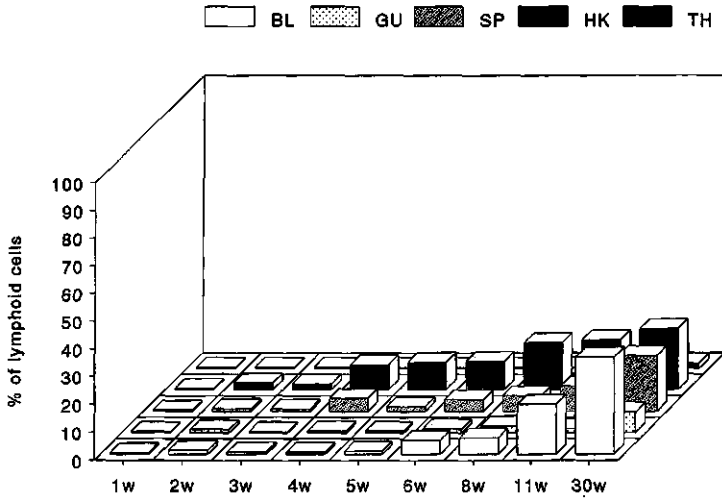
Fig.2 Ontogeny of WCI12$^+$ carp cells. Time-course graph of WCI12$^+$ cells in lymphoid organs detected in immunofluorescence-flow cytometric analysis from 1 to 30 week old carp. BL: blood; GU: gut; SP: spleen; HK: head kidney; TH: thymus.

Fig.3 Ontogeny of WCL9$^+$ carp cells. Time-course graph of WCL9$^+$ cells in lymphoid organs detected by immunofluorescence-flow cytometric analysis from 1 to 30 week old carp. BL: blood; GU: gut; SP: spleen; HK:

The percentage of monocyte/macrophages (WCL15$^+$/WCL6$^-$ cells) in thymus, head kidney, spleen, gut and blood of 1 to 30 week-old is depicted in Fig.5. The percentage of WCL6$^+$ cells has been subtracted because of the cross-reaction of WCL15 with thrombocytes.

Monocyte/macrophages were already present in all organs, except the spleen, of 1 week-old fish and their number gradually increased during development reaching considerable percentages in the eighth week in spleen (46%), thymus (37%), head kidney (37%), blood (33%) and gut (19%). Between 8 and 30 weeks the percentage of WCL15$^+$ 6$^-$ cells increased in gut (up to 38%) and decreased in blood (to approximately 18%), while it did not changed in other organs.

Although statistical data cannot be given since leucocytes of different individuals were pooled throughout the experiment, the presented results show the same tendencies as found in three other similar performed experiments (Table 1).
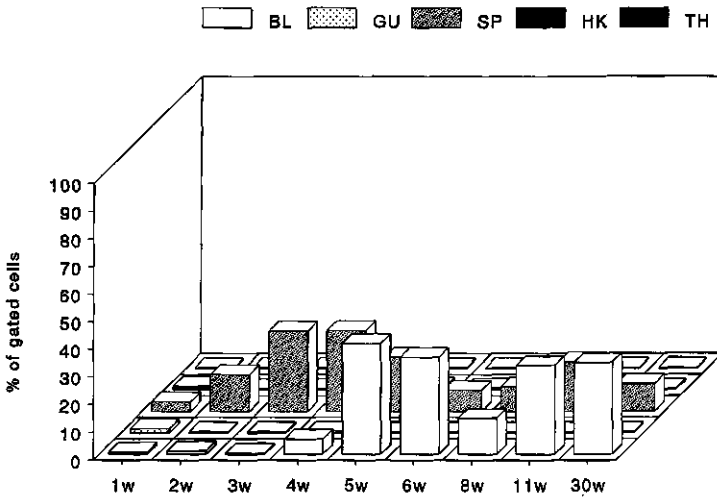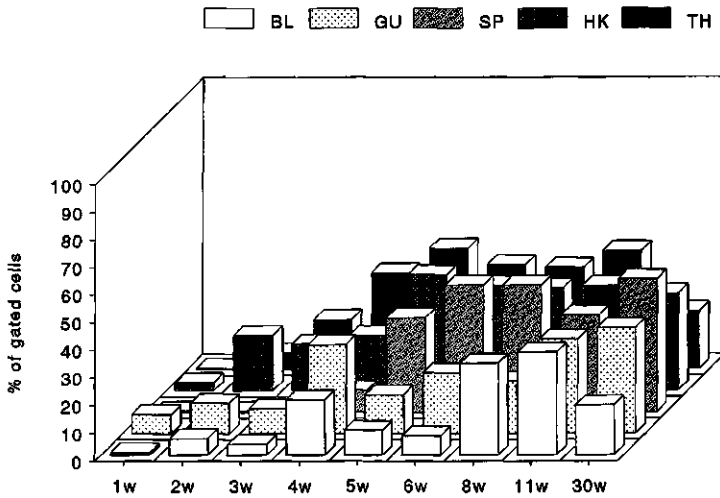

Fig.4 Ontogeny of WCL6$^+$ carp cells. Time-course graph of WCL6$^+$ cells in lymphoid organs detected b immunofluorescence-and flow cytometric analysis from 1 to 30 week old carp. BL: blood; GU: gut; SP: spleen; HK head kidney; TH: thymus.

Discussion

In this study the timing of ontogenetic development of leucocyte subpopulations (B cells, early T-cells, thrombocytes and monocytes/macrophages) in different lymphoid organs of carp is reported.

Fig.5 Ontogeny of WCL15$^+$6$^-$ carp cells. Time-course graph of WCL15$^+$ cells in lymphoid organs detected by immunofluorescence-and flow cytometric analysis from 1 to 30 week old carp. BL: blood; GU: gut; SP: spleen; HK: head kidney; TH: thymus. The values are generated by subtraction of WCL6$^+$ cells from the percentage of WCL15$^+$ cells in spleen and blood.

The development of lymphoid organs in carp seems to be precocious as compared with other teleost species (Grace & Manning, 1980; Fishelson, 1990; Hine, 1992; Abelli *et al.*, 1994). Head kidney develops between the third and the fourth day post-fertilisation

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(p.f.) and thymus a day later, and spleen between the fourth and fifth day. A morphological investigation revealed that lymphocyte-like cells are detectable around the first week in thymus, head kidney and spleen and one day later also in gut and blood (Botham & Manning, 1981).

It should be noted that leucocytes resulted from Percoll separation (between 1.020 and 1.070 g l^{-1}) and they have been studied using a FSC/SCC gate to exclude non-cellular particles and granulocytes. In adults, the same fraction contained lymphoid cells, thrombocytes and monocytes/macrophages (Koumans-van Diepen *et al.*, 1994*a*). Erythrocytes, basophilic granulocytes and most neutrophils of adults have a higher density, but it cannot be excluded that a part of these cells could be present in cell suspensions of younger carp. In fact, preliminary, electron microscopic studies (unpublished) have shown that cell suspensions of blood and spleen of 2-6 week old carp still contain a considerable number of erythrocytes. This observation may explain in early stages the lower number of cells observed inside the gate because the majority of cells (erythrocytes and granulocytes) were present outside this gate. Three monoclonal antibodies reacting with leucocyte subpopulations of the carp were used as leucocyte marker: WCI12 with B cells (Koumans-van Diepen *et al.*, 1995; Koumans-van Diepen *et al.*, 1994c), WCL6 with thrombocytes (Rombout *et al.*, 1996) and WCL9 with early T-cells (Koumans-van Diepen *et al.*, 1994b). The fourth monoclonal antibody used (WCL15) has been described as a putative marker of monocytes and macrophages, but also reacted with thrombocytes and showed a faint reaction with basophils (Weyts *et al.*, 1996). Percoll enrichement used in this study probably avoided the collection of basophils, since they have density higher than 1.07 gr ml^{-1} (Verburg-van Kemenade *et al.*, 1994). Therefore, the subtraction of the percentage of WCL6^{+} cells from the percentage of WCL15^{+}cells gave an indication on the putative percentages of monocyte/macrophages present in each organ.

Ontogeny of B cells

Previous immunofluorescence and immuno-electronmicroscopy studies revealed the presence of B cells and plasma cells in 0.5 to 16 month-old carp (Koumans-van Diepen *et al.*, 1994b, 1995). Present results confirm the first appearance of B cells in the

head kidney around the second week p.f., and their number raises with age (up to 20%). Lower percentages of B cells are detectable in other organs in the first 4-5 weeks of development. Following head kidney, the spleen also shows a significant percentage of B cells (5%) at 4 weeks, and this percentage gradually increases and reaches adult values (approximately 20%) at 30 weeks. In blood, considerable numbers of B cells appear in the sixth week p.f. showing a gradual increase until 35% at 30 weeks. In thymus and gut the proportion of Ig^+ cells remains low, except a rise until 8% between the eleventh and thirtieth week p.f. in the gut. Part of these Ig^+ cells in the gut may be Ig-binding macrophages as previously demonstrated (Rombout et al., 1993). These Ig^+ macrophages are principally found in the intestine and not in cell suspension of other lymphoid organs (Koumans-van Diepen et al., 1994a). Thus, it can be concluded that the majority of Ig^+ cells detected outside the gut are B cells. The low percentage of B cells observed in the thymus during ontogeny confirms that the thymus mainly contains T cells. The primary presence of high percentage of B cells in head kidney and later in spleen of carp suggests that these organs are predominant sites of B cell localisation. At present it is not clear whether the spleen is colonised by B cells from head kidney or it can produce these cells independently at a later stage. The observation that B cells can be recognised from 2 weeks onwards, and that plasma cells have been reported in carp to be present at 1 month onwards (Koumans-van Diepen et al., 1994c; Secombes et al., 1983), indicate a maturation humoral immune system between 2 and 4 weeks of development. The maximum number of plasma cells is found after 3 months, while the percentage of B cells is still growing after 1 year (Koumans-van Diepen et al., 1994c), indicating that the complete maturation of the B cell population takes even longer than 30 weeks.

Ontogeny of early T-cells

WCL9 monoclonal antibody only reacts with a portion of the thymocytes of adult carp (Koumans-van Diepen et al., 1994b). In this study, high percentages of $WCL9^+$ cells (approximately 80%) were found in thymus at 1 week p.f., but considerable numbers were also found in the other lymphoid organs investigated, especially head kidney (approximately 37%). The small size of larvae before the first week p.f. did not

allow dissection of lymphoid tissues, therefore FACS results are not available. However, immunohistochemistry revealed positive cells only in the thymic anlage around the fourth day p.f. (unpublished data). The thymic origin of WCL9 positive cells later found in the head kidney and blood could be speculated and tracing experiments are needed to properly address this hypothesis. Migration of thymocytes to the head kidney has been suggested during the development of carp (Secombes et al., 1983), sea bream (Jósefsson. & Tatner, 1993) and sea bass (Abelli et al, 1996), and isotope-labelled thymocytes could be traced to the head kidney and spleen in older trout.

In this study, the percentage of early T cells in the thymus gradually decreases in the first weeks of development reaching the adult level (40%, Rombout et al., 1997) around the sixth week. Possibly, early T-cells first appear in all lymphoid organs, but they become confined to the thymus at later ages. Consequently, WCL9 appears to be a suitable and unique marker to study early T-cell development in carp. Results obtained in chicken with a monoclonal antibody (CT-1) directed against early T-cells, showed that differentiation antigens were expressed in the thymus and then disappeared following seeding of immature T cells to the peripheral lymphoid organs (Chen et al., 1984). Recently, a considerable marker has been described for sea bass (Scapigliati et al., 1995, Abelli et al., 1996) which is reactive with most T-cells, but also with low percentages of Ig-negative cells in other lymphoid organs of adult fish, suggesting that this antibody can recognise at least a subset of mature T-cells.

Ontogeny of thrombocytes

Fish thrombocytes are responsible for blood clotting in response to injury and are activated by collagen (Ellis et al., 1977; Rowley et al., 1988; Uchida et al., 1992). These features resemble those of mammalian platelets, but fish thrombocytes are nucleated cells and morphologically resemble lymphoid cells (Ellis et al., 1977). Until now no specific antibody was available for fish thrombocytes due to the cross-reaction with T cells and neutrophils (Miller et al., 1987). WCL6 reacts specifically with carp thrombocytes and their precursors (Rombout et al., 1996), for this reason it represents a suitable tool in developmental study in order to highlight the primary site of differentiation of thrombocytes. Thrombocytes in developing carp first appear in the

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spleen from 1 week post-fertilisation (4%) and this percentage strongly increases until the 4th week (approximately 30%). From the fifth week onward, the percentage of thrombocytes decreases in spleen and quickly increases in blood, whereas it is hardly detectable in cell suspensions from the other organs. Similar distribution also occurs in adult carp, where flow cytometry revealed the majority of thrombocytes in PBL (30%) and spleen (10%), and negligible amounts in other organs including the head kidney (Rombout _et al._, 1996). Taken together these results support the hypothesis that spleen is the main thrombopoietic organ in carp.

Ontogeny of macrophages

Monocytes/macrophages are involved in specific and non-specific reactions of the immune system, such as phagocytosis, killing of micro-organisms and antigen presentation (Ellis _et al._, 1977; Unanue _et al._, 1984). In fish, it is generally assumed that circulating monocytes and tissue macrophages belong to the same cell lineage, and that macrophages and neutrophils are the predominantly active cells, being highly phagocytic for both inert and antigenic materials (Manning, 1994; Verburg-van Kemenade _et al._, 1994). Carp macrophages have a different morphological pattern (Rombout _et al._, 1989, 1993; Lamers, 1986; Verburg-van Kemenade _et al._, 1995), Ig- and/or complement-binding capacity (Koumans-van Diepen _et al._, 1994a) and secreting cytokines, such as interleukin-like factors (Verburg-van Kemenade _et al._,1995). Flow cytometry and immuno-electronmicroscopy analyses revealed that WCL15 is reactive with monocyte/macrophage like cells (Weyts _et al._, 1996), but also cross reacts with thrombocytes. The subtraction of the percentage of thrombocytes (WCL6$^+$ cells) has given an indication of the percentage of monocyte/macrophage-like cells. The presence of monocyte/macrophages (WCL15$^+$6$^-$ cells) can be detected in all organs from 1 week p.f. onwards. Previous morphological studies localised macrophage-like cells before the differentiation of any lymphoid organ, suggesting that the yolk sac may be the primary site for macrophage development (Manning, 1994). Immunohistochemical study revealed that localised WCL15-positive cells develop before other leucocyte subpopulations (unpublished data). The percentages of monocyte/macrophages gradually increase from 1 week p.f., reaching a plateau during the 2nd month. From

these results it can be concluded that monocyte/macrophages are present in considerable number in the first month of development. Investigations in earlier stages of development (i.e. before the first week) are in progress to establish the function and localisation of these cells in a period when the specific immmune response is not yet functional.

In conclusion, distinct leucocytes populations appear in carp at different ages and at distinct locations during development. From this study it can be hypothesised that: (1) the thymus is a primary lymphoid organ for T-lymphocytes, (2) the head kidney is the primary lymphoid organ for B-lymphocytes, (3) the spleen is the primary site for thrombopoiesis and (4) monocyte/macrophage-like cells are already present at stage when leucocyte subpopulations still have to develop; and (5) B cell subpopulation appeared later in development compared with other leucocytes. These findings on the maturation of the carp immune system may be used to improve the knowledge of developmental and comparative immunology of vertebrates, and to better define the age of carp vaccination.

Chapter 3

Distribution of macrophages during fish development: an immunocytochemical study in carp (*Cyprinus carpio* L.).

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Abstract

A monoclonal antibody against carp macrophages (WCL15) has been utilised in flow cytometry, immuno-histochemistry and immuno-electron microscopy to assess the distribution of monocytes/macrophages in developing carp lymphoid tissues. In suspensions of living cells WCL15 reacted strongly with cytoplasm and plasmic membrane of macrophages. It also cross-reacted with a subpopulation of thrombocytes, but this reaction could be neglected by double immunostaining in combination with a thrombocyte-specific marker. In Bouin-fixed tissues the antibody distinctly recognised macrophages.

Macrophages were found from day 2 post-fertilisation in head kidney and in the dorsal portion of the yolk sac epithelium. From 1 week onwards macrophages were found scattered in thymus and gut and, during the second week in spleen. Macrophages increased in number in all lymphoid tissues until the 6-8th week post-fertilisation, but they decreased except in thymus, where they became localised mainly in the cortical-medullary boundary, and in white pulp areas of head kidney.

The role of macrophages in allowing an early non-specific defence in young fish and in co-operating during the differentiation processes of T-cells and B-cells is discussed.

Introduction

The study of the immune system of fish is having a great impetus, both for a better knowledge of the evolution of the vertebrate immune system and to improve the control of fish health in aquaculture. Teleost fish show analogy with other vertebrates concerning humoral, cellular and non-specific responses (reviewed by Manning, 1994). Phagocytosis appears to play an important role in the non-specific defence against microbial and microsporidian infections, as well as in the elimination of damaged tissues (Secombes & Fletcher, 1992). Fish phagocytic cells include granulocytes (especially neutrophils), monocytes and tissue macrophages. A large body of evidence suggests that fish macrophages also play an important role in induction of humoral immune responses by presenting antigens and secreting cytokines (Secombes, 1991; Verburg-van Kemenade et al., 1995).

The characterisation and localisation of fish monocytes/macrophages was mainly based on functional (Faisal & Ahne, 1990; Verburg-van Kemenade *et al.*, 1994) and histological observations (Manning, 1994; Koumans-van Diepen *et al.*, 1994). Specific monoclonal antibodies (mAbs) for leucocyte populations are available for T- and B- lymphocytes of different fish species (Koumans-van Diepen *et al.*, 1995; Scapigliati *et al.*, 1995, 1996; Passer *et al.*, 1996; Rombout *et al.*, 1997; Joosten *et al.*, 1997), non-specific cytotoxic cells (Evans & Jaso-Friedmann, 1992) and carp thrombocytes (Rombout *et al.*, 1996); however, mAbs for monocyte/macrophages were still lacking.

In this study a mAb (WCL15) reactive with monocytes/macrophages has been characterised and used to describe the distribution of such cells in lymphoid organs of carp (thymus, head kidney, spleen and gut) from 2 days post fertilisation (p.f.) until 60 weeks p.f. The positive cells were detected by avidin-biotinylated peroxidase immunostaining with nickel enhancement on paraffin sections, by indirect immunofluorescence and by immuno-gold electron microscopy.

Materials and methods

Animals

Wild-type carp, *Cyprinus carpio* (Linnaeus, 1758) from 2 to 420 days p.f. were bred and kept in the laboratories of the Wageningen School of Animal Sciences of the Agricultural University of Wageningen, The Netherlands. They were reared in recirculating, filtered, UV-sterilised water at 23°C and fed with *Artemia salina* nauplii for a period of 2 weeks followed by Trouvit K 30 pellets (Trouw & Co., Putten, The Netherlands) at a daily age-dependent ratio of 10% (2 week-old) decreasing to 2% at 30 weeks.

Before the dissection of head kidney, thymus, gut and spleen, fish were killed with an overdose of buffered 0.03% tricaine methane sulfonate (TMS; Crescent Research Chemicals, Phoenix, USA) in water (w/v).

Production and selection of monoclonal antibodies against carp macrophages.

The mAb WCL15 was raised in order to have a marker against carp macrophage-like cells (Weyts *et al.*, 1997). The antibody was producted and tested in a

permanent leucocyte line originated from carp blood leucocytes (CLC) selected by Faisal & Ahne (1990). The cells of the CLC line shared functional properties with monocytes and macrophages (e.g. adherence to plastic and phagocytosis of iron particles) and were WCL15-positive. The production of antibody is described by Weyts *et al* (1997). Briefly, a macrophage-enriched fraction (density range 1.02-1.07) from one-year-old carp head kidney was utilised to immunise intraperitoneally Balb/c mice. Mouse spleen cells were isolated 3 days after the last treatment, fused with SP2/0-Ag-14 myeloma cells (Schullman *et al.*, 1978) and cultured according to the procedure described by Schots *et al.* (1992). First selection was carried out by immuno-cytochemical labelling of cytocentrifuge slides with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig serum (RAM-FITC, Dakopatts, Glostrup, Denmark) of Ig^+ and Ig^- cell fractions of peripheral blood leucocytes (PBL) obtained after a magnetic sorting procedure previously described (Rombout *et al.*, 1996). Both Ig^+ and Ig^- cell fractions were controlled with a fluorescence-activated cell sorter (FACStar, equiped with a 5 W argon laser tuned at 488 nm, Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) and subsequently cytocentrifuge slides were made for each fraction. Hybridoma clones reactive with a subpopulation of Ig^- cells were selected and further analysed by flow cytometry testing the reactivity of their supernatants on cell suspensions from different lymphoid tissues (head kidney, PBL, spleen and thymus). Among these supernatants, WCL15 was selected as the mAb reactive with monocyte-like cells.

Cell preparations

Cell suspensions from the lymphoid tissues of 60-weeks old carp were prepared in c-RPMI (270 mOsm/Kg) containing 0.1M sodium azide by teasing out each tissue through nylon gauze filter (50 μm mesh size). Blood was sampled from the caudal vein using a syringe containing 0.5 ml heparin solution (10 IU/ml) in c-RPMI. The buffy coat was collected and cell suspensions were layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in c-RPMI to yield densities of 1.02 and 1.07 g/ml. After centrifugation (840 g, 30 min, 4°C), cells between 1.02 and 1.07 density layer were collected, washed twice (680 g, 10 min, 4°C) and resuspended in c-RPMI containing 1% BSA and 0.1M sodium azide.

Double immunofluorescence staining and flow cytometry

For double labelling, cells were incubated in 250 µl suspensions in different tubes with the mAb WCL15 (diluted 1:50, 45 min at 0°C). After rinsing in TBS$^+$, cells were incubated for 30 min with RAM-FITC (Dakopatts, Glostrup, Denmark) diluted 1:100. After washing, cells were then incubated for 30 min at 0°C with normal mouse serum diluted 1:25. Cells were again washed and incubated (45 min at 0°C) with the biotinylated mAbs WCI12 (diluted 1:100) or WCL6 (diluted 1:100). WCI12 reacts specifically with carp Ig heavy chains and Ig-containing cells (Koumans-van Diepen *et al.*, 1994) and WCL6 is specific for carp thrombocytes (Rombout et al., 1996). After washing, cells were finally incubated (45 min at 0°C) with phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates Inc., Birmingham, AL, USA) diluted 1:5, and were analysed with a FACStar flow cytometer using the Data-MATE software analysis package (Applied, Cytometry Systems, Sheffield, UK). All necessary controls for non-specific reactions were carried out. For each analysis 10^4 cells were measured.

Immuno-gold electron microscopy

Fractionated cells were obtained from adult lymphoid tissues and labelled with WCL15 as described above. Labelled cells were washed in c-RPMI and incubated (30 min at 4°C) with 25 nm gold-conjugated goat anti-mouse Ig (Aurion, Wageningen, The Netherlands) diluted 1:5. The cells were washed, resuspended in 1 ml of c-RPMI and centrifuged (1000 g) to yield a compact pellet. The pellet was fixed for 1 hr as previously described (van Diepen *et al.*, 1991). After fixation, the pellets were dehydrated through a series of graded alcohol and embedded in EPON 812 (Fluka Chemie, Buchs, Switzerland). Ultrathin sections (70 nm) were examined with a Philips EM 208 electron microscope.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Scapigliati *et al.*, 1995). Briefly, freshly dissected tissues were immersion-fixed in Bouin's liquid, dehydrated, cleared in toluene and embedded in paraffin. Serial transverse sections (7 µm) were rehydrated, washed in 0.1 M, pH 7.3 phosphate-buffered saline (PBS), followed by 20 min incubation with PBS containing 0.5% hydrogen peroxide to

quench endogenous peroxidase activity. Adjacent sections were incubated (18 hrs at room temperature) with WCL6 (diluted 1:10 in c-RPMI), WCL15 (diluted 1:50 in cRPMI) or c-RPMI (control). Slides were then rinsed three times in PBS and incubated for 1 hr with biotinylated horse anti-mouse Ig serum (Vector, Burlingame, CA, USA) diluted 1:1000 in PBS containing 0.1% sodium azide and 1% BSA. Slides were then incubated for 1 hr with avidin-biotinylated peroxidase complex (ABC, Vector, Burlingame, CA, USA), with the avidin and biotinylated horseradish peroxidase solutions diluted 1:2000 in 50 mM, pH 7.6 Tris-buffered saline (TBS). Sections were incubated in 50 mM Tris buffer containing 0.4% nickel ammonium sulphate, 0.02% diaminobenzidine and 0.015% hydrogen peroxide, then were dehydrated and mounted. From each animal, 5 sets of 2 consecutive sections (10 sections per tissue) of each lymphoid organ were differentially immunostained with WCL6 and WCL15, respectively. An observer who was unaware of the treatments made the counts of immunoreactive cells (nucleated only). Estimates of the number of macrophages present in the various tissues were then calculated by averaging ± standard deviation (SD) the cell numbers from 3-5 animals at each developmental age. WCL6 immunoreactive cells were examined in adjacent sections to estimate the cross-reaction of WCL15 with thrombocytes.

Analysis

Numerical results were analysed by two-tail Student t test for unpaired data. Cell measurements were obtained with a computer-assisted image analysis system [Leitz Aristoplan microscope, TK-1070E colour video camera (JVC, Japan) interfaced through TARGA 16 plus (AT&T) with a 486 PC, and Image ProPlus software package (Media Cybernetics, Silver Spring, MD, USA)].

Results

Characterisation of WCL15

Immuno-electronmicroscopy of Percoll-enriched cell fractions from lymphoid tissues of juvenile carp confirmed that WCL15^{+} cells had the morphological pattern of monocytes or macrophages. WCL15^{+} monocyte-like cells of thymus (not shown) and head kidney (Fig.1 a) contained a considerable number of lysosome-like vesicles,

whereas macrophages showed phagosomes with membranous bodies, especially in the intestine (Fig.1 b). Some thrombocytes in spleen and blood were also positive (not shown).

Flow cytometric double staining confirmed that WCL15 was not reactive with WCI12$^+$cells (Fig. 2 a, b) but cross-reacted with the majority of WCL6$^+$ thrombocytes (Fig.2 a c). In contrast to thrombocytes, macrophages displayed a strong cytoplasmic immunoreaction (Fig. 3). In PBL and splenic suspensions WCL6$^+$ cells had to be subtracted, but in suspensions of other organs the number of WCL6$^+$ cells was negligible.

Immunohistochemical studies in developing carp

Numerical results after ABC-peroxidase staining were expressed as mean ± SD in Tab.1. It was not necessary to subtract WCL6$^+$ cells in tissues, because the immunoreaction on thrombocytes was weak and hardly influenced the count of macrophages. The presence of WCL15$^+$ cells outside the lymphoid tissues was scarce in the nervous system, gills, liver and pancreas.

Head kidney

At day 2 p.f. the paired head kidney anlage consisted of two pronephric tubules. The inter-tubular tissue had no lymphoid appearance until day 3 p.f.. Immunohistochemistry revealed macrophages in carp near hatching time (2 days p.f.) localised in tubular epithelium of head kidney anlage (Fig. 4a) and in the dorsal portion of the yolk sac (Fig. 4b). At day 4 p.f. haematopoietic components appeared in the head kidney without any evident regionalisation. Immunohistochemistry revealed at this age some macrophages (Tab. 1) scattered in the parenchyma. At day 7 p.f. a higher number of macrophages was found in the head kidney, whereas in thymus and gut the number of macrophages was low. From 3 weeks until 5-6 weeks p.f., the head kidney (Fig. 4b) showed a large haematopoietic tissue and numerous macrophages were observed (cell diameter 9 ± 1.6 μm, n=60). After 4 weeks p.f., the number of WCL15$^+$cells per section in head kidney stabilised and reached the adult values (Tab. 1).

Fig.1. Pre-embedding immuno-electron microscopy of cell suspensions from head kidney (a) and gut (b). a) A monocyte shows numerous gold particles on the plasmic membrane (arrows). Bar :750 nm. b) An intestinal macrophage with numerous phagosomes shows gold particles (arrows) distributed around the plasmic membrane (bar :1 μm), which are magnified in the inset (bar :150 nm).
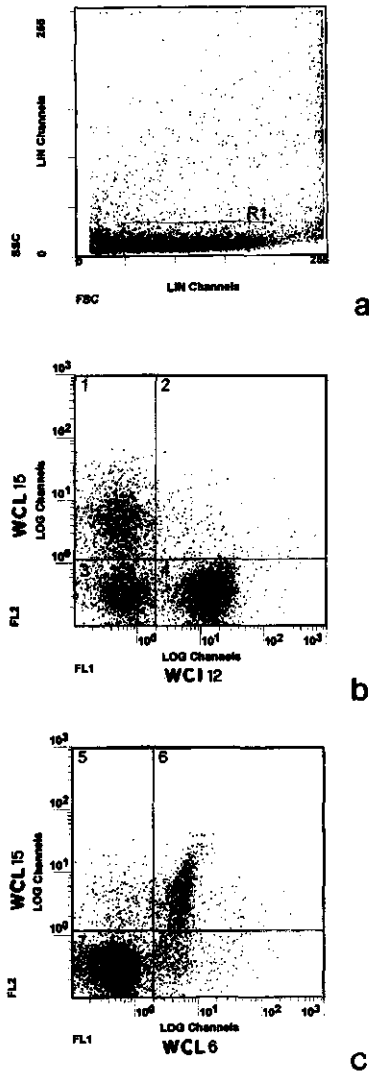
Fig.2 Flow cytometric analysis of juvenile carp PBL. The 90° forward/side scatter (a) shows the gate used to calculate the percentages of immunoreactive cells. b) Double labelling with WCL15, WCI 12 (anti-carp Ig) shows that these antibodies recognise two different leucocyte subpopulations: WCL15$^+$ monocytes/macrophages in quadrant 1 (24.8%) and WCI 12$^+$ in quadrant 4 (44.6%). The proportion of double-positive cells in quadrant 2 (2.7%) is negligible. c) Double labelling with WCL15 and WCL6 (anti-carp thrombocyte) shows that the majority of thrombocytes (17.7%, quadrant 6) are double-positive. Only 8.3% of cells is WCL6$^+$15$^-$ (quadrant 8) and 3.8% of cells WCL6$^-$15$^+$ (quadrant 5)

Regional differences were observed in the intertubular tissue of juveniles from 11-22 weeks onwards, when lymphoid areas became organised around blood vessels of the head kidney and they were identifiable from the myeloid tissue. WCL15$^+$ macrophages (cell diameter 12 ± 2.4 μm, n=80) were mainly localised in the lymphoid areas.

Thymus

At the day 4 p.f. paired thymic anlagen first appeared in the dorsal part of gill chambers, but the first lymphoid appearance was established at the end of day 4 p.f. At the day 7 p.f. the first macrophages were found among thymocytes (Fig. 5a). From week 2 p.f. onwards the thymus bulbed out into the gill chambers and contained

Table 1. Relative density number (N/100000 μm^2) of WCL15-immunoreactive cells in different organs (HK head kidney; SP spleen; gut ; TH thymus).at various days p.f. Each group is the mean ± SD of 3 specimens.

p.f. day	HK	SP	GUT	TH
2	332±17	-	0	-
3	75±21 [a]	-	0	-
4	330±49 [b]	184±16	14±6	0
7	120±73 [c]	73±12	80±13 [c]	41±16
14	30±1 [d]	96±8 [d]	10±4 [d]	66±9
21	29±13	15±1 [e]	24±3 [e]	81±28
28	53±8	57±21	8±1 [f]	49±40
35	36±14	68±7	7±2	40±1
42	53±11	39±9 [g]	38±11 [g]	15±9
56	61±4	72±18 [h]	70±10 [h]	64±1 [h]
132	68±8	42±7 [i]	56±5 [i]	73±8
adult	70±22	51±2	46±5	107±35

Significantly different (P<0.05)

[a] from day 2, ,	[e] from day 14,	[i] from day 132.
[b] from day 3,	[f] from day 21,	
[c] from day 4 ,	[g] from day 35,	
[d] from day 7,	[h] from day 42,	

numerous lymphoid cells and isolated, scattered macrophages. At week 3 p.f. numerous macrophages (diameter 7.5 ± 1.6 μm, n=20) were in the parenchyma, either isolated or in small groups of 2-3 cells (Fig. 5b). Between 4 and 6 weeks p.f., a medullary and cortical regions became visible. In this period, numerous macrophages with a considerable diameter (7-8 μm, Fig. 5d) were mainly distributed in the cortical-medullary boundary (Fig. 5c). Their presence peaked in 8-16 weeks p.f., then at 20 weeks p.f. macrophages decreased in number in the cortical-medullary boundary and became scattered again in the parenchyma (Tab. 1). In juveniles, the localisation of macrophages (size: 12-15 μm of diameter) was similar to those in 20-22-weeks p.f. carp, moreover, a few WCL15^{+} cells apparently were closed to non-pigmented and unreactive melano-macrophage centres (not shown).

Intestinal tract

At 2 days p.f. the intestinal tube started to differentiate as a columnar epithelium ventrally to the first pronephric tubules and dorsally to the yolk sac (Fig. 4a). Immunostaining occasionally revealed macrophages in the dorsal portion of the yolk sac, whereas the intestinal tube did not show any WCL15^{+} cell (Fig. 5a). From the day 4 p.f. some macrophages were recognisable in the growing intestinal epithelium surrounding the yolk sac (Fig. 6a). From the day 7 p.f. onwards, the gut developed folds in which some positive intraepithelial macrophages were observed (Fig. 6b). From 3-6 weeks p.f. onwards, the cellular diameter of macrophages in lamina propria of intestine became larger (15.6 ± 5.8 μm, n=60) than in earlier stages and compared with other lymphoid tissues (Fig. 6e). The number of macrophages reached a peak on 6-8 weeks p.f., then slightly decreased and stabilised from 18 weeks onwards. In juveniles, large macrophages (10-20 μm of diameter) were localised mainly throughout the intestinal mucosa, while smaller WCL15^{+}macrophages were shown in the submucosa (7-10 μm in diameter).

Spleen

From day 4 p.f., the spleen anlage develops close to the dorsal wall of the anterior intestine (Fig. 6a). The developing spleen was initially composed of large haemopoietic cells. Macrophages (size 7-10 μm) became visible at day 4 p.f. (Fig.6a, Table 1). At day 6 p.f. the number of lymphoid cells increased and macrophages were

apparently more numerous. From 2 weeks p.f. many macrophages were observed between lymphoid cells (Fig. 6b). From 3 to 6 weeks p.f., a growing number of macrophages, isolated or in small groups, were observed close to splenic capillaries, however their relative number per area apparently decreased (Fig. 6c, Table 1).

Considerable numbers of WCL6^{+} thrombocytes were visible, however the cytoplasmic reaction of WCL15^{+} macrophages was stronger compared with the reaction of the thrombocyte membranes (Fig. 6c, d) and hence discrimination of positive macrophages was possible. Regional differences in white and red pulp areas could not clearly detected in the spleen before 8-10 weeks p.f., although lymphoid cells began to accumulate. From 6 weeks onwards, the number of macrophages (size 10-20 µm), slightly decreased and remained constant from 18 weeks onwards (Table 1).
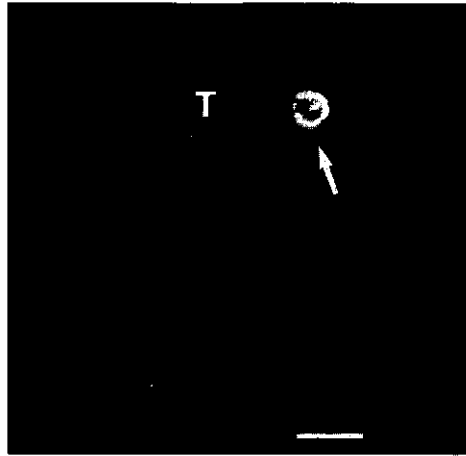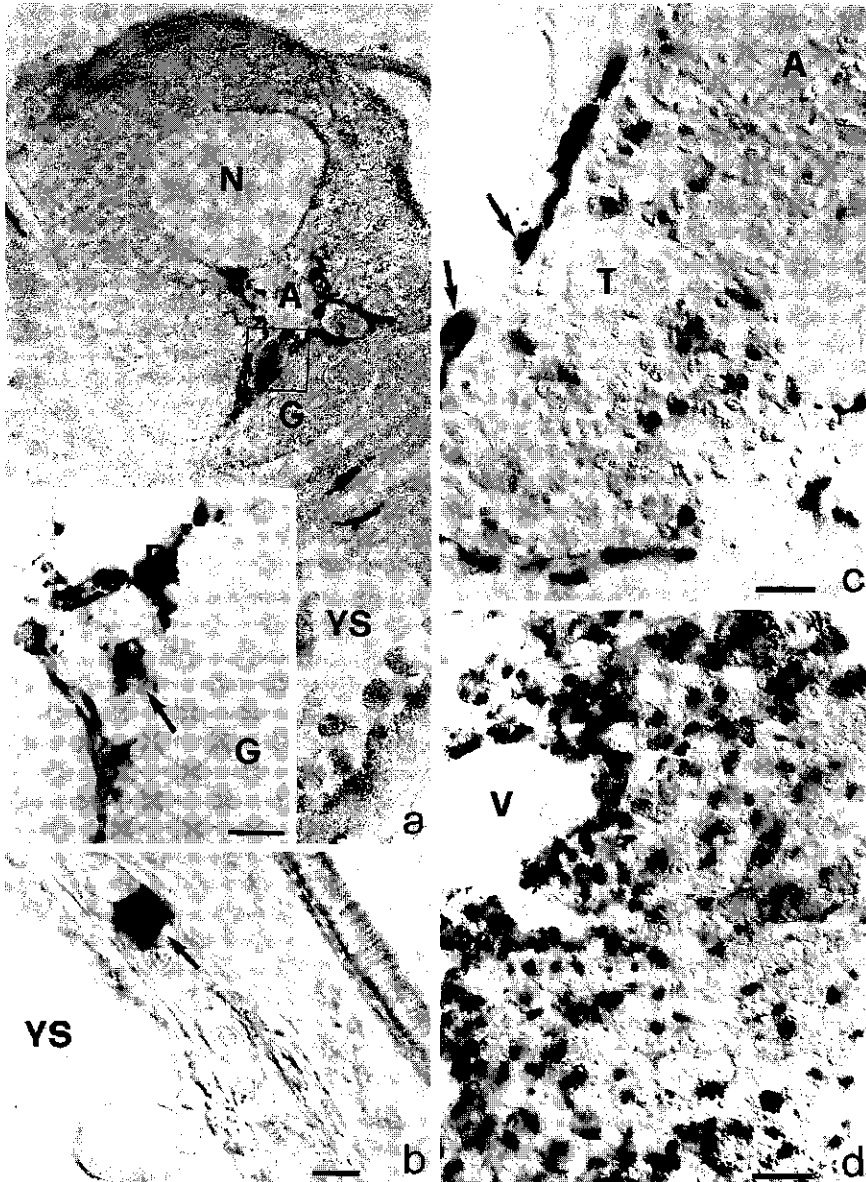

Fig.3. Immunofluorescence staining with WCL15 in PBL suspension shows a marked cytoplasmic reaction in a monocyte (arrow), faint membrane reaction on a thrombocyte (T). Bar: 20 µm.

Fig.4. Ontogeny of monocytes/macrophages in carp head kidney.a) At day 2 p.f. the first two pronephric tubules are just beneath the dorsal aorta (A) and above the gut (G; YS: yolk sac; N: notochord) (x 600). In the epithelium of the kidney tubules a monocyte/macrophage is present (arrow), which is magnified in the inset (bar: 10 µm). Positive cells were larger, devoid of dark granules compared with pigment cells (P) surrounding the head kidney. G: gut. b) At same age (day 2 p.f.) a WCL15 monocyte/macrophage (arrow) could be observed in the dorsal portion of the yolk sac (YS). Interferential contrast. Bar: 8 µm. c) At 3 weeks p.f., numerous monocytes/macrophages (dark cells) are localised in the intertubular tissue, whereas the kidney tubules (T) are devoid of WCL15-positive cells. Arrows: pigment cells; A: dorsal aorta. Interferential contrast. Bar: 20 µm. d) At 5 weeks p.f., the intertubular tissue is rich of WCL15⁺ monocytes/macrophages (dark cells) intermingled with leucocytes V: blood vessel. Interferential contrast. Bar: 20 µm.
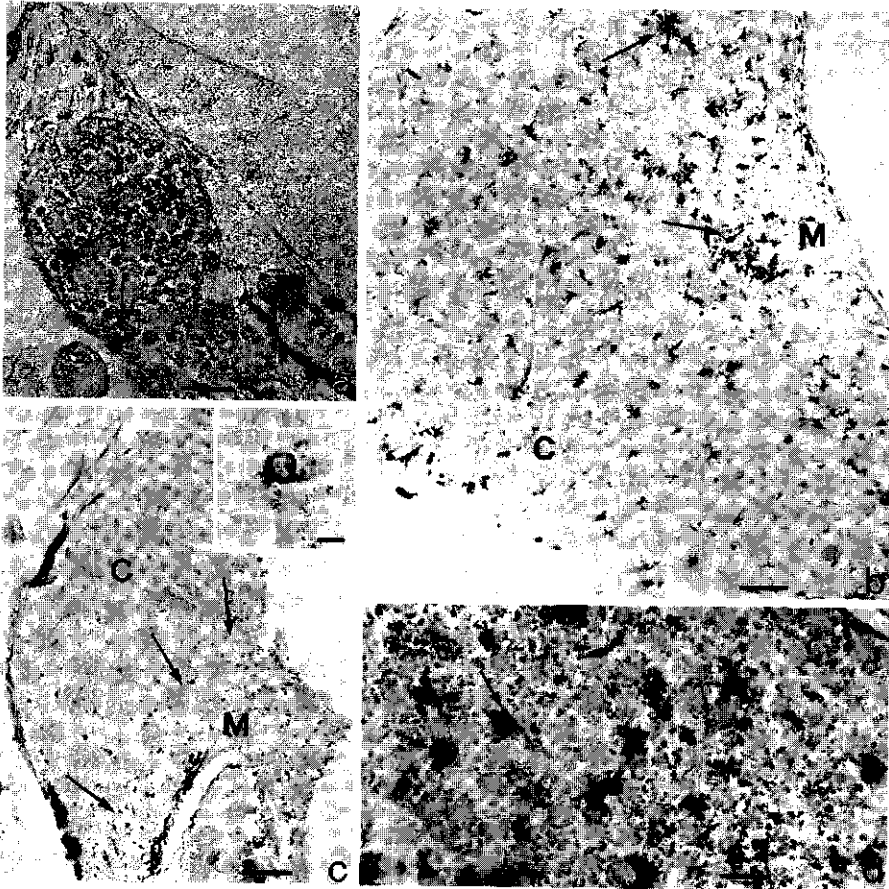
Fig.5. Ontogeny of monocytes/macrophages in carp thymus. **a)** At 4 days p.f. the first anlage of thymus starts to differentiate, first shows immunoreactive macrophages (arrows). Bar: 20 μm. **b)** Between the third and the fourth week p.f. the differentiation first occurs between cortex (C) and medulla (M). Macrophages (dark cells) are scattered in the parenchyma, some of them being concentrated in the cortical-medullary boundary (arrows). Bar: 80 μm. **c)** At week 6 p.f. the thymus has an elaborate morphology and shows numerous macrophages (dark cells) mainly located in the cortical-medullary boundary (arrows) and in the medulla (M, C: cortex,). Bar: 100 μm. A medullary macrophage (dark cell) is shown in the inset. Bar: 5 μm. **d)** Macrophages (arrows) in the medullary region are isolated or in small groups of two to three cells. Bar: 40 μm.
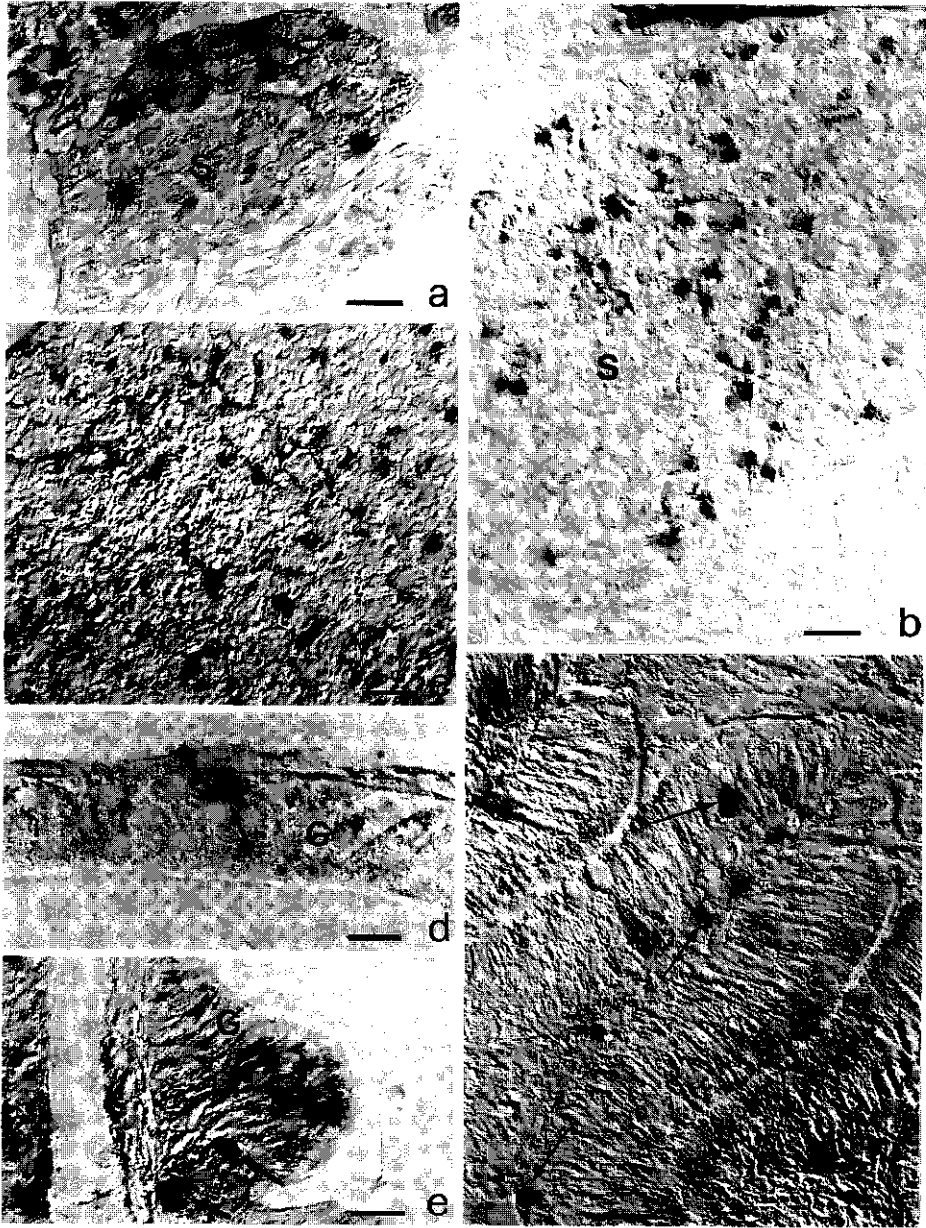
Fig.6. Ontogeny of monocytes/macrophages in carp spleen and intestine. a) At 4 days p.f. the first anlage of the spleen (S) shows WCL15⁻ macrophages (arrows). Interferential contrast, bar: 15 μm. b) During the first week the spleen (S) rapidly grows and numerous WCL15⁻ macrophages (dark cells) are in the parenchyma. Interferential contrast, bar: 30 μm. c) At week 5 p.f. the splenic parenchyma display numerous monocyte/macrophages (dark cells). Interferential contrast, bar: 30 μm. d) At 4 days p.f. the gut (G) consists of a columnar epithelium where WCL15-positive macrophages (arrow) can be seen. Interferential contrast, bar: 30 μm. e) In the first week p.f. the gut epithelium (G) starts to form the first folds and some WCL15-positive macrophages (arrows) are observed. Interferential contrast, bar : 30 μm. f) At 6 weeks p.f., the intestine displays numerous folds containing large macrophages (arrows). Interferential contrast. Bar: 30 μm.

Discussion

Macrophages and neutrophils in fish are the principal phagocytic cells, which phagocytose inert or antigenic materials, exert cytotoxic activity and stimulate lymphocyte proliferation by secreting interleukin-1-like factors (Secombes & Fletcher, 1992; Verburg-van Kemenade _et al._, 1995). As in mammals, fish monocyte/macrophages could play a central role in cell-mediated immunity as they are involved in antigen presentation and regulatory functions (Secombes & Fletcher, 1992; Roitt _et al._, 1993). Previous characterisation of monocytes and macrophages in adult or larval fish was made by functional (Faisal & Ahne, 1990; Sveinbjornsson & Seljelid., 1994; Verburg van-Kemenade _et al._, 1994) or (ultra)structural studies (Zapata & Cooper, 1990; Koumans-van Diepen _et al._, 1994). Both cell types were evidenced and a lineage relationship was suggested, but never proved. A suitable marker for monocytes and macrophages, WCL15, was recently reported by Weyts _et al._ (1997) showing that a permanent cell line originating from carp peripheral blood leukocytes (Faisal & Ahne, 1990) having morphological and functional characteristics of macrophages was reactive with this mAb. In the present study, we utilised WCL15 on cell suspensions of different tissues of juveniles or sections from developing organs of carp, in order to study the timing and localisation of monocyte/macrophages during ontogeny. A more detailed characterisation confirmed that WCL15 reacted with cells having the morphology of carp monocytes and macrophages (Koumans-van Diepen _et al._, 1994) and with WCL6^{+} thrombocytes. The cross-reaction of WCL15 with thrombocytes in non-fixed cell suspension from PBL or spleen could be avoided in fixed paraffin-embedded material, because of the strong cytoplasmic reaction of monocyte/macrophages and the negligible reaction with the membranes of thrombocytes. With immuno-electron microscopy cross-reaction of WCL15 with lymphoid cells and neutrophils could be excluded. Monocyte/macrophages apparently were larger (around 10 μm in diameter, or more) than activated non-specific cytotoxic cells (Meseguer _et al._, 1994; Mulero _et al._, 1994). Moreover, the double staining with WCL15 and WCI12 confirmed the negligible cross-reactivity of WCL15 with B-lymphocytes.

The immunohistochemical results obtained in this study are in agreement with a previous ontogeny study on leucocytes isolated from different organs, where the proportion of WCL15^{+}6^{-} cells (considered as monocytes/macrophages) was estimated from 1 to 30 carp weeks (Romano _et al._, 1997). Since a considerable number of

macrophages was observed in cell suspensions from lymphoid tissues at 1 week p.f., we now have analysed the earlier distribution in lymphoid tissues of macrophages from hatching (2 days p.f) onwards. The first appearance of WCL15$^+$ cells in the head kidney and in the dorsal part of the yolk sac at 2 days p.f. confirms previous morphological observations (Botham & Manning, 1981). Our study could not establish the localisation of macrophage precursors before 2 days p.f.. The yolk sac of fish stores maternal IgM and is apparently an early site of immune competence (reviewed Tatner, 1996), where non-specific defence mechanisms arise, providing fries immune protection before maturation of specific immunity. In addition, the yolk sac is probably the earliest organ exhibiting haematopoietic capacities in fish embryos, as indicated for dogfish (reviewed Zapata *et al.*, 1996) and carp (Botham & Manning, 1981). Most authors agree that the kidney is the major haematopoietic organ and the main site of erythrocyte, granulocyte, B lymphocyte and monocyte differentiation (Ellis, 1977; Zapata, 1979; Bielek, 1981; Botham & Manning, 1981). On the other hand, the observation of erythrocytes and macrophages before development of the kidney suggested the existence of other haematopoietic foci in the embryo (Zapata *et al.*, 1996).

Although it is generally assumed that circulating monocytes are precursors of vertebrate macrophages (Roitt *et al.*, 1993), this has been recently questioned for mammals and birds. In mammals, bipotential precursors for granulocytes and macrophages (Unanue, 1993) are suggested. The mAb WCL15 does not react with carp granulocytes, which suggests that only already differentiated cells could be recognised or that such bipotential precursors are absent in fish.

During the first week p.f. macrophages can be observed in carp thymus. Their number grew reaching adult values from the week 8 p.f., and their distribution changed from scattered (until 3-4 weeks p.f.) to small groups of cells mainly localised in the cortical-medullary boundary (from 5 weeks to 20 weeks p.f.). From 20 weeks onwards, macrophages again dispersed in the parenchyma. Macrophages were localised in "outer-inner" boundary and outer zone of trout thymus by a strong positivity for acid phosphatase and esterase (Castillo *et al.*, 1990). In addition, T-cell proliferation in the thymus of Atlantic salmon and catfish was dependent upon the presence of macrophages (Smith & Braun-Nesje, 1982; Miller *et al.*, 1986). Considering that the thymus seems to be the primary lymphoid organ for differentiation of T lymphocytes (Abelli *et al.*, 1996; Romano *et al.*, 1997), thymic macrophages could play a role in the maturation process, being also involved in the clearance of apoptotic thymocytes.

From day 4 p.f. a considerable number of macrophages appeared in the intestine. Their number increased during larval development, especially in the mucosal epithelium. Intraepithelial hindgut macrophages were larger (10-20 μm) than in the head kidney (7-10 μm), as previously observed in adult carp (Rombout *et al.*, 1993; Koumans van Diepen *et al.*, 1994). Fish intestinal macrophages seem to express antigens on their membrane and their role in scavenging and immunecomplex-binding was suggested (Rombout *et al.*, 1993; Koumans van Diepen *et al.*, 1994), as demonstrated for mammalian intestinal macrophages (Lefrançois & Puddington, 1995). The presence of numerous lymphocytes (mainly T-cells) in the gut-associated lymphoid tissue (Abelli *et al.*, 1997; Joosten *et al.*, 1997) suggests that cellular co-operation can occur with macrophages; however, this function can be neglected in earlier stages of carp because first intestinal lymphocytes were observed after 1 week p.f. (Romano *et al.*, 1997).

Scattered WCL15$^+$cells were observed in spleen from 4 to 7 days p.f.. From week 2 p.f. onwards, numerous WCL15$^+$cells, isolated or in small groups, were found close to splenic capillaries. The presence of macrophages peaked at week 8, just when they were also numerous in thymus, head kidney and gut. As in the head kidney, WCL15$^+$ macrophages were mainly localised in poorly developed lymphoid areas, where the majority of lymphoid cells are accumulated (Botham & Manning, 1981; Abelli *et al.*, 1996) together with WCL15$^-$ melano-macrophages. Although mammalian macrophages have a widespread distribution in non-lymphoid tissues, WCL15$^+$ cells are scarcely present in gills, liver, pancreas and nervous system. The localisation of macrophages in fish central nervous system was previously established (Dowding & Scholes, 1993). The possibility that some specialised tissue macrophages are not recognised by WCL15 is therefore suggested or a different distribution pattern of macrophages in fish.

The size of WCL15$^+$ cells varied among different tissues and increased throughout the development; moreover, morphological differences among thymus, head kidney and hindgut macrophages were demonstrated in juvenile carp by immuno-electronmicroscopy. Therefore, heterogeneity of macrophages is suggested, but it is still unknown whether these differences are correlated with different functions. In mammals, macrophages are heterogeneous, vary in degree of differentiation and have distinctive proprieties in the different tissues (Unanue, 1993). Possibly, similar proprieties are associated to fish macrophages, but functional proof is required.

The early and stable presence of macrophages inside lymphoid tissues (mainly thymus and head kidney) supports the idea that carp macrophages could play a role in

the maturation of fish immune system, being involved in antigen processing and negative selection. A role in the selection of T- and B-lymphocytes exerted by the resident population of monocyte/macrophages in thymus, spleen and bone marrow (mammals) and bursa of Fabricius (birds) has been firmly established (Roitt *et al.*, 1993). In younger fish, macrophages could play a role in secreting growth factors, in non-specific defence and in removing cell debris generated by physiological cell death during development.

Chapter 4

Ontogeny of thymus in a teleost fish *Cyprinus*
carpio L.: developing thymocytes in the epithelial
microenvironment.

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Abstract

A monoclonal antibody, WCL9, specific for membrane molecules of a thymocyte subpopulation was used to detect these cells *in situ* during the ontogeny of thymus. Cryo-sections revealed WCL9$^+$ cells in the rudiment of the thymus (day 4 post fertilisation), thereafter the positive cells were observed exclusively in the cortex from the first appearance of thymic regionalisation (week 4 post fertilisation) until adult age. Whole-mount immunostaining of the thymus with WCL9 revealed the three-dimensional structure of the cortex by specific staining. The presence and distribution of apoptotic cells during thymus development was studied by *in situ* end-labeling of fragmented DNA. From week 4 post fertilisation onwards, apoptotic cells were more frequently detected in the cortex than medulla, suggesting a continuous selection of thymocytes in the cortex. Ultrastructural studies confirmed the presence of numerous cortical apoptotic cells inside macrophages.

Electron microscopy provided evidence for the existence of epithelial heterogeneity in the thymus. During the ontogeny, the differentiation of epithelial cells was followed from the first weeks until the juvenile age. Cell types were classified on the basis of their localisation and cytological characteristics as: i) limiting epithelial cells located in subcapsular, perivascular and peritrabecular zones; ii) reticular epithelial cells situated in medullary and cortical zones; iii) nurse-like cells at the border between the cortex and medulla, iiii) Hassall's-like bodies, localised in the medulla. This study could suggest the occurrence of a wide range of lympho-epithelial interactions thoughout thymocytes differentiation.

Introduction

The major lymphoid organs of teleost are the thymus, kidney, spleen and mucosa-associated lymphoid tissues. The thymus plays an important role in the development of a fully functional immune system, as demonstrated by thymectomy (Nakanishi, 1986; rev. Tatner, 1996; rev. Trede & Zon, 1998). It is composed of differentiating lymphoid cells (thymocytes) within a network of epithelial cells, and is generally organised in cortical and medullary like zones (rev. Manning, 1994). This demarcation was based on histology in different species (rev. Zapata *et al.*, 1996) including the carp (Rombout *et al.*, 1997). However, the presence of a "cortex" and a "medulla" is not a constant feature in fish (rev. Trede & Zon, 1998) and their roles have not been defined (rev. Chilmoczyk, 1992). The presence of a thymic epithelial network

has been shown in several fish species (Zapata *et al*, 1996), but a uniform classification of epithelial elements and their possible functional role(s), has not been formed. An understanding of the functional maturation of the thymus and the existence of negative and positive selection of thymocytes now appears one of the major objectives.

New studies on the development of the immune system have been carried out following the availability of new monoclonal antibodies specific for leucocyte subpopulations, at least for some fish species (Scapigliati *et al.*, 1998). Several antibodies specific for the leucocyte subpopulations of carp are now available (Koumans-van Diepen *et al*, 1994; Rombout *et al.*, 1996, 1997, 1998; Romano *et al.*, 1998). One of these antibodies (WCL9) reacted with membrane molecules of thymocytes localised in the cortex (Rombout *et al.*, 1997). Flow cytometry analysis in developing carp revealed WCL9^{+}cells in high percentages in the first week post-fertilisation ($\sim$ 77 %) in thymus, but also in other organs with minor percentages; subsequently these cells gradually disappeared in all organs, except the thymus ($\sim$ 40 %) (Romano *et al.*, 1997). Thus, defined as an early-thymocyte marker, WCL9 would represent a very suitable tool to study the development of the thymus and to characterise the distribution and localisation of cortex and medulla during ontogeny in carp.

The ontogeny of carp thymus and the development of cortex and medulla were analysed in this study by immunohistochemistry, laser-scanning-confocal microscopy and transmission electron microscopy. Special attention was paid to the appearance and distribution of apoptotic cells and different epithelial cells, in order to correlate their distribution with that of developing thymocytes.

It appears crucially relevant to correlate the development of lymphoid organs with immune responses (Tatner, 1996) to prevent the occurrence of immunological tolerance if vaccination programs start before the immune system is fully developed (Manning et al., 1982; van Muiswinkel *et al.*, 1985; Mughal *et al.*, 1986; Joosten *et al.*, 1995). Although the age-equivalence model of lymphoid development suggests that different fish species attain the same level of immunological maturity at equivalent age (Ellis, 1977; Solomon, 1978; Grace *et al.*, 1981; Botham & Manning, 1981; Schneider, 1983; Jòsefsson & Tatner, 1993), combined morphological and functional studies are required.

Materials and methods

Animals

Wild-type carp, *Cyprinus carpio* (L.) 4 days to 80 weeks old, were bred and kept in the central facilities "De Haar-Vissen" at the Wageningen Agricultural University. They were reared in recirculating; filtered, UV-sterilised water at 23°C and fed with *Artemia salina* nauplii for 2 weeks followed by trout pellets (Provimi, Rotterdam, The Netherlands) at a daily age-dependent ration of 10% of their body weight (2 weeks-old) decreasing to 2% at 30 weeks.

Before dissection of the thymus, fish were killed by an overdose of 0.03% tricaine methane sulfonate (Crescent Research Chemicals, Phoenix, AZ) in water (w/v).

Immunohistochemistry

The monoclonal antibody (mAb) WCL9 (of IgG1 sub class) raised in mouse against carp thymocytes reacts with membrane molecules on a thymocyte subpopulation (Rombout *et al.*, 1997)). Immunohistochemistry was carried out on non-fixed frozen thymus (or whole larvae from 4 days to 4 weeks post fertilisation, p.f.). Serial transverse sections of 7 μm thickness were picked up on poly-L-lysine-coated slides and fixed by cold absolute aceton (15 min at 0°C) and then rinsed by phosphate buffer saline (PBS). The sections were then incubated 1 hour at room temperature (RT) in a humid box with WCL9 as primary antibody (1: 20 in RPMI). The myeloma culture medium was substituted for the primary antibody in control specimens. After two rinses in PBS, the sections were incubated 30 min/RT with a fluoresceinated or rhodaminated rabbit-anti-mouse secondary antibody (RAM-FITC or RAM-TRITC, diluted 1:100, Dakopatts, Glostrup, Denmark). Some cryo-sections were stained with May-Grünwald/Giemsa (Pappenheim method) and Mallory's trichrome to compare the effective staining of cortical thymocytes by WCL9 mAb.

Whole mount immunohistochemistry

Whole thymuses of 4, 8, 16, 22, 30, 80 weeks p.f. were fixed at 4°C with 4% paraformaldehyde in 0.1 M PBS for 12 h and subsequently washed three times in PBS containing 15% sucrose. Specimens were less than 200 μm thick, while for 80 week p.f.

carp consecutive cryo-sections (200 µm thick) were used for analysis. They were incubated in PBS containing 0.2 % Triton X-100 and 10 % normal carp serum for 8 h at room temperature. After three rinses the organs were incubated with WCL9 (undiluted) for 72 h at 4° C. After washing, the thymuses were incubated with RAM-FITC (1:50) for 8 h at 4° C, rinsed in PBS and distilled water (DW) and mounted on slides (with gentle compression) with PBS/glycerol (1:1). The organs were observed (excitation wavelength: 488 nm, emission: 520 nm) with a laser scanning confocal microscope (LSCM, Leica TCS-4D, Germany). LSCM analysis was conducted through acquisition of serial optical sections (6-15 µm focal planes) and three-dimensional reconstruction. Some sections (15 µm thick) were cut on a cryostat and processed for immuno-histochemistry as described above, to compare the staining pattern with that obtained by whole-mount immunostaining.

ISEL of fragmented DNA (apoptosis)

Whole larvae or dissected thymuses were immersion-fixed in Bouin's liquid (7 h at 4° C), dehydrated through a series of graded alcohol at 4° C, cleared in toluene and embedded in paraffin and processed for _in situ_ end-labelling (ISEL) of fragmented DNA, as described previously (Abelli _et al._,1998). Briefly, serial transverse sections were dewaxed, rinsed with buffer containing proteinase K (TE, from _Tritirachium album_, 17.5 U mg^{-1}) and incubated in DW containing 2% hydrogen peroxide to quench endogenous peroxidase activity. Following rinses in DW, sections were washed in TDT buffer (30 mM Tris, 140 mM sodium cacodylate, 1.75 mM cobalt chloride, pH 7.2) and incubated for 2 h at 37° C with DNA-labelling mixture [0.3 I.U. µl^{-1} terminal deoxynucleotidyl transferase (Pharmacia Biotech, Uppsala, Sweden), 2 µM digoxigenin-11-dUTP (2'-deoxyuridine-5'-triphosphate tetralithium salt; Boehringer Mannheim, Germany), 17 µM dATP (2'-deoxyadenosine-5'-triphosphate disodium salt; Pharmacia)] in TDT. Positive controls were obtained by applying deoxyribonuclease I (from bovine pancreas: Pharmacia) before the DNA-labelling step. Terminal transferase was omitted from the DNA-labelling mixture in negative control sections. Slides were washed, using gentle shaking in SSC buffer (30 mM sodium citrate, 300 mM sodium chloride, pH 7) and in 5.6 mM, pH 7.5 Tris-buffered saline (TBS), and incubated for 20 min at room temperature in TBS containing 3% bovine serum albumin (Sigma). Sections were incubated for 90 min at room temperature with a peroxidase-conjugated sheep anti-digoxigenin serum (Fab fragment; Boehringer) diluted 1:1000 with TBS and then for 5

min in 50 mM Tris buffer containing 0.02% diaminobenzidine (Sigma) and 0.015% hydrogen peroxide. After rinsing in TBS, sections were dehydrated, mounted and examined under bright-field illumination.

Two specimens per age were used, and in each specimen 5-10 non-consecutive sections of the thymus were immunostained. Counts of cleary nucleated immunoreactive cells in 1 mm^2 areas of thymus were performed with a computer-assisted image analysis system by an observer unaware of treatments. The number of immunoreactive cells was determined by calculating the mean and standard error of the mean of 2 animals.

Transmission electron microscopy

The whole thymus (larvae) or thymus portions of 1 mm^3 were fixed for 1 h with a mixture of 2% glutaraldehyde, 1% osmium tetroxide and 1% potassium bichromate in 0.1 M sodium-cacodylate buffer (van Diepen et al., 1991). After fixation, the specimens were dehydrated through a series of graded alcohol and embedded in EPON 812 resin (Fluka Chemie, Switzerland). Ultra-thin sections were prepared with a Reichert Ultracut microtome, stained with uranyl acetate and lead citrate and examined with a JEOL 1200 EX II electron microscope.

# Results

Immunohistochemistry

The thymus of carp first appeared as a paired organ above the 2nd and the 3rd branchial arch at the end of day 3 p.f.. The oval thymus bud was surrounded by the pharyngeal epithelium at the external part and by connective tissue at the inner part. WCL9-immunoreactivity was first localised at the end of day 4 p.f. (Fig. 1a) on the membranes of round cells that filled the thymus rudiment. During the first week, the thymus grew strongly and acquired an oval shape. In this period the $WCL9^+$ cells were evenly distributed (Fig. 1b) among the epithelial cells. From the 4th week p.f., the thymus stretched out along the gill chamber (Fig. 1c) and acquired a conical shape (Fig. 1d), with a tip inside the body and a large base just
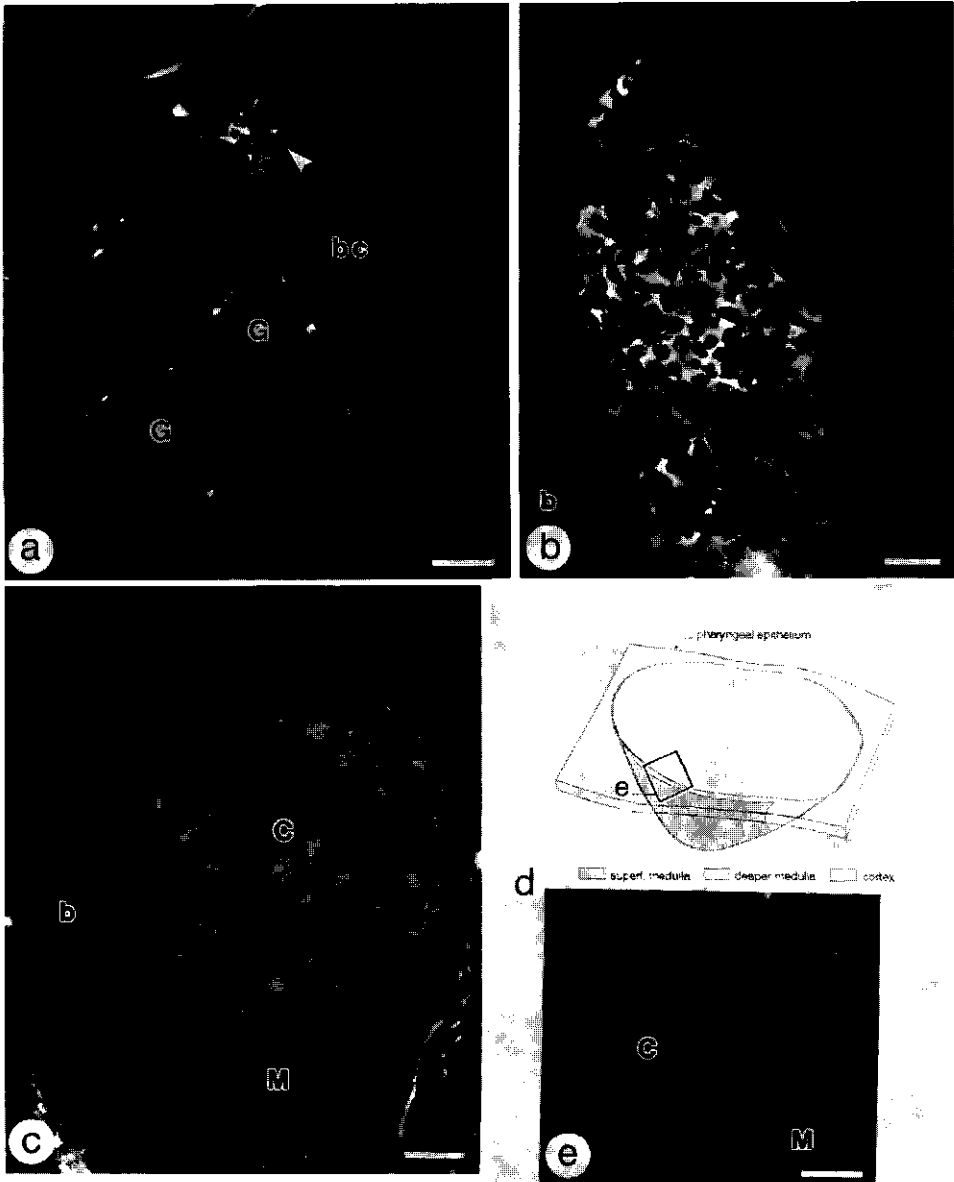
Fig.1. a) A cryo-section at day 4 p.f. shows in the thymic rudiment (arrow) the membranes of rounded cells positive for WCL9. G, gills; bc, branchial chamber, bar 20μm. b) A cryo-section shows the WCL9⁺ thymocytes distributed among the epithelial cells (dark spaces among positive cells) at day 7 p.f.. Note the strong growth of the thymus compared with Fig.1a. (b, branchial chamber; bar 20 μm). c) A cryo-section at 4 weeks p.f. show that the thymus stretched out along the branchial chamber and the first differentiation in cortex (C, WCL9⁺ thymocytes) and medulla (M, WCL9⁺ thymocytes), b, branchial chamber; bar, 80 μm). d-e) A scheme resumes the analysis of whole-mount with WCL9, analysed by LSCM (d). At 4 weeks p.f. thymus acquired a conic shape, with a tip inside the body and a large base beneath the pharyngeal epithelium. WCL9⁺ cells were confined to the superficial cortical part of the thymus. The medulla situated within the organ was here indicated as "deeper medulla" (light grey) and the superface localisation as "superficial medulla" (dark grey). e) A LSCM detail shows part of the superficial cortex (C) and superficial, WCL9⁻, medulla (M). Bar 40 μm.

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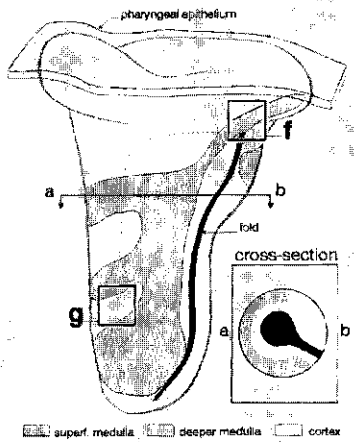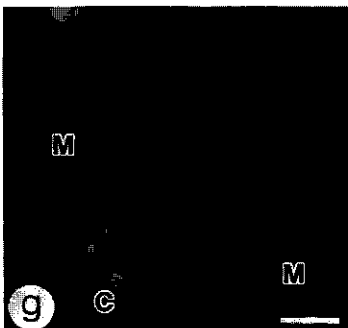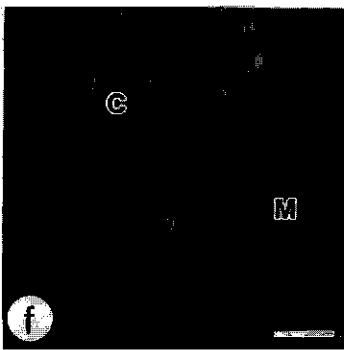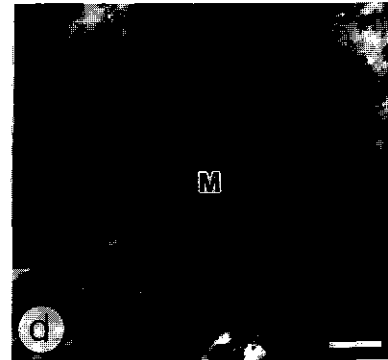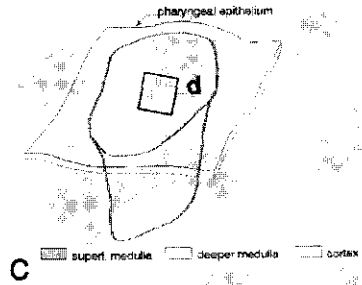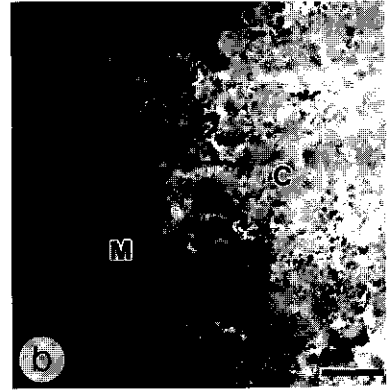
beneath the pharyngeal epithelium. In this period the different density of thymocytes could distinguish the first differentiation in cortex and medulla evidenced by different staining of the zones (not shown). WCL9$^+$ cells were confined in the cortical superficial portion of the thymus (Fig.1d,e). The thymus developed a large, conical inner portion from 8 to 30 weeks p.f., resulting in a lobated morphology in cross sections (Fig.2a,b). The distribution of WCL9$^+$ cells gradually changed during this period. In fact, a portion of the thymus appeared WCL9-negative (medulla), while the superficial and external parts were positive (cortex) (Fig. 2c-g). LSCM analysis revealed an inflexion in the conically shaped thymus from 10 weeks p.f. onwards (Fig. 2e). From 30-40 weeks p.f. onwards, one of the paired thymuses (generally the right one) became relatively smaller, and the other lost the conical shape acquiring a more irregular morphology. The cortex and the medulla intermingled in a very complex fashion (not shown).

Apoptosis

Apoptotic cells were not detected from day 4 p.f. to the first week p.f. (Fig. 3a). In this period the thymocytes showed a similar diameter (6.2 ± 0.8 μm). From the first to the 4th week p.f. the number of apoptotic cells increased (61.4 and 18.6 cells/mm^2 in cortex and medulla, respectively), concentrating in the outer-cortical region of the thymus (Fig. 3b). Apoptotic cells had a diameter similar to non-apoptotic thymocytes (4.27 ± 0.8 μm). From the 4th week until 30-40 weeks p.f., the mean diameter of non-apoptotic lymphoid cells varied following the formation of cortex (3.8 ± 0.4 μm, N=80) and medulla (5 ± 0.8 μm, N=90). At 22 weeks p.f., apoptotic cells were more numerous in the cortex (650 cells/ mm^2) than in the medulla (43 cells/mm^2) and were smaller in the cortex than medulla (2.8 ± 0.5 and 4.0 ± 0.8 μm, respectively, N=50) (Fig. 3c,d). Apoptotic cells were also smaller than non-apoptotic thymocytes (cortex: 4 ± 0.2 μm, medulla: 5.1 ± 0.3 μm, N=60). Interestingly, a slightly higher density of apoptotic cells (700 cells/mm^2) was observed in the area between cortex and medulla. The thymuses from 30 weeks p.f. onwards were in part divided into lobes by connective septa.

Fig.2 a) A cryo cross-section of thymus at weeks 14 p.f. showing the lobate morphology. (M, medulla; C, cortex; B, branchial chamber; f, inflexion (fold) inside the thymus). Bar 40μm. b) A magnification of an adjacent section showing WCL9$^+$ cells in cortex next to the WCL9$^-$cells in the medulla. Bar 60 μm. c-d) LSCM analysis at 8 weeks p.f. shows the WCL9$^-$cells (deeper medulla) while the superficial and external parts were WCL9-positive and represent the cortex (M, medulla, bar40 μm). e-g) LSCM analysis at 22 weeks p.f. reveals an inflexion (continuos and dotted black line) in the conic shaped thymus. The WCL9-positive cortex and the WCL9-negative inside (deeper) and superficial medulla are intermingled in a very complicate fashion, which is shown in scanning sections f) and e), (M, medulla; C, cortex). Bars 40μm.
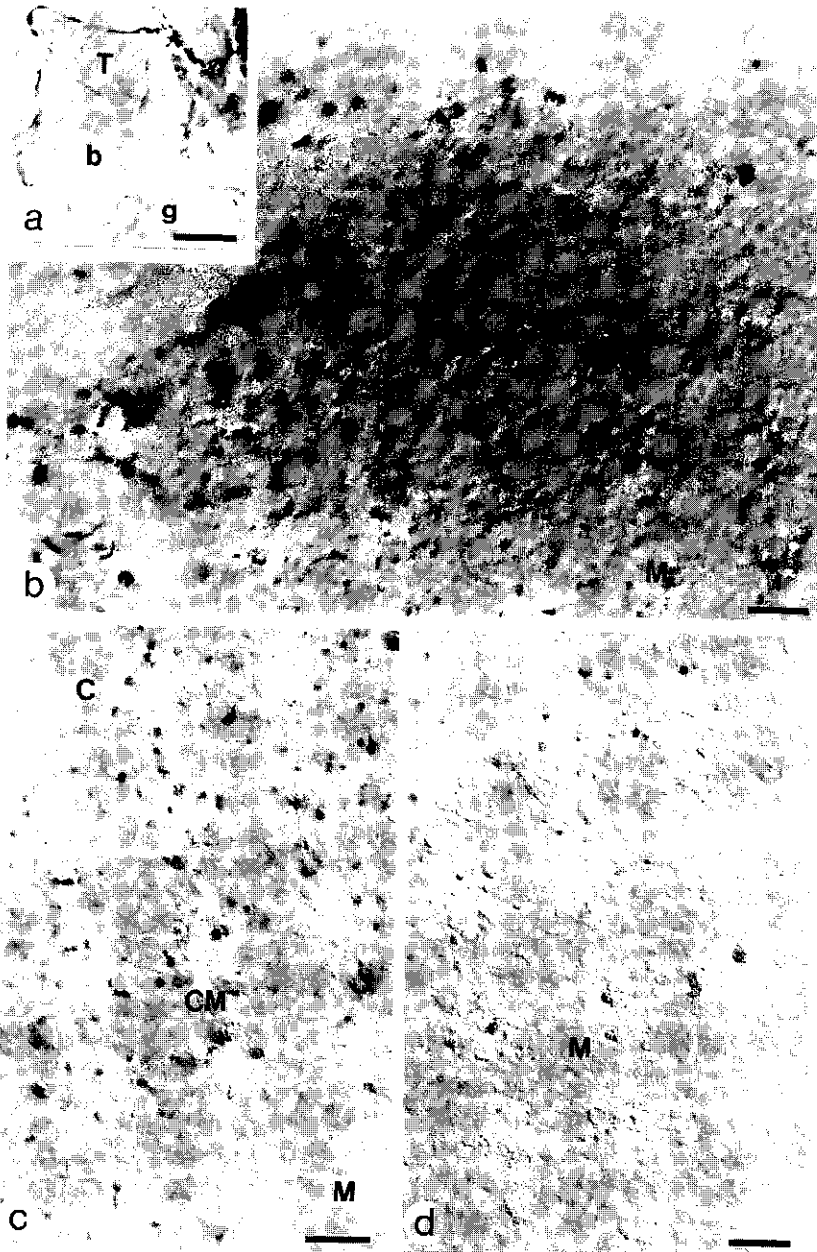
Fig.3. *In situ* end-labelling of fragmented DNA showing apoptotic cells during the thymus ontogeny. a) The thymus (T) at week 1 p.f. lacks any apoptotic cells and is close to the branchial chamber (b). (g, gills; bar 60μm. b) At week 4 p.f. the number of apoptotic cells strongly increased and concentrated in the outer-cortical region of the thymus. (C, cortex; M, medulla; bar60 μm. c-d) At 22 weeks p.f., a slightly higher density of apoptotic cells in the border between the cortex and medulla (CM) was observed (c). d) In the medulla a few apoptotic cells were observed. (C, cortex; M, medulla; bar 60 μm).

During this period the apoptotic cells were smaller in cortex than medulla (diameter 2.6 ± 0.4 μm and 3.6 ± 1 μm, respectively, N=80) and compared with non-apoptotic thymocytes (cortex: 4 ± 0.1 μm, medulla: 5 ± 0.4 μm, N=80). Apoptotic cells were more numerous in the cortex (500 cells/ mm^2) than medulla (110 cells/ mm^2). The distribution pattern of apoptotic cells did not change in the following weeks and up to 80 weeks p.f.

Electron microscopy

Thymocytes

At day 4 p.f., transmission electron microscopy analysis revealed that the thymocytes are grouped just beneath the pharyngeal epithelium. They had an euchromatic nucleus, abundant cytoplasm filled with ribosomes and large mitochondria (Fig. 4a). After 1 week p.f. numerous thymocytes displayed a round nucleus and abundant cytoplasm (Fig. 4e). From 4 weeks onwards, the majority of medullary thymocytes showed a large irregular nucleus and abundant cytoplasm compared with the cortical ones (Fig. 5a,b). In the border between cortex and medulla the thymocytes were similar to cortical cells, showing a more regular nucleus and less cytoplasm than the medullary thymocytes (Fig 6 c-e).

Epithelial cells

The morphology of epithelial cells was analysed during development. In order to unify the terminology used in mammals and birds (van de Wijngaert et al., 1984; Romano et al., 1996), carp epithelial cells were named according to their morphology and localisation. There are limiting cells (LECs), with a pyramidal shape and basal lamina; cortical (c-RECs) and medullary (m-RECs) reticular-epithelial cells; nurse-like cells (NLCs), that completely envelop and contain many small thymocytes and Hassall's body-like structures (HBLS), composed of concentric whorls, where cellular debris filled the centre. Table 1 summarises the observations on appearance of these cells during ontogeny.

At 5-6 days p.f., differentiated LECs were localised in the inner region of the thymus close to the connective tissue, forming a subcapsular zone (Fig. 4d). LECs were characterised by the presence of a basal lamina interposed between these cells and the connective tissue. At this age, the outer part of the thymus remained in continuity with the pharyngeal epithelium and this situation did not change throughout ontogeny. From

4 weeks p.f. onwards, LECs were also arranged along the connectival septa (peritrabecular) and around thymic capillaries forming perivascular areas (Fig. 4b,c) that were localised mainly between cortex and medulla. The number of capillaries increased with age.

From 1-2 weeks p.f., a few RECs still not differentiated into cortical and medullary types were observed (Fig.4e). A clear identification between medullary and cortical RECs was carried out around the 4th week p.f., following the differentiation of the thymus in cortex and medulla. Before 8-10 weeks p.f., the parenchyma was organised in a network with wide spaces (Fig. 5a) but from this period onwards it became gradually more compact (Fig.5c) because of the higher diameter of epitheliocytes and 4-fold increased number of thymocytes.

At week 4 m-RECs were characterised by an irregular/globular shape and the cytoplasm was darker than c-RECs (Fig. 5a,b). During the ontogeny m-RECs became larger, had long cytoplasmic processes and were filled by a smooth endoplasmic reticulum. electron-lucent cytoplasm characterised the c-RECs (Fig. 5b,d). Cytoplasmic processes connecting cells by desmosomes formed a reticular network (not shown). In young larvae (4-8 weeks p.f.), the cytoplasm typically displayed numerous ribosomes, small mitochondria and sparse vesicles. From 8 weeks onwards, the vesicles become larger (0.4-1.0 μm in diameter, Fig. 5d) and more numerous filling the cytoplasm. Numerous macrophages were observed in the cortex and in the cortical-medullary boundary. Some of them contained residues of apoptotic cells (Fig. 6a).

From 4-5 weeks onwards the NLCs were observed in the zone between the cortex and the medulla. Characteristically, NLCs contained numerous small viable thymocytes in their cytoplasmic invaginations (Fig. 6b,c). The number of NLC gradually increased during the ontogeny from infrequent (4 weeks p.f.) to numerous.

HBLS (around 50 μm in diameter) were observed in juveniles from 22 weeks p.f. onwards (not shown). They were characterised by numerous flattened epithelial cells enriched with intermediate filaments, surrounding a lumen often containing cellular debris.

Days p.f.	LECs	c-RECs	m-RECs	NLCs	HBLS
4	-	-	-	-	-
7	+	+/-	+/-	-	-
28	+	+	+	+	-
150	+	+	+	+	+

Table 1. The appearance of epithelial cell types during development of carp thymus: limiting epithelial cells (LECs), cortical (c-RECs) or medullary (m-RECs) epithelial cells, Nurse-like cells (NLCs) and Hassall's body-like structures (HBLS). +: presence, -: absence, +/- presence of undifferentiated type.
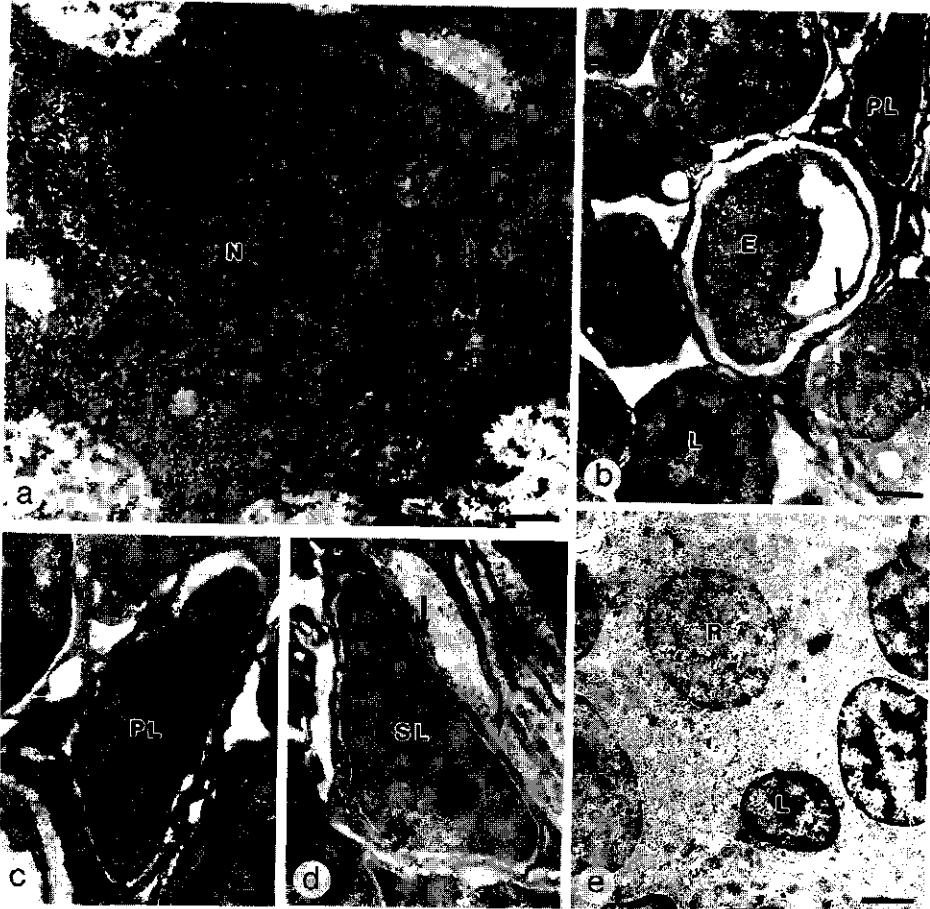
Fig.4. Electron micrographs of the developing thymus. a) An early thymocytes at 4 days p.f. with an euchromatic nucleus (N) and abundant cytoplasm filled with ribosomes and large mitochondria. Bar 500 nm. b-d) LECs are characterised by the presence of a basal lamina (arrows) between them and the connective tissue. b) At 4 weeks p.f. perivascular LECs (PL) surround a capillary (E, endothelium; L, lymphocyte), bar 1 µm. c) The cytology of a perivascular LEC (PL) is shown at higher magnification of (b). Bar 500 nm. d) A subcapsular LEC (SL) was localised close to the external connective tissue. The basal lamina is indicated by arrows, bar 500 nm. e) A poorly differentiated reticular-epithelial cell (R) at the second week p.f., L, lymphocyte; bar 3 µm.
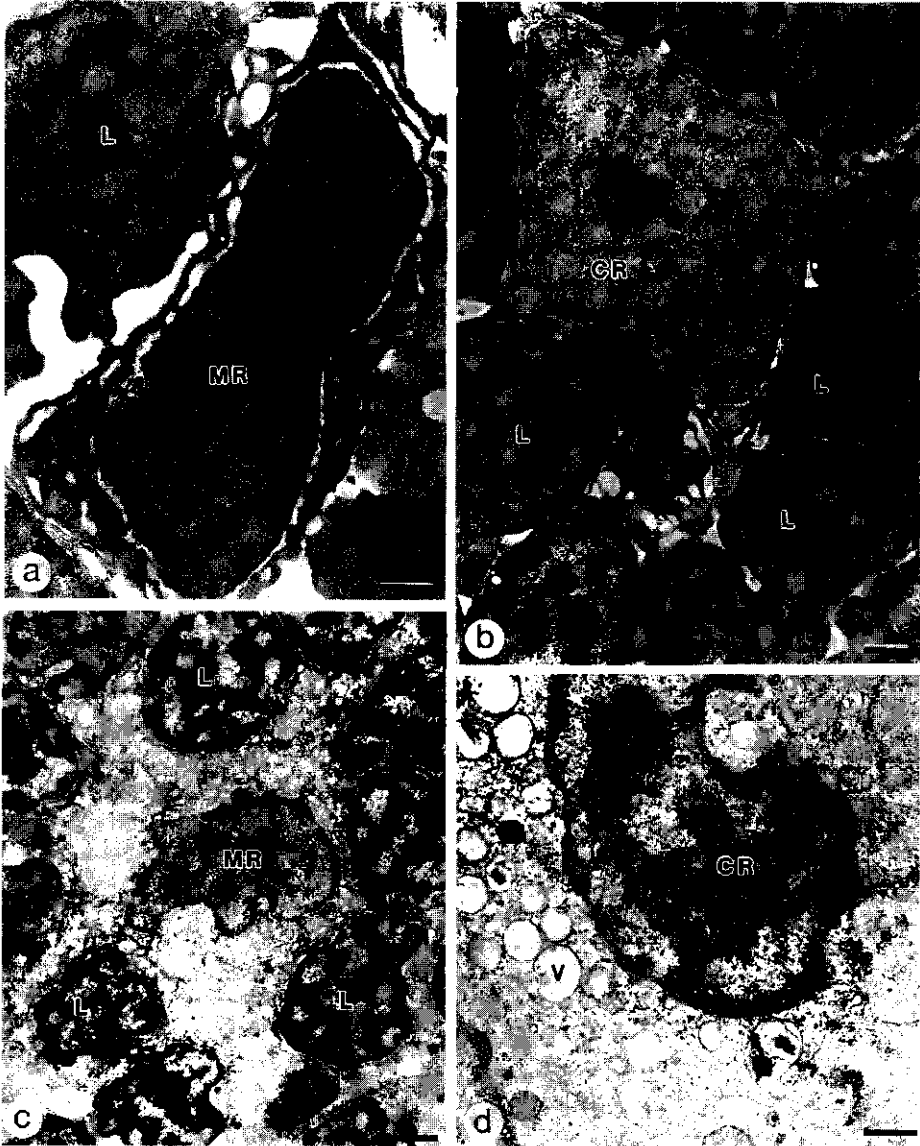
Fig.5. Electron micrographs of the developing thymus, in which cortex and medulla are formed. a-b) Medullary RECs (MR) at week 4 p.f. were characterised by their irregular/globular shape and darker cytoplasm (bar 0.8 μm) compared with cortical ones (CR, bar 1μm). The majority of medullary thymocytes (L) shows a large and irregular nucleus and more cytoplasm than the cortical ones. c) During ontogeny medullary RECs (MR) become bigger with long cytoplasmic extensions. In juveniles, the cytoplasm is filled with a smooth endoplasmic reticulum. (L, lymphocyte, bar 2 μm). d) Cortical RECs (CR) in juveniles are characterised by electron-lucent cytoplasm, the cytoplasm displayed numerous ribosomes, small vesicular mitochondria and vesicles (V) with a floccular content. (L, lymphocyte, bar 500 nm).
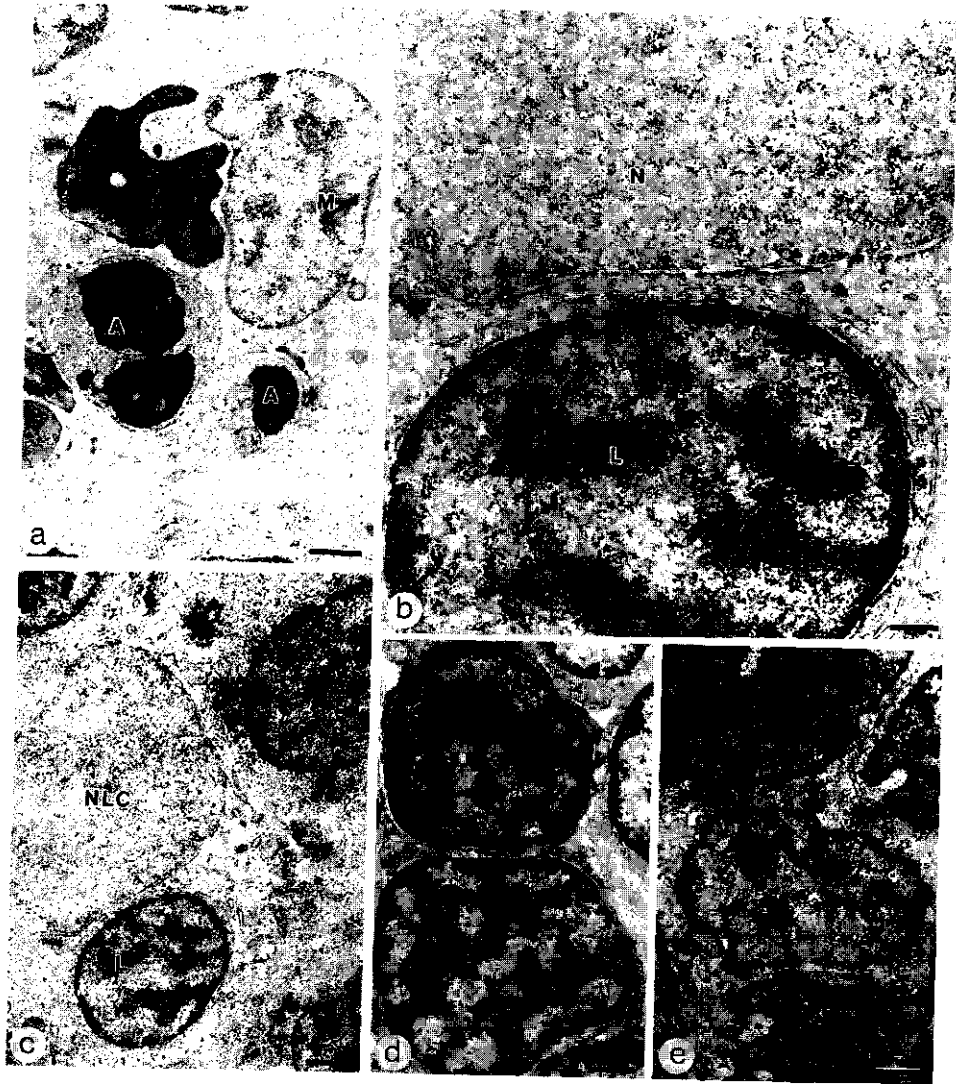
Fig.6. Electron micrographs of the developing thymus. **a)** Numerous *macrophages* (M) were observed in the cortex and in the cortical-medullary boundary, some of them have residues of apoptotic cells (A) in their cytoplasm. Juvenile carp about 1 year of age. Bar 1,5 µm. **b-c)** At 4-weeks p.f. the NLCs were observed between the cortex and the medulla. b) the *invaginations (arrows)* of NLCs contained small viable thymocytes (L) (c). Magnifications: b) bar 200 nm; c) bar 1.5 µm. **d-e)** At 22 weeks p.f. cortical (d) and medullary (e) thymocytes are shown. Bar 0.7 µm.

Discussion

The teleost thymus is thought to be the major organ for storage and maturation of T cells (Manning, 1994), as demonstrated in most vertebrates (Roitt *et al.*, 1993). It seems to be the first organ that acquires lymphocytes during the histogenesis of the lymphoid tissues (Zapata *et al.*, 1996); it has to be proven, however, that the T cells exclusively originate from the thymus.

By using the monoclonal antibody WCL9 (Rombout *et al.*, 1997), it was demonstrated that the carp thymus already contained a high percentage of early-T cells during the first week p.f., but concomitantly, low numbers of positive cells were also observed in the head kidney, spleen, gut and blood (Romano *et al.*, 1997). In this study, immunohistochemistry demonstrated that antigenic determinants recognised by WCL9 first appeared at 4 days p.f. in the thymus. Throughout early development, the percentage of WCL9$^+$ cells gradually decreased and disappeared from lymphoid organs, except the thymus. In juveniles and adults, a consistent population (40-50%) of immature T cells still resided in the thymus (Romano *et al.*, 1997), especially localised in the cortex of the organ (Rombout *et al.*, 1997). The complex development of cortex and medulla during carp ontogeny was studied by staining the cortical thymocytes. Whole-mount immunostaining with WCL9 and LSCM analysis were therefore performed. Previous morphological studies on teleost thymus (Manning, 1994; Abelli *et al.*, 1994) and anti-thymocyte antibodies in the sea bass (Abelli *et al.*, 1996) provided evidence for the presence of a cortex in the outer region of the thymus and a medulla in the inner thymic area. The existence of a cortex and a medulla in carp (Rombout *et al.*, 1997; Botham & Manning, 1981) was also indicated by histology, but their structure was not well defined, because of the lack of specific markers. This is the first immunohistochemical study that specifically deals with the development of cortex and medulla in a teleost fish. The cortex was initially limited to the outer area of the thymus, and during the development it intermingled with the medulla, also reaching the inner thymic region in juvenile carp.

From juveniles to adults, the shape of the thymus changed from a conical to an irregular shape and, in addition, cortex and medulla changed their localisation. These results suggest that carp thymus could also provide the environment for continuous thymocyte maturation in adult life. Further studies are obviously needed to assess the extent of thyme involution in ageing carp and whether hormones could affect sensitive

thymocytes. Indications about sensitivity to cortisone and cortisol of peripheral blood lymphocytes in adult carp are already available (Weyts *et al.*, 1997).

The selection of intrathymic mammalian lymphocytes requires a negative selection of autoreactive T cells based on receptor specificity (Pullen *et al.*, 1989; Boyd *et al.*, 1993). The apoptosis, a programmed suicide mechanism, plays an important role in intrathymic T cell selection (Mc Conkey *et al.*, 1994; Surh & Sprent, 1994) and was demonstrated in mammals (Wyllie *et al.*, 1980), birds (Compton *et al.*, 1990), amphibians (Ruben *et al.*, 1994) and recently also in teleosts (Abelli *et al.*, 1998).

The method used to reveal apoptotic cells *in situ* was previously developed to study the sea bass thymus and the apoptotic process was confirmed by analysis for both high- and low-molecular weight DNA fragments (Grace *et al.*, 1981). In this study, apoptotic cells were not detected in the first rudiment of carp thymus (4 days p.f.) when WCL9$^+$cells are the main population of thymocytes. A conspicuous number of apoptotic thymocytes was found in the cortex concomitantly with the first appearance of cortical and medullary RECs (4 weeks p.f.). In a recent study numerous scattered macrophages were found in the cortex and groups of macrophages were concentrated in the cortical-medullary border of carp thymus (Romano *et al.*, 1998). We frequently observed macrophages filled with apoptotic debris, as described in mammals, that probably represent the deletion of self-reactive thymocytes (Kendall, 1991).

The thymocytes in the outer region of the carp thymus were in direct contact with the pharyngeal epithelium, and consequently with the external environment. The processing of antigens, that could be a step process for the selection of thymocytes, was previously suggested in pharyngeal epithelium of channel catfish (Lobb & Clem, 1987). However, this process is not completely understood and recently was observed a "passive" transfer of ferritin in pharyngeal epithelium close to thymus in developing trout (Castillo *et al.*, 1998).

In the first rudiment of the thymus, undifferentiated thymocytes showed the typical features of immature cells. Differences in thymocyte morphology and size were evident in medulla and cortex of carp thymus as observed in the sea bass (Abelli *et al.*, 1994, 1996). Cortical thymocytes (mainly WCL9$^+$) were characterised by a smaller size compared with medullary thymocytes and by a higher nuclear/cytoplasmic ratio.

The epithelial network seems to be essential for the differentiation of thymocytes in vertebrates. A classification of the epithelial cells was made for mammals (Pullen *et al.*, 1989) and birds (Wilson *et al.*, 1992) by using specific monoclonal antibodies. Previous studies on fish thymus showed the heterogeneity of epithelial cells (Pulsford *et*

_al._, 1991; Castillo _et al._, 1991; Zapata _et al._, 1996). We identified different cell types in carp thymus: LECs, located in subcapsular, perivascular and peritrabecular zones; medullary and cortical RECs; NLCs, situated at the border between the cortex and medulla and HBLS mainly observed in the medulla.

When the LECs appeared from the first week p.f. thymocytes seemed to be more differentiated, containing less ribosomes and mitochondria. Capillaries surrounded by perivascular LECs were especially numerous in the medulla and between the cortical and medullary zones throughout development. LECs were also described in other fish species (Zapata & Cooper, 1990) showing different enzyme patterns compared with other epithelial components (Castillo _et al._, 1990). Their role could be the isolation of the thymic microenviroment during the selection of thymocytes, by establishing a thymus-blood barrier (Zapata _et al._, 1996).

When cortex and medulla are differentiated morphologically, distinct RECs appeared. RECs probably form a framework where thymocytes undergo differentiation and could play a key role in fish like in mammals. In juveniles RECs with small cytoplasmic vesicles surrounded by numerous thymocytes were frequently observed. In carp and sea bass the presence of regulatory hormonal factors like thymulin and thymosyn (fraction V) were detected in RECs (Frohely & Deschaux, 1986).

NLCs were observed between cortex and medulla of the carp thymus, in close association with thymocytes, confirming previous observations in other fish species (Pulsford _et al._, 1991; Flaño _et al._, 1996). Their function is still unknown in fish, while it has been shown that mammalian nurse cells most likely provide the environment for positive selection of cortical thymocytes, being in intimate contact with numerous $CD8^+/CD4^+$ cells, that could enter and exit from the cellular invaginations (Ritter & Crispe, 1992).

In conclusion, there was evidence that some processes of thymocyte selection could take place in carp from 4 weeks p.f. onwards, when the $WCL9^+$ early T cells are restricted to the cortex. It can be speculated that, as it occurs in other vertebrates, the different regions of the carp thymus could play different roles in the maturation of thymocytes.

Chapter 5

Ontogeny of a mucosal T cell subpopulation in carp (*Cyprinus carpio*, L.): immunocytochemical studies.

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Before the dissection of head kidney, thymus, intestine, skin, gills and spleen, fish were killed by an overdose of 0.03% tricaine methane sulfonate (Crescent Research Chemicals, Phoenix, AZ) in water (w/v). From 6 weeks onwards, blood was sampled from the caudal vein using a syringe containing 0.5 ml heparin solution (50 IU/ml) in c-RPMI (RPMI + 10% double-distilled water). From specimens 1 to 5-weeks p.f., blood was collected by cutting the tail. Leucocytes from blood and organs from 6-30 individuals (number decreasing from 30 to 6 with age) were pooled in order to obtain enough cells (2×10^6) for flow cytometric analysis.

Cell suspensions

Cell suspensions from head kidney, thymus, spleen, intestine, skin and gills were prepared in c-RPMI containing 0.1% sodium azide by teasing the tissues through a nylon gauze filter (50 μm mesh). Peripheral blood leucocytes (PBL) were prepared from heparinised blood diluted 1:1 in c-RPMI and centrifuged (15 min at 100 g) at 4°C, subsequently the buffy coat was collected. All cell suspensions were washed once or twice for 10 min at 680 g at 4°C in c-RPMI and resuspended in 2 ml of c-RPMI. The cell suspensions were centrifuged on a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in c-RPMI to yield densities of 1.020 and 1.070 g ml^{-1}. After centrifugation (30 min, 840 g) at 4°C, cells layered at the interfaces between the densities mentioned above were collected and washed twice (10 min at 680g) at 4°C. The pellet was resuspended in c-RPMI with 1% BSA and 0.1% sodium azide.

Monoclonal antibody

The mAb WCL38 was produced by immunising mice with isolated membrane molecules of carp intestinal epithelial cells, which predominantly consisted of Ig-negative lymphoid cells. This mAb was characterised as a marker for mucosal T lymphocytes and belongs to the IgM class (Rombout *et al.*, 1998).

Immunofluorescence and flow cytometry on cell suspensions

Enriched leucocyte suspensions from 1 to 80 weeks p.f. carp were used for immunofluorescence and flow cytometric analysis. Cells were incubated in different tubes (250 µl) with WCL38 (1:100) or with culture medium as a control. Labelled cells were then washed (10 min at 680 g) at 4°C and incubated for 30 min with fluorescein-conjugated rabbit anti-mouse Ig (RAM-FITC, Dako, Glostrup, Denmark) diluted 1:100 in c-RPMI. After washing, 10^4 cells were analysed with a flow cytometer (FACStar, Becton Dickinson, Mountain View, CA, USA), specifically gated for lymphoid cells (Fig.1), using the Data-MATE software analysis package (Applied, Cytometry Systems, Sheffield, UK). The percentage of WCL38^{+} cells was calculated by subtracting the percentage of cells labelled with the secondary antibody (RAM-FITC) only. The percentages of positive cells were calculated from pools of different individuals (n=30 at 1 and 2 weeks p.f.; n=11 at 3 to 5 weeks p.f. and n=5 from 6 weeks p.f. onwards).

Immunohistochemistry

Immunohistochemical analysis was performed on cryo-sections of aceton fixed intestine, gills and skin from carp larvae of 2 days to 80 weeks p.f. by using WCL38. The mAb was used at the same dilution described above. RAM–FITC (1:100) or tetramethyl-rhodamine isothiocyanate labelled rabbit-anti-mouse serum (RAM-TRITC, Dakopatts, Glostrup, Denmark, diluted 1:100) were used as secondary antibodies.

Results

Flow cytometry

Figure 1 shows an example of the forward scatter (FSC) and 90° side scatter (SSC) profiles of Percoll-enriched leucocyte fractions of gills from 1 and 60 weeks old carp, including the gate used to calculate the percentage of immunoreactive lymphoid cells. In thymus, head kidney, gut, spleen, PBL (not shown) and gills the percentage of gated cells increased with age. Table 1 resumes the percentages of WCL38^{+} cells (as
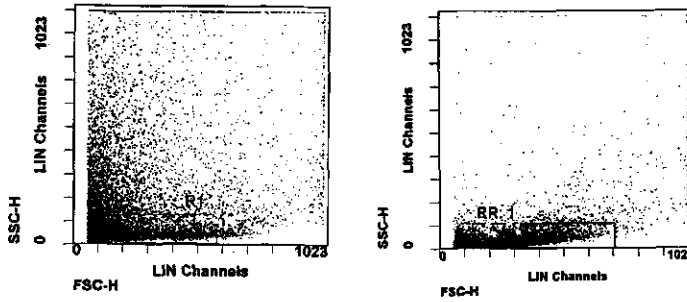
Fig. 1. Forward scatter (FSC) and 90° side scatter (SSC) profile of Percoll enriched leucocyte fractions of gills from 1 and 60 weeks old carp including the gate used to calculate the percentage of immunoreactive lymphoid cells. The percentage of gated cells calculated in the first week p.f. (15.1%, at the left) is significantly lower than the proportion of comparable cells at 60 weeks old (76.1%, at the right).

mean ± standard deviation) obtained from three different offsprings from 1 to 80 weeks p.f.. During the first week the percentage of WCL38$^+$ mucosal T cells (Fig.2, Table 1) was around 12% in gut and gills, and 8% in PBL, while lower percentages were observed in the head kidney, spleen and thymus.

Fig.2 Ontogeny of WCL38$^+$ carp cells. Time-course graph of WCL38$^+$ cells (from one experiment) in lymphoid organs detected in flow cytometric analysis from 1 to 80 weeks p.f. old carp. PBL: blood; SP: spleen; HK: head kidney; TH: thymus.

Table 1. Percentages of WCL38 positive cells in leucocyte fractions of thymus (TH), head kidney (HK), spleen (SP), gut, gills and blood (PBL) of carp, calculated by flow cytometry. Data are expressed as a mean ± standard deviation of three experiments made with different offsprings of the same carp strain at different ages post fertilisation (p.f.).
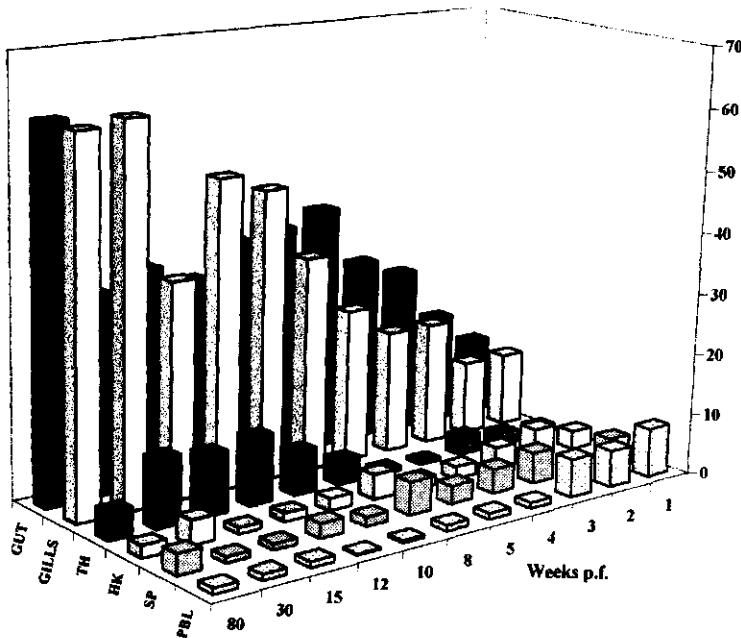
<div align="center">WEEKS p.f.</div>

Organ	1	2	3	4	5	8	10	12	15	30	80
TH	1.6 ± 0.2	2.5 ± 1.2	2.8 ± 1.2	0.4 ± 0.1	0.3 ± 0.4	12 ± 9	7.9 ± 1.4	10.6 ± 8.6	10 ± 6	11 ± 7	4.5 ± 2.1
HK	3.5 ± 2	5 ± 0.1	3.5 ± 1.3	0.2 ± 0.2	1.5 ± 1.3	4.1 ± 3	1.88 ± 0.7	1.4 ± 1.2	0.9 ± 0.2	4 ± 1	2 ± 1.4
SP	4 ± 0.1	1.6 ± 2.2	5.1 ± 1.2	0.3 ± 0.5	3 ± 3.5	5.4 ± 5.2	1.6 ± 0.3	2.4 ± 2	1 ± 0.3	1.0 ± 0.3	3.4 ± 0.3
GUT	12.5 ± 3	18 ± 11	27 ± 1	19 ± 10	40 ± 18	38 ± 6.2	37 ± 17	32 ± 17	35 ± 16	32 ± 18	60 ± 14
GILLS	12 ± 6	12 ± 3	20 ± 1	8 ± 10	26 ± 4	35 ± 10	47 ± 8	50 ± 10	35 ± 5	61 ± 14	60 ± 10
PBL	8 ± 1	5.6 ± 2	6.3 ± 0.1	1 ± 0.2	1.3 ± 1	0.8 ± 4	0.4 ± 0.1	0.2 ± 0.1	1.3 ± 1.8	1 ± 0.1	0.7 ± 0.3

In the latter organ, the percentage of positive cells peaked (around 10%) from 8-15 weeks p.f. and then decreased reaching the adult values of approximately 5%. Interestingly, during ontogeny the percentage of WCL38$^+$cells in PBL decreased to values less than 1%, whereas a slight increase was observed in thymus. In gut and gills the percentages of WCL38$^+$ cells sharply increased and reached the adult values of about 60 % in both tissues.

Immunohistochemistry

Intestinal tract

At 2 days p.f. the intestinal tract was formed by a monolayer of epithelial cells forming a small tube localised dorsally to the yolk sac and ventrally to the dorsal aorta. In the first week p.f., a few WCL38$^+$ cells, were localised in the epithelium as isolated cells or in small groups (Fig. 3a). From 4 weeks onwards, numerous WCL38$^+$cells were found in the mucosa and reactive groups of 4-5 cells in submucosa (Fig. 3c). At 80 weeks, a very high number of WCL38$^+$cells was recognised in intestinal epithelium but also in the lamina propria.

Gills

At the first week rare WCL38$^+$cells were localised in the gill mucosa showing membrane positive spots giving a granular appearance to the cells (Fig. 4a, b). The number of WCL38$^+$cells increased from the first month of development onwards. During the second week p.f. the epithelium of the gills developed around the branchial arches and the first lamellae appeared. From the 4th week p.f. onwards the WCL38$^+$cells were localised in lamellae of the gills (Fig. 5a).
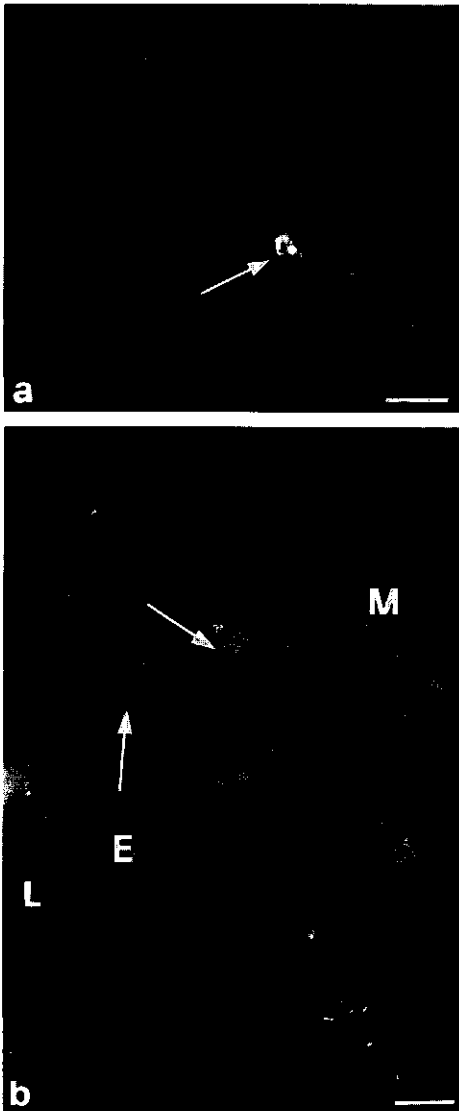
Fig. 3 (a-b). The WCL38 immunostaining of developing intestine. **a)** WCL38$^+$ cells were identified in the intestinal tract at one week p.f. (arrow), bar: 8 μm. **b)** From 4 weeks onwards, numerous WCL38$^+$ cells are shown in the epithelium (E) and in submucosa as reactive isolated cells or in groups of 4-5 cells (arrows). M, muscle layer; L, lumen among the intestinal folds. Bar: 9 μm.
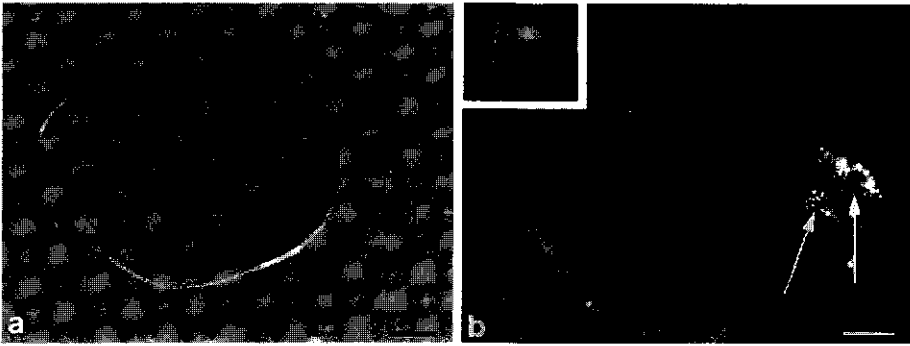
Fig.4 (a-b). WCL38 immunostaining of developing gills. WCL38-immunoreactive cells (were identified in the epithelium (a, interferential micrograph; b, immunostaining) around the gill arch at one-week old carp (arrows). Bars: 10 μm. The inset in b) shows a WCL38-positive cell.

Skin

A few WCL38$^+$ cells were observed in skin from the first week p.f., (not shown). From 4 weeks p.f. onwards WCL38$^+$ cells increased in number in the epidermis (Fig. 5b). Some positive epithelial cells cross-reacted with WCL38 from 4 weeks p.f. onwards.
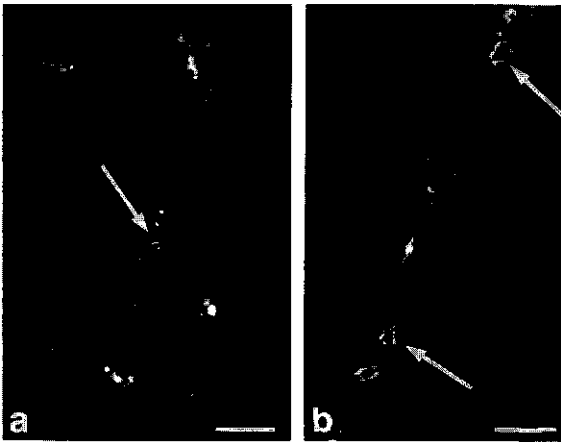
Fig.5 (a-b). WCL38 immunostaining of developing gills and skin from 4 weeks old animals, a) numerous WCL38$^+$cells are shown in the epithelium of gill lamellae (arrows). Bar: 18 μm. b) WCL38$^+$ cells (arrows) in the epidermis showing a faint positivity of epidermal cells. Bar: 10 μm.

Discussion

In fish as in other vertebrates, B-cells (Irwin & Kattari, 1986; Miller *et al.*, 1994; Koumans-van Diepen *et al.*, 1994a), T-cells (Miller *et al.*, 1985; Lin *et al.*, 1992) and non-specific cytotoxic cells (Evans *et al.*,1992) are present in lymphoid organs (Hogan *et al.*,1996) and can also be found within epithelia of gills, skin and gut forming a MALT (Roitt *et al.*,1993). Especially for fish living in a pathogen-rich aquatic environment presence of a MALT is important as a first barrier against non-self

Chapter 6

Immunocytochemical detection and cytomorphology of
lymphocyte subpopulations in a teleost fish *Dicentrarchus
labrax* (L.)

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Abstract

The monoclonal antibodies DLT15 and DLIg3 directed against thymocytes and serum immunoglobulins of the sea bass (*Dicentrarchus labrax*, L.) were used to study cells from thymus, head kidney, spleen, gut-associated lymphoid tissue and peripheral blood leucocytes of this fish by immunofluorescence and pre-embedding immunoelectron microscopy.

Immunofluorescence and flow cytometry of leucocyte fractions revealed a large number of DLT15 positive cells in thymus (~80%) and intestine (~55%) and fewer cells in spleen (~7%), head kidney (~6%) and peripheral blood (~3%). DLT15^{+} cells had two main morphologies, both detectable among thymocytes: a large round and heterochromatic nucleus with light and sparse cytoplasm (type a), and an irregular and heterocromatic nucleus with cytoplasm rich of polysomes and mitochondria (type b). type b was most represented in spleen, head kidney, intestine and blood. We suggest that the type b morphology represent more differentiated lymphocytes.

Flow cytometry revealed numerous DLIg3^{+} cells in head kidney (~33%), spleen (~30%) and peripheral blood leucocytes (~21%) and less positive cells in intestine (~3%) and thymus (~2%). DLIg3-positive cells had morphology of lymphocytes (with large, round nucleus) or macrophages in all tissues. Plasma cells lacked membrane immunoreactivity.

This is the first ultrastructural characterisation of putative T and B lymphocyte subpopulations in a fish species; these subpopulations are differentially distributed in teleost lymphoid organs.

Introduction

Teleost fishes represent almost half of existing vertebrate species and many species are a major source of food. The interests in aquaculture fish-farming has provided the impetus for studies on fish immune system in order to improve methods for vaccination against disease (Tatner, 1983; Manning, 1994). Furthermore, the immune system of fish is of interest from a phylogenetic stand-point, because teleost fish are the first group of animals showing the basic aspects of the immune system of higher vertebrates. However, the field of fish immunology remains one of discovery.

Fish have functional equivalents of T and B lymphocytes, granulocytes, thrombocytes and monocyte/macrophages (Ellis, 1977), whose characterisation is based

on morphological, immunohistochemical and functional criteria (Ellis, 1977; Rijkers *et al.*, 1980; Lamers *et al.*, 1985; Clem *et al.*, 1991). Over the past ten years, the production of specific monoclonal antibodies (mAbs) against fish leucocytes has improved our knowledge of some lymphocyte subpopulations, mainly those of the B cell lineage (Secombes *et al.*, 1983; Miller *et al.*, 1987; Koumans van Diepen *et al.*, 1994a,c, 1995) since mAbs specific for mature T lymphocytes are still lacking. For the sea bass, *Dicentrarchus labrax* (L.), mAbs are now available directed against thymocytes (DLT15; Scapigliati *et al.*, 1995) and immunoglobulin (Ig)-bearing cells (DLIg3; Romestand *et al.*, 1995; Scapigliati *et al.*, 1996; Palenzuela *et al.*, 1996), and these mAbs have been used as probes to detect lymphocyte content in sea bass organs and tissues. DLT15-immunoreactivity of a small percentage of cells in peripheral blood, head kidney and spleen (Scapigliati *et al.*, 1995) and the significant number of gut and skin lymphoid cells of juvenile fish (Abelli, unpublished) have raised the possibility that the antigenically relevant determinants are expressed by at least a subset of mature T cells.

This study describes the fine cytology of DLT15-positive (DLT15$^+$) and DLIg3$^+$ cells. Enriched leucocyte fractions from various lymphoid and non-lymphoid tissues (thymus, spleen, head kidney, intestine and blood) have been analysed to clarify the cytomorphology of immunoreactive lymphocytes and outline possible tissue differences.

Materials and methods

Animals

Sea bass (10-18 months old) were bred and reared in seawater aquaculture in a local fish farm (La Rosa, Orbetello). Fish were killed with 1 mg/ ml tricaine methane sulfonate (MS222, Sigma, St. Louis, MO., U.S.A.) and blood, head kidney, thymus, spleen and intestine were collected.

Cell suspensions

Cell suspensions from head kidney, thymus, spleen and intestine were prepared as previously described (Scapigliati *et al.*, 1995). Cell suspensions were layered over a discontinuous gradient of Percoll (Pharmacia AB, Uppsala, Sweden) diluted in RPMI to

yield densities of 1.02 and 1.07 gr/ml. After centrifugation (30 min at 840 g) at 4°C, cells layered between the densities mentioned above were collected and washed twice (10 min at 680 g) at 4°C. The pellet was resuspended in RPMI with 1% bovine serum albumin (BSA) and 0.1% sodium azide. Peripheral blood leucocytes (PBL) were prepared from heparinised blood (5 ml) diluted 1:1 in RPMI and centrifuged (15 min at 100 g) at 4°C. The buffy coat was collected, resuspended in RPMI and layered as described above.

Indirect immunofluorescence and flow cytometry

The production and characterisation of the mAb DLT15 has previously described (Scapigliati *et al.*, 1995). Briefly, the mAb was obtained by immunising mice with parephormaldehyde-fixed thymocytes (1-yer-old sea bass) andscreening hybrydoma culture media by indirect immunofluorescence (IIF) and fluorescence-activated cell sorter (FACS) analysis of both living and fixed cells. The hybrydoma cell line giving the best stainig was cloned by limiting dilution, screened again, and the clone DLT15 was established. The control of IgG isotype for DLT15 showed that it belonged to the IgG_3 subclass (ISO-I kit, Sigma).

The production and characterisation of the mAb DLIg3 has previously described (Scapigliati *et al.*, 1996). Briefly, the mAb was obtained by immunising mice with sea bass serum immunoglobulins that had been single-step purified by affinity chromatography on protein A-sepharose. The hybrydoma culture media were screened by dot-blot assay, IIF and immunohistochemistry on Bouin-fixed tissues. Western blot analysis showed that DLIg3 slightly recognised the light chain of sea bass IgM. mAb isotyping showed that DLIg3 belong to the IgG_1 subclass (ISO-I kit, Sigma).

The cell suspensions (10^7 cells ml^{-1}) were incubated in 250 µl of undiluted DLT15 or DLIg3 hybrydoma culture supernatants for 45 minutes at 4°C. Labelled cells were then washed (10 min at 680 g) at 4°C and incubated for 30 min with FITC-conjugated goat anti-mouse Ig serum (GAM, Cappel Europe, Turnhout, Belgium) diluted 1:250 in RPMI. After washing, 20 µl of 10^6 cells per ml were cytocentrifuged at 500 g at 4°C and observed with a Zeiss Axiophot microscope. Pictures were taken using Kodak TMAX at 800 ISO. Up to 10000 cells per ml were measured with a flow cytometer (FACScan, Becton Dickinson Immunocytometry System, Mountain View, Calif., USA). The percentage of positive cells was calculated subtracting results

obtained in negative control. Numerical results are expressed as mean ± SEM (standard error of the mean).

Immuno-gold electron microscopy

Labelling with DLT15 and DLIg3 was described above. Labelled cells were washed in RPMI, centrifuged (10 min, 680 g) and incubated with gold-conjugated goat anti-mouse Ig serum (gold particles of 15 nm in diameter, Aurion, The Netherlands) for 30 min at 4°C under gentle agitation. The cells were washed as described, resuspended in 1 ml of RPMI and centrifuged at 1000 g for 10 sec to yield a compact pellet. The pellet was fixed 1 hr with a mixture of 2% glutaraldhyde, 1% osmium tetroxide and 1% potassium bichromate in 0.1 M sodium-cacodylate buffer (van Diepen *et al.*, 1991). After fixation, the pellets were dehydrated through a series of graded alcohols and embedded in Epon 812 resin (Fluka Chemie, Switzerland). Ultrathin (70 nm) sections were prepared with a Reichert Ultracut microtome, stained with uranyl acetate and lead citrate and examined with a 1200 JEOL EX II electron microscope.

Results

Indirect immunofluorescence and flow cytometry

IIF revealed that the thymus the majority of DLT15$^+$ thymocytes were small and round in the thymus (Fig.1a). In head kidney (Fig.1b), intestine (Fig.1c), PBL (Fig.1d) and spleen (not shown), the size of positive cells was more variable. The majority of DLIg3$^+$ cells had round but larger in size than DLT15$^+$ cells (Figs.1 e-h). Some positive cells had the appearance of macrophages (data not shown).

FACS profiles of DLT15 in the thymus and DLIg3 in the spleen are shown in Figure 2. The forward-scatter (FSC) and 90° side-scatter (SSC) profiles are displayed in Fig.2a, d, as the gate selected for quantitative analysis (R2). Cells with low side scatter within the gate were regarded as lymphoid cells, whereas cells with high side scatter were excluded from calculation, because these were mainly non-lymphoid cells as established by electron microscopy of sorted cells (data not shown).

Sharp peaks of DLT15 positive cells in the thymus (Fig.2c) and DLIg3 positive cells in the spleen (Fig.2f) were found, compared with control cells incubated with

myeloma medium alone (Figs.2b,e). The percentage of DLT15 positive cells in the thymus, head kidney, spleen, intestine and blood is shown in Figure 3. DLT15^{+} cells were very numerous in thymus (79.5 ± 10%) and intestine (55.3 ± 2.9%), but less numerous in spleen (7.1 ± 2.9%), head kidney (6.5 ± 3.1%) and PBL (3.1± 1.2%).

DLT 15   **DLlg3**
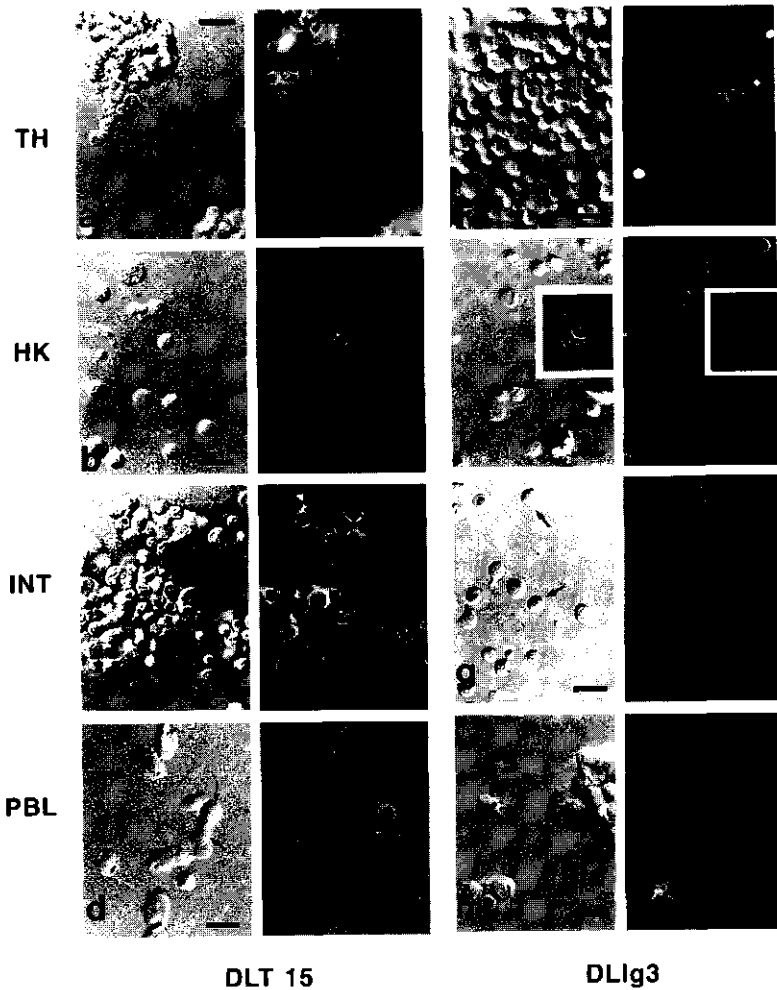
fig.1. Immunofluorescence staining for DLT15 (a-d; left panel) and DLlg3 (e-h; right panel) in leucocyte-enriched fractions of thymus (TH), head kidney (HK), intestine (INT) and blood (PBL). The same fields are shown at interference contrast optics. Bars: 10 μm. f) inset: X 1550.

The percentage of DLIg3$^+$ lymphoid cells in thymus, head kidney, spleen, intestine and blood is shown in Fig.3. DLIg3$^+$ cells were very numerous in head kidney (33 ± 4.6%), spleen (29.9 ± 5.8%) and PBL (21.1 ± 2.9%), whereas they were infrequent in intestine (2.7 ± 1.8 %) and thymus (2.5 ± 2.1%).

DLT15

DLIg3
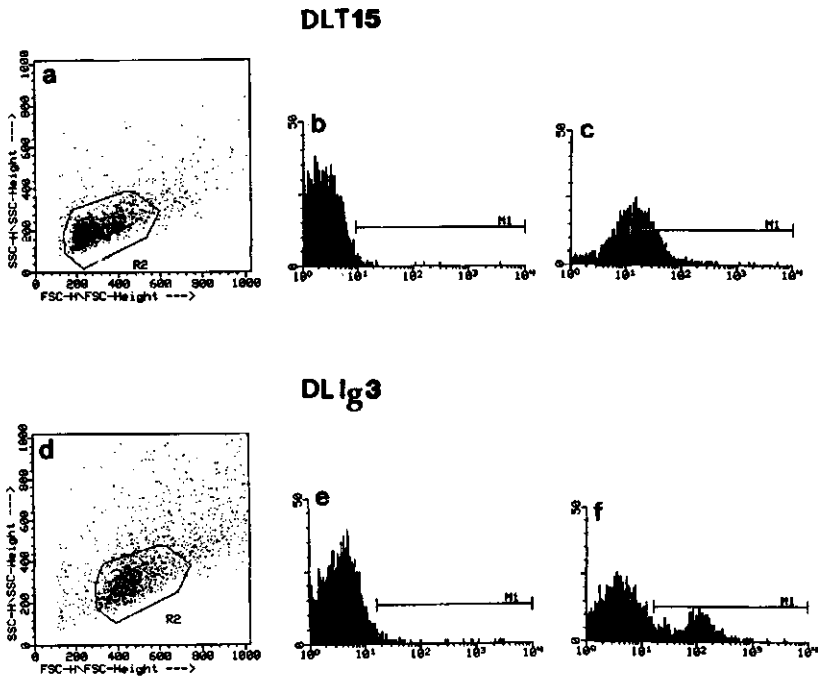
Fig.2 Flow cytometry of leucocyte enriched fractions of thymus (a-c; upper panel) and spleen (d-f; lower panel) labelled with DLT15 and DLIg3 antibodies, respectively. Forward scatter (FSC) and 90°side scatter (SSC) profiles are shown in (a,d), as well as the gate selected for quantitation (R2). FACS profiles revealed major peaks of DLT15$^+$ cells in the thymus (c) and DLIg3 positive cells in the spleen (f), compared with the controls (b,e).
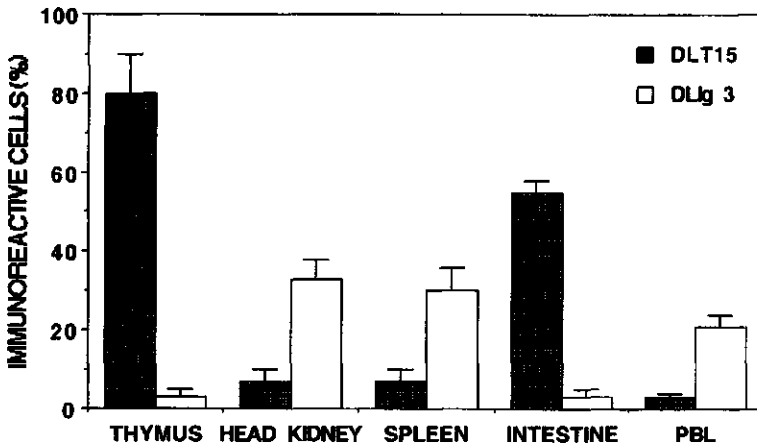
Fig.3 FACS analysis of leucocyte-enriched fractions of different lymphoid organs with DLT15 and DLIg3 mAbs. Columns represent the mean ± standard error of the mean of three experiments.

Immunogold electron microscopy

The majority of DLT15- and DLIg3-immunoreactive cells had ultrastructural features of lymphocytes (large nucleus, small rim of cytoplasm and no granules). DLT15$^+$ cells in thymus showed two main morphologies. Type a cells had a regular, heterochromatic nucleus, often with enlarged perinuclear cisterna (Fig. 4A). The electron-lucent cytoplasm was sparse with poor endoplasmic reticulum. Round mitochondria had poorly developed cristae. Cell diameter of positive cells was 3-4 µm. DLT15 immunostaining was localised to a limited number of spots on the plasma membrane (Fig. 4A). Type b cell (Fig. 4B) had an irregular nucleus that was often lobed.

Fig.4A-F. Immuno-electron microscopy of DLT15$^+$ cells. Two typical morphologies (type a and b) of positive thymocytes are shown (A,B). A) Type a cell has a round heterochromatic nucleus and sparse cytoplasm. Bar: 500 nm. A detail is shown in the inset. Bar: 50 nm. B) the type b cell has a large irregular nucleus and more cytoplasm than that the type a cell. Bar: 500 nm. A detail is shown in the inset Bar: 50 nm. C) Positive cells with irregular heterochromatic nucleus of type b cells are present in head kidney. Bars:500 nm. D) In spleen, similar cells display a multilobated nucleus. Bar: 500 nm. E) Intestinal DLT15$^+$ cells have the same features as the type b cell. Bar: 500 nm. F) Positive cells with irregular heterochromatic nucleus of type b cells are also present in the PBL. Bar: 200 nm. Arrows: gold particles.
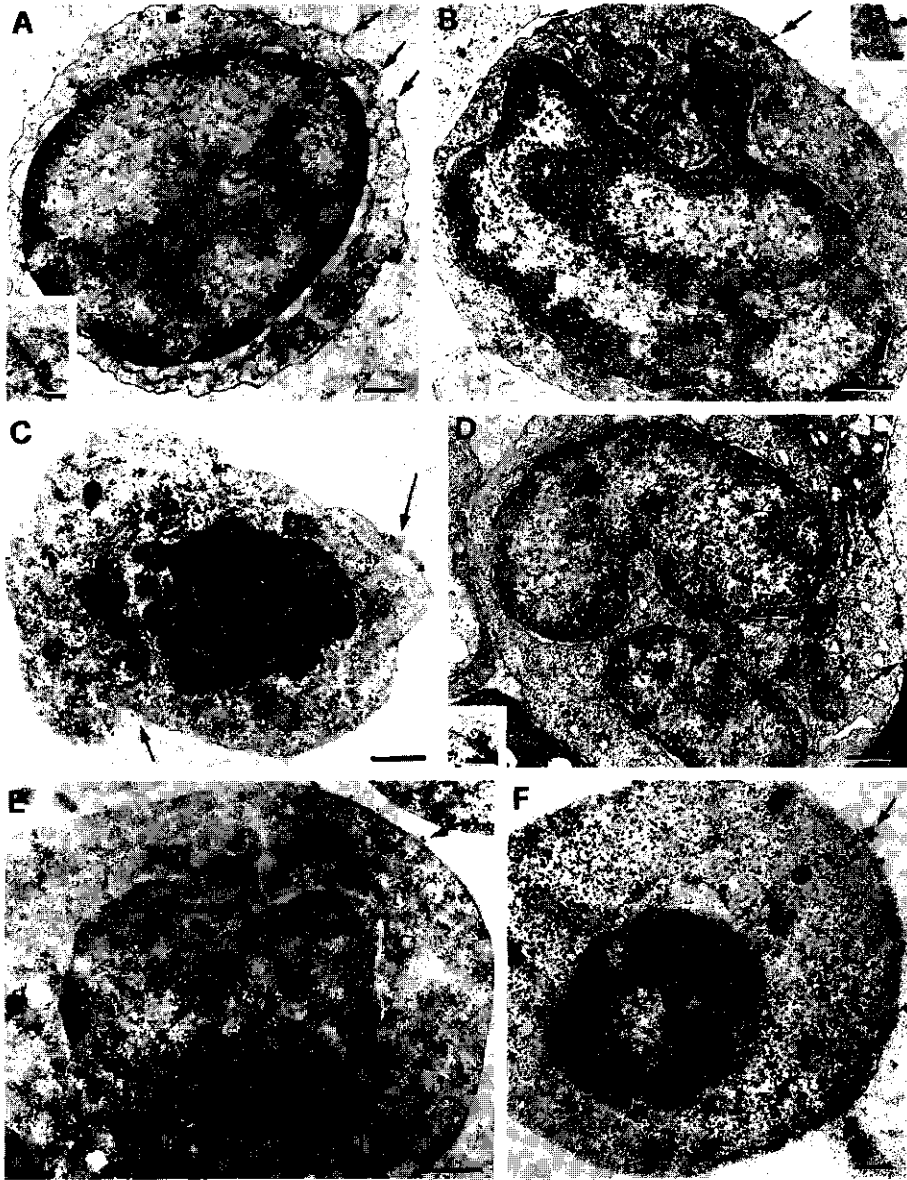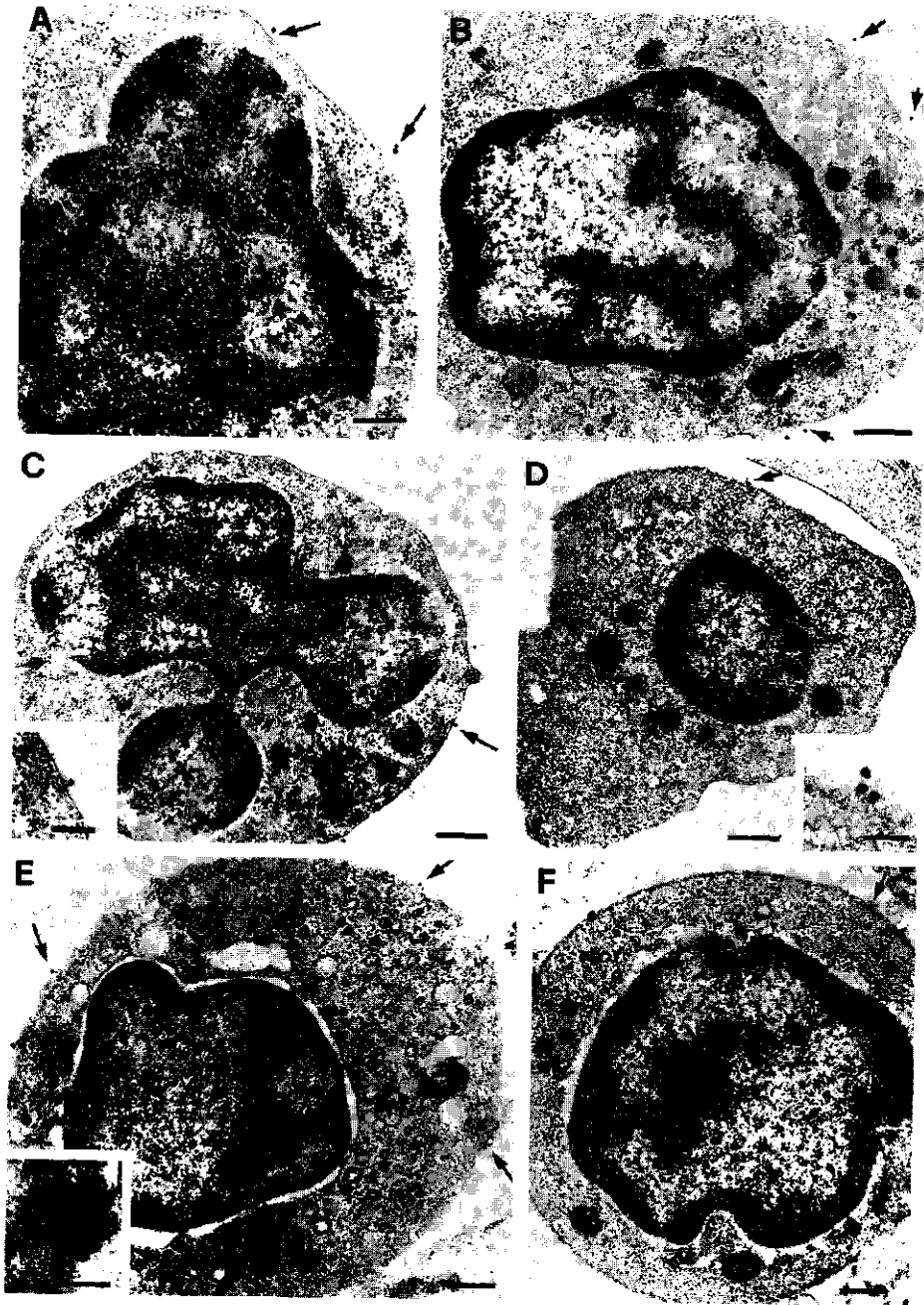
The endoplasmic reticulum was better developed than that in type a cells. Golgi apparatus, many ribosomes and a few lysosome-like vesicles were present. Round or elongated mitochondria with well-developed cristae lay around the nucleus. The size of positive cells was 4-5 μm. DLT15 immunostaining was localised to a limited number of spots on plasma membrane (Fig. 4B) as in the type a cells. In the head kidney, DLT15^{+} cells showed both morphologies with predominance of type b, often with heterochromatic nucleus (Fig. 4C; cell diameter of positive cells ranged 4-4.5 μm). In the spleen, DLT15^{+} cells (Fig. 4D) were present with the morphology described in type b of the thymus, with an even more markedly lobed nucleus. The cell diameter of positive cells ranged 3.6-4.8 μm. Immunostaining occurred was in a few spots on plasma membrane (Fig. 4D). In the intestine, DLT15^{+} cells (Fig. 4E) were predominantly of type b. The cell diameter of positive cells measured around 4 μm; the nucleus was more regularly defined than in thymocytes. The gold particles were distributed in little spots on the membrane (Fig. 4E). Finally, both types of DLT15^{+} cells were found among PBL, with a predominance of type b (Fig. 4F); the cell diameter of positive cells was 3.6-4.5 μm. The nucleus was more heterochromatic compared with immunoreactive thymocytes.

DLIg3^{+} cells were very infrequent in the thymus (Fig.5A). The cell diameter was 4-5 μm. In head kidney, the majority of DLIg3^{+} cells had regular nucleus (Fig. 5b). The cytoplasm had a poorly developed endoplasmic reticulum and Golgi apparatus. Mitochondria with well developed cristae were present, as were lysosome-like vesicles. Cell diameters were 3.5-4.5 μm. In spleen, DLIg3^{+} cells had multilobed, heterochromatic nucleus (Fig. 5C). The cytoplasm had many ribosomes and some lysosome-like vesicles; cell diameters were 3.7-4.5 μm. Gold particles were found in isolate groups on the plasma membrane (Fig. 5C). Cells with typical ultrastructural features of plasma cells (parallel arrays of rough endoplasmic reticulum) were devoid of immunostaining (not shown). DLIg3^{+} cells of PBL possessed a round nucleus and electron-lucent cytoplasm with numerous lysosome-like vesicles (Fig. 5D). The cell diameter ranged 4.7-5 μm. In intestine, few DLIg3^{+} cells, they had a round heterochromatic nucleus were found and sparse cytoplasm containing some lysosome-like vesicles and mitochondria with well developed cristae (Figs. 5F); cellular diameter measure 4-4.5 μm.

Fig.5A-F. Immunoelectron microscopy of DLIg3^{+} cells. Typical Ig-bearing cells in thymus (A) and intestine (F) possess a large nucleus. Bar: 300 nm. In head kidney (B) and PBL (D), the nucleus is more regular and the cytoplasm contains mitochondria and lysosome-like vesicles. Bars: 500 nm. A detail of D is shown in the inset. Bar: 40 nm. In spleen (C), DLIg3^{+} cells have a multilobed nucleus. Bar: 500 nm. A detail is shown in the inset. Bar: 100 nm. In intestine some DLIg3^{+} cells display a morphology similar to immature plasma cells (E). Bar: 500 nm. A detail is shown in the inset. Bar: 100 nm. Arrows: gold particles.

These cells had a morphology comparable with DLIg3^{+} cells in the thymus (Fig. 5A). Some DLIg3^{+} cells in the intestine had cytoplasm filled by vesicles of rough endoplasmic reticulum, sometimes with fine floccular content, and resembled pre-plasma cells (Fig. 5E).

Discussion

Previous ultrastructural studies on lymphoid organs of sea bass have not lead to the unequivocal identification of T and B lymphocytes (Esteban *et al.*,1989; Meseguer *et al.*, 1990, 1991). The production of mAbs against sea bass thymocytes (DLT15) and IgM (DLIg3) has improved our knowledge of the distribution of each lymphocyte subpopulation in some lymphoid organs, as assessed by immunohistochemistry of fixed tissues and flow cytometry on cell suspensions of whole tissues (Scapigliati *et al.*, 1995, 1996; Abelli *et al.*, 1996). Taking advantage of the availability of these reagents, this study has aimed to define the cytomorphology and tissues distribution of mAb^{+} cells in Percoll-enriched leucocyte fractions.

The percentage of DLT15^{+} cells measured by flow cytometry in the leucocyte fraction of the thymus (~80%) did not differ significantly from that obtained from the whole thymocyte fraction (>70%, Scapigliati *et al.*, 1995), whereas leucocyte fractions of head kidney (~6%), spleen (~7%) and PBL (~3%) were significantly enriched compared with cell suspensions obtained from the whole tissues (Scapigliati *et al.*, 1995). These results confirm that DLT15 recognises the majority of thymic lymphoid cells and lymphocyte-like cells in the other lymphoid organs. Furthermore, FACS analysis of enriched leucocyte fraction from the intestine has revealed a considerable presence of DLT15^{+} cells (~55%) secondary only to the thymus. These findings confirm that antigenic determinants recognised by the anti-thymocyte mAb DLT15 are expressed, in juvenile sea bass, not only in thymocytes but also in other lymphocyte-like subpopulation(s) in lymphoid tissues and peripheral blood and especially, the intestine.

Previous immunohistochemical studies in juvenile sea bass have demonstrated DLT15 immunoreactivity in numerous thymocytes, mainly localised in the cortical portion of thymic lobules, whereas stromal components of the thymus lack any immunoreaction (Scapigliati *et al.*, 1995; Abelli *et al.*, 1996). In spleen and head kidney, DLT15-immunoreactive cells are scattered in the parenchyma, either as isolated cells or in small groups, localised in poorly developed areas of white pulp (Scapigliati *et al.*, 1995). Developmental studies have shown that the appearance of DLT15-

immunoreactive cells in lymphoid organs of sea bass larvae occurs in the following sequence: thymus, head kidney, spleen (Abelli *et al.*, 1996).

Immunoelectron microscopy has revealed that DLT15 recognises thymic lymphoid cells with the morphology of lymphocytes at various degree of differentiation. The morphology of DLT15^{+} cells is comparable with that of thymocytes identified in other fish species by means of specific mAbs (Miller *et al.*, 1987; van Diepen *et al.*, 1991). The cytomorphology of lymphoid cells at various degree of differentiation observed in teleost thymus is apparently similar to that of avian (Frazier, 1973) and mammalian (Farr *et al.*, 1985, Roitt *et al.*, 1993). We propose that DLT15-immunoreactive thymocytes can be distinguished according to two main morphologies (types a and b). Type a cells are characterised by round, heterochromatic nucleus and sparse cytoplasm, with little endoplasmic reticulum and few mitochondria.

They are significantly present in thymus (half of the immunoreactive cells) and infrequent in the other lymphoid tissues. Type b cells are characterised by an irregular nucleus that is often lobed and cytoplasm that contains endoplasmic reticulum and Golgi apparatus that is better developed than that in the type a cells. Furthermore, many ribosomes and a few lysosome-like vesicles fill the cytoplasm, together with perinuclear mitochondria with well developed cristae. Type b cells are significantly present in thymus (half of immunoreactive cells) and represent the predominant immunoreactive cell type in spleen, head kidney, intestine and blood.

According to ultrastructural and immunocytochemical findings, we hypothesise that type b cells can represent a more differentiated form of T cells. This consideration is reinforced by the evidence that the thymus of the sea bass is a primary lymphoid organ and that DLT15-immunoreactive cells appear later in secondary lymphoid organs, as shown in previous developmental studies (Abelli *et al.*, 1996). Type a cells can be considered as lymphocytes at lower level of differentiation. The presence of these cells in the thymus and their infrequent occurrence in peripheral lymphoid organs can be interpreted as a leak-age from the thymus of pre-lymphocytes. The question whether negative/positive selection of lymphoblasts might occur at extra-thymic locations can not be addressed at present and is currently under investigation.

The massive presence of DLT15^{+} cells among intestinal leucocytes suggests that an extensive system of cell-mediated immune response protects the intestinal mucosa against penetration of pathogens. Ig-bearing cells positive for DLIg3 are infrequent in the intestine (<3%), indicating that humoral immunity also plays a role in gut-associated lymphoid tissue (GALT) of the sea bass. Studies in carp *Cyprinus carpio* L. have shown

that more than 90% of intestine leucocytes were Ig-negative lymphoid cells recognised by the anti-leucocyte mAb WCT23 (Rombout *et al.*, 1986; 1989), supporting the idea that they are putative T cells. Fish GALT thus appears to have peculiar characteristics compared with other vertebrates (Lefrancois & Puddington, 1995).

DLIg3 antibody was selected to detect the light chain subunits of sea bass IgM (Scapigliati *et al.*, 1996). In the present study, high percentage of Ig-bearing cells was detected among leucocytes of the head kidney (~33%), spleen (~30%) and PBL (>21%), whereas a low percentage has been found in intestine and thymus (~2%). A study in carp (Koumans-van Diepen *et al.*, 1994a) has revealed lower percentages of Ig-positive cells in leucocyte fractions of head kidney and spleen (20%, both), a higher percentage in midgut (5%) and similar percentage in thymus (1-2%), compared with the sea bass. These differences might represent changes in distribution of Ig-bearing cells between the two fish species. The percentage of Ig-bearing cells in PBL of the sea bass (>21%) compared with values reported for carp (33%, Koumans van Diepen *et al.*, 1995); eel (25%; van der Heijden *et al.*, 1995), channel catfish (25%; Ainsworth *et al.*, 1990) and trout (45%; Thuvander *et al.*, 1990) not only once again outlines species differences, but also raises the possibility that the different mAbs would recognise epitopes present in heterogeneous chains of fish IgM, as it has been previously shown in the sea bass (Palenzuela *et al.*, 1996).

Previous immunohistochemical analysis has revealed numerous Ig-bearing cells (DLIg3^{+}) isolated or in small groups in the intertubular tissue of the head kidney and spleen. A few positive cells have also been detected in the mucosa of the middle intestine (Scapigliati *et al.*, 1996).

As shown by immunoelectron microscopy, the majority of DLIg3^{+} cells have lymphocyte cytomorphology with round heterochromatic nucleus (lobated in cells of the spleen) and sparse cytoplasm, as described for B cells of other fish species (Ellis, 1977; Koumans-van Diepen *et al.*, 1994, 1995). Especially in the intestine, DLIg3-immunoreactive cells with numerous vesicles of rough endoplasmic reticulum resemble pre-plasma cells, whereas mature plasma cells lack membrane immuno-gold labelling. Plasma cells can also be identified by IIF staining following detergent (Triton X-100) permeabilisation of cell membranes (unpublished data). These observations indicate that in pre-embedding immuno-electron microscopy, DLIg3 reacts with B cells and immature plasma cells. Similar results have been obtained with mAbs raised against homologous Ig in other fish species (Castillo *et al.*, 1993), indicating that plasma cells store cytoplasmic Ig and lose membrane Ig, as occurs in mammals (Ritter & Crispe,

1992; Roitt *et al.*, 1993), and providing another link between the immune system of fish and higher vertebrates.

Infrequent slightly DLIg3⁺macrophages have been localised in lymphoid organs, mainly in leucocyte suspensions of the spleen and head kidney. These cells were recognised on the basis of characteristic cytomorphology (Meseguer *et al.*, 1991). Previous studies have demonstrated the Ig-binding capacity of fish macrophages, by employing immunofluorescence and immunogold methods. These studies have revealed the presence of a coplement-binding fraction receptor that binds the costant fragment (Fc) part of the Ig-molecule (Haynes *et al.*, 1988; Koumans-van Diepen *et al.*, 1994c).

In conclusion, ultrastructural findings confirm the high specificity of mAbs DLT15 and DLIg3 for various subpopulations of lymphatic cells of the sea bass. These antibodies appear to be promising tools to further exploring the ontogeny, physiology and molecular biology of fish T and B lymphocytes, and for providing new insight into the evolution of specific immunity of vertebrates.

Chapter 7

Immunocytochemical detection of thymocyte antigenic
determinants in developing lymphoid organs of sea bass
Dicentrarchus labrax (L.)

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Abstract

The monoclonal antibody DLT15 specifically recognised antigenic determinants expressed during development of thymic lymphoid cells of the sea bass *Dicentrarchus labrax* (L.). They were first detected at day 30 post-hatching in the thymus of fish maintained at 16 °C; thereafter, a marked increase in the number of immunoreactive cells occurred mostly in the outer region of the thymus. The appearance of DLT15-immunoreactive cells in the lymphoid organs was in the sequence thymus, head-kidney and spleen. The thymus thus appears to be a primary lymphoid organ in the sea bass. Infrequent cells sharing the antigenic determinants expressed on thymocytes were localised in the developing head-kidney and spleen.

Introduction

A large body of evidence indicates that teleosts possess lymphoid organs, immuno-competent cells and immunological activities (antibody production, long term memory and T cell cytotoxicity) similar to those present in other vertebrates (Ellis, 1977; Rijkers *et al.*, 1980; Lamers *et al.*, 1985; Clem *et al.*, 1991). However, further studies are required for the embryological, physiological and comparative characterisation of the fish immune system.

Obvious progress would derive from the development of antibodies recognising fish lymphocyte subpopulations. Specific markers of the B-cell lineage are available (Miller *et al.*, 1987; Thuvander *et al.*, 1990; Israelsson *et al.*, 1991; Navarro *et al.*, 1993; Estevez *et al.*, 1994; Koumans-van Diepen *et al.*, 1994; Romestand *et al.*, 1995; Sanchez *et al.*, 1995; van der Heijden *et al.*, 1995; Scapigliati *et al.*, in press), whereas conventional immunisation of mice with immunoglobulin (Ig)-negative lymphoid cells revealed monoclonal antibodies (mAbs) which were not completely specific for T lymphocytes (Secombes *et al.*, 1983; Miller *et al.*, 1987; Ainsworth *et al.*, 1990). Recently, mAbs have been raised against membrane molecules isolated from carp thymocytes, among which mAb WCL9 was considered to be an early T cell marker (Rombout, unpublished). However, mAbs specific for mature T cells of fish are still not available.

We have recently produced and characterised (by indirect immunofluorescence, FACS, avidin-biotinylated peroxidase immunostaining and immunoblotting) the mAb

DLT15 raised against the thymocytes of the sea bass _Dicentrarchus labrax_ (L.) (Osteichthyes, Percicthydae), which recognised specifically the majority of thymocytes, lymphoid cells scattered throughout the head-kidney and spleen, and a low percentage of peripheral blood cells of juvenile fish (Scapigliati et al., 1995). This antibody was employed in this study to analyse the ontogenetic development of lymphoid cells within the thymus, head-kidney and spleen of the sea bass, with the aim of obtaining new information on the differentiation of T cells in this species.

Materials and Methods

Larvae and juveniles (from hatching to one year-old) of sea bass maintained at 16 °C were sampled to study the development of lymphoid organs. The animals were killed with tricaine methanesulphonate (1 mg/ml) and whole fish or dissected tissues (thymus, head-kidney and spleen) were fixed and embedded. Bouin's or Wood's fixatives (Wood, 1963) and paraffin embedding were utilised for histology. Serial sections of 7 μm thickness were stained with haematoxylin and eosin, May-Grünwald/Giemsa (Pappenheim method) and Mallory's trichrome.

Immunohistochemistry was performed according to a previously described protocol (Scapigliati _et al._, 1995). Tissues were immersion-fixed in Bouin's fluid for 7 h at 4°C, dehydrated through a series of graded alcohols at 4 °C, cleared in toluene and embedded in paraffin. Serial transverse sections of 7 μm thickness were dried at 37°C and placed on glycerin/albumin-coated slides. Deparaffinised sections were rehydrated and washed in PBS (0.1 M, pH 7.3), followed by 20 min incubation with PBS containing 0.5% hydrogen peroxide to quench endogenous peroxidase activity.

The mAb DLT15 was used undiluted or diluted (1:10 to 1:200) in PBS containing 5% normal horse serum, 5% normal sea bass serum and 0.1% sodium azide and incubated for 18 h at room temperature. Normal mouse serum (1:10) or the myeloma culture medium substituted for primary antibody in control sections. The production and characterisation of the mAb DLT15 has been previously described (Scapigliati _et al._, 1995).

Following rinses in PBS, sections were incubated for 1 h at 25°C with biotinylated horse anti-mouse IgG serum (Vector Labs., Burlingame, USA) diluted 1:1000 with PBS containing 0.1% sodium azide and 1% bovine serum albumin, followed by incubation for 1 h with avidin-biotinylated peroxidase complex (ABC, Vectastain® Elite, Vector) with the avidin and biotinylated horseradish peroxidase

solutions diluted 1:2000 in Tris-buffered saline (TBS: 0.05 M, pH 7.6). Following rinses in TBS, sections were incubated in 0.05 M Tris buffer containing 0.4% nickel ammonium sulphate, 0.02% diaminobenzidine and 0.015% hydrogen peroxide. After rinsing in Tris buffer, sections were dehydrated, mounted and examined under bright-field illumination.

In each animal, five non-consecutive sections of thymus, head-kidney and spleen were immunostained with DLT15. Counts of immunoreactive cells (nucleated only) were performed by an observer unaware of treatments. Estimates of the number of immunoreactive cells present in the various tissues at each time point were then calculated by averaging the cell numbers from three animals at each developmental age. Cell measurements were obtained with a computer-assisted image analysis system of three animals at each stage and were pooled to obtain cumulative values of the mean and standard error of the mean. Homogeneity of variances was tested before data processing and numerical results were analysed by one-way ANOVA test.

Results

Histology

At day 27 post-hatching paired thymic anlagen first acquired an obvious lymphoid appearance, according to the previously established chronology of thymic development at 16 °C (Abelli *et al.*, 1994). Thereafter, active lymphopoiesis was accompanied by regionalisation of the gland: numerous lymphoid cells filled the outer thymic region, while they were present to a smaller extent in the inner region. The histology of the thymus was well established in three month-old fish (Fig. 1 a). Neither quantitative nor qualitative differences were observed during the development of the right and left thymic glands.

The inter-tubular tissue of the head-kidney was observed at an early stage of development (day 10 post-hatching). The haemopoietic and lymphoid components of the tissue intermingled without any evident regionalisation through various developmental stages (up to 3.5 months post-hatching). Distinct regional differences were observed in juveniles from 8.5 months onwards, when lymphoid areas close to melano-macrophage centers (MMCs) became organised around blood vessels of the head-kidney and were clearly identifiable from the myeloid tissue (Fig. 1 b). Neither

quantitative nor qualitative differences were observed during development of the right and left portions of the kidney.

The anlage of the spleen was observed at day 17 post-hatching, close to the dorsal wall of the anterior portion of the intestine. The developing spleen was composed of loosely packed haemopoietic cells and appeared mainly erythropoietic. Very scarce lymphoid elements could be detected at day 44 post-hatching. At day 59 post-hatching, a few lymphoid cells were evenly distributed throughout the parenchyma. Distinct regional differences could not be detected in the spleen of 3.5 month-old fish, although lymphoid cells began to accumulate. In one year-old fish, lymphoid areas close to or associated with MMCs were organised around blood vessels of the spleen (Fig. 1 c) and could be identified from erythropoietic tissue.

Immunohistochemistry

ABC-immunostaining of paraffin sections allowed the appearance and localisation of DLT15-immunoreactive cells during development of the thymus (Figure 2), head-kidney (Figure 3) and spleen (Figure 4) to be studied. The number of positive cells in the different tissues is shown in Table 1.

In the thymus, DLT15-immunoreactive cells (diameter 4.5±0.2 µm, range 2.5-6.6 µm, N=60) were first identified at day 30 post-hatching, underneath the pharyngeal epithelium [Fig. 2(a)]. At this age the thymus was predominantly filled with blast-like cells (diameter 3.7-7 µm) (Fig. 2 b). From 35 to 44 days post-hatching, a limited increase in number of positive cells was observed (Table 1), mostly localised in the outer region of the gland.

From day 38 post-hatching onwards, immunoreactive cells were also observed outside the thymus. Such positive cells increased in number from 44 to 59 days post-hatching, when they could be easily detected in areas between the thymus and the head-kidney (Fig. 3 c).

Fig. 1a-c. Histology of thymus, head-kidney and spleen. **a)** The outer and inner regions of the thymus can be readily distinguished by different density of the lymphoid elements in a longitudinal section of a three month-old fish. C: capsule; GC: gill chamber. Pappenheim stain. Bar: 50 µm. **b)** Head-kidney of one-year old sea bass. Lymphoid areas (L) are identifiable from the abundant haemopoietic tissue (H). Lymphoid cells are close to MMCs (arrows). Mallory's trichrome stain. Bar: 50 µm. **c)** Spleen of one year-old sea bass. Lymphoid areas (L) close to MMCs (arrows) surround blood vessels defining poorly developed areas of white pulp. Pappenheim stain. Bar: 20 µm.

Thus, the percentages of Ig$^+$ cells detected by flow cytometry and summarised in Table 1 give only an impression of the presence of B cells in different lymphoid organs of juvenile or adult fish species.

Studies on cell-mediated immunity in teleosts have clarified that fish show T cell responses based upon functional criteria (Rijkers & van Muiswinkel, 1977; Sizemore *et al.*, 1984; Miller *et al.*, 1986, 1987; Clem *et al.*, 1985; Graham & Secombes, 1990; Secombes et al., 1991). Recently, some reports have described four T cell-specific mAbs in three different fish species: carp (Rombout *et al.*, 1997, 1988), sea bass (Scapigliati *et al.*, 1995) and channel catfish (Passer *et al.*, 1996). The distribution of T cells in the main lymphoid organs is shown in Table 2.

Table 2. Percentage of immunoreactive T-cells detected by flow cytometry in leucocyte fractions of different tissues of juvenile and adult teleosts.

mAb	Head kidney	Spleen	Thymus	Intestine	Gills	PBL	Species
WCL9	< 1	< 1	50	< 1	n.d.	< 1	Carp[a]
WCL38	1-3	1-3	1	50-70	50-70	< 1	Carp[b]
DLT15	6	7	78	50-60	50-60[*]	3	Sea bass[c]
CfT1	20	12	73	n.d	n.d	10	Channel catfish[d]

[a] ref. Rombout *et al.*,1997 (16 month-old carp);

[b] ref. Rombout *et al.*,1998 (16 month-old carp);

[c] ref. Romano *et al.*,1997b (10-18 month-old sea bass);

[d] ref. Passer *et al.*,1997 (10-18 month-old catfish);

(*) Romano *et al.*, unpublished.

Regarding the membrane molecules recognised, WCL9 clearly reacted with molecules (150/ 200 KDa) (Rombout *et al.*, 1996) distinct from those recognised by with the three other mAbs, which were all reactive with 35-40 KDa molecules (Scapigliati *et al.*, 1995; Passer *et al.*, 1997; Rombout *et al.*, 1998). In contrast to DLT15 and CfT1, WCL38 recognises a dimeric molecule of 76 KDa. Indications are

available that at least a part of the WCL38^{+} cells (Romano _et al._, unpublished) and the DLT15^{+}cells (Abelli _et al._, unpublished) also show some cytoplasmic reaction. The molecular sequence of the immunoreactive molecules has not yet been elucidated, as well as their role, and hence the function of the immunoreactive T cell (sub)populations. In contrast to the other mAbs, DLT15 could also be used for _in situ_ immunocytochemistry on paraffin sections from lymphoid and non-lymphoid organs (Scapigliati _et al._, 1995). In sea bass head kidney, the T cells were observed in lymphoid areas where erythropoiesis was very scarce (Chapter 7), and in the spleen, T cells were mainly present around blood vessels (Scapigliati _et al._, 1995). In the thymus, DLT15^{+} cells were mainly localised at the periphery of thymic lobuli. Immuno-electronmicroscopy revealed that DLT15^{+} cells have the morphology of vertebrate lymphocytes. Two main types of DLT15^{+} thymocytes could be distinguished in sea bass. One type had more cytoplasm than the other and was very common in spleen, head kidney, intestine and blood, suggesting that this type could represent a more differentiated stage of the cell (Chapter 6).

WCL9, a marker of early T cells, stained a high percentage of carp thymocytes with a high nucleus/cytoplasm ratio. In older carp, WCL9^{+}cells were limited to the thymic cortex (Rombout _et al._, 1997), while WCL38^{+}cells were merely present in mucosal lymphoid organs such as skin, gills and intestine (Rombout _et al._, 1998). In addition, T cells (Ig^{-} cells) not reactive with both mAbs were present in other lymphoid organs, suggesting T cell heterogeneity in carp (Rombout _et al._, unpublished). Moreover, post-embedding immuno-electronmicroscopy revealed at least two different morphotypes of WCL38^{+} cells: agranular lymphoid cells with a high nucleus/cytoplasm ratio and larger granular lymphoid cells, a minor population evidenced in skin and gills (Rombout _et al._, 1998).

In general, it can be stated that B cells are more numerous in the blood, spleen, and head kidney and that T cells dominate in thymus and mucosal compartments (Table 2, Chapter 6). It can be speculated that differences in the distribution of lymphocytes could reflect a different behaviour of immune cells with respect to their homing behaviour and anatomical location (Rombout _et al._, 1989; Klein, 1995).

Appearance of lymphocytes in developing fish

Only a few studies were recently published describing the appearance of leucocyte subpopulations during ontogeny. In carp, two mAbs were used (WCI12 and

WCI4) to detect subpopulations of B cells during the ontogeny (Koumans-van Diepen _et al._, 1995). Studies during carp ontogeny revealed the presence of different B-cell subpopulations in relation to larval development. In 14 days old carp, the main population of Ig-bearing cells was recognised by the mAb WCI4, whereas WCI12$^+$cells were the major B cell population at 56 week-old fish (Koumans-van Diepen _et al._, 1995). In Chapter 2 of this thesis, the presence of WCI12$^+$ B cells was confirmed at 14 days (mainly in the head kidney) and these cells were not detected earlier in other lymphoid organs, suggesting that the head kidney is probably a primary lymphoid organ for B cells. As far as functional aspects are concerned it is important to mention that oral immunisation of 2 or 4 weeks old carp (23°C) induced tolerance, while 2 months old carp were able to develop immunological memory (Joosten _et al._, 1995). In sea bass, IgM-reactive cells were first detected by immunohistochemistry (using mAb 6E11) at day 38 post-hatching (p.h.) in the head kidney of fry reared at 16 to 20 °C (Breuil _et al._, 1997; 38 days p.h. correspondent to 40-41 days p.f., Barnabè _et al._, 1976). The earlier detection (day 18 p.h) by FACS of very few Ig-bearing cells in larval cell suspensions could not be confirmed by immunocytochemistry (Breuil _et al._, 1997). The detection of soluble IgM has also been extended to mature eggs and embryos, and previous research showed that in some fish species mature eggs contain detectable levels of IgM (Clerx, 1978; Yousif _et al._, 1995; Lillehaug _et al._, 1996; Olsen & Press, 1997), which in sea bass could be transmitted to the early embryo (Breuil _et al._, 1997). In sea bass, low numbers of IgM$^+$ cells were detected with the mAbs DLIg3 and DLIg14, at day 49 p.h. in the head kidney, spleen and thymus (very infrequent) of fries reared at 16° C (Picchietti _et al._, 1997). A direct comparison of the results of Breuil _et al._ (1997) and Picchietti _et al._ (1997) appears difficult because of differences in rearing temperatures used, which may also result in discrepancies with respect to the appearance of the thymus as a lymphoid organ: day 27 p.h. at 16° C (Chapter 7) and day 21 p.h. at 16 to 20° C (Breuil _et al._, 1997). However, these findings suggest that the immune system of the sea bass larvae is probably able to mount an immune response when the weaning period is reached around day 50 p.h. (Breuil _et al._, 1997). Although immunisation experiments were not reported for young sea bass, it can be suggested that they have to be at least 50 days p.h. old (16-20°C) or even older to obtain immunological memory. Clinical observations show that sea bass fries are highly sensitive to bacterial diseases during this period and that vaccination from this stage (50 days p.h.) onwards can provide sufficient protection (Breuil _et al._, 1997).

In Chapter 4 the *in situ* presence of immature thymocytes in carp was analysed by using the mAb WCL9, showing the appearance of the thymus at 4 days p.f.. The organ was completely filled with positive cells. In Chapter 2, WCL9⁺cells are also described temporarily in other lymphoid organs, including blood, just after their first appearance in the thymus. These observations suggest the possible migration of early T cells during ontogeny. However, WCL9⁺ cells became completely restricted to the thymus from 5 weeks p.f. onwards. In Chapter 7 the presence of sea bass T cells between thymus and head kidney is described, again suggesting a migration of T cells to other organs. The main difference between WCL9⁺ cells in carp and DLT15⁺ cells in sea bass is the staining of peripheral T cells by DLT15.

In Chapter 4, WCL9 was used to study in developing thymus of carp the *in situ* presence of immature thymocytes in relation to the distribution of apoptotic lymphocytes and epithelial cells. From 4 weeks p.f. onwards, cortex and medulla could be distinguished and contemporary reticular epithelial cells and nurse-like cells appeared. From that age, apoptotic cells were also observed in considerable quantities, suggesting that selection of thymocytes in carp starts around the 4 weeks p.f.. A continuous growth of thymus has been observed with a complex cortex and medulla organisation and in 80 weeks old carp no signs of involution were observed. Whether the development of the carp thymus is different compared with other species, for instance the sea bass, remains to be investigated.

Appearance of monocytes/macrophages in developing fish

Monocytes/macrophages were detected earlier than B cells and thrombocytes (i.e. first week p.f.), suggesting that they are appearing earlier than T and B lymphocytes (Chapter 2). In Chapter 3 this possibility was explored by using the mAb WCL15 in immunohistochemistry and flow cytometry. The analysis of isolated viable cells with flow cytometry revealed a high percentage of WCL15⁺cells in spleen and blood. However, even when cross-reacting thrombocytes were subtracted, a too high percentage of WCL15⁺cells still remained in thymus and intestine (Chapter 2). Recently, indications were obtained that WCL15 can cross-react with part of the thymic and intestinal T cells (Rombout *et al.*, unpublished) and hence WCL15⁺WCL6⁻ cells calculated in Chapter 2 can not be considered all as monocytes/macrophages. The immunohistochemistry on paraffin sections excluded this problem, because only macrophages were stained. This conclusion was drawn from their size, irregular shape

and strong cytoplasmic reaction with WCL15. The *in situ* presence of WCL15^{+} monocyte/macrophages was first observed in the head kidney rudiment, suggesting that this organ could play a central role in the maturation of these cells. Although, the size of WCL15^{+} cells varied in different tissues it increased throughout the development, especially in thymus and head kidney. Immuno-electronmicroscopy revealed morphological differences among thymus, head kidney and hindgut carp macrophages. Therefore, heterogeneity of macrophages was suggested, but it is still unknown whether these differences are correlated with different functions, as observed in mammals (Unanue, 1993). The early and continuous presence of macrophages in lymphoid tissues (mainly thymus and head kidney) supports the idea that macrophages can play a role in the maturation of fish immune system, probably being involved in antigen processing and negative selection of lymphocytes. A role in the selection of T- and B-lymphocytes exerted by the resident population of monocytes/macrophages in the thymus, spleen and bone marrow (mammals) or bursa of Fabricius (birds) has been firmly established in higher vertebrates (Roitt *et al.*, 1993). In younger fish, macrophages could play a role in secreting growth factors, in non-specific defence and removing cellular debris generated by physiological cell death during development.

Appearance of thrombocytes in developing fish

Fish thrombocytes are responsible for blood clotting in response to injury and are activated by collagen (Ellis *et al.*, 1977; Rowley *et al.*, 1988; Uchida *et al.*, 1992; Rombout *et al.*, 1996). Until now only a few mAbs were developed against fish thrombocytes and their specificity was not restricted to thrombocytes due to the cross-reaction with T cells and neutrophils (Miller *et al.*, 1987; Slierendrecht *et al.*, 1995). WCL6 represents the first available mAb specific for carp thrombocytes and their precursors (Rombout *et al.*, 1996). In Chapter 2, WCL6 was used to detect the presence of thrombocytes during ontogeny. The results indicated the presence of thrombocytes in the spleen during the first week p.f. and their percentage increased until the 4th week. From the 5th week onwards the percentage of thrombocytes decreased in spleen and increased in blood, while thrombocytes were hardly detectable in cell suspensions from other organs. This observation suggests that the spleen is the main thrombopoietic organ in carp.

Ontogeny of the mucosal immune system

Evidence of the existence of a mucosal immune system in teleosts comes, among others, from the observation that protection after mucosal immunisation (oral or immersion) was more related to the level of specific Ig in mucosal secretions than in blood (Rombout _et al._, 1989; Fletcher & White, 1973; Lobb & Clem, 1987). Recently, it has been demonstrated that oral vaccination of carp resulted in significantly higher numbers of specific plasma cells in gills and intestine compared with head kidney and blood, while the opposite was observed after injection (Joosten _et al._, 1997). These results strongly indicated the occurrence of a mucosal B cell population. Species differences were reported compared with the intestinal B cells. Abundant B cells of carp were observed in the lamina propria (Rombout _et al._, 1993b), whereas in sea bass only isolated B cells could be found (Abelli _et al._, 1997). In both species high numbers of T cells were observed in the intestinal epithelium. It can be speculated that these T cells play a pivotal role in cellular response to antigens present in intestinal compartment. Throughout development, DLT15-immunoreactive T cells in sea bass were very numerous in the thymus, but immediately after their appearance in the thymus (day 30 p.h.) they were also detected in the intestinal mucosa (Picchietti _et al._, 1997). In carp, the mAb WCL38, specific for mucosal T lymphocytes (Rombout et al., 1998) was used to monitor the distribution of the immunoreactive cells during the ontogeny (Chapter 5). WCL38^{+} cells were detected at the first week p.f. with significant higher numbers in gut and gills than in other lymphoid tissues (thymus, head kidney, spleen and blood). Immunohistochemistry showed that the skin of one-week-old carp already contained scattered WCL38^{+} lymphoid cells, but they became more numerous from the 4th week p.f.. This very early appearance of mucosal T cells and their strong increase in number in the following weeks, is an indication of an important function of these cells in defence against invading pathogens.

While the number of T cells did not show significant variations along the intestine of stomachless carp (Rombout _et al._,1989b, 1998), intestinal T cells were more numerous in the posterior part of the intestine of sea bass, a fish species with a stomach (Abelli _et al._, 1997). The so-called second gut segment has been shown to be the main location for the uptake and transport of antigens in a variety of fish species, i.e. carp (Rombout _et al._, 1985, 1986; Rombout & van der Berg, 1989), trout (Georgopoulou _et al._, 1986) and sea bass (Vigneulle & Baudin-Laurencin, 1981).

Whether the gradient of T cells in the sea bass is related to antigen transport remains to be investigated.

Until now no attention has been paid to the ontogeny of B cells in mucosal tissues, which fact can be related to the absence of a reliable mucosal B cell marker. A mAb specific for secreted mucosal IgM has been described for carp (Rombout et al., 1993a). Unfortunately this antibody did not recognise membrane IgM on B cells. The mentioned differences observed between the distribution of B cells in the gut of carp and sea bass could be explained by the fact that only a fraction of mucosal B cells of the sea bass were recognised by mAbs available (DLIg3 and DLIg14), whereas the mAb WCI12 apparently recognised a large part of the mucosal B cell population in carp.

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List of fish species quoted in the text

Atlantic cod   *Gadus morhua*
Atlantic salmon   *Salmo salar*
Common carp   *Cyprinus carpio*
Channel catfish   *Ictalurus punctatus*
Chum salmon   *Oncorhynchus keta*
Coho salmon   *Oncorhynchus kisutch*
European eel   *Anguilla anguilla*
Gilthead sea bream   *Sparus aurata*
Guppy   *Poecilia reticulata*
Japanese flounder   *Paralichthys olivaceus*
Mozambique mouth brooder (tilapia)   *Oreochromis mossambicus*
Pike   *Esox lucius*
Plaice   *Pleuronectes platessa*
Rainbow trout   *Oncorhynchus mykiss*
Red drum   *Sciaenops ocellatus*
Red sea bream   *Pagrus major*
Rosy barb   *Barbus conchonius*
Sea bass   *Dicentrarchus labrax*
Swordtail   *Xiphophorus helleri*
Turbot   *Scophtalmus maximus*
White sturgeon   *Acipenser transmontanus*
Yellowtail   *Seriola quinqueradiata*

Summary

Teleost fish show the features of an adaptive immune system (antigen-driven cellular and humoral responses) and the expected characteristics of specificity and memory. As regards phylogenetics, therefore, fish represent the first group of animals showing the basic aspects of the immune system of the "higher" vertebrates such as birds and mammals. In addition, a thorough knowledge of the teleost immune system is necessary to develop health-protective strategies to prevent disease under intensive culture conditions. Fish farming at high densities can easily induce infectious diseases at all stages of the production cycle. Therefore, knowledge of the immune system and its development can be very valuable for the protection of fish in aquaculture. Significant achievements derived from the preparation of monoclonal antibodies (mAbs), usually directed against immunoglobulins (Ig) and IgM-bearing cells, and their use, in combination with *in vitro* assays, have proven that teleost possess the functional equivalents of B- and T-lymphocytes, and monocytes/macrophages as accessory cells. In this study, a panel of mAbs against the freshwater common carp (*Cyprinus carpio*, L.) and sea bass (*Dicentrarchus labrax*, L.) leucocytes were used for the characterisation of leucocyte subpopulations during the ontogeny.

The distribution of leucocyte subpopulations was studied in the thymus, head kidney, spleen, gut and blood of developing carp by using mAbs against early T-cells (WCL9), B-cells (WCI12), thrombocytes (WCL6) and macrophage-like cells (WCL15). In the first week post-fertilisation (p.f.), WCL9$^+$ cells were the major population in the thymus ($\pm$ 77 % in leucocyte percentage) and a minor population in the other lymphoid organs; subsequently these cells gradually disappeared in all organs, except thymus ($\pm$ 40%). B-cells appeared in head kidney from the second week p.f., and subsequently in spleen and blood, but their number remained low in thymus and gut. The percentage of thrombocytes increased gradually in spleen from the first week until the fourth week p.f., ($\pm$ 30%) and from that age considerable numbers were found in blood. Consequently, a primary differentiation role of the thymus and head kidney is suggested for the T and B lymphocytes, respectively and the spleen appears to be the primary site for thrombopoiesis. The preliminary analysis with WCL38, a mAb, reacting with mucosal T cells indicated that these cells could play a defensive role at the mucosal surfaces of gut, gills and skin from the first week p.f. onwards. The

proportion of WCL38^{+} cells clearly increased with age in these mucosal tissues and with the exception of the thymus (up to 10%) there was little or no presence in other lymphoid organs.

The mAb WCL9 was used to study the ontogeny of the carp thymus. Special attention was paid to the development of cortex and medulla by using immunohistochemical, confocal-laser-scanning analyses. This study provided evidence that the cortex and medulla start to differentiate from 4 weeks p.f.. The ultrastructural study of the developing thymus confirmed these data and permitted the analysis of the distribution and morphological differences of the epithelial cells. In addition, the *in situ* localisation of thymic apoptotic cells revealed numerous apoptotic cells in the cortex from 4 weeks p.f. and subsequently in the cortical-medullary border, suggesting that these regions are important for the selection and depletion of thymocytes.

A monoclonal antibody against carp macrophages (WCL15) has been used in flow cytometry, immuno-histochemistry and immuno-electron microscopy to assess the distribution of monocytes/macrophages in developing carp lymphoid tissues. In suspensions of living cells WCL15 reacted strongly with the outer membrane of macrophages, but also cross-reacted with a subpopulation of thrombocytes. This cross-reaction could be neglected by double immunostaining in combination with a thrombocyte-specific marker. In Bouin-fixed tissues the antibody distinctly recognised macrophages, probably due to the strong reaction with the cytoplasm of macrophages. Macrophages were found from day 2 p.f. in head kidney and in the dorsal portion of the yolk sac epithelium. From 1 week p.f. onwards macrophages were found scattered in the thymus and gut and, during the second week p.f. in the spleen. Macrophages increased in number in all lymphoid tissues until 6-8 weeks p.f., but then decreased except in the thymus, where they became localised mainly in the cortical-medullary boundary, and in the lymphoid areas of the head kidney. Interestingly, the number of macrophages in thymus grew continuously reaching adult values from the 8 weeks p.f., and their distribution changed from scattered (until 3-4 weeks p.f.) to small groups of cells localised in the border between cortex and medulla (from 5 weeks to 20 weeks p.f.). This information, together with that provided by learnt from the study of the *in situ* distribution of apoptotic cells and WCL9^{+}cells in developing thymus, strongly suggests a role of the thymic macrophages in the maturation process, being also involved in the clearance of apoptotic thymocytes.

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The monoclonal antibodies DLT15 and DLIg3 directed against thymocytes and serum immunoglobulins of the sea bass (*Dicentrarchus labrax*, L.) were used to study T and B cells, respectively, in different lymphoid organs of this fish species by immunofluorescence and pre-embedding immunoelectron microscopy. Immunofluorescence and flow cytometry of leucocyte fractions revealed a large number of DLT15 positive cells in the thymus (± 80%) and intestine (± 55%) and fewer cells in spleen (± 7%), head kidney (± 6%) and blood (± 3%). Regard DLIg3$^+$ B cells, they were numerous in leucocyte suspensions of the head kidney (± 33%), spleen (± 30%) and peripheral blood leucocytes (± 21%) but scarce in intestine (± 3%) and thymus (± 2%). Pre-embedding immuno-electronmicroscopy revealed that DLT15$^+$ thymocytes had two main morphologies, both detectable among thymocytes: a large round and heterochromatic nucleus with light and sparse cytoplasm, and an irregular and heterochromatic nucleus with cytoplasm rich polyribosomes and mitochondria. The last cell type was the major positive cell type in the spleen, head kidney, intestine and blood, suggesting that this cell type represented more differentiated T lymphocyte. DLIg3$^+$ cells showed morphology of lymphocytes, but some macrophage-like cells were also found positive (Ig-binding) in all tissues investigated. However, mature plasma cells lacked membrane immunoreactivity.

During the ontogeny, the DLT15$^+$ cells were first detected *in situ* at 30 days post-hatching in the thymus; followed by a marked increase in the number of immunoreactive cells occurred mostly in the outer region of the thymus. The order of appearance of DLT15-immunoreactive cells in the lymphoid organs was in the sequence thymus, head-kidney and spleen. Infrequent cells sharing the antigenic determinants expressed on thymocytes were localised in the developing head kidney and spleen. Thus, the thymus appears to be a primary lymphoid organ in the sea bass.

Although the mAbs used for these commercially important fish species are different, some similarities were, however, observed in their reactivities. The conclusions and suggestions mentioned in this thesis can be important from the phylogenetic point of view and for the application of vaccination strategies on young fish. In fact, the developmental stage of important leucocyte populations can now be taken into account to determine the best moment for vaccination.

Samenvatting

Net als bij zoogdieren zijn bij beenvissen zowel humorale (productie antilichamen) als cellulaire immuunresponsen (afweer door celcontact) te onderscheiden. Beide vormen van deze zogenaamde verworven immuniteit kenmerken zich door specificiteit en geheugenvorming, waarbij de respons na een tweede contact met hetzelfde antigeen sneller en heftiger verloopt. Phylogenetisch gezien zijn vissen de eerste groep van dieren die deze basale aspecten van het immuunsysteem vertonen. Een goede kennis van het functioneren van het immuunsysteem van vissen is belangrijk om dieren in de intensieve viscultuur adequaat te beschermen tegen pathogenen (b.v. via vaccinatie of immunostimulatie). Al vroeg in de ontwikkeling van gekweekte vissen kunnen infecties grote problemen geven. Daarom is ook kennis over de ontwikkeling van het immuunsysteem noodzakelijk. Hiervoor zijn goede celmarkers, zoals leucocyt-specifieke monoclonale antilichamen essentieel. De eerste geproduceerde monoclonale antilichamen zijn gericht tegen immunoglobuline (IgM) en IgM-producerende cellen van de vis. In combinatie met functionele tests toonden deze antilichamen aan dat vissen zowel over B- als T-lymfocyten beschikken. Daarnaast is duidelijk geworden dat macrofagen ook bij vissen een belangrijke rol spelen bij de initiatie van de immuunrespons. Intussen zijn er ook monoclonale antilichamen beschikbaar gekomen tegen T-lymfocyten en macrofagen. In dit proefschrift zijn diverse van deze antilichamen gebruikt om verschillende subpopulaties van leucocyten van de karper (*Cyprinus carpio*, L.) en de zeebaars (*Dicentrarchus labrax*, L.) te bestuderen.

In de ontwikkelende karper zijn leucocyt-subpopulaties in thymus, kopnier, milt, darm en bloed bestudeerd met monoclonale antilichamen specifiek voor vroege T-lymfocyten (WCL9), B-lymfocyten (WCI12), trombocyten (WCL6) and macrofaag-achtige cellen (WCL15). In de eerste week na bevruchting bevat de thymus voornamelijk WCL9$^+$ thymocyten ($\pm$ 77%), maar deze cellen zijn in lagere aantallen ook in de andere lymfoïde organen aanwezig. In de weken daarna verdwijnen deze cellen uit alle organen behalve in de thymus, waar ongeveer 40% van de thymocyten WCL9$^+$ blijft. B-lymfocyten zijn in de tweede week voor het eerst in de kopnier aantoonbaar en verschijnen daarna ook in milt en bloed. Daarna neemt hun aantal in deze organen langzaam toe, terwijl zij slechts in lage aantallen te isoleren zijn uit thymus en darm. Het aantal trombocyten groeit in de milt geleidelijk (tot $\pm$ 30%) gedurende de eerste 4 weken

na bevruchting. Vanaf dat moment verschijnen er aanzienlijke aantallen in het bloed en daalt het aantal in de milt tot ± 10%. Er kunnen gedurende de gehele ontwikkeling nauwelijks trombocyten uit andere organen geïsoleerd worden. Samenvattend kan gesteld worden dat respectievelijk thymus en kopnier de primaire organen lijken te zijn voor T- en B-lymfocyten, terwijl de milt het orgaan is waar de trombocyten zich ontwikkelen. Met een monoclonaal antilichaam specifiek voor mucosale T-lymfocyten (WCL38) kon worden aangetoond dat deze cellen al vanaf de eerste week in de slijmvliezen van darm, kieuw en huid aanwezig zijn, terwijl zij met uitzondering van de thymus (± 10%), nauwelijks in andere lymfoïde organen voorkomen. Deze resultaten suggereren dat deze mucosale T-lymfocyten al vroeg in de ontwikkeling een afweerfunctie in de slijmvliezen kunnen vervullen.

Het monoclonale antilichaam WCL9 is gebruikt om de ontwikkeling van de karperthymus als primair lymfoïd orgaan te bestuderen. Er is vooral gelet op de ontwikkeling van schors en merg, gebruikmakend van immunohistochemische en confocale laser scanning analyses. Differentiatie van de thymus in schors en merg was niet voor de vierde week aantoonbaar. Vanaf dit stadium zijn ultrastructureel ook de diverse epitheliale cellen te onderscheiden. Tevens verschijnen dan talrijke apoptotische thymocyten in de schors en vervolgens op de overgang van schors en merg. Deze resultaten suggereren dat de thymocyten rond de vierde week van de ontwikkeling geselecteerd en verwijderd worden.

Het monoclonale antilichaam WCL15 is gebruikt om monocyten en macrofagen te lokaliseren in ontwikkelende lymfoïde weefsels van de karper, gebruik makend van flowcytometrie, immunohistochemie en immunoelectronenmicroscopie. Door de kruisreactie van WCL15 met levende trombocyten was een combinatie van WCL15 en WCL6 nodig om een indicatie te krijgen van het aantal macrofagen. Waarschijnlijk door de sterke cytoplasmatische reactie van macrofagen met WCL15 waren macrofagen goed met immunohistochemie op Bouin-gefixeerd weefsel aantoonbaar. Macrofagen werden al 2 dagen na de bevruchting in de kopnier en het dorsale deel van het dooierzakepitheel waargenomen. Vanaf de eerste week worden macrofagen verspreid in de thymus en de darm waargenomen en gedurende de tweede week ook in de milt. In de eerste 6-8 weken neemt het aantal macrofagen in alle lymfoïde organen toe en vervolgens met uitzondering van de thymus weer af. In de thymus zijn grote aantallen aantoonbaar op de overgang van schors naar merg en in de kopnier in de lymfoïde gebieden. Vanaf 8 weken is het volwassen niveau van macrofagen in de

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thymus bereikt, echter de macrofagen concentreren zich van verspreid tot kleine groepjes op de grens van schors en merg vanaf de 5e tot de 20e week. Deze waarneming gecombineerd met de localisering van apoptotische thymocyten suggereert dat de thymus macrofagen een rol spelen in het rijpingsproces, inclusief het opruimen van de apoptotische cellen.

De monoclonale antilichamen DLT15 and DLIg3 die opgewekt zijn tegen respectievelijk thymocyten and serum IgM van de zeebaars, zijn gebruikt om T- en B-lymfocyten in verschillende lymphoïde organen van de zeebaars te bestuderen. Flowcytometrie toonde veel DLT15$^+$ T-lymfocyten aan in de thymus (± 80%) en darm (± 55%) en slechts enkele cellen in de milt (± 7%), kopnier (± 6%) en het bloed (± 3%). DLIg3$^+$ B-lymfocyten waren zeer talrijk in leucocytsuspensies van kopnier (± 33%), milt (± 30%) and bloed (± 21%), maar sporadisch in darm (± 3%) en thymus (± 2%). Ultrastructureel blijken twee types DLT15$^+$ thymocyten aantoonbaar: één met een grote ronde kern en electronenlicht cytoplasma en één met een onregelmatige kern met veel polyribosomen en mitochondriën. Het laatste DLT15$^+$ celtype komt ook frequent voor in milt, kopnier, darm en bloed voor en lijkt een meer gedifferentieerde T lymfocyt te zijn. DLIg3$^+$ cellen zijn morphologisch als lymfocyten te beschouwen, maar sommige macrofaag-achtige cellen kunnen ook positief zijn (Ig-binding). Rijpe plasma cellen blijken in tegenstelling tot de karper geen IgM meer op hun celmembraan te hebben.

Gedurende de ontwikkeling worden DLT15$^+$ cellen *in situ* voor het eerst in de thymus waargenomen 30 dagen na "hatching" (uit het ei komen), gevolgd door een sterke toename in de schors. De volgorde van het verschijnen van DLT15$^+$ cellen in de lymfoïde organen is thymus, kopnier en milt. Met andere woorden de thymus blijkt ook het primaire T-lymfocyten orgaan in de zeebaars te zijn. In kopnier en milt zijn gedurende de ontwikkeling slechts geringe aantalllen DLT15$^+$ cellen aantoonbaar.

Hoewel de monoclonale antilichamen, die gebruikt zijn in deze twee commercieel belangrijke vissen, verschillende determinanten lijken te herkennen, zijn er toch ook veel overeenkomsten in de verspreiding van de immunoreactieve cellen gevonden. De conclusies en suggesties in dit proefschrift zijn niet alleen van phylogenetisch van belang, maar ook van betekenis voor de toepassing van vaccinaties bij jonge vissen. Het immuunsysteem moet namelijk voldoende ontwikkeld zijn om met succes op een dergelijke behandeling te kunnen reageren.