Germ cell development

in larval and juvenile carp (*Cyprinus carpio* L.)

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Germ cell development in larval and juvenile carp (*Cyprinus carpio* L.)

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This thesis describes the development of larval and juvenile gonads of a teleost fish, the common carp, with special attention to the differentiation of the primordial germ cells. The early gonadal development has received relatively little attention, hitherto, as the research on fish reproduction has been focussed mainly on germ cell maturation in adult fish species. The early development of gonads and germ cells, however, is highly important, as in that period a.o. sex determination occurs.

In the early larval period the gonadal tissue is gradually formed around the primordial germ cells (PGCs) which are already located at the sites of the future gonadal primordia. The PGCs in the developing larval gonads increase in size between week 2 and week 4 after fertilization. Concomitantly, ultrastructural changes occur in the cytoplasm and nuclei of these cells, which is supposed to be a preparation for the rapid proliferation of PGCs after week 6.

An important part of the investigations was devoted to the production of monoclonal antibodies (MAbs). In general, MAbs may recognise differentiation stages accurately. This enables the investigation of the factors which may influence such differentiation processes. The MAbs were raised against primary and early secondary spermatogonia. The procedures are presented how germ cells were isolated from testis tissue and how the percentage of these cells (diameter > 10 μm) could be enriched with a percoll gradient from an initial 4% in the early spermatogenic testis to more than 70%.

One of the produced MAbs recognised antigens, appearing first in the cytoplasm and subsequently on the surface membrane of the enlarging PGCs in the larval gonads between week 2 and week 4. This supports the hypothesis that the enlarging PGCs are differentiating cells, possibly in preparation for the fast proliferation of PGCs after week 6. Four of the produced MAbs reacted with PGCs from the onset of PGC proliferation (after week 6) up to and including early (primary) spermatogonia in the adult testis (or oogonia in ovaries). This indicates that specific differentiation antigens are present on these cells. These MAbs may be used for the recognition of primary spermatogonia after isolation of germ cells for cell culture which is necessary for the investigation of factors which stimulate or inhibit specific steps, a.o. the onset of meiosis.

Furthermore, the possible stimulation of gonadal development in larval or juvenile carp by injection with homologous pituitary extract containing a.o. the gonadotropic hormone (GTH) was investigated. It was found in juvenile carp that the gonads were greatly enlarged and that precocious spermatogenesis occurred after injection of pituitary extract (confirming data obtained in other fish species after injection of GTH). However, also gonadal development and PGC proliferation in larval fish was stimulated. Such effect of pituitary extract on larval gonads and germ cells has not been reported previously. The evidence obtained indicates a function of GTH in this stimulation. The question, however, whether one of the MAb-recognised determinants is involved awaits elucidation.

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Stellingen

1 De opinie dat de gonade-ontwikkeling in de larvale karper slechts wordt gekenmerkt door een geleidelijke toename van het aantal geslachtscellen en gonade-cellen is onjuist.
Dit proefschrift

2 De larvale karper vormt een geschikt model voor studie naar de rol van celoppervlakte macromoleculen bij de proliferatie van geslachtscellen.
Dit proefschrift

3 De SEM-immunogoud labeling, zoals beschreven in dit proefschrift, bevestigt dat een goede celmorfologie behouden kan blijven na immunocytochemie op ongefixeerde cellen.
Dit proefschrift

4 Het centrale somatische celtype, dat in de larvale gonade rondom de primordiale geslachtscellen wordt aangetroffen, kan worden beschouwd als de voorloper van de cellen van Sertoli in de latere testis.
Kanamori et al., 1985; dit proefschrift

5 Het karakteristieke perinucleaire electronendichte materiaal ("nuage") in geslachtscellen heeft een functie bij de mitochondriënvermeerdering tijdens de proliferatie van geslachtscellen.
Eddy, 1975; Clérot, 1976

6 In de weinig eenduidige naamgeving van pre-meiotische geslachtscellen bij vissen in wetenschappelijke publicaties komt het collectief nog beperkte inzicht in de differentiatie van deze cellen tot uiting.

7 Het feit dat hybridoma-produktie met spermatozoa of spermatogonia van de karper als immunogeen nog geen monoclonale antilichamen heeft opgeleverd die embryonale primordiale geslachtscellen van de karper herkennen, hoeft niet in te houden dat op dit type cel geen specifieke celoppervlakte antigenen voorkomen.

8 Naarmate geautomatiseerde informatiesystemen in de gezondheidszorg een directere relatie met het medisch proces hebben (bv. kennissystemen voor artsen), is kwantificering van de verandering in de werkwijzen als gevolg van de invoering meer gewenst.
R. van der Loo. 1995. Evaluatie van geautomatiseerde informatiesystemen in de gezondheidszorg; verslag van een promotie-onderzoek. TMI. 24(1):30-37.

Aart van Winkoop
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Wageningen, 22 mei 1995.

Cover design: Wim Valen

Cover illustration: Immunogold staining with monoclonal antibody of primary spermatogonia isolated from testis tissue of carp. x 1500
Front cover: polarized light epi-illuminescence with Zeiss antiflex objective.
Back cover: bright field illumination of the same cells, counterstained with haematoxylin and eosin.

Dankwoord

Mijn grote dank gaat uit naar mijn promotor, prof. Lucy Timmermans, die het oorspronkelijke idee voor het onderzoek leverde, daarnaast met haar enthousiasme en kennis de uitvoering ervan in goede banen leidde en tevens met veel inzet toezag op, en bijdroeg aan, de uiteindelijke afronding in de vorm van publicaties en dit proefschrift.

John Dulos, Ronald Booms en Henk Schipper bedank ik voor de goede uitvoering van de talrijke experimenten waaraan ze meewerkten en ook voor de humor waarmee ze één en ander telkens weer wisten te relativeren. Erkentelijk ben ik ook voor de deskundige ondersteuning die Jos van den Boogaart diverse malen heeft geboden, voor de goede aquariumvoorzieningen en adequate visverzorging door Sytze Leenstra en zijn medewerkers, en voor het tekenwerk en hulp bij het maken van de foto's door Wim Valen. Bijzondere dank gaat ook uit naar Anke Hana en Hilda Valk voor het vele en acuraat uitgevoerde typewerk.

Veel collegas's en studenten binnen de vakgroep EDC en ook daarbuiten waren bij het onderzoek betrokken, gaven adviezen, werkten stimulerend door hun belangstelling; bij de afronding van dit proefschrift wil ik jullie daarvoor hartelijk bedanken!

Aart van Winkoop

Wageningen, mei 1995.

CONTENTS

General introduction

The production of gametes is essential for the maintenance of all higher animal species. Fish gametogenesis has been investigated mainly in adult animals with mature gonads in which precursor germ cells have developed into ripe spermatozoa or egg cells. Relatively little attention has been given to germ cell development in larval and juvenile fish. Yet, detailed knowledge of germ cell differentiation in earlier fish development is a prerequisite for the understanding of sex differentiation and ultimately for optimization of fish reproduction.

This thesis is devoted to the study of the life history of fish germ cells. It is asked whether particular differentiation steps can be distinguished during germ cell development. With several recent techniques, including the use of monoclonal antibodies, and morphological and functional approaches the germ cells of the common carp (*Cyprinus carpio* L.) have been examined. This fish offers the advantage of a relatively short embryonic development, implicating that, except for the initial formation of primordial germ cells, the complete earlier development of germ cells and gonads occurs in free swimming larvae and juveniles. This in contrast to mammalian species, where the major part of the comparable development of germ cells and gonads takes place during intra-uterine life and therefore will be less accessible for experimental approaches. Moreover, the common carp is a species that can be reared easily in the laboratory.

In this introduction an overview is given of the preceeding studies carried out in our department on this subject, including a short account on the results with anti-carp-sperm monoclonal antibodies obtained sofar. Moreover, the aim and outline of this thesis will be described.

Development of germ cells and gonads in carp

A survey of germ cell development in carp cultured under standard conditions at 23 °C (Parmentier & Timmermans 1985) is presented hereafter. The ages of the animals are given as "after fertilization" (a.f.).

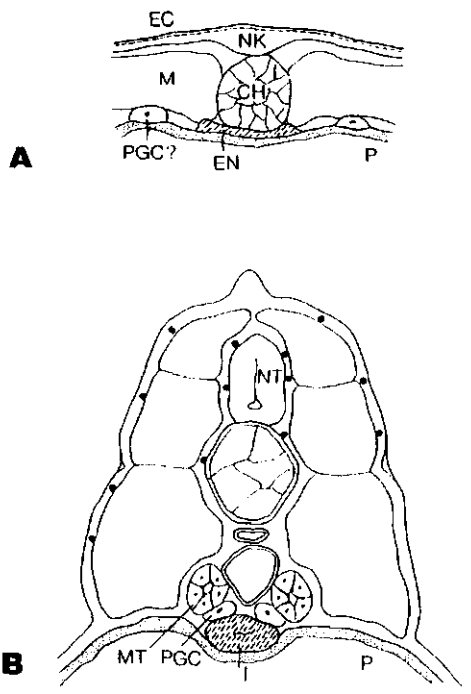

Figure 1. **Localization of PGCs during embryogenesis. In *Barbus conchonius* (25°C; 12 and 24 hours after fertilization). A, 2-somite stage (12 hours); the three germ layers can be clearly distinguished and the notochord is just formed. The inner layer of the ectoderm becomes thicker in the midline, forming the neural keel. A small number of larger cells, probably PGCs, are visible between mesoderm and periblast. B, embryo of 24 hours; the gut, mesonephric tubules, and blood vessels have been formed. PGCs are located between mesonephric tubules and gut. CH, notochord. EC, ectoderm. EN, endoderm. I, Intestine. M, mesoderm. MT, mesonephric tubule. NK, neural keel. NT, neural tube. P, periblast (yolk syncytial layer). PGC, primordial germ cell. In carp a similar stage as represented in Figure 1B is reached after 48 hours.**

Embryonic period

In carp, as in the closely related rosy barb, *Barbus conchonius* (Timmermans & Taverne 1989, Gevers *et al.* 1992), the primordial germ cells (PGCs) can be recognized with certainty during embryogenesis from the early somite stage onwards (Timmermans 1987). At that developmental stage the PGCs are characterized by their relatively large size, large nucleus and their specific location between mesoderm and periblast (also named Yolk Syncytial Layer) (Figure 1A). As soon as the gut is formed, the PGCs can be found between the mesonephric tubules and gut (Figure 1B).

Larval and juvenile period

In newly hatched carp larvae at day 3, the PGCs are located at the dorsal wall of the coelomic cavity, at the site of the future gonadal ridges (Figure 2). Their numbers are small and remain stable at approximately 20 to 40 per animal for several weeks (Figure 3), whereas other tissues do proliferate actively (Timmermans 1987).

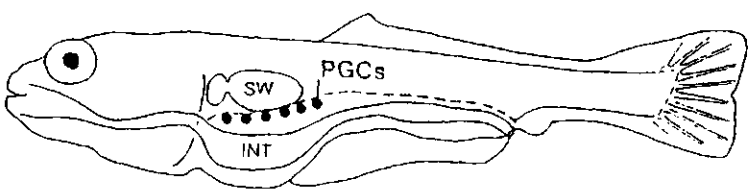

Figure 2. **Localization of PGCs at the dorsal wall of the coelomic cavity in a 10 mm larva of *Cyprinus carpio*, eight days after fertilization. INT, intestine. PGCs, primordial germ cells. SW, swim bladder (From Timmermans & van Winkoop 1993).**

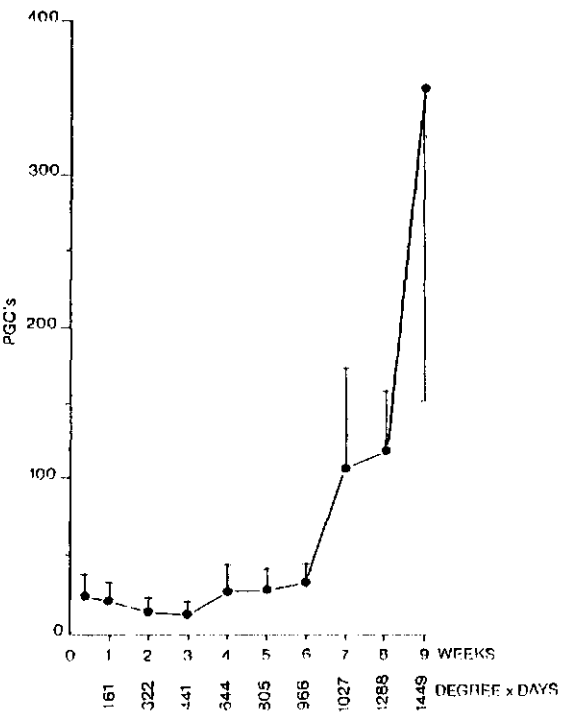

Figure 3. **Numbers of germ cells per animal during early development of *Cyprinus carpio (23 °C)*. Each group represents the mean of 5-6 animals. Bars represent standard deviation. Note that up to and including the age of 6 weeks the germ cells do not increase in number while other systems undergo during that period a rapid differentiation and growth (From Parmentier & Timmermans 1985).**

Initially, each PGC is enveloped by one or two somatic cells which form a continuous layer with the dorsal peritoneal cells (Figure 4A), but gradually during the larval period the gonadal primordia arise around the PGCs followed by invasion of stromal cells (Figure 4B). The gonads develop according to the differentiated type (Yamamoto 1969), i.e. the indifferent gonads (Figure 4B) develop directly into either immature female (Figure 4C) or immature male (Figure 4D) gonads. These can be distinguished from each other from week 10 after fertilization (a.f.) onwards (Parmentier & Timmermans 1985).

At that developmental stage the female gonad is characterised by a flattened sickle-shaped form and is attached at two sides to the coelomic wall, mediodorsally by a thin mesogonium and laterally by a thin extension, thus forming an enclosed cavity, the ovarian sac (Figure 4C); the male gonad is slender, club or arrow-shaped and attached to the coelomic wall by one, rather thick, mesogonium (Figure 4D) (Parmentier and Timmermans 1985).

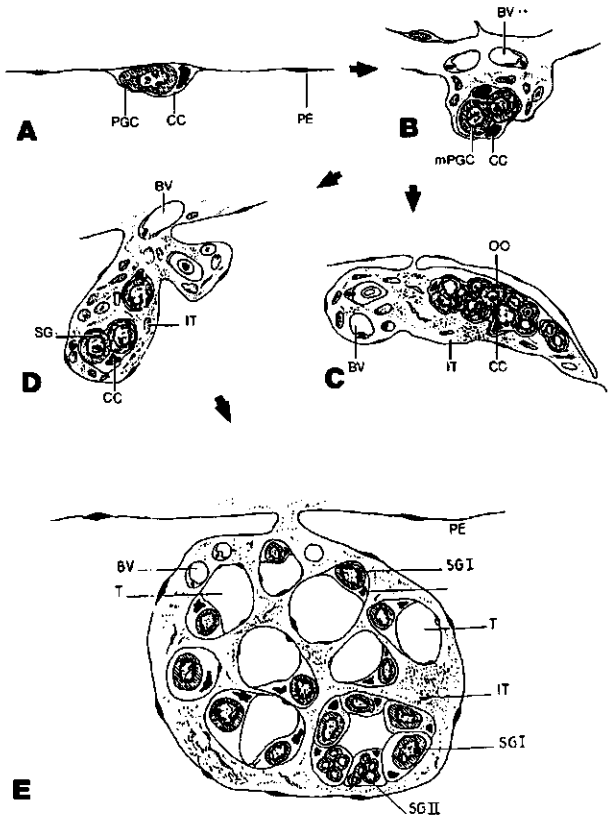

Figure 4. **Schematic drawings of the genital ridge, the indifferent, the immature female and male gonad during development of *Cyprinus carpio* (23 °C, not to scale). A. Genital ridge at one week, with primordial germ cell (PGC) surrounded by a somatic cell. B. Indifferent gonad at eight weeks. C. Female gonad at 15 weeks. D. Male gonad at 15 weeks. E. Male gonad at 19 weeks. BV, blood vessel. CC, cyst cell. IT, interstitial tissue. mPGC, mitotic primordial germ cell. OO, oogonia. PE, peritoneum. SG, spermatogonia. SGI, primary and SGII secondary spermatogonia. T, tubule (After Parmentier and Timmermans, 1985).**

Oogenesis starts at week 16 within cysts bordering the ovarial sac, containing individual primary oogonia, groups of secondary oogonia or early prophase oocytes and gradually appearing larger oocytes enveloped by follicle cells. Spermatogenesis starts at week 19 with the formation of tubules, each made up of cysts surrounding a central lumen (Figure 4E). At first the cysts predominantly contain the early spermatogenic stages, but soon the later stages develop (Parmentier and Timmermans 1985).

Testis

The adult carp testis (Parmentier *et al.* 1984) is a paired organ, located at the dorsal side of the coelomic cavity, ventrolateral to the swim bladder. At the caudal end it terminates into a short unpaired vas deferens. The organ is covered by a peritoneum and contains irregularly shaped tubules (Figure 5A), separated by interstitial tissue containing blood vessels, cells of Leydig and peritubular cells (Figure 5B).

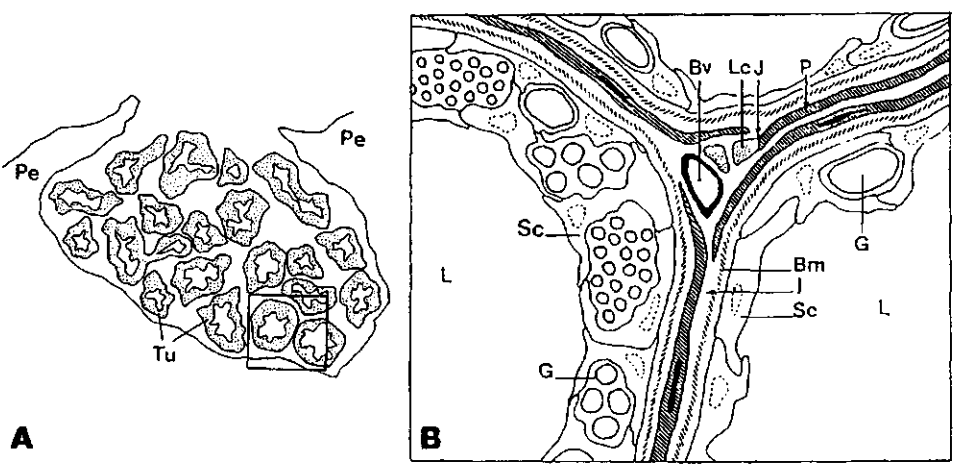

Figure 5. **Schematic drawings of a cross section through the testis of *Cyprinus carpio*. A. The testis is composed of irregular and branching tubules, separated by interstitial tissue. Pe, peritoneum. Tu, cross sections through testis tubules. B. Parts of three testicular tubules of *Cyprinus carpio*. Germ cells are arranged in cysts, enclosed by Sertoli cells, along the walls of the tubules. Bm, basement membrane. Bv, blood vessel. G, germ cell. I, interstitial tissue. J, junctional complex. L, lumen of a tubule. Lc, Leydig cell. P, peritubular cell. Sc, Sertoli cell. (A, from Parmentier *et al.* 1985; B, from Timmermans *et al.* 1993).**

In the walls of the tubules, germ cells are arranged in cysts that are enclosed by cells of Sertoli (Figure 5B). Thus, the Sertoli cells form the boundary of each tubule and they are covered at the outer side by a basement membrane (Figure 5B). They encircle also the tubular lumen and towards the lumen they are closely adhering to each other by tight junctions (Parmentier *et al.* 1985). Large primary spermatogonia, each individually located in a cyst enclosed by Sertoli cells, are found throughout the tubules near the basement membrane (Figure 5B). Within each cyst, the primary spermatogonia proliferate into groups of secundary spermatogonia, which subsequently differentiate into spermatocytes that undergo meiosis and develop into spermatids and mature spermatozoa. Germ cells within a cyst develop synchronously. The spermatozoa are released from the cysts into the lumina of the tubules, and consist each of a round head, a very small midpiece and a long flagellum. The tight junctions between Sertoli cells surrounding the lumina of the tubules provide a blood-testis barrier around the ripe spermatozoa in the lumina, i.e. a physiological barrier to biological macromolecules (Parmentier *et al.* 1985). Moreover, the peritubular cells in the interstitial tissue appear to possess myoid properties, probably related to sperm transport (Timmermans *et al.* 1993).

The testis of carp obtains the features of the adult organ between week 21 and 25 of development (23°C), at the end of the juvenile stage (Parmentier & Timmermans 1985). Under standard culture conditions all germ cell stages are abundantly present at the age of 7 months; at the age of 10 months the size of the testicular tubules has enlarged considerably and large amounts of spermatozoa have been released in the central lumina of the tubules, whereas at the age of 14 months the tubules contain nearly exclusively spermatozoa and only few cysts are present with primary or early secondary spermatogonia (Timmermans *et al.* 1993).

The process of spermatogenesis has been studied in great detail in mammals, especially in the mouse. The number of mitotic divisions of each stem cell or A_1 spermatogonium is known to be 5, and results in type B spermatogonia. These will each divide into two primary spermatocytes. Subsequently, the latter will enter meiosis and will form successively the (haploid) secondary spermatocytes, the spermatids and the spermatozoa (Oakberg 1956).

Moreover, the descendants of one spermatogonium A_1 remain connected to each other by cytoplasmic bridges until the spermatozoa ultimately are released into the lumina of the tubules.

In carp and other fish species the descendants of one primary spermatogonium remain together within a cyst. Nevertheless, also in carp these descendants are interconnected with each other by cytoplasmic bridges (Timmermans, unpubl. observations). Such cytoplasmic bridges between precursor germ cells were also described for the guppy *Poecilia (Lebistes) reticulata* (Billard 1984).

Billard (1984) and other authors use the terms type A and type B spermatogonia for germ cells in testes of fishes. Billard (1969) has estimated the number of division of each stem cell (or primary spermatogonia) in the guppy. However, such estimation was not made in carp due to the fact that in adult carp, contrary to *Lebistes (Poecilia)* species, all germ cell stages are present throughout the testis. For that reason, in the present study the term primary spermatogonium is used for germ cells which are each separately located in a cyst enclosed by Sertoli cells and the term secondary spermatogonia when more than one spermatogonium is present within a cyst. Moreover, the term "early spermatogonia" will apply to primary and early secondary spermatogonia with a diameter > 10 μm and the term "late spermatogonia" to spermatogonia with a diameter < 10 μm.

Ovary

The adult carp ovary (Parmentier *et al.* 1984) is an elongated and paired organ in a position similar to the testis. It develops ventrodorsal-oriented lamellae, protruding into the ovarian sac and covered by the germinal epithelium (Figure 6A). The core of the lamellae consists of connective tissue with blood vessels. Solitary primary oogonia and groups of secondary oogonia or early-prophase oocytes are distributed regularly throughout the gonad and are surrounded by cyst cells, resembling the Sertoli cells in the testis (Figure 6B). Tight junctions are present between the cells that form the boundary towards the ovarian sac (Parmentier *et al.* 1985). Around the growing oocytes a thin follicular layer, the granulosa, is present. The oocytes are also enclosed by a second layer, the theca.

The ovary obtains its adult features from week 16-18 onwards, a few weeks earlier than the testis (Parmentier & Timmermans 1985).

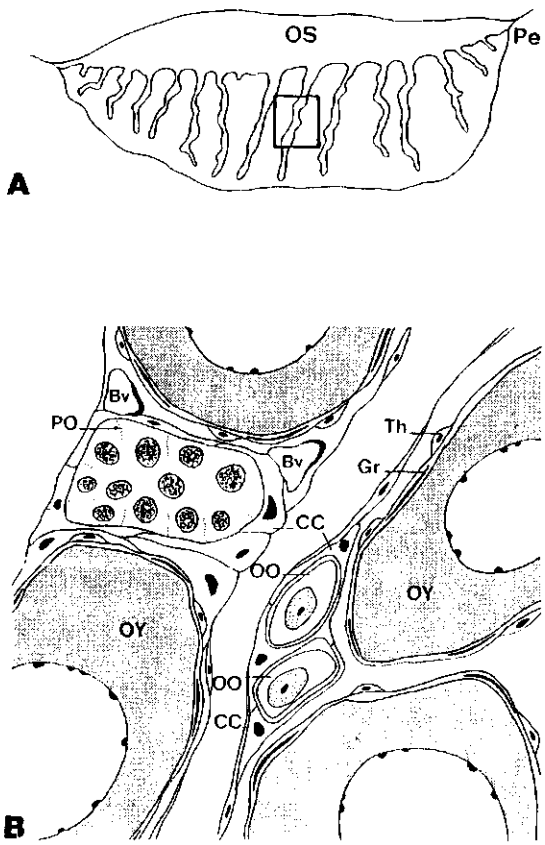

Figure 6. **Schematic drawings of a cross section through the ovary of *Cyprinus carpio*. A. Lamellae projecting into the ovarian sac. OS, ovarian sac. Pe, peritoneum. B. Detail of two lamellae. Bv, blood vessel. CC, cyst cell. Gr, granulosa. OO, oogonium. OY, follicular oocyt. PO early prophase oocytes. Th, theca cell. (From Parmentier *et al.* 1985).**

Recognition of germ cell stages with monoclonal antibodies

Specific molecules, amenable to immunological analysis, arise in the outer membrane of differentiating germ cells during spermatogenesis in juvenile and adult gonads. It has been suggested that these changes in membrane composition during spermatogenesis are associated with the processes of cellular differentiation and cell interactions (Millette 1979). The same author postulated that the defined antigens can serve as markers of germ cell differentiation and may convey understanding of certain differentiation steps. Polyclonal and monoclonal antibodies have been used extensively to study such germ cell differentiation antigens in mammalian species.

Most antibodies were raised against spermatozoa in order to define sperm-specific antigens that are relevant to fertility and in most cases they recognised antigens confined to (specific domains of) the surface membrane of spermatozoa (for a review, see Eddy 1988, and recent reports of Naz *et al.* 1993; Watanabe *et al.* 1992).

Polyclonal and monoclonal antibodies raised against spermatogenic cells of juvenile or ripe testes, recognised germ cell specific antigens on the outer surface and sometimes in the cytoplasm of precursor germ cells. These antigens mostly arised after the onset of meiosis, i.e. on prophase spermatocytes (For a survey, see Watanabe *et al.* 1992). Polyclonal antibodies mostly are part of complex antisera which may recognise several different antigens. The use of monoclonal antibodies (MAbs) does permit analyses of individual antigens (Kohler and Milstein 1976). With germ cell specific MAbs it was found that a number of antigens, appearing on prophase spermatocytes, remained expressed on subsequent germ cell stages including spermatozoa (Bechtol *et al.* 1979; Bechtol *et al.* 1980; Gaunt 1982; Bechtol 1984; Fenderson *et al.* 1984; Bechtol *et al.* 1986; Haneji & Koide 1987; Isahakia 1988; Kurpisz *et al.* 1988; Sobis *et al.* 1988). In the study of Koshimizu *et al.* 1993, a stage-specific antigen was detected, expressed only on the outer surface of zygotene and early pachytene spermatocytes and not on later stages.

Little information is available at present with respect to germ cell specific antigens present on premeiotic germ cells in mammals. O'Brien and Millette (1984) reported that (polyclonal) rabbit antisera, raised against type B spermatogonia and preleptotene spermatocytes, recognised antigens on these germ cells, but those antigens were also present

Table 1. Immunohistochemical reactions of monoclonal antibodies on testis and ovary from carp

Monoclonal antibodies	Testis				Ovary			
	unfixed		with Bouin's fixative		unfixed		with Bouin's fixative	
	spermatozoa	precursor sperm cells	spermatozoa	precursor sperm cells	oogonia and small oocytes[1]	large oocytes[2]	oogonia and small oocytes[1]	large oocytes[2]
WCS 1	+	+	−	−	−	−	−	−
WCS 3	+	+	+	+	+	−	+	−
WCS 7	+	±	−	−	−	−	−	−
WCS 11	+	+	−	−	−	−	−	−
WCS 12	+	+	−	−	−	−	−	−
WCS 14	+	+	+	+	+	−	+	−
WCS 16	+	−	−	−	−	−	−	−
WCS 17	+	+	+	+	+	−	+	−
WCS 27	+	±	−	−	−	−	−	−
WCS 28	+	+	+	+	+	−	+	−
WCS 29	+	+	+	+	+	−	+	−

+ = positive reaction; − = negative reaction; ± = weak reaction; 1 = early prophase oocytes; 2 = oocytes surrounded by follicle cells

(From Parmentier et al. 1984)

on typical somatic cells. Recently, Dissel-Emiliani *et al.* (1993) obtained a MAb, raised against a testicular membrane fraction from 18-day post-coitum (p.c.) rat testis, that specifically reacted with gonocytes in the fetal testis, without significant cross-reactivity with other tissues. The MAb was reactive with rat gonocytes from 17-day p.c. until the day of birth, but not at later stages. Finally, in mouse embryos several antigens have been detected on pregonadal germ cells or PGCs, i.e. F9, EMA, SSEA, M1/22 25 and others (see Eddy and Hahnel 1983; Hahnel and Eddy 1986). However, in all cases these antigens disappeared shortly afterwards, most of them were also present on somatic cells.

In non-mammalian species, MAbs have only been used in carp to study germ cell differentiation. Due to the lack of methods to isolate developing germ cells from the gonads, spermatozoa were used to raise MAbs, as they could be easily obtained by hand-stripping adult males (Parmentier *et al.* 1984). The MAbs were germ cell specific, i.e. they did not crossreact with somatic cells. Contrary to mammals where antigenic determinants, specific for spermatozoa, appeared only after the onset of meiosis (see references above), all anti-carp spermatozoa MAbs, except one, recognised antigenic determinants already present on premeiotic stages. The results are presented in Table 1.

The table shows that all MAbs react with spermatozoa and that all but one react with precursor germ cells in unfixed frozen testis sections. In sections from Bouin fixed tissue a panel of 5 MAbs reacted with spermatozoa and precursor germ cells. These 5 MAbs reacted also with oogonia and early prophase oocytes, but not with follicular oocytes. In later studies the hybridoma producing the MAb WCS 14 appeared to be unstable and was lost.

Immunohistochemistry on sections of developing gonads (Parmentier & Timmermans 1985) revealed that the antigenic determinant recognized by the MAb WCS29 was already present on PGCs of newly hatched larvae, whereas other markers appeared at the onset of PGC proliferation at the age of 7 weeks (recognized by WCS 3 and WCS 17) or on spermatogonia at the onset of spermatogenesis at 19 weeks (recognized by WCS 28). The antigenic determinants detected by these MAbs remained present on all subsequent precursor

germ cell stages until and including ripe spermatozoa. They also appeared on oogonia at the onset of oogenesis after 16 weeks, but disappeared on follicular oocytes. These MAbs provided a new and valuable tool to study the differentiation of the primoridal germ cells in fish.

Aim and outline of this thesis

The research described in this thesis was designed to gain insight in (primordial) germ cell differentiation processes during larval and juvenile development of carp. As a main line of the investigation, it was decided to try to detect marker molecules for (primordial) germ cell development by raising MAbs against surface antigenic determinants.

At first, a new method was worked out to isolate the germ cells from carp gonads. In addition, an immunogold staining method was adapted for the reaction of MAbs with isolated unfixed germ cells, allowing the application of immunocytochemistry in combination with the precise identification of isolated germ cells. In chapter 2, these methods are described and were used to demonstrate the localisation of antigenic determinants recognized by anti-spermatozoa MAbs, raised in a previous study, on the surface membrane of spermatozoa as well as precursor germ cells of carp (chapter 2).

Moreover, a method was devised to purify germ cell subsets from carp gonads. The earliest germ cell stage that could be obtained in sufficient numbers, i.e. early spermatogonia from early spermatogenic testes, constituting 4 - 5% of the precursor germ cells in those gonads, were purified and used as immunogen for the production of MAbs (chapter 4).

Meanwhile an ultrastructural study was conducted on PGCs and gonads in larval carp, in preparation for subsequent studies with MAbs on the differentiation of PGCs (chapter 3). A detailed analysis was made of the mitotic silent period of the PGCs, up to and including week six a.f., in order to investigate whether ultrastructural changes occur in that period which can be related to the expression of MAb-detectable antigens.

In a further study, selected anti-spermatogonia MAbs were used to examine changes in the expression of surface markers on germ cell stages during gonadal development, with emphasis on phenotypic changes of PGCs during larval development (chapter 4).

In chapter 5, a comparison was made of anti-spermatogonia MAbs and anti-

spermatozoa MAbs. It was investigated whether on the surface membrane of carp spermatozoa specialized domains occur, differing in antigenicity from each other, as in mammals. This was performed with the WCS-MAbs and the WCG-MAbs, using a new combination of the modified immunogold-staining assay (see chapter 2) with scanning electron microscopy.

In chapter 6, carp germ cell differentiation antigens were biochemically analysed, using anti-carp spermatozoa MAbs and carp spermatozoa protein extracts in immunoblotting assays.

In chapter 7 the possible functional importance of the recognized germ cell differentiation antigens was addressed. As a first approach, an endocrinological study was performed on the role of pituitary hormones during the development of germ cells and gonads, with particular reference to the stimulation of the (onset of) PGC proliferation during larval development.

The main conclusions of this thesis will be given in chapter 8.

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Surface location and stage specificity of differentiation antigens on germ cells in the common carp (*Cyprinus carpio*), as revealed with monoclonal antibodies and immunogold staining.

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SUMMARY

During development of juvenile and young adult carp (*Cyprinus carpio* L., Teleostei) three differentiation stages were distinguished in the testis: the pre-spermatogenic, the early spermatogenic and the advanced spermatogenic testis. Carp testis tissue of these stages was dissociated by enzymatic digestion and viable testis cells with well preserved morphological features were obtained. The surface location and stage-specificity of differentiation antigens on these germ cells was investigated using monoclonal antibodies (MAbs) raised against carp spermatozoa. Binding of MAbs to cells was visualized with immunofluorescence as well as in the immunogold staining assay. Both methods revealed that antigenic determinants defined by seven MAbs were located on the outer surface of testis cells. Four MAbs, i.e. WCS 3, 17, 28 and 29, reacted with germ cells from both pre-spermatogenic testes (WCS 28 weakly) and spermatogenic testes. The antigenic determinants defined by three other MAbs, i.e. WCS 7, 11 and 12, appeared only after the onset of spermatogenesis. In the immunogold staining assay a post-fixation and nuclear staining procedure was developed which allowed identification of isolated germ cells, revealing clearly, for all seven MAbs, that the determinants were expressed on germ cells but not on somatic cells and, for WCS 7, 11 and 12 only, that the determinants first appeared on small spermatogonia prior to meiosis. A survey of the immunogold assay on the binding of the seven MAbs with isolated germ cells from ovaries, is included.

INTRODUCTION

Spermatogenesis in vertebrates comprises mitotic proliferation of spermatogonia, meiosis and the formation of spermatozoa. Whereas for mammalian species detailed knowledge on these processes in the testis is available, relatively little is known on spermatogenesis in lower vertebrates.

It has been suggested that during spermatogenesis specific molecules, amenable to immunological analysis, arise in germ cells. Such antigens can serve as differentiation markers and may convey understanding of certain differentiation steps (Millette 1979). In

mammalian species, monoclonal antibodies (MAbs) have been used extensively to study such germ cell differentiation antigens (Bechtol *et al.* 1979; Bechtol *et al.* 1980; Gaunt 1982; Bechtol 1984; Fenderson *et al.* 1984; Moore *et al.* 1985; Bechtol *et al.* 1986; Lee & Wong 1986; Haneji & Koide 1987; Isahakia 1988; Kurpisz *et al.*1988).

Monoclonal antibodies have also been raised against spermatozoa of the common carp (Parmentier *et al.* 1984) and have been used to study germ cell differentiation in fish (Parmentier & Timmermans 1985).

In carp, these MAbs, recognizing germ cell specific antigenic determinants, defined surface membrane antigens on carp spermatozoa in immunofluorescence tests on smears of these fully differentiated cells (Parmentier *et al.* 1984). However, whether these differentiation markers were also located at the outer surface of earlier germ cell stages remained to be answered, although such a location was suggested from immunocytochemical analysis on histological sections of the gonads (Parmentier *et al.* 1984; Parmentier & Timmermans 1985).

The present study deals with the visualization of differentiation antigens on the surface of carp precursor germ cells. Carp testis cells were isolated according to a new method and freshly isolated cells were processed in respectively immunofluorescence and immunogold staining (IGS) assays. The latter technique, modified from the original procedure for prefixed human leucocytes (De Waele *et al.* 1981) was applied on fresh cells. Postfixation of these cells enabled the identification of the isolated germ cells and was used to follow the stage-specificity of antigen expression in more detail. A short survey of the IGS assay on isolated ovary germ cells is included.

MATERIALS AND METHODS

Animals

Specimens of *Cyprinus carpio* L. were reared under standard laboratory conditions (cf. Parmentier & Timmermans 1985). Animals of average size were selected at indicated stages and anaesthetized with 0.01 % tricaine methane sulfonate (Sandoz, Basel, Switzerland) before killing. Testes were dissected and immediately placed in cold phosphate-buffered saline (PBS;

pH 7.4) consisting of 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.0 mM $Na_2HPO_4.2H_2O$ and their weight was determined.

Isolation of gonadal cells

For testis cell isolation, the direct sequential enzyme incubation technique as applied commonly in rodents (Romrell *et al.* 1976; O'Rand & Romrell 1977) was used initially. In subsequent experiments, carp testes were dissociated using the cold enzyme-preincubation principle (Freshney 1987). Early thread-like gonads were cut into five pieces, whereas larger testes were cut into small blocks measuring ca. 2 mm^3. Tissue fragments, up to 20 mg, were incubated in a centrifuge tube in 1 ml RPMI 1640 culture medium, buffered with 25 mM HEPES (pH 7.4) and supplemented with 0.1 % collagenase type II and 0.05 % trypsin type III (Sigma, St. Louis, Mo., USA; cat. nr. C-6885 and T-8253), for 16 hours at 4 °C and subsequently for one hour at 23 °C. The enzyme solution was removed and the tissue was washed by sedimentation with 2 ml dissociation solution (23 °C), consisting of 137 mM NaCl, 5.4 mM KCl, 10 mM Na_2HPO_4, 5 mM NaH_2PO_4, 11.1 mM glucose, 0.04 % ethylenediaminetetraacetic acid (EDTA) and 0.5 % bovine serum albumin (BSA) (pH 7.4). The testis fragments were disrupted completely by repeated pipetting (50 times) through a Pasteur pipet. The isolated cells were collected by centrifugation (200 x g, 7 min), washed twice and resuspended in cold PBS. Samples of the cell suspensions were counted in duplicate in a haemocytometer using a x40 phase-contrast objective. Cell viability was determined by trypan blue (0.1 %) exclusion (Tennant 1964). For the isolation of ovary cells the same procedure was applied as for testis cells.

Immunostaining

Freshly isolated cells were used either in the indirect immunofluorescence test or in the indirect immunogold staining test (modified from De Waele *et al.* 1981). Both procedures were carried out at 4 °C and in the presence of 0.02 M sodium azide.

The isolated cells were attached by centrifugation to 13 mm glass cover slips pretreated with 0.05 % aqueous poly-L-lysine, placed in wells of a tissue culture plate (2.5 x 10^5 cells/16 mm well), to prevent aggregation of cells during antibody incubation. After

reduction of the volume of PBS to 200 μl per well, first antibody was added. Anti-carp spermatozoa MAbs (Parmentier *et al.* 1984) were used [1:1000 final dilution of ascites fluid in PBS with 10 % newborn calf serum (PBS/NCS)]. Ascites fluids produced by subclones with the same specificity as their parental clones were used. Control incubations, in which the first antibody was substituted for myeloma ascites, were used in each of the assays. After 45 minutes, the cells, in 200 μl solution, were washed five times by addition and subsequent removal of one ml PBS per well. Second antibody was then added. For immunogold staining, goat-anti-mouse IgG, or IgM, coupled to gold particles of 30-nm size (GAM/IgG/G30 or GAM/IgM/G30; Janssen, Olen, Belgium; final dilutions 1:5 in PBS/NCS), and for immunofluorescence goat-anti-mouse Ig coupled to fluorescein (GAM/Ig/FITC; Nordic, Tilburg, The Netherlands; final dilution 1:40 in PBS/NCS), were used. After incubation for 45 min, the cells were washed in PBS as described above.

In the immunofluorescence test, the cover slips were mounted on microscope slides using PBS-glycerol (1/9, v/v), containing 1 mg/ml p-phenylenediamine to prevent photobleaching of the fluorochrome (Johnson & Noqueira Araujo 1981). In the immunogold staining test, cells were processed for light microscopy as described below. Immunofluorescence was analyzed using a Zeiss photomicroscope with incident-fluorescence optics. Immunogold labeling was examined with the same microscope equipped with polarized light epi-illuminescence optics (De Mey 1983) and a x63 Antiflex objective (cat nr. 421800; Zeiss, Oberkochen, FRG). Photographs were taken on Agfapan 400 and 100 film respectively.

Preparation of cells for microscopy

Cells on cover slips were fixed for 30 min at 4 °C in Benda's solution, consisting of a mixture of 2 % OsO_4, 1 % chromic acid and acetic acid (4:15:0.1 v/v) (Romeis 1968). The cells were washed in tap water (10 min), 70 % ethanol (10 min) and aqua dest (2 x 2 min) respectively, and stained with Mayer's haemalum and eosin. Cover slips with dehydrated cells were mounted via xylene on microscope slides using DPX mounting medium (BDH Chemicals, Poole, UK).

Histology

Gonadal tissue for examination by light microscopy was fixed in Bouin's solution for 24 h, dehydrated, and embedded in Paraplast Plus (Polysciences, Warrington, USA). Sections with a thickness of 5 μm were stained with Mayer's haemalum and eosin.

RESULTS

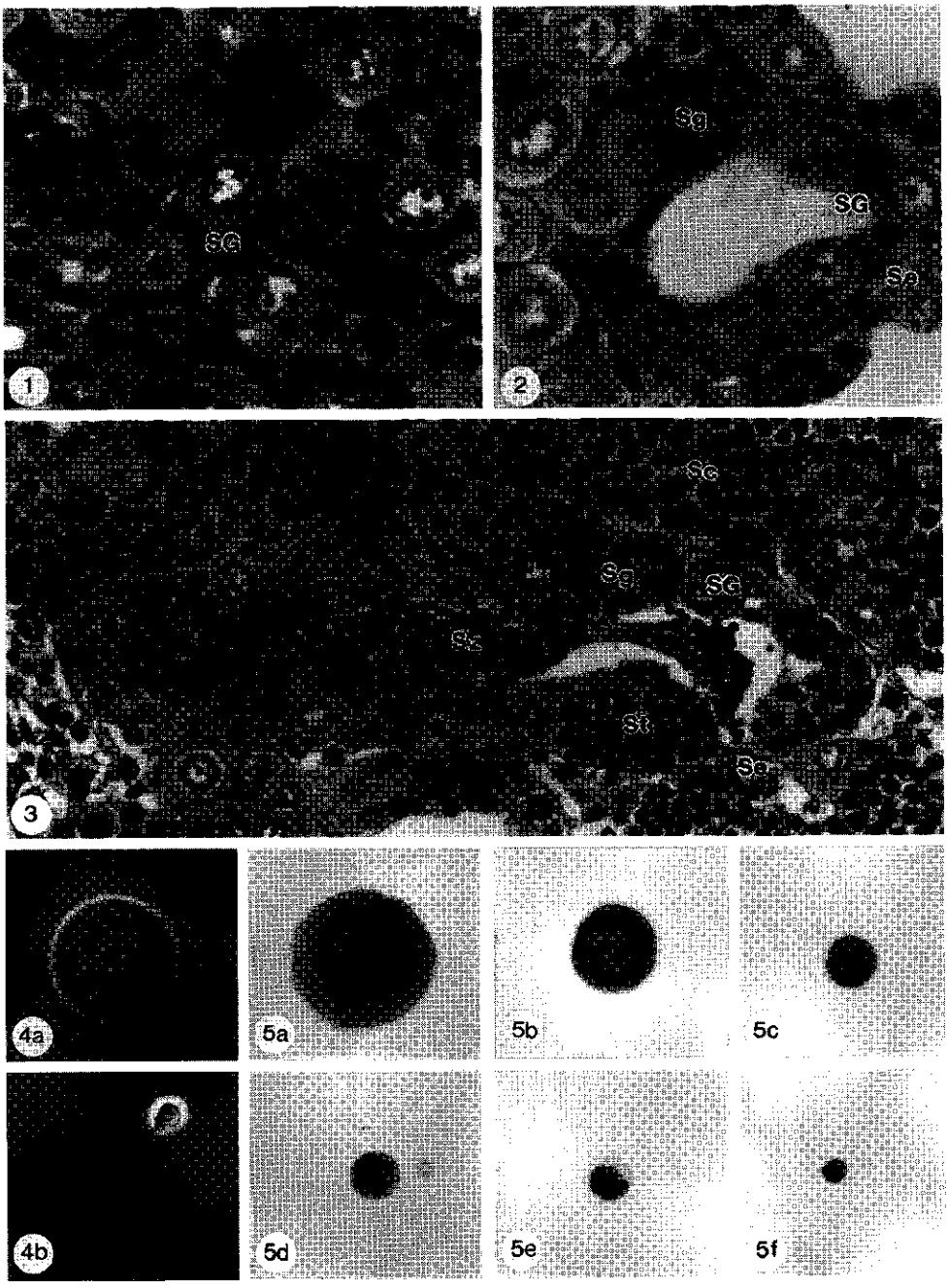
Testis developmental stages

Histological examination of the testis in carp, at stages between week 16 and week 30 of development, revealed three successive testis stages. In the *pre-spermatogenic* testis (week 16 - week 20), only one type of germ cell was observed. These spermatogonia were each surrounded by one or more somatic cells (Fig 1). In the *early spermatogenic* testis (week 19 - week 26), branched irregularly shaped seminiferous tubuli had begun to develop and spermatogenesis had started within germinal cysts lining the tubular walls. These cysts were composed of somatic cells, termed Sertoli cells, encompassing spermatogenic stages. Within a cyst either a single germ cell, i.e. a primary spermatogonium, was enclosed or a group of germ cells, i.e. secondary spermatogonia or, occasionally, spermatocytes or spermatids. Spermatozoa were still absent (Fig 2). In the *advanced spermatogenic* testis (from week 23 onwards), the size and number of tubuli and germinal cysts had distinctly increased. Moreover, spermatozoa had been formed and had been released from the germinal cysts into the tubular lumina (Fig 3).

A distinct asynchrony was noted in the onset of spermatogenesis. Whereas at week 19 in some animals early spermatogenic testes were present, others showed no signs of germinal cyst formation. Advanced spermatogenic testes were in some cases observed at the age of 23 weeks. From week 26 onwards, however, spermatozoa production was noted in each of the animals examined.

Isolation of testis cells

The direct sequential enzyme incubation method as applied in rodents to isolate testis cells (Romrell *et al.* 1976; O'Rand & Romrell 1977) allowed only the release of low


1
SG
2
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3
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Sg
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5b
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4b
5d
5e
5f

numbers of germ cells from carp testes. Particularly, large spermatogonia were sparse. Therefore, a new method was worked out in which fragments of testes, after cold preincubation with collagenase and trypsin, were digested at room temperature and mechanically disrupted in a solution containing EDTA. In this procedure, 1 mg/ml (0,1 %) collagenase and 0,5 mg/ml (0,05 %) trypsin were used. Apparently all types of testis cells could be easily released from the three testis stages described above. Clumps of cells were rare. Using phase-contrast microscopy, large spermatogonia ($\geq$ 10 μm) as well as spermatozoa were readily noticed among the isolated testis cells (Fig 4), but other, smaller, spermatogenic cells could not be identified.

It was noted that lower concentrations of trypsin were sufficient for the effective dispersal of advanced spermatogenic testes. In Table 1, the results are shown of a representative assay in which parts of an advanced spermatogenic testis (week 28) were dissociated under different conditions. As little as 0,13 mg/ml (0,013 %) trypsin appeared to be sufficient to obtain optimal numbers of cells, including large spermatogonia, from this testis stage. Omittance of EDTA (0,04 %) from the dissociation solution resulted in a 75 % reduction of the total number of isolated cells (Table 1), indicating the effectiveness of this chelating agent in the dissociation procedure.

Figures 1-5,

Figs 1-3. Testis developmental stages. SG, large spermatogonia; Sg, small spermatogonia; Sc, spermatocytes; St, spermatids; Sz, spermatozoa; Se, Sertoli cells; I, interstitial tissue. Bouin, H/E, x 1000

Fig 1. Pre-spermatogenic testis at week 16. Spermatogonia, each surrounded by one or two somatic cells, are distributed through the interstitial tissue.

Fig 2. Early spermatogenic testis at week 22 showing a cross section through a testis tubule. Cysts with germ cell stages are present lining the tubular wall and surrounding the tubular lumen.

Fig 3. Advanced spermatogenic testis at week 28 showing a section through a testis tubule. Note that the cysts have enlarged and that spermatozoa are released into the tubular lumen.

Fig 4a,b. Phase contrast micrographs of isolated germ cells from advanced spermatogenic testis. a. Spermatogonium, b. Spermatozoon. x 1500

Fig 5a-f. Cytopreparations of isolated germ cells from an advanced spermatogenic testis. a-c. Spermatogonia; d. and e. Spermatocytes; f. Spermatid. x 1500

Table 1. **Dissociation of carp testis tissue**

Dissociation conditions[a]			Isolated cells[b]			
Coll. (%)	Trypsin (mg/ml)	EDTA (%)	Total number $\times 10^{-6}$	SG (%)	Sz	Viability[c]
1.0	–	0.04	49.2	0.4	48.8	85.4
1.0	0.06	0.04	82.2	2.7	48.7	90.5
1.0	0.13	0.04	58.4	6.8	42.8	89.7
1.0	0.25	0.04	55.0	4.4	36.4	88.0
1.0	0.50	0.04	65.2	4.6	42.0	86.2
1.0	0.50	–	18.0	6.6	36.7	93.4

[a] Parts of an advanced testis (ca. 20 mg each) were incubated with different concentrations of collagenase and trypsin and dissociated with or without EDTA
[b] *Isolated cells were counted in duplicate in a haemocytometer* using phase contrast microscopy
[c] Viability was determined by the trypan blue exclusion test
SG = large spermatogonia
Sz = spermatozoa

Table 2. ***Isolation of carp testis cells during the onset of spermatogenesis***

Fish age (weeks)	Fish weight (g)	Testis weight (mg)	Isolated cells[a] Total number $\times 10^{-6}$	SG (%)	Sz	Viability[b]
16[c]	6.5 ± 0.3	n.d.	0.4[d]	0.7	0.0	80.7
18	8.0 ± 0.9	n.d.	0.5[d]	0.8	0.0	79.3
20	21.2 ± 2.5	n.d.	1.9 ± 0.8	0.4	0.0	92.4
22	22.3 ± 6.1	3.3 ± 1.7	2.1 ± 1.4	2.8	2.3	85.2
24	32.7 ± 9.2	6.1 ± 2.5	3.7 ± 0.9	2.8	1.2	90.4
26	40.6 ± 7.7	8.1 ± 2.0	3.6 ± 1.7	2.0	2.8	96.3
28	65.5 ± 6.6	15.9 ± 5.1	15.0 ± 12.3	0.6	21.3	93.7
30	81.0 ± 6.4	29.0 ± 9.2	24.0 ± 6.3	1.6	14.5	92.8

[a] Isolated cells were counted in duplicate in a haemocytometer using phase contrast microscopy
[b] Viability was determined by the trypan blue exclusion test
[c] Each group represents the mean of 4–5 animals
[d] Testes of five fish were pooled
SG = large spermatogonia
Sz = spermatozoa
n.d. = not determined

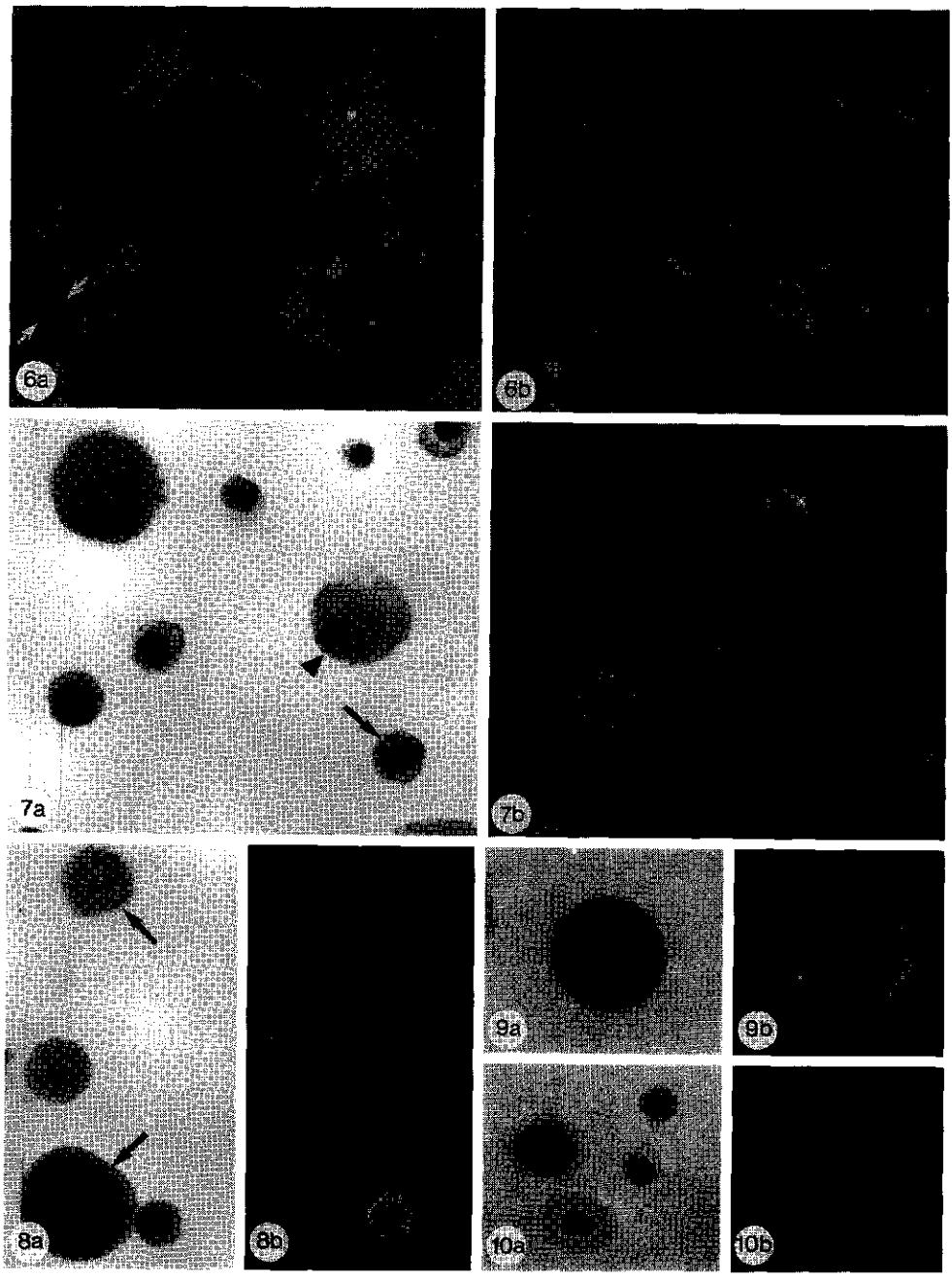
In order to determine the relation between testis weight and number of testis cells during the onset of spermatogenesis, testes of 4 to 5 animals were dissociated at bi-weekly intervals from week 16 up to and including week 30. As is shown in Table 2, the average testis weight as well as the average number of testis cells per fish increased markedly during this period. A sharp increase was noted from week 26 onwards, concomittantly with a distinct increase in the number of spermatozoa per fish. Among animals within each of the age-groups, the testis weights as well as the numbers of isolated testis cells per fish varied markedly (Table 2), confirming the observations on sectioned material (described above).

In cell suspensions of pre-spermatogenic testes (week 16, week 18) the number of spermatogonia was 3 - 4 x 10^4 per fish, approximating the numbers determined from serially sectioned pre-spermatogenic testes (data not shown). After the onset of spermatogenesis, the number of large spermatogonia per animal increased gradually with age, reaching about 7 x 10^5 per fish at week 30. Since the number of other testis cells increased too, the proportion of the large spermatogonia remained low, ranging from 1.5 % to 4.5 % per fish throughout the observation period.

Morphology of testis cells

The morphology of the isolated testis cells was well preserved after tissue dissociation (cf. Fig 4). All major spermatogenic stages, as identified in testis sections (cf. Figs 1 - 3), were observed in cytopreparations, i.e. upon processing isolated cells for routine light microscopy (Fig 5). No distinction, however, could be made in testis cell suspensions between primary and early secundary spermatogonia, since these cells shared most morphological features and the testis architecture was lost.

Spermatogonia obtained from pre-spermatogenic testes varied in size from 25 - 11 μm. The diameter of spermatogonia in suspensions prepared from advanced spermatogenic testes ranged from 25 μm to 5 μm, in agreement with the size difference between primary and late secondary spermatogonia in testis sections (Fig 3). The nucleus of the larger spermatogonia contained about six distinct nucleoli. However, in a small proportion of these cells (< 1 %), measuring 13 - 14 μm, only a single prominent nucleolus was present. It was noted that during spermatogonial development the size of nucleoli, as well as the size of perinuclear


6a
6b
7a
7b
8a
8b
9a
9b
10a
10b

dense bodies, described for germ cells of carp and rosy barb in a previous study (Timmermans & Taverne 1989), decreased markedly (Fig 5 a-c).

Other spermatogenic stages identified in cytopreparations were spermatocytes (5 - 7 μm, Fig 5 d, e), spermatids (3 - 5 μm, Fig 5f) and spermatozoa (head diameter 2,3 μm, tail length 38 - 44 μm, cf. Fig 4b). These cell types were readily distinguished from somatic testis cells (cf. Fig 7a).

Reactivity of testis cells with anti-sperm monoclonal antibodies

Initially, the indirect immunofluorescence test was used to study the binding of MAbs to testis cells. Freshly isolated cells were used. Distinct reactions of the MAbs WCS 3, 17 and 29, and a weaker reaction of WCS 28, were observed with spermatogonia in cell suspensions from pre-spermatogenic testes (week 16). No reacitivity was observed with other MAbs (data not shown). In contrast, most cells in cell suspensions from advanced spermatogenic testes (week 26 - week 30) showed distinct reactivity with WCS 3, 17, 29, as well as WCS 28, and with WCS 7, 11 and 12 (Fig 6). Immunostaining was not observed with

Figures 6-10.

Fig 6a,b. Indirect immunofluorescence reaction of WCS 3 on isolated germ cells from advanced spermatogenic testis. a. Phase contrast micrograph; b. Epi-illuminescence. Note that a few cells (arrows), presumably representing somatic testis cells, are negative. Similar reactions were obtained with WCS 17, 28 and 29 and WCS 7, 11 and 12. x 1200

Fig 7a,b. Indirect immunogold staining of WCS 3 on advanced spermatogenic testis cells. a. Bright field illumination; b. Polarized light epi-illuminescence. Note that germ cells, including a smaller spermatogonium containing a mitotic figure (arrow), are clearly immunostained, whereas a somatic testis cell (arrowhead) is negative. Similar reactions were obtained with WCS 17, 28 and 29. x 1200

Fig 8a,b. Indirect immunogold staining with WCS 12 on spermatogonia. Larger spermatogonia (arrows) are negative, whereas smaller spermatogonia are positive. Similar reactions were obtained with WCS 7 and WCS 11. a. and b. as in Fig 7. x 1500

Fig 9a,b. Indirect immunogold staining with WCS 3 on a large spermatogonium containing a mitotic figure. a. and b. as in Fig 7. x 1500

Fig 10a,b. Indirect immunogold staining with WCS 3 on carp spleen cells. Note that the cells are negative. a. and b. as in Fig 7. x 1500

WCS 1, 16 and 27 (not shown).

Whereas the results of these immunofluorescence tests indicated a surface location of the antigenic determinants defined by seven anti-carp sperm MAbs on precursor germ cells, the rather poor morphological preservation of cells in the immunofluorescence test did not allow conclusive answers towards the reactive stages.

To improve the identification of antibody-treated cells, a procedure was worked out in which cells, after the second antibody step, were fixed and stained for light microscopy. A first approach, in which a second antibody conjugated to peroxidase was used (described by Bechtol *et al.* 1980) gave slightly improved, but still unsatisfactory, results (not shown). From our observations we deduced that, most likely, incubation in a solution containing diaminobenzidine and H_2O_2, necessary in this procedure for enzyme visualization, caused a marked cellular detoriation.

Subsequently, the enzyme reaction was avoided by using a second antibody conjugated to gold particles followed by fixation and nuclear staining. The results of this IGS assay, as modified from De Waele *et al.* (1981) who used a prefixation method, revealed clearly a specific reaction of WCS 3, 17 and 29 with all germ cells in cell suspensions from pre-spermatogenic testes as well as from advanced spermatogenic testes (Fig 7). WCS 28 reacted in a similar way, except that germ cells from pre-spermatogenic testes were usually more faintly stained (Table 3). Germ cells from pre-spermatogenic testes, and large spermatogonia from advanced spermatogenic testes, showed no reactivity with WCS 7, 11 and 12, but these MAbs reacted clearly with smaller spermatogenic cells, identified undoubtedly as small spermatogonia, and with successive spermatogenic stages (Table 3, Fig 8). Apparently, a transition in antigen expression occurred during spermatogonial development in spermatogonia measuring 9 - 11 μm; larger spermatogonia were unreactive, whereas all smaller spermatogonia were labeled (n = 200). Somatic testis cells were not stained with these MAbs.

The IGS method revealed clearly that large spermatogonia containing mitotic figures expressed the antigenic determinants defined by WCS 3, 17, 28 and 29 (Fig 9). Small spermatogonia with mitotic figures were reactive with these four MAbs, as well as with WCS 7, 11 and 12 (not shown). Hence, it seems likely that the antigenic determinants defined by

Table 3. Survey of immunogold staining reactions with anti-sperm MAbs on germ cells[a] isolated from carp gonads

	Prespermatogenic testis	Advanced Spermatogenic Testis					Ovary			
MAb	SG	SG	Sg	Sc	St	Sz	OG	Og	EPO	OC
WCS 3	±	+	+	−	+	±	±	+	+	−
WCS 17	−	+	±	±	+	−	+	+	−	−
WCS 28	±	± or +	−	−	+	+	± or +	+	+	−
WCS 29	+	+	+	−	−	+	+	+	+	−
WCS 7	−	−	+	+	±	−	−	+	−	−
WCS 11	−	−	+	+	−	+	−	+	−	−
WCS 12	−	−	+	+	−	+	−	+	−	−
WCS 1		−	−	−	−	−				
WCS 16		−	−	−	−	−				
WCS 27		−	−	−	−	−				

[a] No reaction was observed on somatic cells obtained from either testis, ovary, or spleen
SG = large spermatogonia; Sg = small spermatogonia; Sc = spermatocytes; St = spermatids; Sz = spermatozoa; OG = large oogonia; Og = small oogonia; EPO = early prophase oocytes; OC = follicular oocytes; MAb = monoclonal antibody

these seven anti-carp spermatozoa MAbs, once expressed, are retained on the surface of spermatogonia during mitotis.

No immunogold staining was observed with WCS 1, 16 and 27 on germ cells and somatic cells obtained from pre-spermatogenic and advanced spermatogenic testes (Table 3).

Isolation of ovary cells and reactivity with antisperm monoclonal antibodies.

Ovary cells were isolated from gonads of 25 weeks in which all oogonic stages were present. The results of the IGS assay on fresh cells combined with postfixation and nuclear staining are listed in Table 3. They revealed that the MAbs WCS3, 17, 28 and 29 reacted with large and small oogonia and with early prophase oocytes but not with follicular oocytes. The MAbs WCS7, 11 and 12 reacted only with small oogonia and not with other female germ cells. The MAbs WCS1, 16 an 27 were not tested. No reaction was observed with somatic cells from the ovary.

In control incubations included in each assay, in which myeloma ascites was substituted for anti-sperm MAb, no reactions were observed. In addition, the anti-carp sperm MAbs did not react with spleen cells of carp treated in the same way as testis cells (Fig 10).

DISCUSSION

This study shows that differentiation antigens as defined by seven anti-carp spermatozoa MAbs (Parmentier *et al.* 1984) are expressed on the outer surface of precursor germ cells in carp. This conclusion is based on treatment of freshly isolated carp gonadal cells in immunofluorescence and immunogold staining assays. Moreover, new postfixation and nuclear staining procedures applied after antibody labeling in the immunogold assay, allowed us to identify immuno-stained germ cells clearly and to establish from which developmental stage the MAbs reacted with the germ cells.

In this study, testis development in carp was examined histologically, revealing that the onset of spermatogenesis was preceded by the formation of tubules and was marked by the appearance of enlarged tubules and germinal cysts, occurring at about the age of 19 weeks. These data confirmed previous observations on animals bred under similar conditions (Parmentier & Timmermans 1985). Viable germ cells with well retained morphological features were isolated from developing carp testes. Our new germ cell isolation method considerably improved a previous approach (Secombes *et al.* 1986) and allowed the efficient release of all major spermatogenic stages, including early spermatogonia. Methods have been described for the isolation of testis cells from the rainbow trout *Salmo gairdneri*, but these emphasized the isolation of either haploid cells (Iatrou *et al.* 1978) or somatic cells (Loir 1988) from large, mature testes. Our method even allowed the isolation of germ cells from thread-like pre-spermatogenic testes.

In the immunofluorescence test, the seven MAbs, i.e. WCS 3, 7, 11, 12, 17, 28 and 29, reacted with surface constituents on cells from testes in which spermatogenesis had started. Four of these MAbs, i.e. WCS 3, 17, 29 and 28 (the latter weakly) reacted also with cells from pre-spermatogenic testes.

Although for the MAbs WCS 1, 16 and 27 a positive reaction with carp spermatozoa had been demonstrated previously (Parmentier *et al.* 1984), no such reactivity was detected with isolated precursor germ cells in the present study. In the case of WCS 16, the previous data showed that the reactivity was restricted to spermatozoa, (Parmentier *et al.* 1984). That study, however, did reveal reactions of both WCS 1 and WCS 27 with precursor germ cells

in sections of carp gonads. It is supposed that the antigenic determinants defined by these MAbs on precursor germ cells are not resistant to the cell isolation procedure.

In the present study, we worked out an immunogold staining technique in which immunolabeling was performed on freshly isolated cells, rather than on prefixed cells as used by De Waele et al. (1981). By using postfixation and nuclear staining, the antibody-treated testis cells could be clearly identified. The data learned that each of the seven positive MAbs reacted with germ cells and not with somatic testis cells. Moreover, the antigenic determinants defined by the MAbs WCS 7, 11 and 12 were not expressed on the surface of early, large, spermatogonia but did appear on small spermatogonia after the onset of spermatogenesis and were retained thereafter during meiosis and spermiogenesis.

Previously, immunocytochemistry on histological sections (Parmentier & Timmermans 1985) revealed reactions of WCS 29 from the primordial germ cell (PGC) stage in the indifferent gonadal primordium of newly hatched larvae onwards. WCS 3 and WCS 17 marked the onset of PGC proliferation in larvae at the age of 7 weeks, whereas WCS 28 appeared in spermatogonia at the onset of spermatogenesis (Parmentier & Timmermans 1985). In the present study, a weak reactivity of WCS 28 was already observed with germ cells isolated from testes at week 16, which might be explained by a higher sensitivity of the immuno assays on freshly isolated cells. Each of these four MAbs also reacted with isolated precursor germ cells in female specimens, but not with follicular oocytes, which confirms previous results (Parmentier *et al.* 1984; Parmentier & Timmermans 1985). In contrast, reactivity with WCS 7, 11 and 12 has previously been thought to be male-specific (Parmentier *et al.* 1984). This was deduced from immunofluorescence reactions on frozen sections of adult carp gonads. The positive reactions observed in the present study with isolated small oogonia might be explained by a higher sensitivity of the immuno assays on freshly isolated cells.

In studies on germ cell differentiation antigens in mammals, the localization of antigens defined by MAbs on precursor germ cells was studied with immunofluorescence tests on isolated testis cells. In such assays, the cell surface location of differentiation antigens was readily demonstrated on germ cells of rodents (Gaunt 1982; Fenderson *et al.* 1984; Moore *et al.* 1985; Lee & Wong 1986; Haneji & Koide 1987) and a primate (Isahakia 1988).

However, the rather poor morphological preservation of antibody-treated cells hampered their immediate identification.

To identify immuno(un)reactive stages, indirect approaches have been used. Thus, the reactivity of germ cells was analyzed using immunocytochemistry on sections of testes processed for routine histology (Bechtol 1984; Kurpisz 1988; Isahakia 1988). In other studies immunofluorescence reactions were performed on groups of cells with known identity, either isolated from testes at known prepubertal stages (Gaunt 1982; Moore *et al.* 1985; Lee & Wong 1986; Haneji & Koide 1987) or obtained by velocity sedimentation separation of testis cells (Fenderson *et al.* 1984). The results of both types of assays indicated that in rodents as well as in primates surface differentiation antigens specific for germ cells were expressed in nearly all cases from meiotic stages onwards.

In studies on carp the first approach has also been exploited, however, data on stage-specificity could not be obtained for WCS 7, 11 and 12 in this way due to negative reactions of these MAbs with Bouin-fixed testis sections (Parmentier *et al.* 1984). The second approach was applied in the present study. This provided us with the basic outlines of the reactivity patterns of the MAbs, and, in particular, revealed that WCS 7, 11 and 12 did not react with germ cells from pre-spermatogenic testes whereas these MAbs showed positive reactions with cells from testes after the onset of spermatogenesis. The further acquisition of information on the stage-specificity of the MAb reactions was prevented, however, by the asynchronous onset of spermatogenesis in carp. This was clearly resolved by the application of the IGS assay on fresh cells, followed by fixation and staining, which has now been used for the first time for germ cells in vertebrates.

The significance of the different temporal expression patterns now known remains to be studied. As some of the MAbs (WCS 3, WCS 17) appear to mark the onset of proliferation in early germ cells in carp (Parmentier & Timmermans 1985), the patterns observed with WCS 7, 11 and 12 might be related to a particular differentiational event in spermatogonia. In this respect, recent observations on the restriction of developmental potential at some stage during spermatogonial development in the teleost species *Oryzias latipes* are highly interesting (Hamaguchi 1988). As mentioned previously (Secombes *et al.* 1987), MAbs binding specifically to the surface of germ cells are regarded as a very

powerful tool to control gonadal maturation in fish culture.

ACKNOWLEDGEMENTS

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Ultrastructural changes in primordial germ cells during early gonadal development of the common carp (*Cyprinus carpio* L., Teleostei)

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Cell Tissue Res 267: 337-346 (1992)

SUMMARY

A description is given of primordial germ cell (PGC) differentiation and gonadal development in carp from hatching until the age of 6 weeks. This period was chosen as the PGCs are mitotically silent before they start to proliferate rapidly after week 6. The PGCs increased in size between week 2 and week 4 after fertilization. Ultrastructurally, the perinuclear dense bodies present in PGCs from hatching onwards increased in size and formed the 'cement' between mitochondria. Moreover, from week 2 onwards, an elaborate Golgi-apparatus was present in PGCs, indicating synthetic activity that may be related to PGC enlargement. During the observation period, gonadal tissue was gradually formed around the PGCs. From the age of 4 and 5 weeks onwards, two somatic cell types could be distinguished; the central type had a light appearance and was closely associated with the PGCs, the other type being darker and forming the peripheral layer of the developing gonads. Thus, during the period of mitotic quiescence, the PGCs and the gonads actively differentiate in preparation for the fast PGC proliferation that occurs after 6 weeks.

INTRODUCTION

Early in the embryonic development of fish, primordial germ cells (PGCs) arise at an extragonadal location, as found in other vertebrate groups (see Nieuwkoop & Sutasurya 1979; Eddy 1984). After the arrival of these cells at the gonadal area, further differentiation proceeds within the microenvironment of the developing gonads. Whereas, in higher vertebrate groups, the process of early gonadogenesis has been intensively studied, far less attention has been devoted to the early formation of gonads in fish. In the latter group, studies were mostly concentrated on the later process of sex differentiation.

PGCs, in the majority of the fish species studied, are mitotically inactive during the period before they reach the gonadal area (see Hardisty 1967). The onset of PGC proliferation occurs almost immediately, or within days, after gonadal colonization in a number of teleost species, i.e., Cyprinodontes (Wolf 1931; Dildine 1936; Gamo 1961; Satoh & Egami 1972; Hamaguchi 1982), *Micropterus* (Johnston 1951), and *Tilapia* (Nakamura &

Takahashi 1973; Yoshikawa & Oguri 1978). However, in other species a prolonged period of intragonadal mitotic rest, lasting for weeks or months, has been noted, i.e., in Salmonidae (Böhi 1904; Mrsic 1923; Lebrun *et al.* 1982), *Cottus* (Hann 1927), Percidae (Mezhnin 1978; Roblin & Bruslé 1983) and Cyprinidae (Timmermans & Taverne 1989). Species of the latter group are particularly suited for investigation of the mechanism underlying the onset of intragonadal PGC proliferation, since the events accompanying this process are separated in time.

In a previous study, it has been shown that germ cell development proceeds in a specific way in the common carp, a cyprinic fish. (Parmentier & Timmermans 1985). At hatching, on the third day after fertilization, the PGCs are located at the dorsal wall of the coelomic cavity, i.e., at the sites of the future gonads; they are encompassed by a single layer of peritoneal cells. Distinct gonadal ridges are formed from week 3 onwards. The number of PGCs remains low and mitoses have not been observed until after the sixth week, when the PGCs start to proliferate rapidly. Ovaries and testes can be distinguished from week 10 onwards, and oogenesis and spermatogenesis started at week 16 and week 18, respectively.

Morphological studies on early intragonadal PGCs in Cyprinids are uncommon (Stromsten 1931; Takahaski 1977; Parmentier & Timmermans 1985), and have only been performed at the light microscopical level. Moreover, no specific attention has been given to PGCs before the onset of proliferation. Therefore, a light and electron microscopical study has been undertaken on PGCs of carp during the mitotically silent period in relation to the formation of the somatic gonadal tissue.

MATERIALS AND METHODS

Animals

The common carp, *Cyprinus carpio* L., was reared in the laboratory under standard conditions with a light period from 0800 - 2000 hours and a water temperature of 23°C (cf. Parmentier & Timmermans 1985). Larvae were selected from a group of average size at days 3 (newly hatched), 7, 14, 21, 28 and 35 after fertilization. Their mean standard body lengths

were, respectively, 4, 8, 9, 11, 13 and 16 mm. The animals were anaesthetized in 0.01% (w/v) tricaine methane sulfonate (TMS; Crescent Research Chemicals, Arizona, USA) prior to decapitation.

Light microscopy

Decapitated larvae were fixed in Bouin's solution for 24 h and embedded in Paraplast Plus (Polysciences, Warrington, USA). The region comprising the gonadal primordia was serially cross-sectioned at a thickness of 5 μm. Complete series of sections were stained with Crossmon (5 animals per group). In addition, larvae were sectioned sagittally.

The total number of primordial germ cells (PGCs) in individual larvae was determined by the examination of all serial sections, and represented more than 100 per stage. The size of each PGC was determined by measuring the largest cell diameter in cross-sections using a x63 objective and a calibrated eyepiece micrometer. The number of nuclei of somatic gonadal cells surrounding individual PGCs, per animal, was determined in cross-sections containing the nucleus of a PGC. The total number of somatic cell nuclei counted, divided by the number of PGCs per specimen, was termed the somatic cell index.

Electron microscopy

Five decapitated larvae per age-group were fixed in a mixture containing 1% OsO_4, 2% glutaraldehyde and 1% potassium dichromate, buffered with 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 0°C, dehydrated and embedded in Epon 812. Anterior portions of the gonadal primordia were serially cross-sectioned at a thickness of 1 μm. Sections were stained with toluidine blue and examined with a light microscope to identify PGCs. Ultrathin gonadal cross-sections, comprising the nucleus of a PGC, were contrasted with saturated uranyl acetate and lead citrate and examined in a Philips EM 400 electron microscope. Per group, 4 - 13 PGCs were photographed.

In 1-week and 4-week-old larvae, the numbers of mitochondria were counted in median sections of PGCs (3 sections per cell), containing the nucleus. This was carried out in 11 PGCs of 1-week-old and 7 PGCs of 4-week-old specimens.

RESULTS

In the present study, the ages of larval stages of carp are given from fertilization onward. Hatching occurred at day 3.

Primordial germ cells

Light microscopy

The PGCs had an ovoid shape and were easily recognized by their large size and irregular nucleus, throughout the observation period (Figs 1-4).

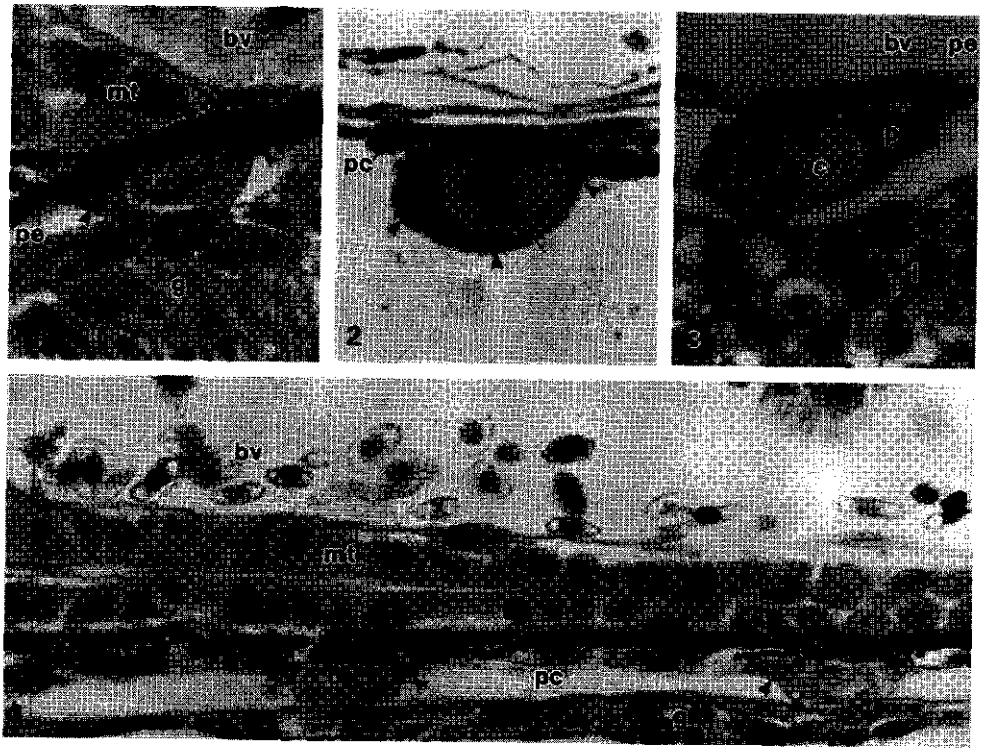


Table 1. Germ cells and somatic cells during early gonadogenesis in carp

Developmental stage[a]	Number of PGCs (mean ± SE [range])	Somatic cell index[b] (mean ± SE)
3 days	21 ± 10 (8–33)	0.8 ± 0.2
1 week	17 ± 11 (2–32)	1.4 ± 0.3
2 weeks	17 ± 7 (9–31)	2.2 ± 0.9
3 weeks	13 ± 4 (6–17)	7.4 ± 1.2
4 weeks	22 ± 14 (5–45)	15.0 ± 3.3
5 weeks	22 ± 15 (0–43)	34.8 ± 8.0

[a] Each group represents the mean of 5 animals
[b] The average number, per fish, of somatic cell nuclei per gonadal cross-section containing a PGC nucleus

The average number of PGCs per animal (13 - 22) remained constant from day 3 to week 6 (Table 1). Mitotic figures were not observed in PGCs. Their number varied markedly among individual animals, from a few cells to more than 40, at the different developmental stages (Table 1). In one specimen, at week 5, no PGCs were found. In individual animals, the PGCs were usually distributed unequally over the left and right gonadal areas. However, when all animals in the 6 age-groups were considered, the total numbers of PGCs at the left and right locations were 288 and 290, respectively.

Figures 1-4.

Fig 1. Cross-section through a gonadal area at day 3 showing a PGC (*arrow*) accompanied by two somatic cells (*arrowheads*). *bv* blood vessel; *g* gut; *mt* mesonephric tubule; *pe* peritoneum. Bouin/Crossmon. x 1900

Fig 2. Cross-section through a gonadal area at day 18. Note that the number of somatic cells (*arrowheads*) surrounding the PGC (*arrow*) has increased. *pc* peritoneal cell. OsO_4/glutaraldehyde/toluidine blue. x 2050

Fig 3. Cross-section through a gonadal area at week 5. The PGC (*arrow*) is encompassed by pale-staining central cells (*c*) surrounded by darker peripheral cells (*p*). *bv* blood vessel; *l* liver; *pe* peritoneum. Bouin/Crossmon. x 1230

Fig 4. Longitudinally sectioned gonadal area at day 3, showing PGCs (*arrows*), each accompanied by somatic cells (*arrowheads*). Note that gonadal tissue is still absent between the two successive PGCs. Abbreviations as in Fig 1. *pc* peritoneal cell. Bouin/Crossmon. x 1640

Despite the mitotic resting state, the average size of the PGCs increased during the observation period, viz. from 16.5 μm at weeks 1 and 2 to 21.0 μm at weeks 4 and 5 (Fig 5). At each developmental stage, PGCs of different sizes (cf. Fig 5) were distributed randomly along the long-axes of the gonadal areas in individual animals.

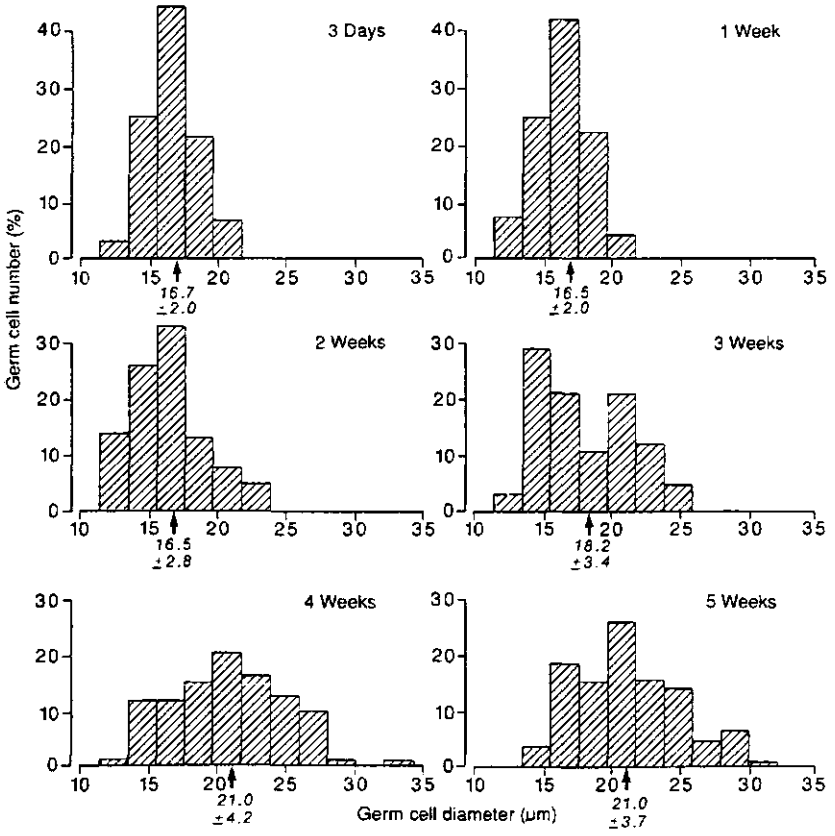
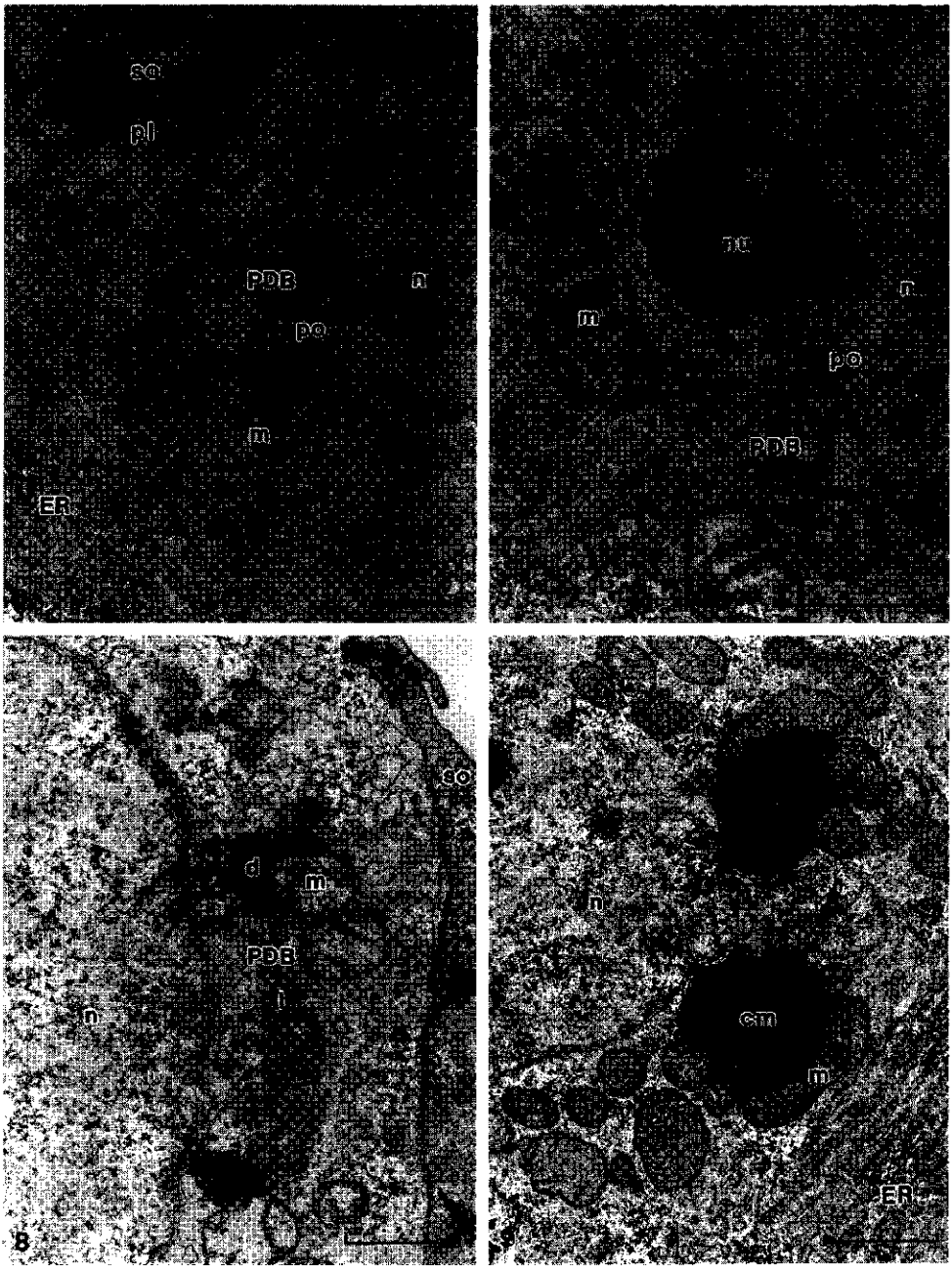


Figure 5. **Size distribution of PGCs at successive larval stages. Five embryos were examined at each stage, and the total number of PGCs counted was more than 100 per stage. Note that PGC sizes increase between week 2 and week 4. *Arrow* mean diameter ± SE.**

Electron microscopy

At day 3 and week 1, i.e., shortly after the arrival of PGCs at the gonadal area, these cells had a smooth surface membrane without cytoplasmic protrusions. The nucleus was situated centrally; it had an irregular outline (Fig 6) and many nuclear pores, occurring in clusters in its envelope (Figs 6,7). The nucleoplasm was homogeneous; a single nucleolus was located near the nuclear membrane and consisted of densely aggregated, electron-opaque granules, except for a few small electron-lucent areas (Fig 7). The cytoplasm contained several prominent perinuclear dense bodies (PDBs), composed mainly of a fine loose fibrillar component and ribosome-like particles (Figs 6,7). These PDBs were located in close proximity to the nuclear pores and were associated with mitochondria. The endoplasmic reticulum was smooth, had a distinct vesicular appearance and was widely distributed throughout the cytoplasm (Fig 6). Free ribosomes were numerous, some ribosomes being present as polysomes. The Golgi apparatus was small or absent. However, in one larger PGC in a newly hatched animal, a more elaborate Golgi apparatus was observed, indicating synthetic activity in this cell (not shown). Yolk-like inclusions with a highly electron-dense appearance were also occasionally observed.

During the observation period, distinct changes occurred in the cytoplasm of the PGCs, resulting in differences in the ultrastructure at weeks 4 and 5. In PGCs at that age, the nucleolus had increased considerably in size and was ring-shaped because of the presence of a large electron-lucent area. Occasionally, a second nucleolus was present in the same cell (not shown). In the cytoplasm, some of the PDBs had an amorphous and more electron-dense structure. PDBs of this type were found from week 2 onwards and increased gradually in size. At week 3, these PDBs formed small aggregates with mitochondria (Fig 8). At week 4 and week 5, still larger aggregates of mitochondria were present associated with these dark-staining PDBs (Fig 9). In PGCs at week 4 and week 5, the endoplasmic reticulum consisted of elongated smooth cisternae arranged in parallel with the nuclear membrane (Fig 9). Occasionally, rows of mitochondria were attached to these cisternae (not shown). Quantitation of mitochondria in PGCs of 1-week-old and 4-week-old larvae showed a distinct increase in their number from an average of 19 ($\pm$ 10) to 60 ($\pm$ 10) per median section through a PGC. However, at week 4, their size was smaller than at week 1. An elaborate Golgi apparatus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located in one area of the cytoplasm (Fig 10) was regularly observed. Coated vesicles were present at several locations in the cytoplasm at all stages examined. In the older larvae, however, they were located more frequently near the surface membrane; coated vesicles were occasionally attached to the surface membrane (Fig 11).

Morphogenesis of the gonadal primordium

Light microscopy

At day 3 and week 1, the PGCs were each accompanied by one or two peritoneal cells (Fig 1). Distinct gonadal ridges were not observed at these early stages, i.e., the germ-cell-free spaces between the successive PGCs in a rostro-caudal direction in the gonadal area contained only flattened peritoneal cells (Fig 4). At week 2, and more distinctly at week 3 (Fig 2), larger numbers of somatic cells were present around PGCs. An antero-posterior gradient was noticed, i.e., anteriorly located PGCs were usually surrounded by larger numbers of somatic cells. At week 3, small capillaries were present at the medio-lateral sides of the gonadal structures, but only in the region of the anterior PGCs. Gradually, the gaps between successive PGCs became filled with somatic cells in an antero-posterior direction.

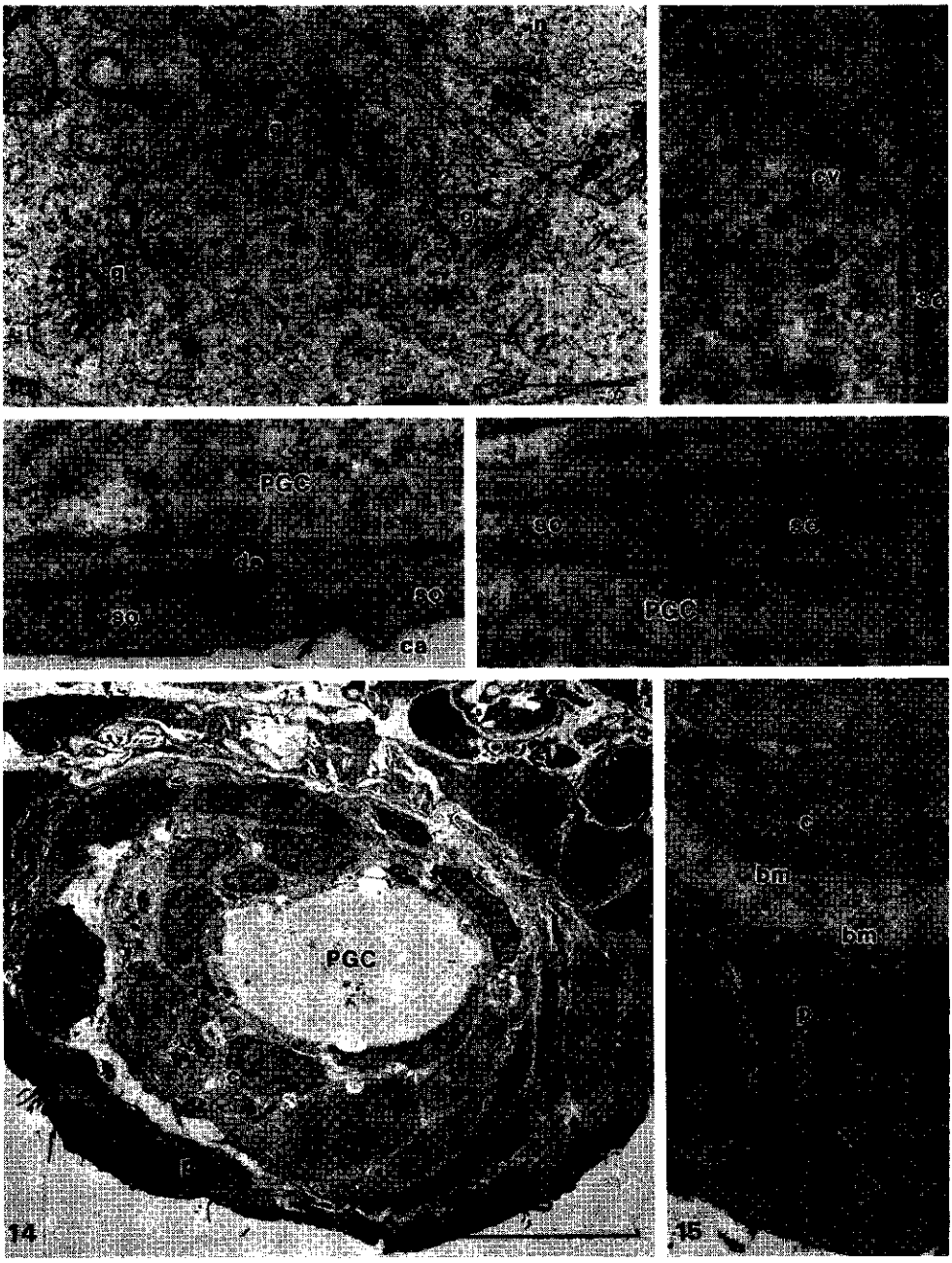
Figures 6-9. Electron micrographs of cross-sections through PGCs.

Fig 6. PGC at the age of 3 days, showing part of the nucleus (*n*) with nuclear pores (*po*) in the nuclear membrane. The cytoplasm contains smooth endoplasmic reticulum (*ER*). Perinuclear dense bodies (*PDBs*) are visible in association with mitochondria (*m*) and are located near the nuclear pores. *pl* surface membrane; *so* dorsal extensions of enveloping somatic cells. Bar = 1 μm

Fig 7. Detail of a PGC at the age of 7 days, showing part of the nucleus (*n*) with nucleolus (*nu*) and *PDB* material in the cytoplasm. *m* mitochondria; *po* nuclear pores. Bar = 1 μm

Fig 8. A PGC at the age of 3 weeks, showing two types of *PDB* material, light (*l*) and dark (*d*), the latter adhering to a mitochondrion (*m*). Note junctional complexes at the *right* (*arrows*) between the extensions of surrounding somatic cells (*so*). *n* nucleus. Bar = 1 μm

Fig 9. Electron micrograph of a PGC at the age of 4 weeks. Note the dark cement (*cm*) in association with the mitochondria (*m*). The endoplasmic reticulum consists of elongated smooth cisternae arranged in parallel with the nuclear membrane (*ER*) *n* nucleus. Bar = 1 μm.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From week 4 onwards, continuous gonadal ridges were present. The somatic gonadal tissue was organized into central cells surrounding the PGCs and more darkly staining peripheral cells forming the remaining part of the gonadal primordium (Fig 3). Blood capillaries had invaded the gonadal primordia (Fig 3).

In order to determine the increase in size of the gonadal primordia during early development, the number of nuclei of somatic gonadal cells was counted in each cross-section comprising the nucleus of a PGC. This enabled the comparison of somatic cell numbers throughout the observation period. As is shown in Table 1, the average of these cell numbers per animal, termed the somatic cell index, doubled approximately each week. Since mitotic figures were found in somatic gonadal cells from week 2 onwards, the increase in size of the gonadal primordia may be ascribed, at least in part, to mitotic proliferation of the somatic gonadal cells. At week 5, the mitotic figures were most numerous. They were observed in each of the larvae and in both the central and peripheral somatic gonadal cells. As shown in Table 2, the number of mitotic figures was considerably higher in the anterior portions of the gonadal primordia. The percentage of mitotic cells at this stage was 0.5. Despite the

Figures 10-15.

Fig 10. Elaborate Golgi-apparatus (*g*) with many vesicles in a PGC at the age of 2 weeks. *n* nucleus. Bar = 1 μm

Fig 11. Coated vesicles (*cv*) in the cytoplasm of a PGC at the age of 5 weeks. Note that one of these vesicles has fused with the plasma membrane and opens towards the exterior. *so* somatic cell. Bar = 0.1 μm

Fig 12. Junctional complex consisting of a tight junction (*arrow*) and a desmosome (*de*) between extensions of somatic cells (*so*) surrounding a *PGC* at the side of the coelomic cavity (*ca*). Bar = 0.5 μm

Fig 13. Contact between extensions of somatic cells (*so*) at the dorsal side of a *PGC* showing that no junctional complex is present. Bar = 0.5 μm

Fig 14. A PGC and enveloping somatic cells at the age of 5 weeks. The nucleus of the PGC is not in focus. Note that the lighter central cells (*c*) are surrounded by a layer of darker peripheral cells (*p*). Bar = 10 μm

Fig 15. Detail of enveloping somatic cells at the age of 5 weeks showing that both the lighter central cells (*c*) and the layer of peripheral cells (*p*) rest on a basement membrane (*bm*) with connective tissue in between. Bar = 1 μm

Table 2. **Metaphases in somatic cells of gonadal primordia at week 5**

Region of gonad	Fish				
	1*	2	3	4	5
Anterior					
0– 20%	21	11	16	11	37
20– 40%	12	5	8	14	38
40– 60%	9	3	5	8	22
60– 80%	7	1	5	3	13
80–100%	6	2	4	4	11
Posterior					
Length of gonad in mm:	1.4	1.5	1.4	1.2	2.5

* No PGCs were present

absence of PGCs in fish 1, the numbers of mitotic cells (cf. Table 2) and the average number of somatic cells was similar to that of other fishes.

Electron microscopy

Schematic drawings of cross-sections through PGCs and surrounding somatic cells at week 1, 3 and 5 are presented in Fig 16. Details are shown in Figs 12, 13, 14 and 15.

At day 3 and week 1, the PGCs were completely surrounded by cytoplasmic extensions of peritoneal cells (Fig 16a,b). Up to and including week 3, such extensions were joined by junctional complexes at the side of the coelomic cavity (Fig 12, see also Fig 8). The cytoplasmic extensions covering the dorsal surface of the PGCs were not joined by junctional complexes (Fig 13). From week 3 onwards, the PGCs became surrounded gradually by larger numbers of somatic cells (Fig 16b). As shown in Figs 14 and 16c, at week 4 and 5, two types of somatic gonadal cells were discerned, i.e., lighter central cells, surrounding the PGCs completely, and darker peripheral cells, forming the remaining part of the gonadal primordium. At several places, areas between central and peripheral cells were filled with extracellular matrix material (Fig 14). Furthermore, a basement membrane almost completely encompassed the central cells, and another was deposited against the peripheral cells (Fig 15). The somatic cells were joined by junctional complexes along the outer margin of the gonadal primordium, bordering the coelomic cavity; desmosomes were present at specific places between the cytoplasmic processes of the central cells.

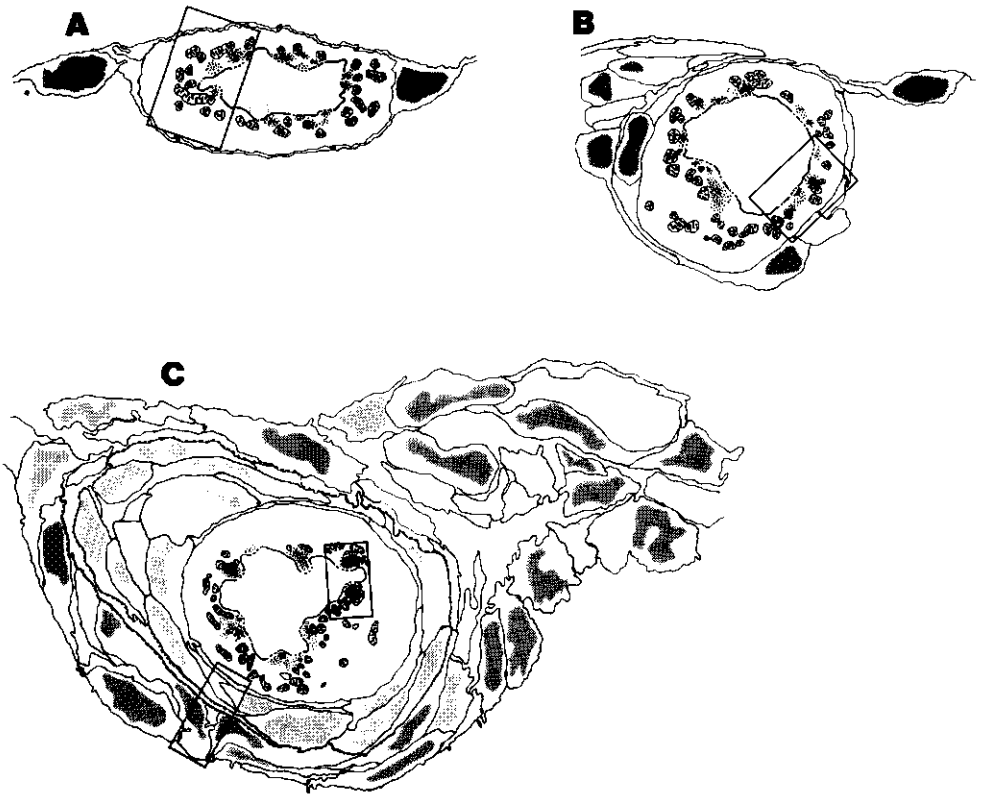

Figure 16 A-C. Schematic drawings of cross-sections through gonadal primordia at the ages of 1, 3 and 5 weeks, showing the increase in numbers of somatic cells surrounding a PGC. **A.** In the first week, the PGC is encompassed by extensions of one or two somatic cells; these form junctional complexes at the side of the coelomic cavity, but not at the dorsal side. The *outlined* area represents Fig 6. **B.** In the third week, the number of somatic cells enveloping a PGC has increased. The *outlined area* represents Fig 8. **C.** In the fifth week, a central layer of lighter cells is located close to the PGCs and surrounded by a layer of darker peripheral cells. The *upper outlined area* represents Fig 9; the lower *outlined areas* indicates the location of Fig 15, which is taken from another section).

DISCUSSION

Light microscopy

In the present study, we have shown that PGCs increase in size between week 2 and week 4 after fertilization, viz. during the mitotic quiescent period prior to the onset of proliferation. A mitotic quiescent period of PGCs from hatching until the age of 6 weeks has been reported for carp by Parmentier & Timmermans (1985). Their data have thus been confirmed, and are also in agreement with data obtained in other cyprinids, i.e. *Carassius auratus* (Stromsten 1931) and *Barbus conchonius* (Timmermans & Taverne 1989).

In addition, the numbers of PGCs in carp vary considerably between individual specimens, confirming data obtained in other teleost species (see review by Johnston 1951). Moreover, the numbers of PGCs in the left and right gonadal primordia are unequal in individual larvae. A preponderance of germ cells on the right side has been observed in several teleost species (see Johnston 1951). Hamaguchi (1983, 1986) reports that in six related *Oryzias* species, PGCs colonize the right gonadal area exclusively. In carp, however, when the numbers of PGCs for the right and the left gonadal primordia are summed separately for all specimens studied, their total is the same, indicating that no preference for a particular gonadal area exists.

The increase in cell size during the mitotic quiescent period occurs asynchronously within the PGC population of carp gonadal primordia. At present, however, no explanation is available for the asynchronous onset of this differentiation. No reports are known dealing with PGC enlargement during the mitotic quiescent period. However, a critical reading of the literature reveals that cell growth of intragonadal PGCs prior to the resumption of proliferation has been noted, but not emphasized, in other teleosts (Hann 1927; Johnston 1951).

The cell enlargement indicates that a differentiation step occurs within the PGCs, probably representing a preparation for the rapid proliferation after week 6. Such differentiation might be accompanied by the insertion into the surface membrane of new macro-molecules that can act as antigens for (monoclonal) antibodies. A previous study using monoclonal antibodies (MAbs) raised against carp spermatozoa has shown that new surface

markers appear on germ cells at specific developmental stages, i.e., the onset of proliferation after week 6 and the start of spermatogenesis at week 19, whereas one sperm-cell-specific antigen is present on PGCs from hatching onward (Parmentier & Timmermans 1985). Recently, MAbs have been raised against isolated carp spermatogonia and one of them appears to react with PGCs from week 4 onwards (Van Winkoop & Timmermans 1992). This corroborates the supposition that the increase in cell size represents a differentiation step within the PGCs.

To our knowledge, no other studies on the differentiation of PGCs in fish have been carried out using MAbs. In mammals, several antigens have been recognized on the surface membrane of murine PGCs, e.g. F9, EMA 1, SSEA 1, M1/22.25 antigens and others (see review of Eddy & Hahnel 1983). However, unlike the case in lower vertebrates such as fishes, the PGCs of mammals proliferate actively during migration and colonization of gonadal primordia (Hardisty 1967).

Electron microscopy

Primordial germ cells (PGCs)

During the observation period, remodelling occurs in PGCs at the ultrastructural level, including modifications of the nucleolus and of several cytoplasmic organelles. The fine structure of PGCs in carp is equivalent to that described for PGCs in other teleosts (Satoh 1974; Hogan 1978; Bruslé & Bruslé 1978; Bruslé 1980; Hamaguchi 1982, 1985). However, annulate lamellae, reported for PGCs of other teleosts, are not found in carp PGCs. Coated vesicles, on the other hand, present in carp PGCs have not been described in PGCs of other teleosts. Coated vesicles have been reported in PGCs of *Xenopus* (Wylie *et al.* 1976); they are suggestive of metabolic exchanges with the gonadal environment.

Although the ultrastructure has hitherto been described only briefly for pregonadal PGCs in cyprinids (Timmermans & Taverne 1989, in *Barbus conchonius*), germ cells in the adult gonads of several cyprinids have been studied more extensively with the electron microscope (Schjeide *et al.* 1972; Clérot 1976, 1979). Clérot (1976) has described the presence of PDB-material or 'nuage' and intermitochondrial 'cement'. These terms have been introduced before by André and Rouiller (1957). A large body of literature is devoted to

PDBs (see reviews by Beams & Kessel 1974; Eddy 1975). More recently, this material has been described in teleosts with respect tot the germ cells of *Oryzias* (Satoh 1974; Hogan 1978; Hamaguchi 1982, 1985, 1987), *Mugil* (Bruslé & Bruslé 1978) and *Poecilia* (Billard 1984). The exact function of the PDBs and the cement material is not known. However, most authors consider that the cement-aggregated groups of mitochondria are centres for mitochondrial multiplication (see Eddy 1975; Clérot 1976). Evidence has been obtained in cyprinids, by incorporation experiments with ^{3}H-uridine and ^{3}H-amino acids, that the cement contains RNA and protein (Clérot 1979).

In the PGCs of carp, PDBs were present after hatching at day 3. In subsequent weeks, they increased in size, part of the material obtained a darker appearance and became aggregated with mitochondria from week 2 onwards. The number of mitochondria also increased. These data are in accordance with the above mentioned supposition. It may be supposed that, in carp PGCs, the cluster of cement-aggregated mitochondria serves as a site of multiplication in preparation for the rapid proliferation at the age of 6 weeks. Changes in PDBs have been described for PGCs of *Oryzias latipes* during the migratory period before their arrival at the gonadal area (Hamaguchi 1985). However, no formation of mitochondrial cement has been reported, which is in agreement with our data on pregonadal PGCs in newly hatched carp larvae and with those obtained in *Barbus conchonius* (Timmermans & Taverne 1989).

When comparing PGCs at week 1 with those at weeks 4 and 5, other changes also occur in the cytoplasm. Whereas at week 1, the amount of ER is low, at weeks 4 and 5, it increases, the ER being arranged in cisternae, in parallel with the nuclear membrane. Moreover, from week 2 onwards (and in one case at week 1), an elaborate Golgi-complex is present in the cytoplasm, indicating high metabolic activity. Although small Golgi-complexes are reported for PGCs of *Oryzias* (Satoh 1974; Hogan 1978), the presence of an elaborate Golgi-complex has not previously been reported. Its presence is thought to indicate increased cell activity in relation to PGC enlargement.

Gonadal development

During the observation period, gonadal tissue was gradually formed around the PGCs;

this occurred in an antero-posterior fashion, as observed before (Stromsten 1931; Parmentier & Timmermans 1985). From week 4 onwards, two somatic cell types could be clearly distinguished. One type had a lighter appearance and was located centrally around the PGCs, the other type was darker and formed the peripheral layer in the gonadal anlage. Both cell types were located on a basal lamina and became further separated from each other by a layer of connective tissue. A similar phenomenon has been reported by Kanamori *et al.* (1985) for *Oryzias latipes*. According to these authors, the central cells are the precursors of Sertoli cells or of follicle cells in the later testis or ovary. Because mitotic figures have been observed in both somatic cell types, it follows that their numbers increase at least partly by division.

Apparently, gonadal development is not stimulated by the PGCs, for in one gonadal primordium lacking PGCs, the numbers of mitotic and somatic cells were similar to those in PGC-containing primordia. On the other hand, the gonadal primordia probably have no inducing influence on the PGCs, as gonadal development occurs in an antero-posterior fashion and the PGCs with increasing cell size are distributed randomly.

In conclusion, the increase in PGC size and the marked changes at the ultrastructural level indicate that complex phenotypic changes occur in PGCs prior to the resumption of proliferation. The gonadal primordia develop apparently independently of PGC differentiation and form central and peripheral somatic cells that increase in number before the onset of PGC proliferation.

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Phenotypic changes in germ cells during gonadal development of the common carp (*Cyprinus carpio*). An immunohistochemical study with anti-carp spermatogonia monoclonal antibodies.

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SUMMARY

A procedure is described in which large early spermatogonia were isolated from carp testes and purified from an initial 4-5% recovery up to 60-70% using equilibrium density centrifugation on a continuous Percoll gradient. Mice were immunized with these spermatogonia via the intrasplenic route. Six hybridoma cultures, producing monoclonal antibodies (MAbs) reacting selectively with germ cells, were selected and further analysed. Reactivity with five of these MAbs was observed on primordial germ cells (PGCs) in the developing indifferent gonads at the onset of proliferation, i.e., the age of 7 weeks. One MAb, encoded WCG 6, appeared to define a new surface marker on PGCs being gradually expressed on the surface membrane between the age of 2 and 4 weeks, concomitantly with an increase in size of these mitotically silent cells. The reactivity of germ cells with five of the MAbs disappeared completely (WCG 7, 12, 15, 21) or nearly completely (WCG 6) during spermatogenesis, providing a striking difference from patterns obtained with MAbs raised previously against carp spermatozoa. Differences between male and female germ cells were not observed with the WCG-MAbs during gonad development, indicating that a common set of surface antigens is shared between germ cells of both sexes up to and including spermatogonia and oogonia.

INTRODUCTION

Spermatogenesis in the testis of fish comprises mitotic proliferation of spermatogonia, meiotic cell divisions and spermiogenesis (Nagahama 1983; Billard 1986) and resembles in its major outlines the process of spermatogenesis in the mammalian testis. However, there are also differences between spermatogenesis in fish and mammals. These are reflected most clearly in the different morphology of fish spermatozoa (Mattei 1970) and in the characteristic arrangement of spermatogenic cells in germinal cysts in the testis of fish (Grier 1981). Although the available descriptive studies on gametogenesis in fish provide general information, many questions still remain, particularly with respect to the period of gonad

Abbreviation: WCG, Wageningen carp spermatogonia antibody

development before the onset of spermatogenesis.

During spermatogenesis specific macromolecules, which are recognizable by monoclonal antibodies (MAbs), may arise in germ cells. MAbs may be considered to be highly selective stains as each of them reacts specifically with (a determinant of) one type of macromolecule; such macromolecules or antigens may serve as differentiation markers and lead to the understanding of certain differentiation steps (Millette 1979). In mammals MAbs have been used extensively to study germ cell differentiation antigens (Bechtol *et al.* 1979, 1980, 1986; Gaunt 1982; Bechtol 1984; Fenderson *et al.* 1984; Moore *et al.* 1985; Haneji & Koide 1987; Isahakia 1988; Kurpisz *et al.* 1988), resulting in a number of MAbs recognizing germ cells mainly from meiotic stages onwards.

In order to study germ cell differentation in fish, MAbs have been raised against spermatozoa of the common carp, *Cyprinus carpio* L. (Parmentier *et al.* 1984). Using four of these MAbs (WCS 3, 17, 28, 29), differentiation markers, specific for germ cells, were recognized on meiotic and premeiotic male germ cells, as demonstrated with immunohistochemical tests on sections of more advanced stages of testicular development. In ovaries the reaction occurred only with oogonia and early prophase oocytes (Parmentier *et al.* 1984). During development these MAbs reacted with germ cells from different stages onwards, i.e., the time of hatching (WCS 29); 7 weeks after fertilization concomitantly with the onset of germ cell proliferation (WCS 3, 17); and from the start of spermatogenesis or oogenesis (week 19 or week 16 respectively, WCS 28; Parmentier & Timmermans 1985). These four MAbs and three others (WCS 7, 11, 12), the latter recognizing antigenic determinants only from the stage of late spermatogonia onwards, also reacted with isolated precursor germ cells (van Winkoop & Timmermans 1990).

The question was raised whether on premeiotic germ cells in carp yet other specific antigenic determinants, which are not expressed on spermatozoa and hence not detected so far, do occur. Such determinants may include markers for differentiating primordial germ cells (PGCs) in developing embryos and larvae; or markers for sex distinction or determination before or after male and female gonads can be distinguished from each other at any time from 10 weeks after fertilization (Parmentier & Timmermans 1985). In the present study, phenotypic changes were examined in germ cells with a new set of MAbs

produced by using spermatogonia of carp as immunogen. Six MAbs that immunostained germ cells selectively have been analysed with particular reference to their reaction with germ cells in testes and ovaries and with PGCs in developing gonads.

MATERIALS AND METHODS

Animals

The common carp *Cyprinus carpio* L. was reared under standard laboratory conditions (cf. Parmentier & Timmermans 1985). Animals of average size were selected at the age of 22-30 weeks and anaesthetized with 0.01% tricaine methane sulphonate (TMS, Crescent Research Chemicals, Arizona, USA), before killing. Moreover, groups of 10 larvae were also used, which were selected at weekly intervals from a group of average size, at the age of 1 week up to 16 weeks. For routine histology, gonadal and other tissues and larval stages were fixed in Bouin's fluid, dehydrated and embedded in paraffin. Serial 5-μm-thick sections were mounted on albumin-coated slides and stained with Mayer's haemalum and eosin.

Purification of early spermatogonia and immunization of mice

Gonads were dissected from the adult specimens and kept in ice-cold phosphate-buffered saline (PBS; pH 7.4) and testis cells were isolated as described by van Winkoop & Timmermans (1990). The cells were washed twice and resuspended in ice-cold PBS with 0.1% bovine serum albumin (BSA) and layered onto preformed continuous density gradients of 33.3% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in PBS with 0.1% BSA (modified from Schumacher *et al.*, 1978). Equilibrium density centrifugation of carp testis cells in continuous Percoll gradients at 500 x *g* for 30 min allowed the separation of primary and early secondary spermatogonia, named early spermatogonia, from the other germ cells, due to their larger size (> 10 μm). The density gradient fractions containing early spermatogonia were collected and diluted fivefold. The cells were washed three times and resuspended in PBS. The viability of cells was determined by the trypan blue exclusion test (Tennant 1964) and exceeded 95%. The method resulted in final cell suspensions containing 60-70% early spermatogonia (size > 10 μm). Other cells, included in low percentages in the

final suspensions were : late spermatogonia (< 10 μm), spermatocytes and somatic testis cells; spermatozoa were virtually absent. Six-weeks-old female Balb/c mice were injected intrasplenically (Spitz *et al.* 1984) with 2.5 - 1 x 10^6 purified (60-70 %) early spermatogonia of carp in 0.05 ml PBS. Seventy hours later the mice were killed, their spleens removed, and cell suspensions were made by gently pressing the organs through a nylon gauze filter.

Preparation of monoclonal antibodies

Monoclonal antibodies against carp spermatogonia (WCG) were prepared as described by Egberts *et al.* (1982). Briefly, hybridomas were obtained from a fusion between mouse Sp2/0-Ag14 plasmacytoma cells and splenocytes from Balb/c mice immunized with carp spermatogonia. Spent media from hybrid clones were tested for reactivity with carp germ cells by an indirect immunofluorescence test on frozen sections of carp testis. Selected cultures were cloned by limiting dilution (see Goding 1980). The resulting subclones were tested for the secretion of antibody like the parental hybridoma cell lines. Stable hybridoma subclones showing germ cell specificity were used to prepare ascites fluids (see Goding 1983). These were tested in the same way as the parental clones and, in addition, assayed by indirect immunofluorescence and indirect immunoperoxidase on Bouin-fixed sections of carp gonads and indirect immunogold staining on isolated cells (see below).

Immunohistochemical staining

Indirect immunofluorescence on frozen sections or sections of tissue fixed in Bouin's fluid was performed as described previously (Parmentier *et al.* 1984). The sections were incubated with hybridoma supernatant or ascites fluid in a humidified atmosphere for 45 min and then washed in PBS. Subsequently a 1:40 diluted solution of rabbit anti-mouse immunoglobulin antiserum coupled to fluorescein isothiocyanate (RAM/Ig/FITC; Nordic, Tilburg, The Netherlands) was applied for another 45 min at room temperature. After washing with PBS, the slides were mounted in a solution of PBS/glycerol (1/9, v/v) containing 1 mg/ml *p*-phenylenediamine to prevent photobleaching of the fluorochromes (Johnson and Noqueira Araujo 1981).

The indirect immunoperoxidase test on sections fixed in Bouin's fluid was performed

according to the same protocol as the immunofluorescence method, except that the second antibody was rabbit anti-mouse immunoglobulin conjugated to peroxidase (RAM/Ig /PO; Nordic, Tilburg, The Netherlands). Slides were washed in PBS and then incubated for 10 min with 0.05 *M* diaminobenzidine (DAB, Sigma) in 0.05 *M Tris*/HCl buffer (pH 7.6), containing 0.01% H_2O_2. After washing the sections in distilled water they were counter-stained with Mayer's haemalum and mounted.

Indirect immunogold staining on isolated cells was performed as described (van Winkoop & Timmermans 1990). Controls including an antigen-negative hybridoma supernatant, myeloma ascites fluid and PBS, were consistently negative in each of the assays. Immunofluorescence was analysed using a Zeiss photomicroscope with incident-fluorescence optics. Immunogold labelling was examined with the same microscope equipped with polarized light epi-illuminescence optics (De Mey 1983) and a x63 Antiflex objective (cat no. 421800; Zeiss, Oberkochen, FRG). Photographs were taken on Agfapan 400 and 100 film respectively.

Immunological characterization of monoclonal antibodies

Hybridoma ascites fluids were characterized with respect to mouse immunoglobulin heavy chains in an Ouchterlony double diffusion test using purified goat immunoglobulins (Meloy, Springfield, Va., USA) specific for IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 mouse immunoglobulins, and rabbit immunoglobulins (Central Laboratory of the Dutch Red Cross, Amsterdam, The Netherlands) specific for IgM mouse immunoglobulin.

RESULTS

Purification of early spermatogonia

The extent of purification of the early spermatogonia, comprising primary and early secondary spermatogonia with a size $>10\mu$m, depended on the composition of the initial cell mixture that was used. It was observed that testes in which spermatozoa formation had started recently (at week 26-30 after fertilization) were most suited for spermatogonia purification. In particular when testes were selected with a relatively high percentage of early

spermatogonia (>4 %) and a low amount of spermatozoa (<3%), the density gradient separation procedure resulted in final cell suspensions containing 60-70 % early spermatogonia. These cells showed good retention of morphological features.

Immunohistochemistry with germ cells in the testis

A fusion between Sp2/0-Ag14 plasmacytoma cells and spleen cells from mice immunized with early carp spermatogonia, using the intrasplenic route, resulted in six stable hybridoma clones. The clones produced MAbs of the IgM class that reacted selectively with germ cells and not at all or with only one or two somatic cell types (Table 1). These MAbs have been applied on testes at the age of 22-30 weeks, as these contained all precursor spermatogenic stages in abundance (Fig 1a).

Four different patterns of immunostaining were obtained with these six MAbs on frozen testis sections using the indirect immunofluorescence test (Table 1). With WCG 1, surface membranes of all germ cell stages including spermatozoa were immunostained clearly; with WCG 6 the surface membranes of precursor germ cells were stained distinctly, whereas the

Table 1. Reactivity of monoclonal antibodies (MAbs) raised against carp spermatogonia with gonad cells and cells in somatic tissues[a]

MAb	Heavy chain type	Precursor germ cells in testes[b]	Somatic cells in				
			Testes	Liver	Spleen	Kidney	Brain
WCG 1	μ	All[c,d]	–	–	–	–	–
WCG 6	μ	prc[c,d]	–	–	–	Glomeruli	Nerve Fibres
WCG 7	μ	Sg[c,d]	–	–	–	Glomeruli	Nerve Fibres
WCG 12	μ	pSg	–	–	–	–	–
WCG 15	μ	pSg	–	–	Leukocytes	–	–
WCG 21	μ	pSg	–	–	–	–	–

[a] The reactivity of MAbs was examined on frozen tissue sections in the indirect immunofluorescence test (see Materials and methods)
[b] Positive reactions were also obtained with oogonia in frozen sections from ovaries
[c] Similar reactions were obtained on sections of Bouin-fixed testes in both indirect immunofluorescence and indirect immunoperoxidase tests
[d] Positive reactions were obtained on isolated precursor germ cells in the indirect immunogold staining test
WCG, Wageningen carp spermatogonia; Sg, spermatogonia; pSg, primary spermatogonia; prc, precursor germ cells; –, no reaction

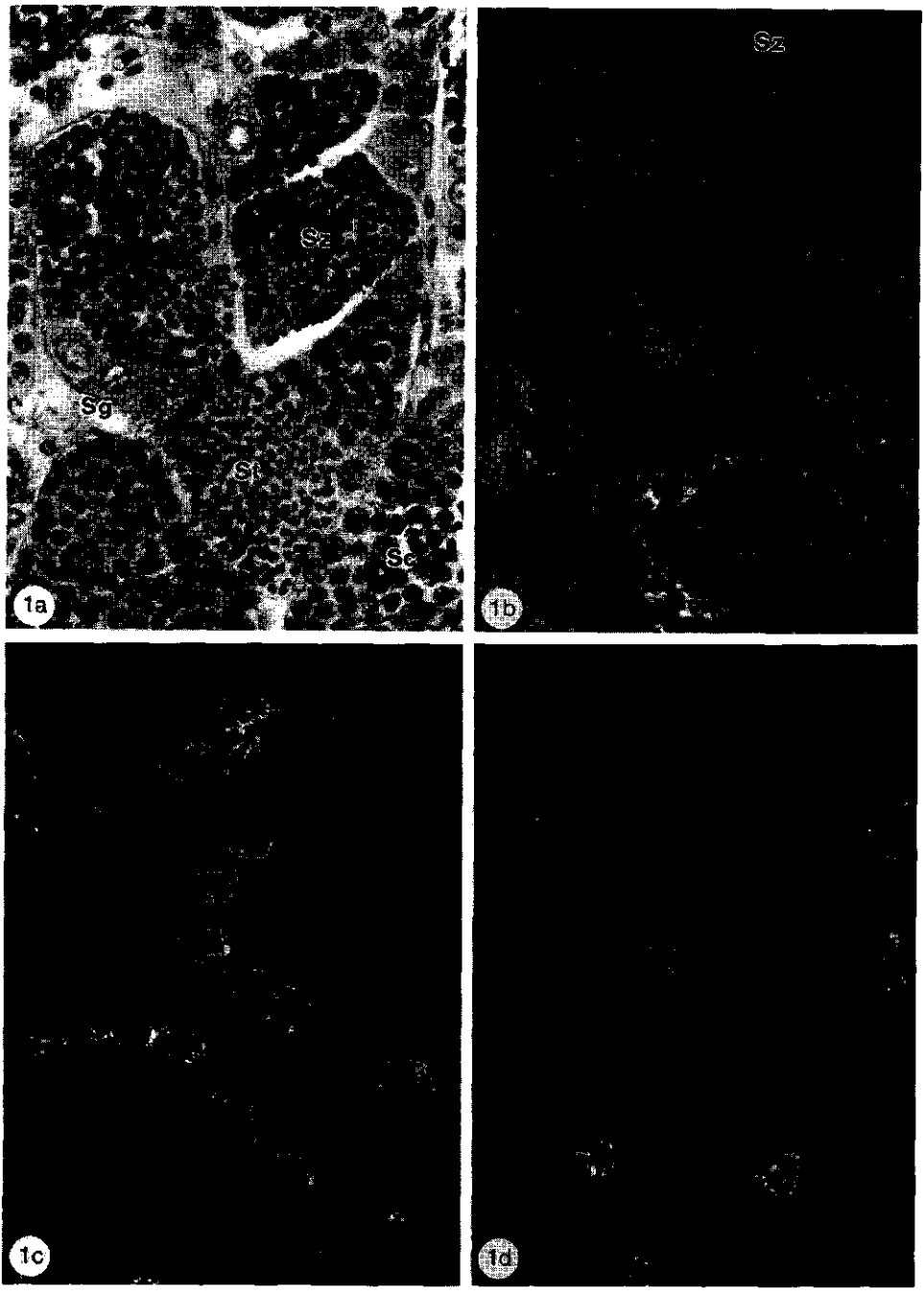
spermatozoa reacted very weakly or not at all (Fig 1b). In contrast, with WCG 7 only cells located close to the walls of the seminiferous tubules and probably representing groups of larger spermatogonia, were distinctly immunostained, whereas later germ cell stages were sparsely labelled and spermatozoa were negative for the MAb (Fig 1c). Moreover, very restricted reactions were observed with WCG 12, 15 and 21. Each of these three MAbs reacted with germ cells located individually near the walls of the seminiferous tubules, apparently representing primary spermatogonia; other spermatogenic stages were negative (Fig 1d).

In order to allow characterization of the germ cell stages reacting with the MAbs, immunostaining was also applied on sections of Bouin-fixed gonads. In indirect immunofluorescence as well as in indirect immunoperoxidase tests, positive reactions were obtained with WCG 1, 6 and 7, but not with the other MAbs (Table 1). The reaction patterns obtained with WCG 1, 6 and 7 corresponded to the patterns observed in frozen testis sections (cf. Fig 1b, c). The much improved cellular identification in sections of Bouin-fixed testes confirmed that WCG 1 reacted with all germ cell stages including spermatozoa, WCG 7 reacted intensely with primary and small groups of secondary spermatogonia, and very weakly or not at all with successive spermatogenic stages. WCG 6 on the other hand reacted distinctly with all germ cell stages with the exception of spermatozoa.

To investigate whether the antigenic determinants defined by the six MAbs were expressed on the outer surface of germ cells, freshly isolated testis cells were treated in the indirect immunogold assay. Distinct reactions were seen with WCG 1, 6 and 7 on germ cells, but not on somatic testis cells (Table 1), confirming the surface location of the antigenic determinants suggested from the reactivity of germ cells in testis sections (see above). However, with WCG 12, 15 and 21, no immunostaining was observed on isolated testis cells (Table 1).

Immunohistochemistry with germ cells in developing gonads

Representative micrographs of the developing gonad at weeks 4, 7 and 8 are given in Figs 2a, 3a and 4a, showing that gonadal tissue, which is still weakly developed at week 4, has increased in subsequent weeks. At weeks 7 and 8, more than one PGC was observed in


Sz
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a cross section, due to their increase in number after week 6. In 1-week-old larvae, PGCs had colonized the gonadal area at the dorsal wall of the coelomic cavity. No immunostaining was seen on the germ cells at this stage with any of the six MAbs (Table 2). In larvae at week 4 of development, immunostaining of PGCs was seen with WCG 6, but not with the other MAbs (Table 2, Fig 2). However, distinct immunostaining with each of the six MAbs

Table 2. Reactivity of MAbs raised against carp spermatogonia with cells in developing gonads[a]

MAb	PGCs in gonadal area	PGCs in gonadal primordium	Germ cells in indifferent gonad	Germ cells in immature testis[c]
	Week 1	Week 4	Week 8	Week 16
WCG 1[b]	–	–	±	+
WCG 6[b]	–	+	+	+
WCG 7[b]	–	–	+	±
WCG 12	–	–	+	+
WCG 15	–	–	+	+
WCG 21	–	–	+	±

[a] The reactivity of MAbs was examined on frozen tissue sections in the indirect immunofluorescence test

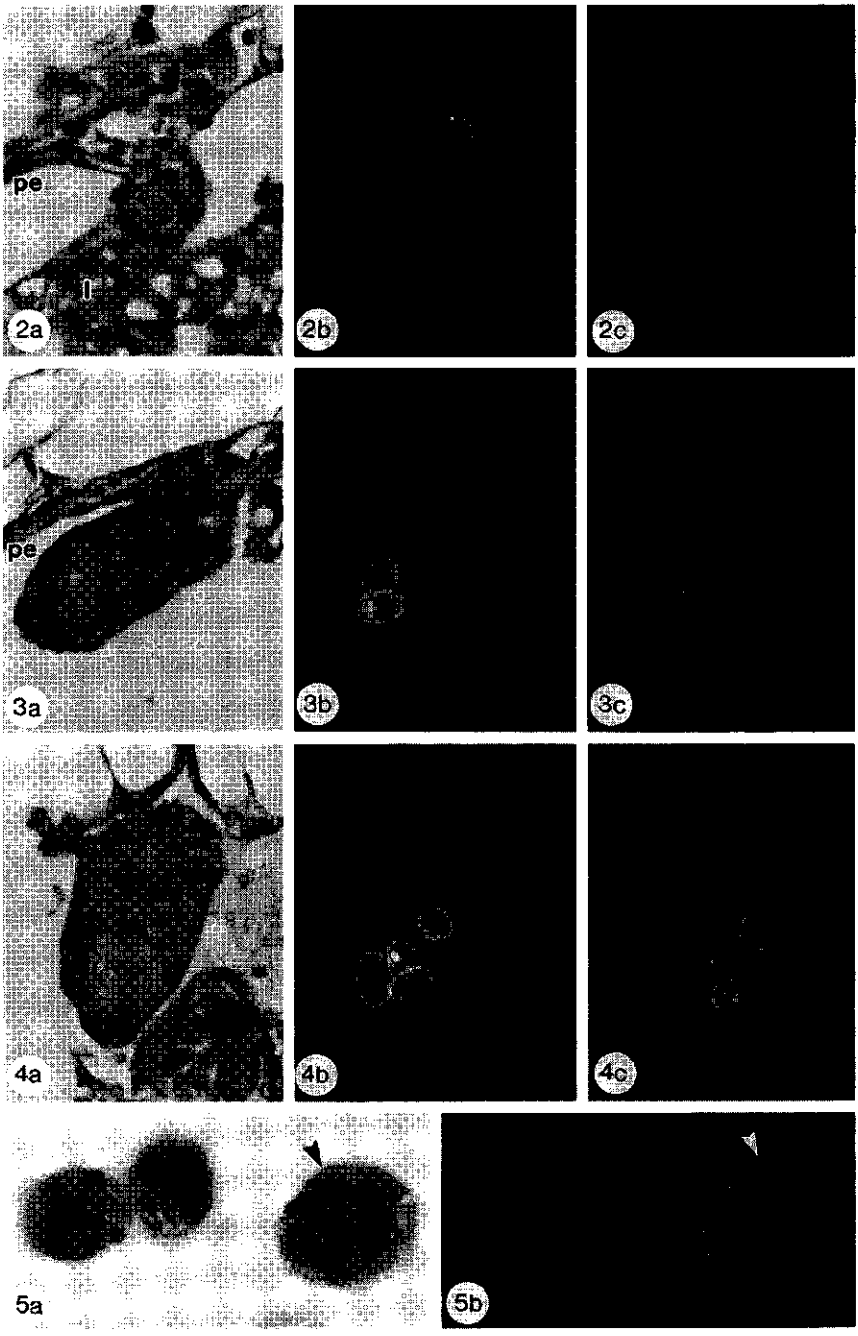
[b] Similar reactions were obtained on sections of Bouin-fixed gonads; positive immunostaining reactions were also obtained on isolated PGCs in the indirect immunogold staining test

[c] At week 16 oogenesis had already started; oogonia of this stage were also immunostained

WCG, Wageningen carp spermatogonia; PGCs, primordial germ cells; –, no reaction, ±, positive reaction

Figure 1.

a. Section of Bouin-fixed spermatogenic testis. Note that all major spermatogenic stages are present. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; Sz, spermatozoa. Haematoxylin and eosin (H/E) staining. b-d. Frozen sections of spermatogenic testis incubated with monoclonal anti-carp spermatogonia antibodies. b. Immunofluorescence with WCG 6 on apparently all germ cell stages except the spermatozoa (Sz), which react weakly or not at all. c. WCG 7: only cells close to the walls of seminiferous tubuli, i.e., early spermatogonia, are distinctly stained. d. WCG 12: immunostaining restricted to a subset of early spermatogonia. The same reaction was obtained with WCG 15 and 21. x 400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was observed on PGCs from week 7 onwards (Table 2, Figs 3 and 4), i.e. after the onset of proliferation in the until then mitotically silent PGCs. Moreover, a positive reaction was observed on germ cells in pre-spermatogenic testes and in early ovaries at week 16 (Table 2).

Incubation of cells, isolated from week 4 gonadal primordia, with WCG 6 in the indirect immunogold staining test confirmed that the defined antigenic determinant was already expressed at the outer cell surface of PGCs at this stage (Fig 5a, b). WCG 1 and WCG 7 reacted with isolated germ cells from week 7 onwards (Table 2).

No reactivity was observed in controls, using an antigen-negative hybridoma supernatant, myeloma ascites fluid or PBS instead of the first antibody.

Relation between size of PGCs and reactivity with WCG 6 in developing gonads

Indirect immunoperoxidase staining with WCG 6 from week 1 until week 5 revealed, in addition to unlabelled PGCs, two types of immunostaining PGCs. The latter showed either an immunoreactive cytoplasmic patch, or both cytoplasmic and surface membrane staining (Fig 6a). The three types of PGCs were, when present in individual animals, distributed randomly along the length-axes of the gonadal areas. Figure 7 illustrates that PGCs with a

Figures 2-4. **Consecutive cross sections of indifferent gonads incubated with (b) WCG 6 and with (c) WCG 7 in the indirect immunofluorescence test. a. H/E staining of the same section as (c) in Fig 2 and as (b) in Figs 3 and 4; pe, peritoneum; l, liver. Bouin's fixative.**

Fig 2. Cross-sections at the age of 4 weeks, showing one primordial germ cell (PGC; arrow) disctinctly immunostained with (b) WCG 6. Note that no immunofluorescene is obtained with (c) WCG 7; x 600

Fig 3. Cross-sections at the age of 7 weeks, showing two PGCs (arrows), distinctly immunostained with (b) WCG 6 and (c) WCG 7; x 500

Fig 4. Cross-sections at the age of 8 weeks, showing four PGCs (arrows), distinctly immunostained with (b) WCG 6 and (c) WCG 7; x 500

Figure 5. **Cells isolated from gonadal primordia at the age of 4 weeks and incubated with WCG 6 in the indirect immunogold staining test. a. Bright field illumination, H/E staining; b. Polarized light epi-illuminescence of the same cells. Note the distinct immunostaining of the surface of a PGC (arrow), whereas somatic cells are negative. x 2000**

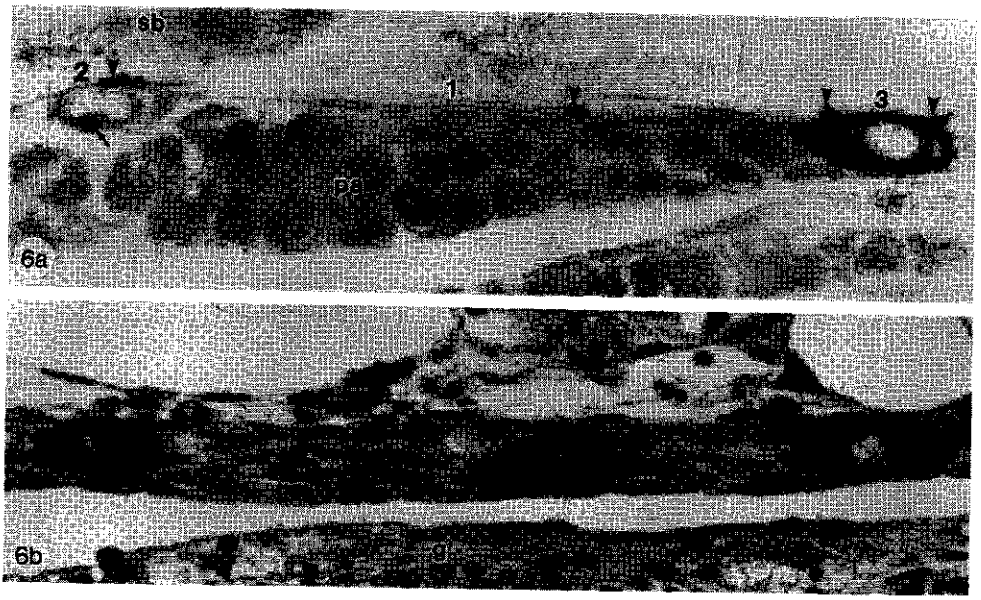

Figure 6.

a. Different types of immunostaining PGCs at week 3. A longitudinal gonadal section is shown after incubation with the monoclonal antibody WCG 6 in the immunoperoxidase test. (*1*) PGC showing no immunoreactivity, confirmed in adjacent sections. The circumference of the PGC is indicated by *small arrowheads*. (*2*) PGC with an immunoreactive cytoplasmic patch (arrow). (*3*) PGC with both cytoplasmic and surface membrane immunoreactivity; *large arrowheads* indicate pigment cells. sb, *Swim* bladder; pa, pancreatic tissue. Bouin's fixative; x 1250

b. Three PGCs with cytoplasmic and surface membrane immunoreactivity in a longitudinal gonadal section at week 4. g, Gut (anterior is at left in the micrographs). Bouin's fixative; x 1250

positive cytoplasmic patch were found in the first week and increased in number up to and including week 3. PGCs with a WCG 6-positive surface membrane were found from week 2 onward. The percentage of these cells, being low at weeks 2 and 3, had increased sharply at weeks 4 and 5 to 84 and 96% respectively (Fig 7; cf. Fig 6b). As at the age of 3 weeks nearly half of the PGCs showed immunoreactivity, which was partly restricted to cytoplasmic

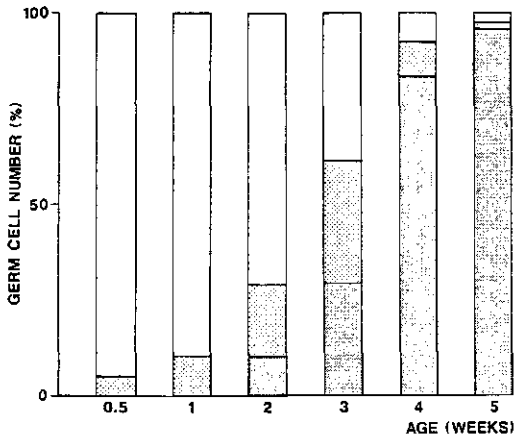

Figure 7. Percentages of the three types of immunostaining PGCs at successive larval stages; *unstippled*, unreactive PGCs; *lightly stippled*, PGCs with immunoreactive cytoplasmic patch; *densely stippled*, PGCs with cytoplasmic and surface membrane immunostaining. Five embryos were examined at each stage respectively, and the total number of PGCs counted was more than 100 per stage. Note that the percentage of PGCs with surface membrane immunoreactivity increases distinctly between week 2 and week 4.

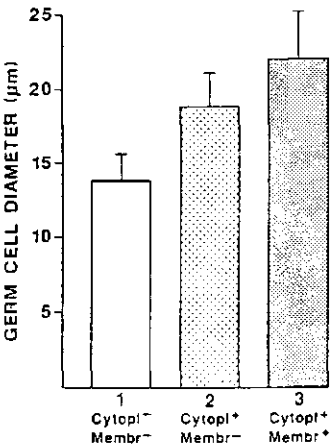

Figure 8. Correlation between cell size and extent of immunostaining in PGCs at week 3. PGCs with surface membrane immunoreactivity are distinctly larger than PGCs with cytoplasmic patch or unstained PGCs. *Stippling* as in Fig 7. The cell types 1, 2 and 3 correspond to those indicated in Fig 6a.

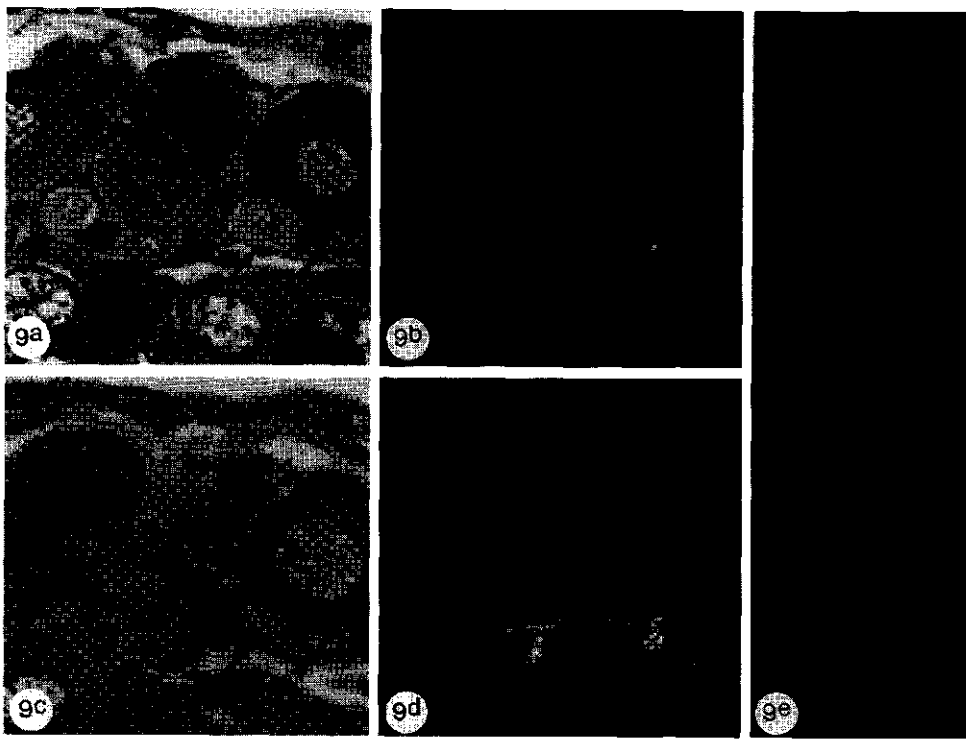

Figure 9.

a-d. **Sections of Bouin-fixed ovaries. Consecutive cross-sections incubated with (b) WCG 7 and with (d) WCG 6 in the indirect immunofluorescence test. a. H/E staining of the same section as (b); c. H/E staining of the same section as (d). Arrows in (a) and (c) indicate oogonia, which are immunostained in (b) and (d). Note that in (d) one more advanced stage is immunoreactive, which remains unstained in (b) (*arrowheads*); x 500**

e. **Frozen section of ovary, immunostained with WCG 12. Immunostaining is restricted to a subset of early oogonia. The same reaction was obtained with WCG 15 and 21; x 400**

patches and partly including the surface membrane, the sizes of the unlabelled and the immunostaining PGCs were compared within this age group. Figure 8 shows that a positive correlation existed between cell size and the extent of immunostaining; WCG 6-unreactive PGCs were the smallest, PGCs with only cytoplasmic patches were of intermediate size and PGCs with a positive plasma membrane were of the largest size.

Immunohistochemistry with germ cells in the ovary

The results of the indirect immunofluorescence test applied to frozen sections of *C. carpio* ovaries (Table 1) revealed that WCG 1, 6 and 7 reacted distinctly with oogonia, but not with growing follicular oocytes and somatic ovary cells, whereas WCG 12, 15 and 21 reacted specifically with only a small proportion of the cells. Presumably the latter cells represent primary oogonia (Fig 9e). Moreover, contrary to the immunostaining with WCG 12, 15 and 21, WCG 1, 6 and 7 reacted with oogonia in Bouin-fixed sections (Fig 9a-d) and with isolated oogonia.

DISCUSSION

In the present study a procedure is described for the separation and purification of early spermatogonia (>10 μm in size), constituting 4-5% of isolated testis cells and resulting in a final percentage recovery of 60-70%. To our knowledge, such a procedure has not been described hitherto for fishes. Spermatogonia have been separated from cell suspensions of prepubertal mice testes using velocity sedimentation (Bellvé *et al.* 1977) and from cell suspensions of immature rat testes using centrifugal elutriation combined with equilibrium density centrifugation (Bucci *et al.* 1986), resulting in purities of 76-91% and 51-76% respectively.

In carp, contrary to guppy (Billard 1984), successive generations of A and B spermatogonia could not be defined with certainty, due to the fact that cysts with earlier spermatogenic stages and cysts with more advanced spermatogenic stages are distributed randomly in the testis tubules. Therefore in the present study, the term early spermatogonia is used for germ cells with a size $>$ 10 μm, these were isolated from cysts with primary

spermatogonia, each surrounded separately by Sertoli cells, and from cysts with groups of early secondary spermatogonia. The term late spermatogonia is used for spermatogonia with a size < 10 μm, originating by division from the early secondary spermatogonia.

With six MAbs raised against early spermatogonia, germ cells were immunostained selectively, i.e., they reacted not at all or only with one or two somatic cell types. Four MAbs (WCG 7, 12, 15 and 21) reacted exclusively, and one MAb (WCG 6) nearly exclusively, with precursor germ cells and not at all or nearly not at all with spermatozoa. This indicates that the MAbs recognize antigenic determinants which differ from the determinants that immunostain with MAbs raised against carp spermatozoa (Parmentier *et al.* 1984). Three of the anti-spermatogonia MAbs did not react with isolated germ cells, although in testis sections they seemed to react with cell membranes. Probably they react with membrane constituents that are not resistant to the germ cell isolation procedure.

During development, five of the six WCG MAbs (WCG 1, 7, 12, 15 and 21) reacted with PGCs from the age of 7 weeks onwards, concomitantly with the onset of PGC-proliferation. In a previous study it was observed that two WCS MAbs (WCS 3 and 17, raised against carp spermatozoa) reacted also with PGCs from the onset of proliferation (Parmentier & Timmermans 1985). This might indicate that at that stage at least two new antigenic determinants appear in the outer plasma membrane of PGCs, one of them disappearing during early spermatogenesis (recognized by WCG 7, 12, 15 and 21), the other remaining inserted in the plasma membrane of stages up to and including ripe spermatozoa (recognized by WCG 1, WCS 3 and 17). However, further research is needed to reveal the functional significance of these antigenic determinants.

One of the monoclonal antibodies, WCG 6, reacted from an earlier developmental stage than the other WCG MAbs, during a period in which the PGCs are mitotically inactive (Parmentier & Timmermans 1985). It was observed that the expression of the WCG 6 determinant occurs concomitantly with an increase in size of the PGCs, between the ages of 2 and 4 weeks. The cell enlargement has been observed before, occurring together with ultrastructural changes in the cytoplasm of PGCs (van Winkoop *et al.* 1992). These data indicate strongly that the PGCs are not in a resting stage during the mitotically silent period, but that a differentiation step occurs and is distinctly recognized by the WCG 6 MAb; this

might represent a preparation for the rapid proliferation occurring after week 6. However, the chemical nature and the function of the antigenic determinant, which is recognized by the WCG 6 MAb, remains to be elucidated.

With the exception of the studies on carp, reactions of MAbs with premeiotic germ cells have only been reported for XT-2, a MAb raised against mouse testis cells. XT-2 showed detectable levels of reactivity in absorption studies with mouse testes at postnatal day 8 when the germ cells present are largely spermatogonia (Bechtol *et al.* 1979). Interestingly, the reactivity of this MAb decreased on spermatids and was not detectable on epididymal spermatozoa. Furthermore, a heterologous antiserum has been prepared against type B spermatogonia of mouse which, after absorption with somatic cells, selectively immunostained type A spermatogonia and successive spermatogenic stages, but not spermatozoa (Millette & Bellvé 1980). From these data it can be concluded that in mouse, similar to carp, differentiation antigens appear on early germ cell stages, which disappear before the mature spermatozoa are formed.

In immunostained, Bouin-fixed sections observed reactivity for several MAbs, which was especially clear during development and also around the start of spermatogenesis, occurred as a positive patch present in the cytoplasm of germ cells in addition to a positive reaction on the outer cell membrane (cf. Fig 6). Such cytoplasmic patches also have been observed in mammalian PGCs, and were suggested to be the sites of synthesis of these MAbs (Heath & Wylie 1981). It may be supposed that the MAbs are synthesized within Golgi-complexes. This is supported by the observation in a previous ultrastructural study that an elaborate Golgi-complex is present in PGCs from the age of 2 weeks (van Winkoop *et al.* 1992). Moreover, the view that surface membrane components are assembled within the Golgi apparatus is now generally held (Alberts *et al.* 1989).

All six anti-spermatogonia MAbs appeared to react also with oogonia in the ovary although not with follicular oocytes. Such reactivity with germ cells of both sexes has been observed before with the anti-spermatozoa MAbs (Parmentier *et al.* 1984; van Winkoop & Timmermans 1990). It can be concluded that male and female germ cells in carp up to and including spermatogonia and oogonia share a common set of surface membrane antigens. This is corroborated by the fact that sex reversal may occur under natural circumstances and

can be induced during fish development (cf. Reinboth 1983, 1987) and that testis-ova can be generated from spermatogonia (Shibata and Hamaguchi 1988). Nonetheless, our sets of monoclonal antibodies may provide tools for the elucidation of processes underlying the differentiation steps indicated by the appearance or disappearance of the respective antigenic determinants.

ACKNOWLEDGEMENTS

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Recognition of surface antigens on spermatozoa of the common carp (*Cyprinus carpio* L., Teleostei) using monoclonal antibodies and scanning electron microscopy

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Eur J Morphol 33: 51-58 (1995)

SUMMARY

The distribution of antigenic determinants, recognized by seven anti-carp spermatozoa monoclonal antibodies (MAbs) and two anti-carp spermatogonia MAbs were studied using fresh, unfixed carp spermatozoa. The location of the antigenic determinants was analysed in light- and in scanning electron microscopy, in the latter with the backscattered imaging mode.

With all seven anti-carp spermatozoa MAbs a similar regular distribution of the immuno-gold labeling was present on heads, midpieces and tails of carp spermatozoa, whereas with the anti-carp spermatogonia MAbs only low or no labeling was observed.

The regular distribution of antigenic determinants, as observed with the anti-spermatozoa MAbs in carp, is in agreement with data on agglutination of spermatozoa by autoantibodies in *Salmo gairdneri* and data on distribution of antigenic sites on spermatozoa in *Xenopus laevis*. It differs from data in mammals in which the presence of specialized domains on spermatozoa was shown. These results are discussed.

INTRODUCTION

Spermatozoa in mammals exhibit regional heterogeneity in the macromolecular composition of the plasma membrane. Several sharply delineated domains can be distinguished: the head, with the acrosomal, equatorial and postacrosomal region, and the tail with the middle piece, the principal piece and the end piece. These domains have been characterized with lectins and with different antisera and monoclonal antibodies. It has been suggested that the heterogeneous distribution of macromolecules has functional significance (for review see Eddy, 1988).

In some animal groups, where fertilization of the eggs occurs through a micropyle, the acrosome in spermatozoa is lacking (for a review, see Baccetti & Afzelius 1976). The question raises, whether surface domains, as demonstrated for mammalian spermatozoa, do occur on sperm in these groups.

Spermatozoa of the common carp, *Cyprinus carpio*, lack an acrosome (Billard 1970; Udo 1980) like other teleost species (Mattei 1970; Billard 1986; Bacetti *et al.* 1984).

Monoclonal antibodies (MAbs) have been raised against spermatozoa of the common carp (Parmentier *et al.* 1984). They recognize antigenic determinants on spermatozoa and on precursor germ cells in carp gonads, i.e., they react exclusively with germ cells and not with other cells and tissues in carp (Parmentier *et al.* 1984; Parmentier & Timmermans 1985).

During development of carp a number of the MAbs react with differentiating germ cells from different developmental stages onward (Parmentier & Timmermans 1985), indicating that they might recognise different antigens. However, immunofluorescence reactions on prefixed smears of spermatozoa suggested that the antigenic determinants, defined by these anti-spermatozoa MAbs, were all uniformly expressed on the heads of the spermatozoa and not on the tails (Parmentier *et al.* 1984).

In the present study, freshly isolated carp spermatozoa were used. The binding of the MAbs to these spermatozoa was visualized by both indirect immunofluorescence and indirect immunogold staining techniques. To obtain a higher resolution, immunogold stained sperm were observed also with the scanning electron microscope (SEM), using the backscattered electron imaging (BEI) mode (de Harven *et al.* 1984). This approach permits the specific correlation of the presence of a given antigen with the surface morphology of cells.

The patterns obtained with the anti-spermatozoa MAbs were compared with those obtained with two MAbs recently raised against carp spermatogonia (van Winkoop & Timmermans 1992). In addition, the structure of the spermatozoa was studied with transmission electron microscopy (TEM).

MATERIALS AND METHODS

Collection of sperm

Mature spermatozoa were collected from adult carp *Cyprinus carpio* L., as described (Parmentier *et al.* 1984). The spermatozoa were washed three times in phosphate-buffered saline (PBS), pH 7·4, and resuspended in PBS with 0·02 M sodium azide. The viability of the cells, as determined in the trypan blue exclusion test (Tennant 1964), exceeded 99 %.

Immunocytochemistry

Freshly isolated spermatozoa were treated in indirect immunofluorescence and indirect immunogold staining assays for analysis by light microscopy as described (van Winkoop & Timmermans 1990), with the exception that fixation after the immunogold staining was carried out in the present study with 2% glutaraldehyde in 0,1 M sodium-cacodylate buffer (pH 7.4), to enable direct comparison with SEM treated cells.

The protocol for the indirect immunogold staining assay for analysis by SEM was essentially the same as the immunogold assay for light microscopy, except that spermatozoa were attached to Nucleopore polycarbonate filters (Agar Scientific Ltd., Stansted, Essex, England,; cat. nr. G 3816) coated on two sides with carbon, instead of glass cover slips. After immunogold staining the cells were fixed as mentioned above, dehydrated and critical point dried with CO_2. The filters were mounted on aluminium stubs and coated with carbon in a vacuum evaporator.

Both immunofluorescence and immunogold labeling for light microscopy were analyzed as described (van Winkoop & Timmermans 1990). The SEM immunogold stained cells were analyzed with a Philips 535 scanning electron microscope using the secondary electron emission (SEI) mode and the backscattered electron imaging (BEI) mode. The accelerating voltage was 20 kV.

Antibodies

Seven MAbs raised against carp spermatozoa (i.e., WCS 3, 7, 11, 12, 17, 28, 29; Parmentier *et al.* 1984), and two anti-carp spermatogonia MAbs (i.e., WCG 6 and WCG 7; van Winkoop & Timmermans 1992) were used. Ascites fluids of hybridoma subclones were used at a final dilution of 1 : 1000 in PBS with 10% newborn calf serum (NCS). In each assay, controls were included in which the first antibodies were replaced by PBS or myeloma ascited fluid, diluted 1 : 1000 in PBS with 10% NCS.

For immunofluorescence, the second antibody was either rabbit-anti-mouse IgG, or rabbit-anti-mouse IgM, coupled to fluorescein isothiocyanate (RAM/IgG/FITC and RAM/IgM/FITC; Nordic, Tilburg, The Netherlands). These were used at a final dilution of 1 : 40 in PBS with 10% NCS. For immunogold staining, either goat-anti-mouse IgG, or

goat-anti-mouse IgM, coupled to 30 nm gold particles (AuroProbe EM GAM/IgG/G30 and GAM/IgM/G30 probes; Janssen Pharmaceutica, Beerse, Belgium) were used, at a final dilution of 1 : 10 in PBS with 10% NCS.

Electron microscopy

Testes from adult carp and freshly ejaculated spermatozoa were processed for electron microscopy. The tissues and cells were fixed for 1 h in 4% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.2) and postfixed in 1% osmium tetroxide (1 h). After dehydration and embedding in Epon 812, ultrathin sections were contrasted with saturated uranyl acetate and lead citrate and examined using a Philips EM 400 electron microscope.

RESULTS

Structure of spermatozoa

The structure of carp spermatozoa as seen in thin sections with TEM is schematically represented in Figure 1. The mean diameter of the head is 2,3 μm and the tail length is 38-44 μm. The roundish head of the spermatozoon is attached eccentrically to the tail. The nucleus is full of dense granular chromatin. No acrosomal complex or vesicles are present anteriorly to the nucleus, where the plasma membrane is always in close contact with the nuclear envelope. Two centrioles are found in a posterior indentation of the nucleus. The distal centriole functions as the basal body for the axoneme in the tail. The midpiece of the spermatozoon contains a small number of mitochondria and encircles the proximal end of the tail in a collarlike fashion (length 0.6-1 μm). This is also clearly visible in a scanning electron micrograph (Fig. 2).

Indirect immunofluorescence and immunogold staining in light microscopy.

Incubation of freshly isolated carp spermatozoa with anti-carp spermatozoa monoclonal antibodies (MAbs) and subsequently with FITC-conjugate revealed distinct immunofluorescence on, apparently, the entire cell surface of the spermatozoa when WCS 3, 7, 11, 12, 17 and 28 were used (Fig. 3A). A similar, but less intense, staining was

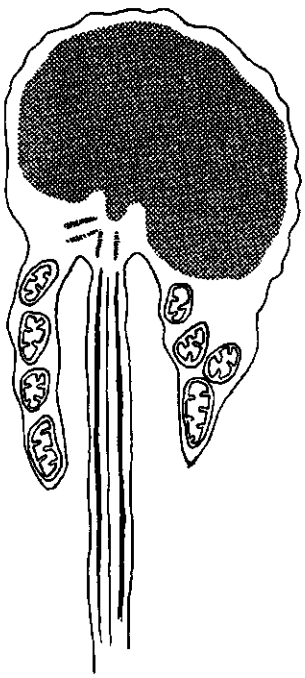
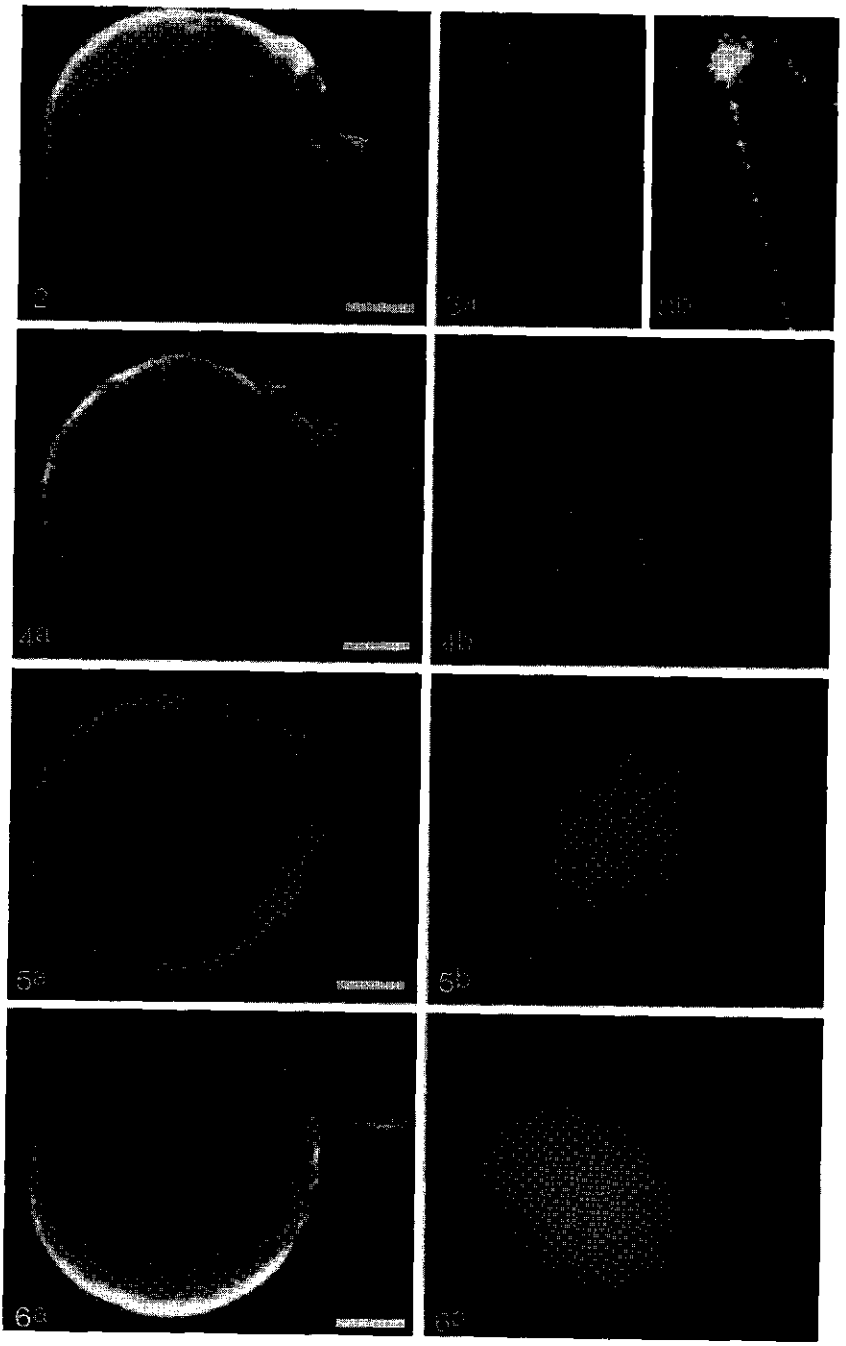


Figure 1. **Schematic representation of carp spermatozoon. 1, head; 2, middle piece; 3, tail (principal piece).**

observed with WCS 29 (not shown). With the immunogold method a positive reaction was obtained too with the surface of the entire spermatozoa, using the seven MAbs (Fig 3B). However, only scarse (WCG 6) or no labeling (WCG 7) was observed with both immunomethods when anti-carp spermatogonia MAbs were used (not shown). No label was detected on the spermatozoa in control incubations, in which PBS or myeloma ascites fluid was substituted for anti-carp MAbs.


2
3a
3b
4a
4b
5a
5b
6a
6b

Indirect immunogold staining in scanning electron microscopy (SEM)

The method worked out in the present study was based on the previously described light microscopical immunogold staining method, devised for precursor germ cells of carp (van Winkoop & Timmermans 1990). SEM analysis in the secondary electron imaging (SEI) mode revealed that the morphology of the spermatozoa, as seen at the onset of the antibody incubation steps (Fig 2), was well preserved during the immunogold staining procedure (Figs 4A, 5A, 6A). Moreover, no apparent cell loss did occur.

Figures 2-6.

Fig 2. Carp spermatozoon processed for SEM prior to immunogold staining and examined in the secondary electron imaging (SEI) mode. Note the collar-like middle piece (arrow). Bar = 0,5 μm

Fig 3a. Carp spermatozoon incubated with monoclonal anti-spermatozoa antibody WCS 3 in the indirect immunofluorescence test, revealing bright immunostaining on the entire surface of the cell. Similar immunofluorescence was obtained with WCS 7, 11, 12, 17 and 28. Immunofluorescence was weaker with WCS 29 and absent or nearly absent with WCG 7 and WCG 6. x 2000

Fig 3b. Carp spermatozoon incubated with monoclonal antibody WCS 28 in the indirect immunogold test, showing gold labeling on the entire surface of the cell. Similar immunogold labeling was obtained with WCS 3, 7, 11, 12 and 17. Immunogold labeling was lower with WCS 29 and absent or nearly absent with WCG 7 and WCG 6. x 2000

Fig 4. Carp spermatozoon incubated with WCS 3 and subsequently treated with colloidal gold-conjugate. a. SEI mode. It appears that the cell morphology is well preserved. However, colloidal gold particles cannot be identified with certainty. b. Backscattered electron imaging (BEI) mode. Gold particles can now be seen clearly and appear as white dots on the head, middle piece and tail. Similar immunogold labeling was obtained with WCS 7, 11, 12, 17 and 28, but labeling was lower with WCS 29. Bar = 0,5 μm

Fig 5. Carp spermatozoon incubated with WCG 6 and colloidal gold-conjugate. a. SEI mode, for description see Figure 4. b. BEI mode. Only a few gold particles are present. Note that they are regularly distributed on head and midpiece. Immunogold-labeling was absent with WCG 7. Bar = 0,5 μm

Fig 6. Control immunogold staining (in which irrelevant MAb was substituted for specific MAb). a. SEI mode. b. BEI mode. A similar picture was obtained with WCG 7. Note that gold particles are virtually completely absent. Bar = 0,5 μm

Incubation of spermatozoa with respectively WCS 3, 7, 11, 12, 17 and 28, revealed regular immunogold labeling of the heads, midpieces and tails of the spermatozoa (Fig. 4B). Much lower labeling of these structures was seen with WCS 29 (not shown). Gold labeling was very low after immunolabeling with the anti-carp spermatogonia MAb WCG 6 (Fig. 5B). Gold particles were absent or very rare on spermatozoa treated with WCG 7 or in control incubations in which the first antibody was replaced by PBS or myeloma ascites (Fig. 6B).

DISCUSSION

The morphology of carp spermatozoa, as observed from TEM sections in the present study, is in agreement with the descriptions of Billard (1970) and Kudo (1980) in carp and with those in other cyprinid species (Bacetti *et al.* 1984; Fribourgh *et al.* 1970). We agree with the comments of Fribourgh *et al.* that no cellular elements are present in the midpiece and no mitochondria in the tail as was reported for carp by Fujimura *et al.* (1956). The descriptions mentioned concerned morphological studies with electron microscopy. None of them was directed at membrane constituants.

In the present study it was shown with light microscopy and with immunogold labeling and SEM, using seven anti-carp spermatozoa MAbs, that antigenic determinants were regularly distributed on head, midpiece and tail of carp spermatozoa. To our knowledge this is the first study in which SEM is used for the visualisation of immunogold labeling of spermatozoa.

In a former study it was observed that during development the seven anti-spermatozoa MAbs reacted also with precursor germ cells, but not with somatic cells (Parmentier *et al.* 1984). However, they reacted from different developmental stages onward, i.e., the time of hatching (WCS 29), the onset of germ cell proliferation (seven weeks after fertilization (a.f.), WCS 3, 17), around the start of spermatogenesis (19-23 weeks a.f., WCS 28), and at the appearance of small secondary spermatogonia (WCS 7, 11 and 12) (Parmentier & Timmermans 1985; van Winkoop & Timmermans 1990). Although the similar distribution of the antigenic determinants recognised by the seven MAbs on spermatozoa might indicate that they belong to the same antigen, their appearance at different developmental stages

suggests that they are part of different antigens. However, although monoclonal antibodies are often specific to particular molecules, they may also recognize epitopes shared by many molecules (see e.g. Villaroya & Scholler 1986). This has to be elucidated by further studies. Moreover, in the present study only low or no gold labeling was observed on the outer surface of carp spermatozoa with two anti-spermatogonia MAbs. This confirms data of a previous light microscopical study, in which it was observed that the determinants recognised by these MAbs are nearly completely or completely restricted to precursor germ cell stages (van Winkoop & Timmermans 1992).

In a former light microscopical study, using indirect immunofluorescence on formalin fixed spermatozoa, a positive reaction with the anti-spermatozoa MAbs was observed only on the sperm heads (Parmentier *et al.* 1984). This restricted staining is probably due to the use of prefixation, for in the present study, using suspensions of unfixed spermatozoa, the entire spermatozoa were immunostained. These results are in agreement with data on agglutination of spermatozoa by autoantibodies in another teleost species, viz. the rainbow trout *Salmo gairdneri* (Secombes *et al.* 1984). However, whether among the MAb-defined antigenic determinants on spermatozoa of carp some represent autoantigens, awaits further elucidation.

In mammals, a large number of MAbs have been raised against spermatozoa (see Eddy, 1988). Most of them recognise antigenic determinants on one or more specialized regions, i.e. anterior acrosome, equatorial segment, post-acrosomal region, middle piece and tail, respectively. Only a few MAbs reacted with whole living spermatozoa, i.e. 2D6 (Gaunt *et al.* 1983; Jones *et al.* 1985, in the rat) and HM 1.0, HM 2.0 (Moore & Hatman 1984, in the hamster). Although the seven anti-spermatozoa MAbs in carp reacted with the whole surface, it cannot be excluded that antigens are present on the surface membrane of carp spermatozoa, still undetected sofar, which are grouped in domains.

Data on surface domains in spermatozoa of non-mammalian animals are scarse. To our knowledge, data have only been presented for the anuran amphibian *Xenopus laevis*, indicating that the surface of spermatozoa in this species also lacks topographical domains (Bernardini *et al.* 1989). According to these authors, the homogeneous distribution of particular macromolecules in the plasma membrane of spermatozoa, as found in their

ultrastructural and lectin-binding studies, does not exclude a role in fertilization. Although in carp the sharply delineated onset of expression of the spermatozoa antigens during early development, i.e., in primordial germ cells (Parmentier & Timmermans 1985) or also in spermatogonia and oogonia (van Winkoop & Timmermans 1990), may argue for their role in precursor germ cell differentiation, a function of these antigens during fertilization cannot be excluded.

ACKNOWLEDGEMENTS

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Further characterization of differentiation antigens expressed on the surface of germ cells of the common carp (*Cyprinus carpio* L.). A study with anti-carp spermatozoa monoclonal antibodies.

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SUMMARY

Four monoclonal antibodies (MAbs), raised against carp spermatozoa and reacting exclusively with germ cells from different developmental stages onward, showed first reaction as patches within the cytoplasm. These patches, especially numerous in (pre)spermatogonia around the onset of spermatogenesis, probably represent Golgi cisternae, indicating synthesis of new surface membrane constituents.

Histochemical analysis reveals that two MAbs (WCS 17 and WCS 29) probably bind to glycoproteins, the other two (WCS 3 and WCS 28) to lipoproteins or proteins proper. With all four MAbs a number of bands was visualised after Western blotting, suggesting that the antigens, recognised by each of them may be part of several differently sized macromolecules.

It is discussed that the four MAbs likely do not recognize autoantigens. It is suggested that they recognize differentiation antigens appearing on the germ cell surface at specific differentiation steps during development.

INTRODUCTION

Fishes are suitable models to study early intragonadal germ cell development. This especially concerns cyprinids (Timmermans 1987), for in these species, the onset of gonadal development as well as primordial germ cell (PGC) proliferation occurs after hatching in the free-swimming larvae. In the commmon carp, *Cyprinus carpio* L. the numbers of PGCs are small at hatching and remain small for several weeks, i.e. six weeks (Parmentier & Timmermans 1985). After six weeks a fast proliferation occurs in the still indifferent gonads; female and male gonads can be distinguished from each other after the age of 10 weeks, oogenesis starts at 16 weeks and spermatogenesis at 19 weeks (Parmentier & Timmermans 1985).

A prerequisite for cell differentiation in PGCs, as in other cell types, will be gene activation, resulting in the synthesis of new proteins. Likely, part of these molecules will be

inserted into the surface membrane, either as proteins or as glyco- or lipoproteins. Such macromolecules may serve as antigens which can be recognised with monoclonal antibodies (MAbs) raised against germ cells.

The production of MAbs, raised against carp spermatozoa has been described (Parmentier *et al.* 1984). It was observed that several MAbs, reacting exclusively with germ cells and not with somatic cells, recognised not only antigenic determinants on spermatozoa but also on precursor germ cells in male and female gonads. In ovaries, these MAbs reacted only with oogonia and early prophase oocytes, but not with follicular oocytes (Parmentier *et al.* 1984). One set of MAbs reacted with (primordial) germ cells during development from different stages onward, i.e. from hatching (WCS 29), from the start of proliferation of PGCs after 6 weeks (WCS 3, 17) and from the onset of gametogenesis (WCS 28), i.e. oogenesis at 16 weeks and spermatogenesis at 19 weeks (Parmentier & Timmermans 1985). Another set of MAbs (WCS 7, 11 and 12) reacted with late spermatogonia and subsequent stages in the spermatogenic testis (van Winkoop & Timmermans 1990). It has been shown that these seven MAbs reacted not only with germ cells in histological sections, but also with germ cells isolated from gonadal tissue, thereby confirming that they recognise antigenic determinants from macromolecules inserted into the surface membranes (van Winkoop & Timmermans 1990).

More recently, a third set of MAbs (6) has been raised against isolated and purified spermatogonia (van Winkoop & Timmermans 1992). Five of these MAbs reacted nearly exclusively with precursor germ cells and not with spermatozoa, indicating that they recognise antigen(s) which differ from those recognized by the anti-spermatozoa MAbs.

A common feature of the detected differentiation antigens is their initial expression in the cytoplasm of germ cells as a patchy pattern (Parmentier & Timmermans 1985; van Winkoop & Timmermans 1992), before their expression on the surface membrane.

It is the aim of this study to further characterize the differentiation antigens expressed on carp germ cells. Firstly, closer analysis was made of the intracellular staining pattern obtained with these Mabs by combining a study at the ultrastructural level with light microscopical immunostaining.

Secondly, a biochemical analysis of surface antigens recognized by MAbs will be decribed. In this first approach the analysis has been confined to spermatozoa as the latter can be easily obtained in rather large amounts, contrary to spermatogonia. The MAbs WCS 3, 17, 28 and 29, reacting with germ cells from early larval stages onward, before the onset of spermatogenesis, were used in this analysis.

MATERIAL AND METHODS

Animals

The common carp, *Cyprinus carpio* L., was reared in the laboratory under standard conditions at a water temperature of 23°C (cf. Parmentier and Timmermans 1985).

Gonad preparation for histology and electron microscopy

Carp specimens were selected from a group of medium body size at the age of 19 weeks and anaesthetized with 0.01% (w/v) tricaine methane sulphonate (TMS; Crescent Research Chemicals, Arizona, USA) before killing. Gonads were dissected out and parts were either frozen in liquid nitrogen to prepare frozen sections or fixed in Bouins' solution, dehydrated, embedded in paraffin, and serially sectioned. Sections of frozen or Bouin-fixed testes were used for immuno-histochemistry.

Other parts of the same gonads were processed for electron microscopy. These parts were fixed in a mixture containing 1% OsO_4, 2% glutaraldehyde and 1% potassium dichromate buffered with 0.1 M sodium-cacodylate buffer (pH 7.2) for 2 h at 0°C, dehydrated and embedded in Epon 812. Ultrathin sections were contrasted with saturated uranyl acetate and lead citrate and examined using a Philips EM 400 electron microscope.

Immunohistochemical staining

Slides with Bouin-fixed gonadal sections (for light microscopy) were incubated with 50 μl ascites fluid (diluted 1 : 1000 in PBS containing 10% newborn calf serum) from the monoclonal antibodies (MAbs) WCS 3, 17, 28 and 29 respectively, for 45 min in a humidified atmosphere and then washed with PBS. Control incubations, in which the first

antibody was substituted by PBS or myeloma ascites were used in each assay. Afterwards, 50 μl of a 1 : 40 diluted solution of rabbit-anti mouse immunoglobulin antiserum, conjugated to horse radish peroxidase (RAM/Ig/HRP; Nordic, Tilburg, The Netherlands) was applied for 45 min. After washing in PBS, the slides were incubated for 10 min with 0.05 M diaminobenzidine (DAB, Sigma) in 0.05 M Tris/HCl buffer (pH 7.6) containing 0.01% H_2O_2. The slides were then washed in distilled water, counterstained with Mayer's hematoxylin and mounted.

Slides with frozen sections were immunostained with the same MAbs as first antibodies and RAM/Ig/FITC (Nordic, Tilburg, The Netherlands) in a 1 : 40 dilution as second antibody. After washing with PBS the slides were mounted in a solution of PBS/glycerol (1/9, v/v) containing 1 mg/ml p-phenylenediamine to prevent photobleaching of the fluorochromes (Johnson & Noqueira Araujo 1981). The same control incubations as with Bouin-fixed sections were used. The immunoperoxidase and immunofluorescence reactions were studied with a Nikon Microphot FXA microscope equipped with incident-fluorescence optics.

Collection of sperm

Adult carp were injected intramuscularly with 0.4 mg/kg body weight carp pituitary extract (Hydroquest International, Rosemont, USA). Twelve hours later the fish were dry stripped. The spermatozoa were washed three times and suspended in phosphate-buffered saline (PBS), pH 7.4 at 4°C.

Preparation of spermatozoa protein extract and immunochemical identification of antigens

In a first trial carried out in 1987 (spermatozoa extract prepared according to the procedures of Fenderson *et al.* (1984) and Wolf et al. (1983); SDS-PAGE analysis and immunoblotting essentially according to Laemmli (1970) and Towlin *et al.* (1979), respectively), only a band with the low Molecular weight (Mw) of 8-12 kD was found with the MAbs WCS 3 and WCS 28, whereas no antibody binding was observed with WCS 17 and WCS 19. From these results it was concluded that the concentration of spermatozoa

membrane antigens was too low to allow visualization with the then available methods. As recently new methods for the isolation of cell membrane proteins have become available, resulting in higher yields of surface membrane proteins, and also a sensitive immunoluminescence method has been developed, the preparation of spermatozoa lysate and the immunoblotting procedures were repeated.

Preparation of protein extract from spermatozoa

Membrane lysates of spermatozoa were prepared essentially according to the method used by Koumans-van Diepen *et al.* (1995) for carp leucocytes. The spermatozoa ($1x10^9$) were centrifuged for 10 min at 680 x (4°C) and resuspended in 10 ml destilled water in order to allow them to swell. Thereafter, the cells were fragmented in a small Potter tube for 10 min. on ice and the supernatant was centrifuged for 1 h at 100.000 x g (4°C). The membranes (pellet) were washed in PBS without bovine serum albumin (BSA) and subsequently incubated for 5 d (4°C) with PBS without BSA + 3% CHAPS (3-[3-cholamido-propyl-dimethylammonio]-1-propanesulphonate) to which the protease inhibitors PMSF (phenyl-methyl-sulphonyl-fluoride, 1 mM, and ethylmaleinid, 1 mM) had been added. The pellet was then centrifuged for 1 h at 100.000 x g (4°C). The supernatant (in PBS + CHAPS) was stored in small volumes at -20°C and thawed shortly before use.

Dot blot analysis

Dot blot analysis was performed essentially according to the method of Koumans-van Diepen *et al.* (1995) for carp leucocytes. A sheet of nitrocellulose (NC; BA 85, Schleicher & Schuell, Dassel, Germany) was incubated in a 96-well dot blot apparatus (minifold SRC-96; Schleicher & Schuel, Dassel, Germany) with 200 μl PBS for 15 min. at room temperature (RT). The spermatozoa protein extract, after thawing and washing in PBS, was added in volumes of 100 μl to the wells in fivefold at dilutions of 1/2, 1/4, 1/8, 1/16 ... etc, until 1/4096 per well and allowed to settle during 10 min. The NC sheet was then blocked in PBS + 1% acetylated BSA for 1 h at RT and subsequently overnight at 4°C. After washing in PBS 5 ml of the following MAbs, in PBS + 1% NCS, were each added to one dilution series of spermatozoa lysate: WCS 3 (1 : 500), WCS 17 (1 : 400), WCS 28 (1 :

400) and WCS 29 (1 : 500) for 1 hr at RT. AS a control PBS + 1% NCS was used instead of the first antibody. After washing thrice in PBS + 0,05% Tween 20, and once in PBS, the conjugate RAM/Ig/HRP (Dako) was added, diluted 1 : 1000 in PBS + 1% NCS for 45 min. at RT. Following one wash in PBS + Tween 20 and 3 washes in 0.05 M Tris/HCl buffer (pH 7.5), the blots were stained for 10 min with 0.05 M DAB (Sigma) in 0.05 M Tris/HCl buffer (pH 7.6) containing 0.01% H_2O_2, then washed in distilled water and dried.

Western blotting and immunochemical identification of antigens

Samples of spermatozoa lysate were diluted with 200 μl loading buffer (5 ml 0,5 M tris/HCl, pH 6.8; 0.8 g SDS; 4 ml glycerol; 0.2 ml 1,5% bromophenol white, 320 mg DTT[di-thio-triethol]; 0.8 ml distilled water) and distilled water (60 μl) and heated for 2 min. at 96°C. They were then analysed by SDS-PAGE and transferred onto nitrocellulose (BA 85; Schleicher & Schuell, Dassel, Germany) at RT. Transfers were cut and one of the lanes with sample was stained with Coomassie brilliant blue. (Prestained) SDS-PAGE Molecular Weight Standards (Biorad Laboratories, Richmond, CA, USA) were used. As a control carp thymocyte lysate (prepared in the same manner as spermatozoa lysate) was used for SDS-PAGE analysis and transfer to NT sheets.

Transfers were saturated in TTBS (0.02 M Tris-HCl, pH 7.4-7.6, 0.05 M NaCl, 0.05% Tween 20) for 1 h at RT, washed twice in TTBS and each incubated with 5 ml of one of the MAbs WCS 3 (1:500), 17 (1:400), 28 (1:400) or 29 (1:500) for 1 h at RT. Subsequently, transfers were washed thrice in TTBS and incubated with RAM/Ig/HRP (Dako) for 1 h at RT, washed thrice in TTBS, followed by removal of excess fluid. Thereafter, the blots were incubated with ECL-Western blotting detection reagents (Amersham Life Science) for 1 min., wrapped in transparant foil and exposed for 15 sec to Fuji RX film. The film was then dried and photographed.

Periodate treatment

In order to investigate whether carbohydrate or protein determinants are recognised, microscopic slides with frozen sections of carp testis were treated for 10 min. with cold acetone (4°C), washed in sodium acetate buffer (pH 4.5), and incubated for 1 h at RT with

10 or 50 mM (or for 22 h with 100 mM) periodate in 50 mM sodium acetate buffer (pH 4.5). After two washes in acetate buffer, the slides were treated with 10 mM sodium borohydride in PBS (pH 7.2) and finally washed with PBS. The treatment with sodium borohydride reduces aldehyde groups generated by periodate oxidation to alcohols and prevents non-specific cross-linking of antibody to antigens (Woodward et al. 1985). The slides were then thrice washed with PBS, treated with 10% NCS in PBS for 10 min (RT) to prevent unspecific binding of antibody, washed in PBS and immunostained with WCS 3, 17, 28 or 29 and FITC labeled conjugate as described.

To investigate whether protein determinants were affected by the periodate treatment, control sections were immunostained with anti-desmine antibody as first antibody, recognising desmine in peritubular cells of carp testis (Timmermans et al. 1993). As a control the first antibodies were omitted.

RESULTS

Reactivity of anti-spermatozoa MAbs with germ cells in testes

The four MAbs WCS 3, 17, 28, 29 reacted with surface membranes of ejaculated spermatozoa, with spermatozoa and precursor germ cells in sections of frozen or Bouin-fixed adult testes and with isolated (precursor) germ cells, thereby confirming previous results (Parmentier *et al* 1984; Winkoop & Timmermans 1990).

In addition to immunostaining of surface membranes of precursor germ cells, often an immunoreactive cytoplasmic patch was seen in frozen or Bouin-fixed sections after staining with these four MAbs (Fig 1). It was noticed that in particular in testes shortly after the onset of spermatogenesis, when proliferating spermatogonia were relatively numerous, these cyptoplasmic patches were located frequently in a mirror image-like fashion in two adjacent large spermatogonia (Fig 1). By using electron microscopy, Golgi complexes were found to be arranged in a strikingly similar way in such adjacent spermatogonia on both sides of the contacting plasma membranes (Fig 2), suggesting that the immunostained cytoplasmic patches in the germ cells represent Golgi complexes and, hence, reflect either the synthesis or processing of antigenic components destined for the germ cell surface.

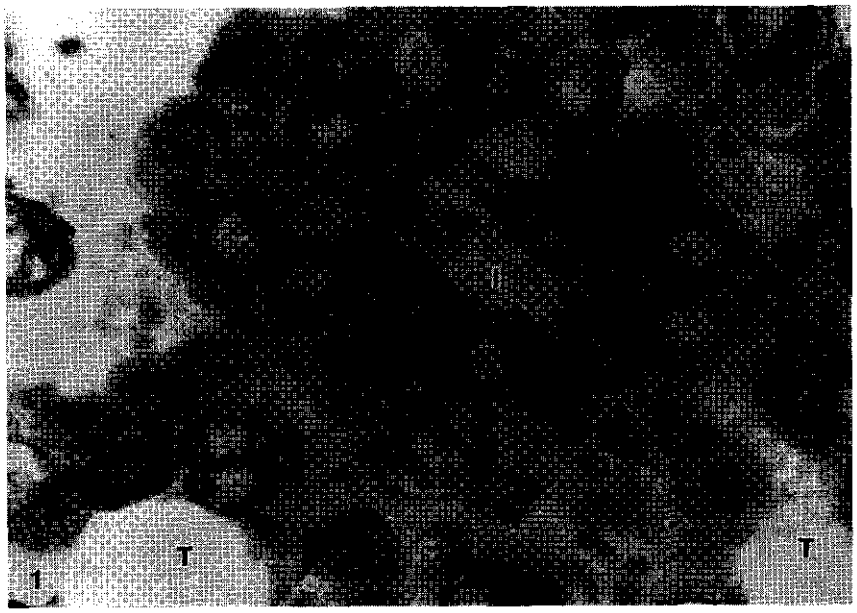
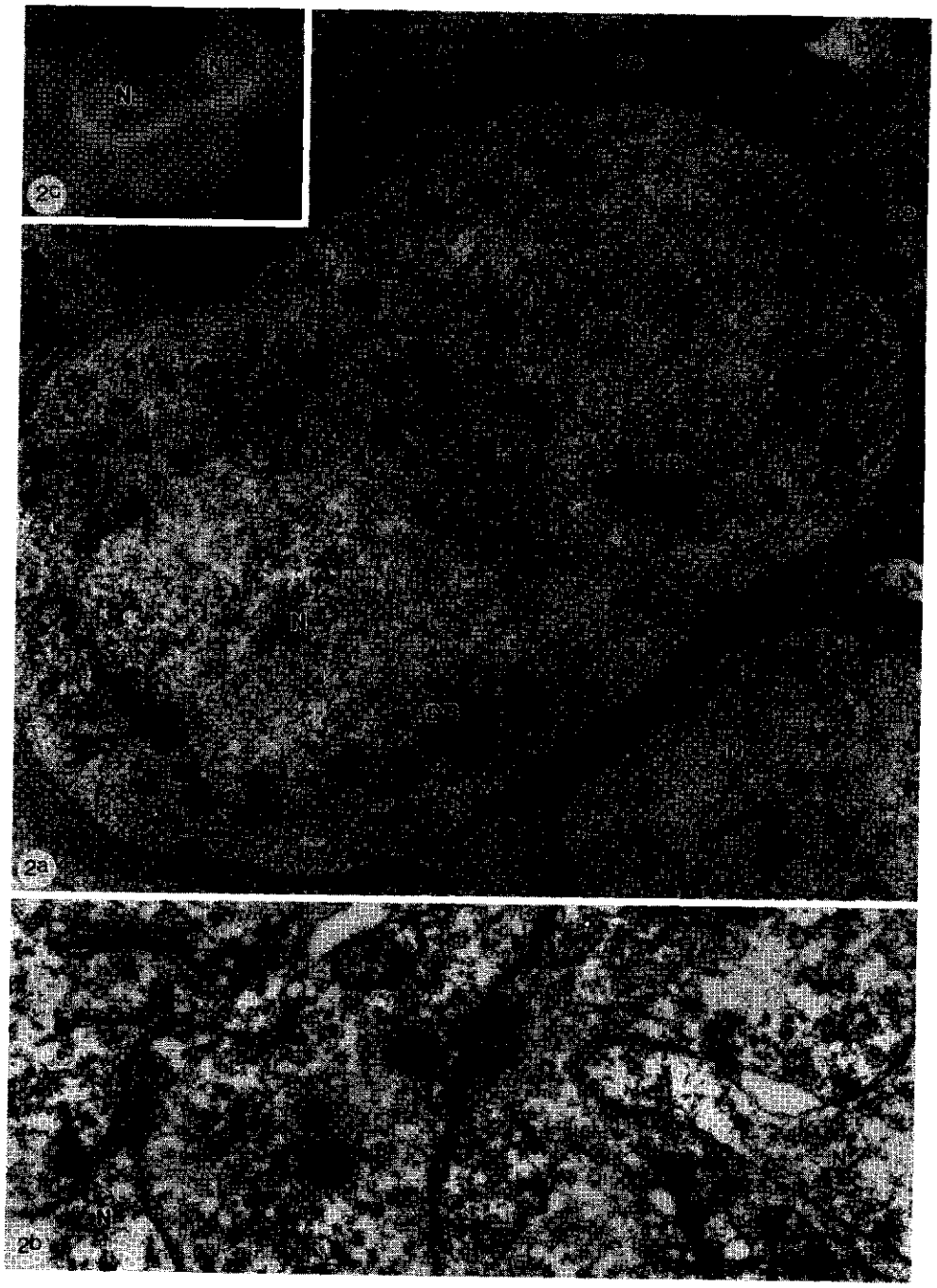


Figure 1. **Section of Bouin-fixed testis of carp at week 19, incubated with WCS 3 in the immunoperoxidase test, revealing positive cytoplasmic patches (arrows) in two adjacent spermatogonia. A similar reaction was obtained with WCS 17, 28 and 29. T, testis tubule; I, interstitial tissue. x 2000**

Immunobiochemical characterization

Spermatozoa protein extract was prepared according to a recent procedure, developed for the extraction of surface membrane proteins from carp leucocytes (Koumans-van Diepen, 1993). Coomassie Blue staining revealed that Mw of spermatozoa proteins in SDS-PAGE gels ranged between 22 and 143 kD (Fig 3).

Figure 2. **a. Ultrastructure of two adjacent spermatogonia in spermatogenic testis of carp at week 19. Note that the Golgi complexes (arrowheads) are located in a mirror image-like fashion on both sides of the contacting cell membrane. N, nucleus; PDB, perinuclear dense bodies; Se, cell of Sertoli. x 7500 b. Detail of the indicated part of Fig 2a showing the Golgi complexes of both spermatogonia more clearly (arrowheads). x 120.000 c. Detail of a light microscopical picture of two adjacent spermatogonia showing immunopositive cytoplasmic patches (arrows) after immuno-peroxidase staining with WCS 3. x 2000**


N
2c
N
2a
N
2b

Dot blot analyses with the indirect immunoperoxidase test confirmed the presence of antigens recognized by WCS 3, 17, 28 and 29 in the spermatozoa protein extract. In immunoblot analyses with the immunoperoxidase test positive bands were obtained with all four MAbs, although these bands were very weakly stained. For that reason the recently developed immunoluminescence method was used for the immunoblot analyses. With the latter method a set of at least 10 distinct bands were observed with WCS 3 and WCS 28 (Fig 3), ranging from a Mw of approximately 30 kD to a Mw > 200 kD. With WCS 29 a slightly different set of 10 bands was immunostained within the same range of mW, and with WCS 17 a set of at least 5 immunoreactive bands in approximately the same range of Mw. However, the bands, recognized by WCS 29 and WCS 17 were distinctly weaker stained than those recognized by WCS 3 and WCS 28. Moreover, in repeated immunoblottings with WCS 3 and also with WCS 28 positive bands with Mw of approx 10-15 kD were intensely stained. A similarly located band was weakly stained by WCS 17.

As a control, immunoblots of carp thymocyte membrane proteins were used. The results showed that those immunoblots were not stained with WCS 3 and WCS 28, whereas a set of bands was faintly stained with WCS 17 and WCS 29 (Fig 3).

Effect of periodate treatment

To investigate the possible carbohydrate nature of the recognized epitopes cryosections of frozen carp testis were pre-incubated with periodate. The results show that binding of the four MAbs was not reduced after treatment with 10 mM periodate (Table 1). After treatment with 50 mM periodate immunofluorescence with WCS 17 and WCS 29 was distinctly reduced but not with WCS 3 and WCS 28 (Table 1; Fig 4). After treatment with 100 mM periodate no immunofluorescence was observed with WCS 17 and WCS 29, whereas WCS 3 and WCS 28 showed only a weak fluorescence (Table 1). With the antidesmine antibody immunofluorescence was not diminished after treatment with 50 mM periodate, but distinctly reduced after treatment with 100 mM periodate (Table 1).

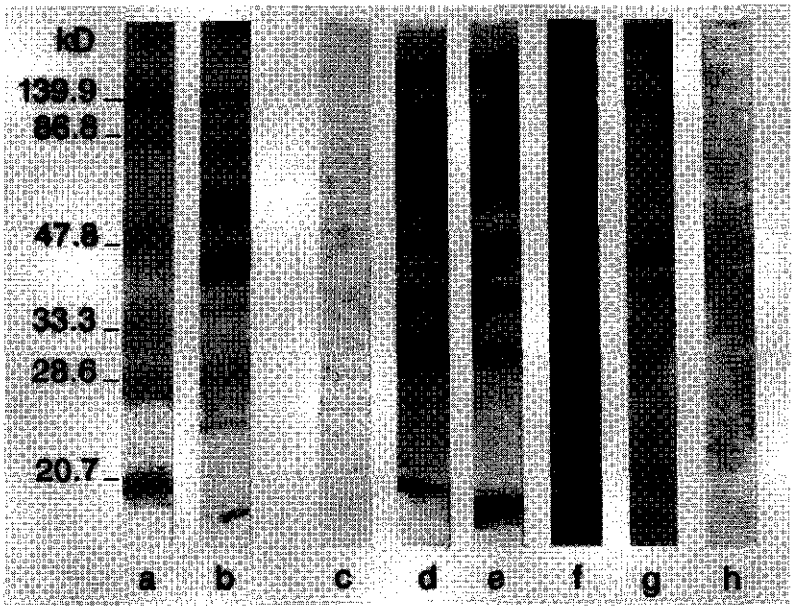
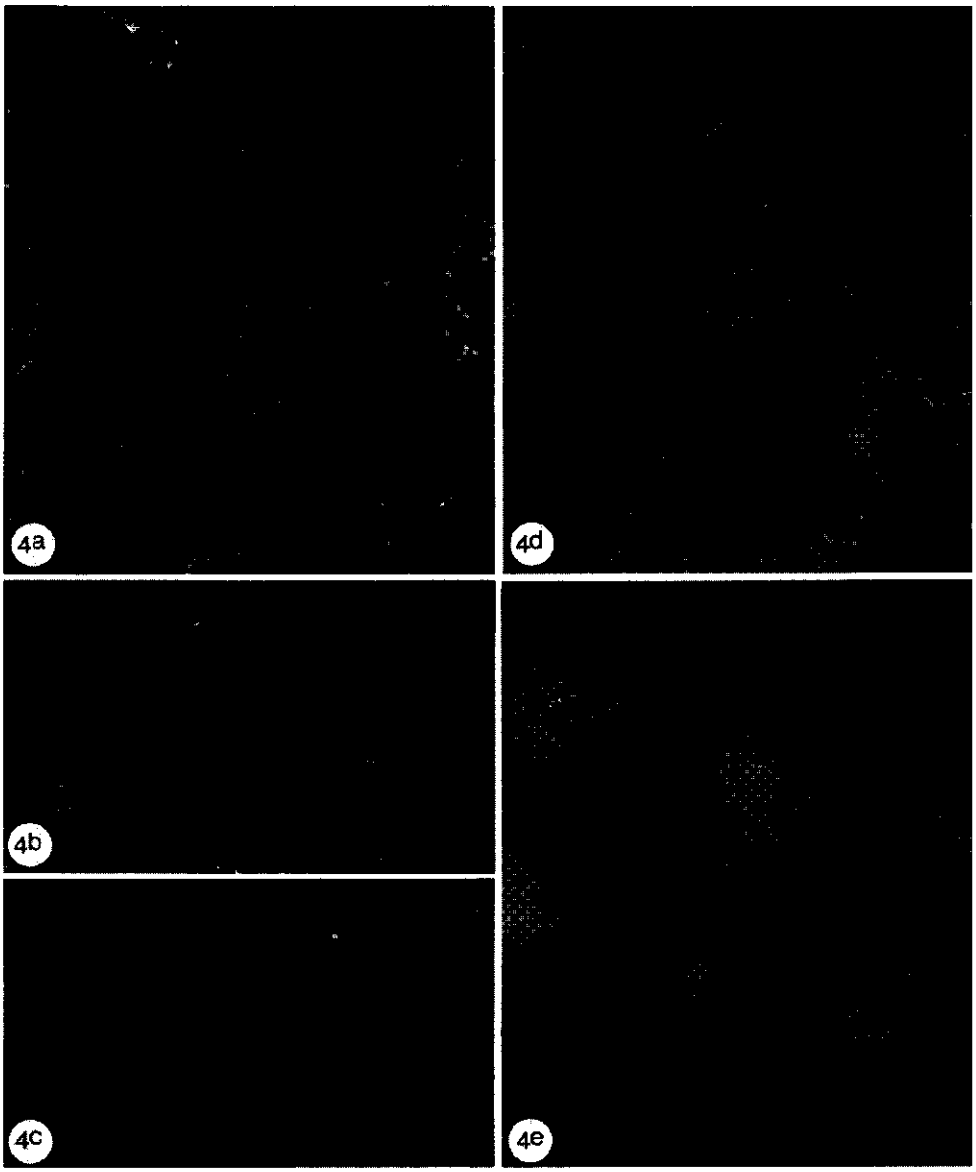


Figure 3. **Immunoblotting analysis of carp spermatozoa lysate with anti-spermatozoa MAbs WCS 3 (lane d), WCS 28 (lane e), WCS 17 (lane f) and WCS 29 (lane g). Left: numbers indicate Mw values of reference proteins in kD (lane a). Lane b shows the protein pattern on the blots as visualized with Coomassie brilliant blue. Lane c is an immunoblotting of thymocyte lysate with WCS 3 (a similar blotting was obtained with WCS 28) and lane h an immunoblotting with WCS 29 (a similar blotting was obtained with WCS 17).**

Table 1. **Effect of periodate on the reactivity of monoclonal antibodies with germ cells in the testis of carp as visualized with immunofluorescence.**

MAbs	Control	Periodate treatment		
		10 mM	50 mM	100 mM
WCS 3	++	++	++	+
WCS 17	++	++	+	-
WCS 28	++	++	++	+
WCS 29	++	++	+	-
anti-desmine	++	++	++	+

MAbs = Monoclonal Antibodies; WCS = Wageningen Carp Spermatozoa.


4a
4d
4b
4c
4e

DISCUSSION

Histochemistry

In immuno-stained, Bouin-fixed sections it was observed for several MAbs, especially clear during development and around the start of spermatogenesis, that a positive patch is present in the cytoplasm of germ cells in addition to a positive reaction on the outer cell membrane. Such cytoplasmic patches have been observed in mammalian PGCs too, and they were supposed to be the sites of synthesis of these MAbs (Heath & Wylie 1981). In cysts containing two spermatogonia the MAB-positive patch was located in a mirror image-like fashion. In EM-sections, prepared from similar developmental stages it appeared that Golgi-complexes are present in a mirror image-like fashion in cysts containing two spermatogonia. This indicates strongly that the MAb-positive patches represent Golgi-cisternae. Since it is generally accepted that new membrane constituents are processed within Golgi-complexes in these cells (Alberts *et al.* 1989, 1994), the patches point to active processing of new membrane constituents containing the differentiation antigens recognized by the WCS MAbs.

Biochemistry

The results show that with the sensitive immunoluminescence method a set of at least 10 distinct bands, ranging from Mw of approximately 30 kD to > 200 kD was recognized in immunoblots of carp spermatozoa protein extract by WCS 3 and WCS 28. A slightly different set of bands was immunostained, although more weakly, by WCS 29 and by WCS 17 in the same range of Mw. It is possible that the antigenic determinants recognised by each

Figure 4. **Immunofluorescence staining of carp testis after treatment with periodate (frozen sections). a-c. Immunostaining with WCS 29. d. e. Immunostaining with WCS 28. a. d. Control sections, not treated with periodate. b. e. Treated with 50 mM periodate; note that fluorescence is greatly reduced in (b) but not in (e). c. Treated with 100 mM periodate, showing that no binding with antibody occurs. With WCS 17 the same results were obtained as with WCS 29 and with WCS 3 the same results as with WCS 28. x 450**

of them are part of several differently sized macromolecules. This has to be investigated further. No antigens were recognized by WCS 3 and WCS 28 in immunoblots from carp thymocyte surface membrane lysate and only a set of very weak bands with WCS 29 and WCS 17. This provides evidence that the recognized macromolecules in the immunoblots of carp spermatozoa lysate are specific for carp spermatozoa. It confirms previous studies on unfixed frozen and on fixed tissue sections in which it was shown that the anti-carp spermatozoa MAbs react exclusively with germ cells (Parmentier *et al.* 1984, Parmentier & Timmermans 1985).

The results of the present study show that immunostaining of frozen testis sections by WCS 17 and WCS 29 was greatly diminished after pretreatment with 50 mM periodate and completely inhibited after pretreatment with 100 mM periodate. However, with WCS 3 and WCS 28 and also with the anti-desmine antibody, immunostaining was not inhibited but only diminished after pretreatment with 100 mM periodate and not at lower mM periodate. These results may indicate that WCS 17 and WCS 29 recognize glycoproteins (Woodward *et al.* 1985) and that the two other MAbs recognize protein macromolecules or liproteins.

To our knowledge only three studies have been carried out on the biochemical analysis of spermatozoa membrane proteins in a fish species (Loir *et al.* 1990; Lou *et al.* 1990; Labbé & Loir 1991, in trout) and only two reports have appeared on anti-spermatozoa antibodies in a fish species (Lou & Takahashi 1991, Mochida et al. 1994) in addition to the work on carp. In order to obtain information on the sperm surface properties, Lou & Takahashi raised conventional antibodies against spermatozoa of the Nile tilapia. These spermatozoa consist, as in carp (van Winkoop & Timmermans 1995) of a round head without an acrosome, a short midpiece and a tail (Lou & Takahashi 1989a). The latter authors observed by immunoblotting analyses of isolated spermatozoa membrane extract, that at least 15 constituent protein macromolecules were recognized as surface antigens by their antibodies. Six polypeptides, corresponding with the major surface antigens, were identified as autoantigens by autoantibodies purified from male Nile tilapia specimens which had been previously immunised with allogeneic spermatozoa (Lou *et al.* 1989).

As a step towards determining the functions of sperm autoantigens in fish, Mochida *et al.* (1994) raised MAbs against Nile tilapia spermatozoa autoantigens, which had been isolated

with autoantibodies prepared according to the method of Lou *et al.* (1989). Four MAbs were produced, recognizing polypeptides with Mw of approximately 27, 40, 80 and 120 kD, i.e. well within the range of Mw, recognized by the four anti-carp spermatozoa MAbs. Nor Lou and Takahashi, nor Mochida *et al.* investigated a possible glycoprotein nature of the antigens recognized by their antibodies.

In mammals it is known that spermatozoa and precursor germ cells from the early pachytene stage onward are shielded from the individual's immune apparatus by a blood-testis barrier formed by Sertoli cells, connected to each other by tight junctions (Dym & Fawcet 1970; Dym & Cavicchia 1979). It is known that in these sheltered tubular compartments autoantigens arise on precursor germ cells and spermatozoa. Most of the antibodies raised against mammalian spermatozoa, recognize antigens which appear on germ cells from the pachytene stage or from later spermatogenic stages onward and these antigens are considered to be autoantigens (for a review, see Eddy 1988). It is supposed that these autoantigens play a role in the fertilization process. Consequently, the antibodies recognizing these antigens may be used to prevent fertilization. Indeed, Primakoff (1988) raised antibodies to human spermatozoa autoantigens which prevented fertilization, thus proving their ability as possible antifertility vaccines.

In a number of teleosts the presence of a blood-testis barrier has been demonstrated (Abraham *et al.* 1980, in *Aphanius dispar*; Parmentier *et al.* 1985 in *Cyprinus carpio*; Shibata & Hamaguchi 1986, in *Oryzias latipes*; Lou & Takahashi 1989b, in Nile tilapia) In these fish species this barrier is also formed by tight junctions between Sertoli cells, but, contrary to mammals, only spermatozoa and late spermatids are shielded from the blood. Consequently, autoantigens will be present only on these germ cell stages. As could be expected, immunostaining of Nile tilapia testis sections with three of the MAbs raised against tilapia autoantigens was restricted to late spermatids and spermatozoa (Mochida *et al.* 1994). However, one MAb, TAT_{10}, reacted also with early spermatogonia, but according to Mochida *et al.* this reaction was localized in the cytoplasm of these germ cells.

The four anti-carp spermatozoa MAbs, used in the present study, recognize antigenic determinants on the surface membrane from prespermatogenic stages onward, three even on primordial germ cells from early larval stages onward, in which germ cells are accessible for

macromolecules from the blood (Timmermans *et al.* 1985). Consequently it may be concluded that the four carp MAbs do not recognize autoantigens and that the antigens recognized by these MAbs differ from those recognized by the four anti-Nile tilapia autoantigens MAbs. The recognized carp antigens are supposed to be differentiation antigens appearing on the surface membrane of the germ cells when new differentiation steps occur. The available MAbs will allow to isolate and sequence the recognized antigen(s) enabling the investigation of their possible function(s). This will be the object of future studies.

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Stimulation of gonadal and germ cell development in larval and juvenile carp (*Cyprinus carpio* L.) by homologous pituitary extract

A. van Winkoop, L.P.M. Timmermans and H.J.Th. Goos

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SUMMARY

The data presented show that in larval carp, gonadal size has increased distinctly after treatment with homologous pituitary extract (PE). Moreover, the precocious onset of primordial germ cell proliferation and of sex differentiation into male and female gonads was induced. Body weight of treated and control specimens did not differ significantly from each other throughout the experiment. Treatment of juvenile carp with homologous PE led also to an increase in gonadal size without concomitant increase in body weight. Induction of precocious spermatogenesis was observed too. GTH-levels in control and PE-treated carp were monitored by means of a homologous radioimmunoassay, showing that in PE-treated carp the GTH-level was distinctly elevated. The possible role of pituitary hormones in larval and juvenile gonadal development is discussed.

INTRODUCTION

The importance of the role of pituitary gonadotropins in germ cell maturation in the adult gonads of fish is generally accepted. However, much less is known about their role in gonadal and germ cell development during larval and juvenile stages. Non-mammalian vertebrates, especially fish, developing very early through a pelagian (or free swimming) stage are outstanding models to study the onset of development of the brain pituitary-gonadal axis, as such species can already be subjected at embryonic stages to experimental treatments. This in contrast to mammalian species, where comparable development takes place during intra-uterine life.

In the freshwater teleost, the common carp *Cyprinus carpio* L., the gonads and germ cells develop in a particular way. When the animals are cultured under standard conditions at 23°C, primordial germ cells (PGCs) are already present at the sites of the "gonadal ridges" in newly hatched larvae at day 3 after fertilization (Parmentier & Timmermans 1985). Gradually, somatic gonadal tissue appears around the PGCs and the gonadal primordia start to increase in size at about week 3 after fertilization. However, the number of PGCs remains constant up to week seven (Parmentier & Timmermans 1985). During this mitotic resting

period of PGCs, they distinctly increase in size and intracellular differentiation takes place prior to proliferation (van Winkoop *et al.* 1992). These changes in PGCs are accompanied by the appearance of cell surface differentiation antigens, as demonstrated by immunocytochemistry using monoclonal antibodies (Parmentier and Timmermans 1985; van Winkoop & Timmermans 1992). The indifferent gonads gradually change into immature female or male gonads, which can be detected by light microscopy from about week 10. The process of oogenesis starts at week 16 and spermatogenesis at week 19 (Parmentier & Timmermans 1985).

In the present study it was investigated whether pituitary extract stimulates gonadal and germ cell development in larval carp. Induction of precocious spermatogenesis in juvenile carp was studied for comparison. Effects of pituitary extract on larval fish have not been studied hitherto. The larval stages were treated, using the protocol effective in juvenile stages, from week 4 onwards, when the gonadal primordia contained mitotically resting PGCs only. The effects on the development of these early gonadal stages and on the proliferation of the PGCs were studied.

MATERIALS AND METHODS

Animals

Specimens of *Cyprinus carpio* L. were reared at the laboratory under standard conditions at 23°C (Parmentier & Timmermans 1985). Salinity, pH and nitrogen levels were monitored daily. For the experiments specimens were selected from groups of medium body size.

Treatment with carp pituitary extract (PE)

Four injection experiments with carp PE were performed. The extract was prepared by suspending carp pituitary powder (Crescent Research Chemicals, Phoenix, Arizona, U.S.A.) in phosphate-buffered saline (PBS; pH 7.4) for two hours at a concentration of 16 mg/ml. Undissolved particles were removed by centrifugation (500 x g) and the extract was stored in small volumes at -20°C. For each experiment samples from the same pituitary extract

were used. Samples were thawed shortly before injection.

In experiment 1 animals were injected two times a week with carp PE (the equivalent of 2 μg undissolved pituitary powder/g body weight) from week 13 after fertilization (a.f.), when the testis was still immature and contained no signs of spermatogenesis. The treatment ended at the end of week 21. Control animals, placed in separate tanks, received PBS. At weekly intervals ten animals per group were killed. Plasma samples were collected from each fish for RIA analysis and gonads were examined histologically.

In experiment 2, animals were treated three times a week with a dose of 40 μg/g body weight from week 13 until week 21. Controls received PBS. At week 17 and 21 ten animals per group were killed and the gonads were examined histologically, RIA analysis was not performed.

In experiment 3, animals were treated from week 13 until week 19, using the same protocol as in the second experiment. At weekly intervals ten animals per group were killed. Plasma samples were collected from each fish for RIA analysis and gonads were examined histologically.

In all three experiments, carp pituitary extract was injected intraperitoneally between 16.00 and 17.00 h. Animals were sacrificed and blood samples taken from each individual animal, always 48 h after the last injection. Anaesthesized animals were bled from the tail vein. Blood samples were stored over crushed ice and centrifuged. The blood plasma was then collected and stored at -20°C until analysis.

In experiment 4, carp larvae were treated from week 4 until the end of week 8 with the same doses as in the second and third experiment. At week 4, 5 and 8, groups of ten larvae were fixed. Due to the small size of the specimens blood was sampled at week 8 only and pooled from 10 animals.

Examination of gonads

Prior to decapitation, animals were anaesthesized with 0.01 % tricaine methane sulfonate (MS 222; Sandoz, Basel, Switzerland). Due to the small size of the gonads in the larval and juvenile specimens, gonadal length and weight could not be measured. Gonads, still attached to dorsal parts of the trunks, were processed for light microscopy. Tissues were fixed in

Bouin's fluid, dehydrated and embedded in paraplast. Cross-sections (5 μm) of the anterior and mid-regions of the juvenile gonads, at 50 μm intervals, were stained with Crossmon. The larval gonads were completely serionally sectioned and stained.

Quantification

The average surface of gonadal cross-section per animal was determined. Five separate cross-sections in the rostral and five in the mid-regions of the gonads (from the larval gonads only sections from the middle region), were projected with an Olympus FHT projection microscope on a Calcomp 9100 digitizing tablet (resolution 40 lines/mm) connected to a Macintosh IIfx. The outlines of the projected sections were traced with the cursor of the digitizing tablet. An object micrometer was used for calibration. From each age group gonads were measured. At week 4, 5 and 8, all germ cells present in the gonads were counted in serial sections in 5 animals per age group.

GTH measurement

Plasma GTH levels were determined for each individual fish by radioimmunoassay (RIA) according to the method of Breton *et al.* (1971), using carp-GTH for labeling and as standard hormone and an antibody against the ß-chain of carp-GTH at a final dilution of 0.5 x 10^{-5}. The GTH content in the samples of pituitary extract was measured with RIA and calculated at 2.7 mg/ml (i.e., per 16 mg pituitary powder, see treatment with carp pituitary extract). Thus in the experiments the amount of PE extract injected was equivalent to 2.7 μgGTH/μl. Consequently, in experiment 2, 3 and 4, 6.75 μgGTH/g body weight was injected. The sensitivity of the RIA ranged from 0.80-64 ng GTH/ml. Purified carp GTH and ßGTH were a gift from Dr. E. Burzawa-Gérard.

Statistics

Because of unequality of variances between treated groups and controls a nonparametric test (Wilcoxon) was used to estimate the significance of the measured differences.

RESULTS

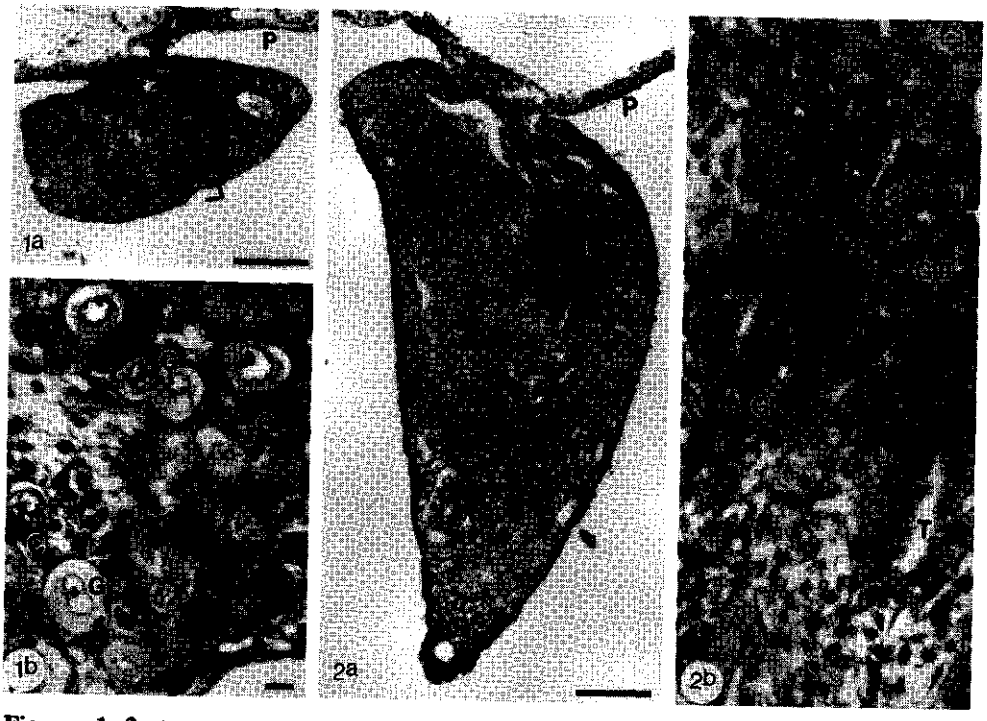
In the present study the ages of the developmental stages of carp will be given as "after fertilization" (a.f.).

Four periods can be distinguished during gonadal development in carp: a. *formation of the gonadal primordium* (week 1-5), during which somatic cells assemble around the mitotically silent PGCs (Fig 4a, b). b. *indifferent gonad* (week 6-10), characterized by the appearance of stromal cells and blood vessels together with the onset of a fast PGC proliferation. In cross sections of gonads often more than one PGC can be observed and each is individually surrounded by cyst cells (Fig 4c). c. *male and female immature gonads*, in which the still threadlike gonads gradually increase in size, but gametogenesis has not yet started. [The germ cells, now named pre-oogonia or pre-spermatogonia are scattered through the gonadal tissue, each separately surrounded by cyst cells (Fig 1). This period lasts from week 10-16 in females and from week 10-19 in males.] d. *Early maturing gonads*, in which gonadal size increases distinctly and gametogenesis has started. [In the testis the onset of spermatogenesis is corroborated by the appearance of tubules in which the walls are lined by cysts containing spermatogenic stages (Fig 2).]

Effects of pituitary extract (PE) on juvenile carp

In the first experiment a dose equal to that used for stripping of mature carp (2 μg/g body weight, Weil *et al.* 1986) was given. In RIA it was shown that the GTH content in blood samples from PE-treated fish remained low and did not surpass the level in the control fish. In series of cross sections no significant differences in gonadal diameters were found between treated fishes and controls and no precocious spermatogenesis was observed.

The second experiment was a pilot experiment in which the injected doses of pituitary extract were elevated twentyfold (40 μg/g body weight). In this experiment, it was observed in series of cross sections that at the age of week 17, four weeks after the onset of the injections, precocious spermatogenesis had been induced. Gonadal diameter had increased distinctly in all treated specimens and in three out of five specimens tubules had been formed containing cysts with spermatogenic stages (not shown). The controls contained only pre-



Figures 1, 2. Cross sections through carp testes at week 18.

Fig 1. Untreated, showing many prespermatogonia (G) each individually surrounded by cyst cells (C) scattered through the interstitial tissue (I). Fig 1a. General view. Fig 1b. Detail of Fig 1a.

Fig 2. Treated with homologous pituitary extract. Note the considerable increase in surface area. Tubules have been formed and spermatogenesis has started. Fig 2a. General view. Fig 2b. Detail of Fig 2a, showing cross section through tubules (T), cysts containing primary spermatogonia (GI), and cysts with secondary spermatogonia (GII). I, interstitial tissue; P, peritoneum. Figs 1a, 2a, x 130 Scale bar: 100 μm, Figs 1b, 2b, x 620. Scale bar: 10 μm.

spermatogonia, each separately surrounded by cyst cells.

The third experiment was a repeat of the second experiment, but the effect of PE-treatment was followed in detail in the course of the experiment. In cross sections it was observed that from one week after the onset of treatment the gonadal size had increased significantly in comparison with controls (Figs 1, 2, 3). At the ages of 14-17 weeks, the increase in gonadal size, as determined by increase in surface area of cross sections (Fig 3), was largely due to fluid filled spaces in the gonads. At the age of 18 weeks, fluid filled spaces were no longer observed, although the increase in surface area was considerable. Moreover, in three out of five experimental specimens spermatogenesis had started precociously, whereas in controls only pre-spermatogonia were present, showing that spermatogenesis had not yet started (Figs 1, 2).

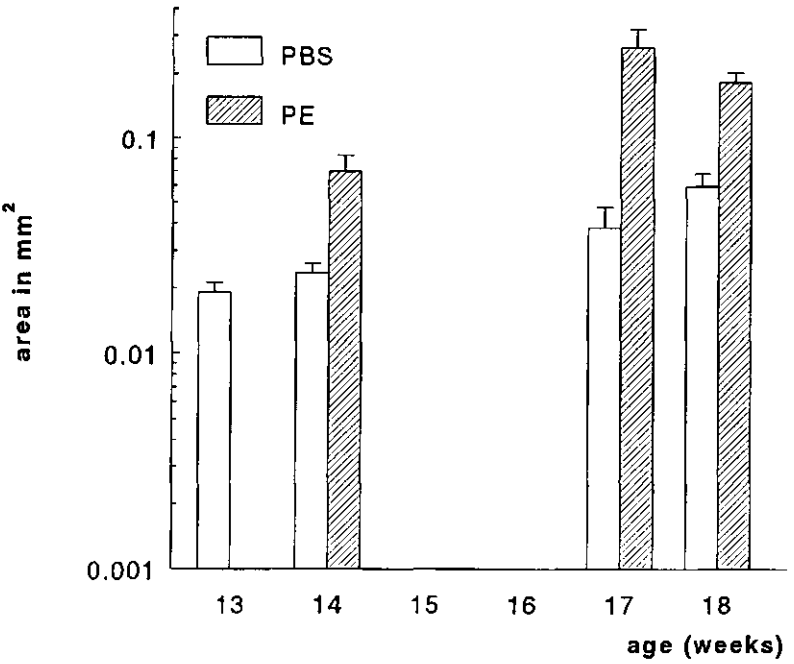


Figure 3. **Increase in gonadal size of juvenile carp after treatment with homologous pituitary extract as measured in cross sections through carp gonads at week 13 to week 18. Note that the surface area had already increased one week after the onset of treatment. For numbers of animals see tables 1 and 2. Significance: week 14, $P < 0.005$; weeks 17, 18, $P < 0.01$.**

Although gonadal diameter had increased distinctly, the body weight of experimental and control animals did not differ significantly from each other throughout the experiments (table 1). In all experimental specimens of juvenile fish, when measured from one week after the onset of treatment, the plasma level of GTH was elevated 5-20 fold in comparison with controls (table 2).

Effects of pituitary extract on larval carp

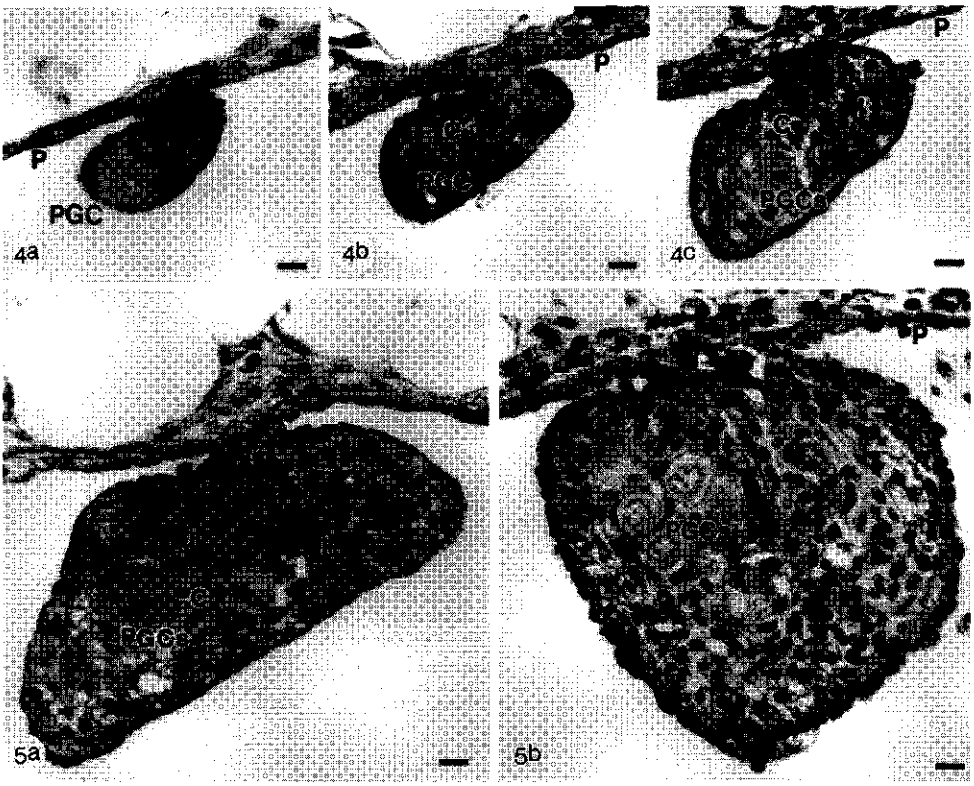
In the larval stages, not only had the gonadal size increased (Figs 4, 5, 6), but so had the mean number of PGCs per fish (Fig 7). A distinct increase in germ cell number was observed at the age of 5 weeks, indicating that the onset of PGC proliferation had started precociously. At the age of 8 weeks, the number of PGCs had increased considerably. Moreover, two out of five experimental specimens at the age of 8 weeks possessed female gonads, recognised by two attachment sites at the dorsal wall of the coelomic cavity instead of one mesogonium in males (not shown). This means that gonadal development in larval specimens was accelerated considerably, since in normal developing carp male and female sex can only be distinguished after week 10.

As in juvenile carp the body weight of experimental and control larvae did not differ significantly from each other (Table 3). Similarly, in larval specimens, in which blood

Table 1. **Body weight of male juvenile carp after injection of pituitary extract**

	Body weight (g)			
Age in weeks af	n	Control	n	Injected with pituitary extract
13	6	11.98 ± 0.83		
14	5	12.82 ± 2.39	5	13.55 ± 3.15[1]
15	6	19.70 ± 1.95	4	18.11 ± 3.53[1]
16	5	19.76 ± 0.91	3	25.10 ± 2.36[1]
17	4	23.75 ± 1.06	6	26.46 ± 3.08[1]
18	8	39.86 ± 11.80	5	40.21 ± 2.54[1]

n = number of animals; [1]not significant; animals received pituitary extract from adult carp in PBS at a dose of 40 µg/gram body weight, controls received PBS only.



Figures 4-5. **Cross sections through gonadal primordia at week 4 (Fig 4a), week 5 (Figs 4b, 5a) and through gonads at week 8 (Figs 4c, 5b). The gonads contain primordial germ cells (PGCs) surrounded by cyst cells (C). Fig 4. untreated; Fig 5. treated with homologous pituitary extract. Note the increase in surface area and in the number of PGCs after treatment. I, stromal tissue; P, peritoneum. x 520. Scale bars: 10 μm**

sampling was only possible at the age of 8 weeks, GTH plasma level was elevated considerably in the treated group (80.23 ng/ml in PE-treated animals versus 1.44 ng/ml in controls).

Table 2. **Plasma concentrations in male juvenile carp after injection of pituitary extract**

	Plasma GTH concentration (ng/ml)			
Age in weeks af	n	Control	n	Injected with pituitary extract
13	6	4.89 ± 1.67		
14	5	4.25 ± 1.14	5	75.26 ± 33.42**
15	6	4.57 ± 1.23	4	31.09 ± 9.08**
16	5	3.99 ± 3.73	3	29.87 ± 7.78*
17	4	3.83 ± 1.15	6	45.09 ± 22.07*
18	8	4.29 ± 1.43	5	22.26 ± 18.81**

n = number of animals; *p < 0.05; **p < 0.005; animals received pituitary extract from adult carp in PBS at a dose of 40 µg/gram body weight, controls received PBS only.

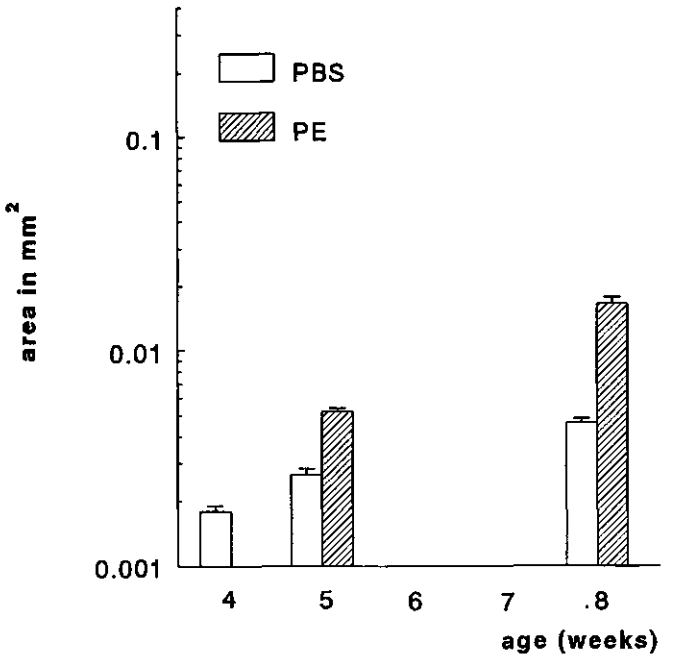


Figure 6. **Increase in gonadal size of larval carp, after treatment with homologous pituitary extract from week 4 onward, as measured in cross-sections through carp gonads at week 4 to week 8. Note that the surface area had already increased one week after the onset of treatment. For each diagram gonads of 5 larvae were measured. Significance: weeks 5 and 8, P < 0.005.**

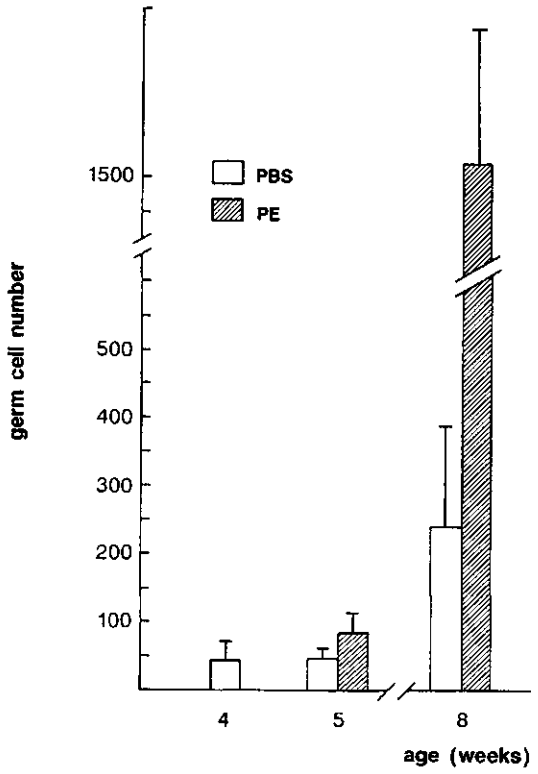


Figure 7. **Increase in numbers of primordial germ cells at week 5 and 8, after treatment with homologous pituitary extract from week 4 onward. Note that the numbers had increased already one week after the onset of treatment. For each diagram gonads of 5 larvae were used. Significance: weeks 5 and 8, $P < 0.005$.**

Table 3. **Body weight of larval carp after injection of pituitary extract**

	Body weight (g)			
Age in weeks af	n	Control	n	Injected with pituitary extract
4	10	0.30 ± 0.10		
5	10	0.44 ± 0.05	10	0.44 ± 0.12[1]
8	10	1.14 ± 0.12	10	1.15 ± 0.17[1]

n = number of animals; [1]not significant; animals received pituitary extract from adult carp in PBS at a dose of 40 µg/gram body weight, controls received PBS only.

DISCUSSION

The normal development of gonads and germ cells in larval and juvenile carp, as observed in the present study, is in agreement with data of previous studies on carp (Parmentier & Timmermans 1985; van Winkoop & Timmermans 1990).

In the present study it was shown that treatment of carp larvae with homologous pituitary extract (PE) stimulates gonadal development and germ cell proliferation at that early age (Experiment 4). The treatment resulted in a precocious onset of PGC proliferation at the age of 5 weeks, instead of after 6 weeks in controls, and to precocious sex differentiation at the age of 8 weeks, instead of after 10 weeks in controls. To our knowledge this is the first report of a stimulatory effect of PE on larval gonadal development.

The results of experiments 2 and 3 show that treatment of juveniles with homologous PE stimulates gonadal development. Precocious spermatogenesis was induced at the age of 17 weeks (experiment 2) and 18 weeks (experiment 3) instead of 19 weeks as in normal development of carp. Thus, gonadal development in juveniles is accelerated by the treatment.

The induction of spermatogenesis in juvenile male teleosts has been described for a number of species, using either heterologous pituitary extracts, purified GTH or human chorionic gonadotropin (HGC) (see Lam 1982, for a review and recent papers by Miura *et al.* 1991a, c, d, in Japanese eel).

The question may be asked whether a particular factor in the pituitary extract is responsible for the observed effects on the juvenile and larval gonads in carp. The fact that at the examined stages the plasma levels of GTH were considerably higher in the experimental animals than in controls might argue in favour of GTH. This is corroborated by the fact that body weight from treated and control animals did not differ significantly from each other throughout the experiments, suggesting that the effect may not be caused by a generally acting hormone like growth hormone. Effects of thyroid hormone on gonads of juvenile fish have been described (see review by Leatherland 1987), although their direct involvement in the onset of gametogenesis is uncertain. Preliminary data on the effects of thyroxine on juvenile carp showed that gonadal size is not influenced, whereas an effect on

gametogenesis was observed (unpublished results). However, the effects on larval carp were not studied hitherto.

With respect to juveniles the possibility for GTH to be the activating agent is supported by previous investigations, showing that injections with HCG (Miura *et al.* 1991a, c, in the eel) or with purified preparations of pituitary GTH, resulted in precocious spermatogenesis in teleosts. In this respect it is of importance that recently two types of GTH (GTH-1 and GTH-2) have been described in salmon (Suzuki *et al.* 1988a; Swanson *et al.* 1989) and in carp (Van der Kraak *et al.* 1992). In salmon GTH-1 is the predominant GTH in both pituitary and plasma during vitellogenesis and spermatogenesis, whereas GTH-2 levels exceed GTH-1 at the time of ovulation and spermiation (Suzuki *et al.* 1988b; Kawauchi *et al.* 1989). Moreover, GTH-1 producing pituitary cells have been detected immunocytochemically prior to GTH-2 cells during trout ontogeny (Nozaki *et al.* 1990), and expression of GTH-1 subunit genes occurred earlier than those of GTH-2 subunit genes (Naito *et al.* 1991). Consequently, it may be hypothesised that precocious spermatogenesis in carp is induced mainly by a GTH-1 like factor in the carp pituitary extract. In favour of this hypothesis might be that in order to obtain the observed effects in the present study, higher concentrations of pituitary extract were needed, than the doses used for stripping of mature carp (Weil *et al.* 1986). The commercially obtained pituitary extract used, was prepared from adult carp pituitaries, probably containing predominantly GTH-2 and less GTH-1.

With respect to the precocious PGC proliferation and sex differentiation in larval fish, GTH may play a role. In the present experiment the GTH plasma concentration at 8 weeks was considerably higher in experimental animals compared to controls. GTH containing cells are present in the pituitary gland of carp larvae from the age of 3 weeks onward (as observed in sections immunostained with anti-cGTHß, van Winkoop *et al.* 1987). Moreover, in a study by Van den Hurk (1982) in rainbow trout, it was shown that GTH-cells appear before the onset of sex differentiation. The question which hormone, or combination of hormones, induces precocious PGC-proliferation and sex determination will be the object of a future study.

There exists convincing evidence that in precocious spermatogenesis, induced by gonadotropin treatment, the gonadotropin does not exert its action in a direct way, but that

androgens are involved, in particular 11-ketotestosterone (see review by Fostier *et al.* 1987 and recent data of Miura *et al.* 1991b) produced by somatic testicular cells. It may be expected that steroid hormones are involved also in accelerated development of larval gonads. This view is supported by the fact that sex-reversal can be obtained during early gonadal development of fish by exogenous administration of steroid hormones (for a review, see Hunter & Donaldson 1983; and recent studies by Van den Hurk *et al.* 1989; Davis *et al.* 1990; Colombo & Grandi 1990). Also, in carp successful sex-reversal has been induced by treatment with steroid hormones (Komen *et al.* 1989; Bongers *et al.* 1991) showing that steroid hormones have an effect on early gonadal development in carp.

The present study indicates that gonadal differentiation and the onset of gametogenesis in the carp is subjected to increased levels of pituitary hormones. GTH, probably being the most important hypophysial factor in these processess, has been given more attention than other pituitary hormones. This was possible because of the availability of reliable and accurate homologous radioimmunoassay

This investigation will be the basis of studies of the gonadal differentiation and the onset of gametogenesis in relation to the functional development of the brain-pituitary-gonad axis in the carp.

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Summary and conclusions

The investigations described in this thesis were aimed at the development of germ cells in the common carp (*Cyprinus carpio* L., Cyprinidae, Teleostei). In this cyprinid fish the embryonic phase is relatively short and the formation of germ cells and gonads occurs almost entirely in free-swimming larvae and juveniles. Comparable processes take place in mammals predominantly during the intra-uterine phase and, hence, are less accessible. In the investigations emphasis was given to the development of germ cells in the larval and juvenile stage. That period is important since then sex-differentiations occurs and also the preparation for gametogenesis. Moreover, in fish investigations on germ cell differentiation during the larval and juvenile stage are relatively sparse. The experiments were performed with various cell biological techniques adapted for carp cells, a.o. isolation and purification of "early" spermatogonia from gonads and immunogold staining of unfixed isolated germ cells. In particular monoclonal antibodies (MAbs) were raised and applied.

The relevance of the investigation is the increase in fundamental knowledge on germ cell differentiation, which can attribute to improved fish culture conditions. MAbs are an important tool in this investigation, since they open new possibilities for the accurate recognition of differentiation stages and also allow detailed studies on the way by which cell differentiation can be influenced.

The approach with MAbs was supplementary to previous research on the differentiation of fish germ cells, in particular in carp. MAbs were raised against carp spermatozoa (Parmentier *et al.* 1984) with the aim to investigate whether antigenic determinants recognised on spermatozoa were also expressed on precursor germ cells in adult gonads and during gonadal development (Parmentier & Timmermans 1985). This was based on the assumption that germ cell development involves changes in gene expression, resulting in the appearance of new cell membrane macromolecules that can be detected with immunological methods. In histological preparations of carp gonads the anti-spermatozoa MAbs indeed recognised antigenic determinants, both on spermatozoa as well as on precursor stages (Parmentier *et al.* 1984). The antigenic determinants were only expressed on germ cells and were present from specific moments during development onwards, i.e. on PGCs from hatching of carp larvae at day 2 or 3 after fertilization (MAb WCS 29), on PGCs from the

onset of proliferation at week 6 after fertilization (WCS 3 and 17) and from the start of spermatogenesis and oogenesis (WCS 28) (Parmentier & Timmermans 1985, see table 1).

In **Chapter 2** of this thesis it is described how the histological investigations of Parmentier *et al.* (1984) were supplemented with research on the expression of antigens on the cell surface of germ cells, isolated with adapted techniques from gonads. The isolated cells were, according to a new protocol, exposed to immunogold-staining without pre-fixation, allowing optimal antigenic reactivity to be combined with optimal identification of the isolated cells. The methods were mainly applied to testis tissue (resp. pre-spermatogenic, early spermatogenic and advanced testes). Antigenic determinants recognised by selected anti-spermatozoa MAbs were revealed in the same way on both the surface of spermatozoa and isolated precursor germ cells, including cells from the pre-spermatogenic testis (WCS 28 gave a weak reaction at that stage). In addition, positive reactions were obtained with oogonia from ovaria, but not with follicular oocytes. With the immunogold-staining method also, for the first time, evidence was given for the appearance of a new antigenic determinant, recognised by the MAbs WCS 7, 11 and 12, during spermatogenesis and oogenesis prior to meiosis on (isolated) small proliferating spermatogonia resp. oogonia (see table 1).

In preparation for further studies with MAbs on the differentiation of PGCs, subsequently a detailed (ultra)structural investigation was carried out on the earliest development of PGCs and gonads in carp larvae (**Chapter 3**). It was shown that, although the number of PGCs remains constant up to week six after fertilization, the volume of these cells increases distinctly between week 2 and week 4. Using electronmicroscopy, distinct changes in the PGCs were observed. In the cell nucleus the nucleolus increased distinctly in size. In the cytoplasm appeared from week 2 onwards ever increasing perinuclear dense bodies, that were gradually localised as intermitochondrial cement between the mitochondria and most likely act during the formation of new mitochondria; the number of mitochondria increased distinctly during the experimental period. From week 2 onwards extensive Golgi complexes were found and from week 4 stacks of endoplasmatic reticulum cisternae were observed.

Table 1. Recognition of antigenic determinants by monoclonal antibodies on germ cells of carp, during the larval and juvenile development and during spermatogenesis.

	Indifferent gonad; larval			Pre-sperm. testis; juvenile	Spermatogenic testis; juvenile/adult a)		
MAbs*)	1w	4w	7w	10w - 16w	19w	22w	sp.zoa
WCS 29	+1)	+	+	+	+	+	+
WCS 3	-	-	+	+	+	+	+
WCS 17	-	-	+	+	+	+	+
WCS 28	-	-	-	-	+	+	+
WCS 7	-	-	-	-	-	+2)	+
WCS 11	-	-	-	-	-	+2)	+
WCS 12	-	-	-	-	-	+2)	+
WCG 1	-	-	+	+	+	+	+3)
WCG 6	-	+	+	+	+	+	-
WCG 7	-	-	+	+	+	+4)	-
WCG 12	-	-	+	+	+	+4)	-
WCG 15	-	-	+	+	+	+4)	-
WCG 21	-	-	+	+	+	+4)	-

*) The WCS-MAbs were produced by Parmentier et al. (1984).

a) In ovaria the WCS-MAbs 3, 17, 28, 29 react only with oogonia and early prophase oocytes; the WCS-MAbs 7, 11 en 12 and the WCG-MAbs 6 - 21 only with (part of) the oogonia.

1) reaction from hatching onwards.

2) reaction starts in cysts with small spermatogonia

3) reaction similar to WCS 3 and 17.

4) reaction restricted to early (primary) spermatogonia, WCG 7 reacts also with secundary spermatogonia.

MAbs = Monoclonal antibodies; w = week; + = positive reaction; - = no reaction; WCS = Wageningen Carp Spermatozoa; WCG = Wageningen Carp Spermatogonia.

The gonadal tissue developed gradually after week 2 around and between the PGCs in rostro-caudal direction. A normal gonadal development was found in a specimen were PGCs were absent. From week 4 onwards two types of somatic cells were present, both bright stained central cells and dark stained periferal cells. It has been supposed that the central cells are precursors of the later Sertoli cells in the testis and cyst or follicle cells in the ovary. The changes that occur in the PGCs and gonads have been interpreted as preparations for the fast proliferation of the PGCs from week 6 onwards.

In **Chapter 4** it is described how new MAbs were raised in order to trace those antigenic determinants that are selectively expressed on precursor germ cells and not on spermatozoa and, hence, can not be detected with anti-spermatozoa MAbs. Isolated early spermatogonia were used as immunogen. The primary and early secundary spermatogonia (diam. $> 10\ \mu m$) were purified from testis cell suspensions. Immunisations were carried out via the intra-splenic method in mice, which allowed the use of relatively low numbers of cells. Using immunohistochemistry and a set of 5 selected MAbs, new antigenic determinants were demonstrated on spermatogonia and oogonia in the adult and juvenile gonads and on PGCs in the larval stage, but not or virtually not on spermatozoa (table 1). With 4 MAbs (WCG 7, 12, 15 and 21) the reaction was restricted to "early" (primary) spermatogonia (WCG 7 reacted also with early secondary spermatogonia) and in the ovary to a subset of oogonia. However, after isolation of the germ cells from the gonads the antigenic determinants recognised by WCG 12, 15 and 21 were not demonstrable.

With MAb WCG 6 a new antigenic determinant was demonstrated that first appeared in the cytoplasm of PGCs between week 2 and week 4 after fertilization and subsequently on the surface of these cells. The observed obvious correlation between the appearance of this determinant and increase of PGC diameter was interpreted as a preparation for the fast proliferation after week 6. Strikingly, the expression of WGC 6 appeared on PGCs located at random sites in the gonads and not in a rostro-caudal direction.

In **Chapter 5** the new immunogold-staining method (chapter 2) is combined with scanning electron microscopy for a detailed analysis of the localisation of cell surface antigens on spermatozoa. This method clearly revealed that antigenic determinants recognised by anti-spermatozoa MAbs are expressed on spermatozoa and those recognised by anti-

spermatogonia MAbs are not, or virtually not. This confirmed the difference between both types of detected antigenic determinants. In contrast to mammals, on carp spermatozoa a localisation of antigens in different domains of the surface membrane could not be demonstrated with the available MAbs. Each of the recognised antigenic determinants was regularly distributed on the head, midpiece and tail.

In **Chapter 6** the biochemical characterisation of antigens recognised by MAbs was initiated. Carp spermatozoa were taken for analysis, as sufficient antigen extract could be obtained from these cells, and four selected MAbs raised against spermatozoa. Immunoblotting revealed with all four MAbs a corresponding set of positive bands with Mw between 30 and >200 kD. The antigens recognized by two MAbs probably are glycoproteins. From the discussion it is concluded that the four MAbs define differentiation antigens. The functional significance remains to be clarified.

The results of the studies with MAbs on the differentiation of germ cells, are summarised in table 1.

In **Chapter 7**, investigations on the functional significance of antigens recognised by the MAbs were initiated by examining the influence of external factors, with particular reference to pituitary hormones, on the larval development of germ cells and gonads. It is generally accepted that gonadotropic hormones function during maturation of spermatozoa and egg cells. A possible role during larval and juvenile development has gained little attention. It is known however that in certain fish species spermatogenesis can be induced already at the juvenile stage by addition of GTH. In this chapter it is shown that in carp, using homologous pituitary extract, precocious spermatogenesis can be induced in the juvenile testis. Application of the same method for carp larvae revealed that addition of pituitary extract resulted in the stimulation of larval gonadal development and precocious proliferation of PGCs. The evidence obtained indicated a function of GTH in this stimulation. The question, however, whether one of the MAb-recognised determinants is involved remains to be answered.

Conclusions:

1. An important result of the research described in this thesis is the preparation of five monoclonal antibodies (MAbs), raised against isolated and purified primary and early-secundary spermatogonia, recognising determinants of differentiation antigens on precursor germ cells. The recognised antigens are not (or virtually not) present on spermatozoa and, hence, differ from antigens that were recognised in previous investigations using anti-carp spermatozoa MAbs. They define differentiation steps in larval and juvenile germ cells, providing a basis for further functional studies.

2. WCG 6, one of the anti-spermatogonia MAbs, recognises an antigenic determinant that first appears, within primordial germ cells (PGCs) in larval gonads, in the cytoplasm and thereafter on the cell membrane of mitotically inactive PGCs. The appearance is correlated with an increase in the PGC diameter, suggesting a function related to the onset of the fast proliferation of PGCs from week 6 after fertilization onwards.

3. Four other MAbs (WCG 7, 12, 15, 21) recognise antigenic determinants that appear in the cell membrane of PGCs at the onset of PGC proliferation from week 6 onwards. These determinants are retained on descendants of the PGCs until after the onset of the spermatogenesis and oogenesis, but at that stage they only occur on primary and early-secondary spermatogonia and oogonia (WCG 7) or on a subset of the primary spermatogonia and oogonia (WCG 12, 15, 21). The function is not known and remains to be elucidated.

4. In germ cells of male and female carp during larval and juvenile development similar differentiation processes occur. This is deduced from the fact that the antigens recognised by MAbs raised against carp spermatozoa (WCS) or spermatogonia (WCG) are expressed on precursors germ cells of both sexes. None of the MAbs reacted exlusively with (precursors of) male germ cells.

5. The somatic gonadal tissue develops in carp in a rostro-caudal direction. In contrast, the early differentiation of PGCs, as characterised by the appearance of the WCG 6 determinant and the increase in cell diameter, does not occur in the rostro-caudal direction. This suggests that cell-cell contact between PGCs and somatic cells is not a prerequisite for PGC differentiation. In relation to this it is also interesting that larval gonads appear to develop normally in the absence of PGCs.

6. An important result of the investigation is that GTH-containing (homologous) pituitary extract stimulates the development of larval gonads and induces the proliferation of PGCs. Moreover, precocious spermatogenesis was induced in juvenile carp. An influence of GTH on larval gonads and PGCs has not been demonstrated previously. It is suggested, though evidence remains to be obtained, that WCG 6-carrying antigen functions as a receptor for factors induced by GTH.

Samenvatting en conclusies

Het in dit proefschrift beschreven onderzoek richtte zich op de ontwikkeling van geslachtscellen in de karper (*Cyprinus carpio* L., Cyprinidae, Teleostei). Bij deze cyprinide vis is de embryonale fase relatief kort en vindt de vorming van geslachtscellen en gonaden vrijwel geheel plaats in vrijzwemmende larven en juvenielen. Vergelijkbare processen treden bij zoogdieren voornamelijk op tijdens de intra-uterine fase en zijn daardoor minder toegankelijk. In het onderzoek werd de nadruk gelegd op de ontwikkeling van geslachtscellen in het larvale en juveniele stadium. Die periode is belangrijk daar dan de sex-differentiatie plaatsvindt en tevens de voorbereiding op de gametogenese. Bovendien is bij vissen nog weinig onderzoek gedaan naar de geslachtsceldifferentiatie tijdens de larvale en juveniele fase. Bij het onderzoek werd gebruik gemaakt van diverse celbiologische technieken, aangepast voor karpercellen, o.a. isolatie en purificatie van "vroege" spermatogonia uit gonaden en immunogoud kleuring van ongefixeerde geïsoleerde geslachtscellen. In het bijzonder werden monoclonale antilichamen (MAbs) opgewekt en toegepast.

Het belang van het onderzoek is het vermeerderen van fundamentele kennis over, en het verdiepen van inzicht in, de geslachtsceldifferentiatie, hetgeen kan bijdragen tot optimalere broed- en kweekomstandigheden. MAbs vormen een belangrijk hulpmiddel bij dit onderzoek, omdat hiermee nieuwe mogelijkheden worden geopend voor het nauwkeurig herkennen van de diverse differentiatiestadia. Daardoor wordt het mogelijk gedetailleerd onderzoek te doen naar de wijze waarop die differentiatie kan worden beïnvloed.

De benadering met MAbs vormde een aanvulling op eerder onderzoek naar de differentiatie van geslachtscellen bij vissen, en karpers in het bijzonder. Hierbij waren MAbs opgewekt tegen karperspermatozoa (Parmentier *et al.* 1984) met het doel om de expressie van de op spermatozoa herkende antigene determinanten te onderzoeken op voorloper-geslachtscellen in de rijpe gonaden en tijdens de gonade-ontwikkeling (Parmentier & Timmermans 1985). Daarbij werd uitgegaan van de veronderstelling, dat de differentiatie van geslachtscellen gepaard gaat met veranderende gen-expressie, resulterend in het verschijnen van nieuwe macromoleculen op de celmembraan, die met immunologische methoden kunnen worden gedetecteerd. In histologische preparaten van karpergonaden bleken de opgewekte anti-spermatozoa MAbs inderdaad antigenene determinanten te herkennen, zowel op

spermatozoa als op voorloper-stadia ervan (Parmentier *et al.* 1984). De antigene determinanten kwamen uitsluitend op geslachtscellen voor en bleken aanwezig te zijn vanaf specifieke momenten tijdens de ontwikkeling, n.l. respectievelijk op primordiale geslachtscellen (PGCs) vanaf het uitkomen van de karperlarven op dag 2 of 3 na bevruchting (MAb WCS 29), op PGCs vanaf de start van de proliferatie vanaf week 6 na bevruchting (WCS 3 en 17) en vanaf de aanvang van de spermatogenese en oogenese (WCS 28). In de testis bleven deze determinanten aanwezig op de latere stadia, inclusief de spermatozoa; tijdens de oogenese verdwenen ze op folliculaire oocyten (Parmentier & Timmermans 1985, zie tabel 1).

In **hoofdstuk 2** van dit proefschrift is beschreven hoe het histologische onderzoek van Parmentier *et al.* (1984) werd aangevuld met de studie van de expressie van antigenen op het celoppervlak van voorlopergeslachtscellen, die hiertoe met gemodificeerde technieken werden geïsoleerd uit de gonaden. De geïsoleerde cellen werden, middels een nieuw ontwikkeld protocol, getest met een immunogoud-kleuringsmethode zonder pre-fixatie, waarin optimale antigene reaktiviteit kon worden gecombineerd met optimale identificatie van de geïsoleerde cellen. De methoden werden hoofdzakelijk toegepast op testisweefsel (resp. pre-spermatogene, vroeg-spermatogene en geavanceerde testes). Antigene determinanten, herkend door geselekteerde anti-spermatozoa MAbs, werden op deze wijze aangetoond op het celoppervlak van zowel spermatozoa als van geïsoleerde voorlopergeslachtscellen, inklusief cellen uit de pre-spermatogene testis (WCS 28 gaf op dat stadium een zwakke reaktie). Geïsoleerde somatische cellen uit testes en geïsoleerde miltcellen vertoonden geen reactie. Met geïsoleerde oogonia uit ovaria werden eveneens positieve reakties verkregen; echter niet met folliculaire oocyten. Met de immunogoud-kleuringsmethode kon bovendien voor het eerst worden aangetoond dat tijdens de spermatogenese en de oogenese, voorafgaand aan de meiose, op kleine of "late" prolifererende spermatogonia, resp. oogonia, een nieuwe antigene determinant verschijnt, die wordt herkend door de MAbs WCS 7, 11 en 12 (zie tabel 1). Op basis van het tijdstip van verschijnen zou kunnen worden verondersteld dat de nieuwe antigene determinant betrokken is bij de start van de meiose. Dit dient echter nader te worden uitgezocht.

Tabel 1. **Expressie van antigene determinanten herkend door monoclonale antilichamen op geslachtscellen van de karper, tijdens de larvale en juveniele ontwikkeling en tijdens de spermatogenese.**

	Indifferente gonade; larvaal			Pre-sperm. testis; juveniel	Spermatogene testis; juveniel/adult a)		
MAbs*)	1w	4w	7w	10w - 16w	19w	22w	sp.zoa
WCS 29	+1)	+	+	+	+	+	+
WCS 3	-	-	+	+	+	+	+
WCS 17	-	-	+	+	+	+	+
WCS 28	-	-	-	-	+	+	+
WCS 7	-	-	-	-	-	+2)	+
WCS 11	-	-	-	-	-	+2)	+
WCS 12	-	-	-	-	-	+2)	+
WCG 1	-	-	+	+	+	+	+3)
WCG 6	-	+	+	+	+	+	-
WCG 7	-	-	+	+	+	+4)	-
WCG 12	-	-	+	+	+	+4)	-
WCG 15	-	-	+	+	+	+4)	-
WCG 21	-	-	+	+	+	+4)	-

*) De WCS-MAbs werden geproduceerd door Parmentier et al. (1984).

a) In ovaria reageren de WCS-MAbs 3, 17, 28, 29 alleen met oogonia en vroege profase oocyten; de WCS-MAbs 7, 11 en 12 en de WCG-MAbs 6 - 21 alleen met een (deel der) oogonia.

1) reaktie vanaf uitkomen larven.

2) reaktie begint in cysten met kleine spermatogonia

3) reaktie komt overeen met WCS 3 en 17.

4) reaktie beperkt tot vroege (primaire) spermatogonia, WCG 7 reageert ook met vroege secundaire spermatogonia.

MAbs = Monoclonale antilichamen; w = week; + = positieve reaktie; - = geen reaktie; WCS = Wageningen Carp Spermatozoa; WCG = Wageningen Carp Spermatogonia.

Ter voorbereiding op verdere studies met MAbs naar de differentiatie van geslachtscellen werd vervolgens een gedetailleerd (ultra)structureel onderzoek uitgevoerd naar de vroegste ontwikkeling van PGCs en gonaden in karperlarven (**hoofdstuk 3**). Uit het onderzoek bleek dat, hoewel het aantal PGCs tot de zesde week na bevruchting konstant blijft, het volume van deze cellen sterk toeneemt tussen week 2 en week 4 (toename diameter van 16,5 $\pm$ 2,8 in week 2 tot 21,0 $\pm$ 4,2 in week 4). Met behulp van electronenmicroscopie werden duidelijke veranderingen in de PGCs waargenomen. In de celkern nam de nucleolus sterk in omvang toe. In het cytoplasma verschenen vanaf week 2 steeds prominentere "perinuclear dense bodies". Deze structuren, ook wel "nuage" genoemd, werden geleidelijk als intermitochondriaal cement tussen de mitochondriën gelokaliseerd, en fungeren waarschijnlijk bij de vorming van nieuwe mitochondriën; het aantal mitochondriën nam aanzienlijk toe in de onderzochte periode. Vanaf week 2 werden uitgebreide Golgi-complexen gevonden en vanaf week 4 trad stapeling van endoplasmatisch reticulum-cisternae op.

Het gonadeweefsel ontwikkelde zich geleidelijk na week 2 rond en tussen de PGC's in rostro-caudale richting. Een normale gonade-ontwikkeling bleek ook op te treden bij een specimen waarin geen PGCs werden aangetoond. Vanaf week 4 waren twee typen somatische cellen aanwezig, lichter gekleurde centrale cellen, die de PGCs direkt omgeven, en donker gekleurde perifere cellen. Verondersteld wordt dat de centrale cellen voorlopers zijn van de latere Sertoli cellen in de testis en cyste- of follikelcellen in het ovarium. De veranderingen die optreden in de PGCs en gonaden worden geïnterpreteerd als voorbereiding op de snelle proliferatie van de PGCs, die na week 6 van start gaat.

In **hoofdstuk 4** wordt beschreven hoe nieuwe MAbs werden opgewekt met het doel antigene determinanten op te sporen, die selektief op voorlopergeslachtscellen voorkomen en niet met anti-spermatozoa MAbs kunnen worden gedetecteerd. Hiertoe werden geïsoleerde primaire en vroege secundaire spermatogonia als immunogeen gebruikt. Deze spermatogonia (diam. $> 10\mu$) werden met een nieuwe methode gezuiverd uit testis-celsuspensies. Immunisaties werden uitgevoerd via de intra-milt methode in muizen, waarbij met relatief geringe aantallen cellen kon worden volstaan. Immunohistochemisch werden met een set van 5 geselekteerde MAbs nieuwe antigene determinanten aangetoond, die voorkomen op

spermatogonia en oogonia in de volwassen en de juveniele gonaden en op PGCs in de larvale fase, maar niet, of vrijwel niet, op spermatozoa (tabel 1). Bij vier MAbs (WCG 7, 12, 15 en 21) bleef de reaktie in de testis zelfs beperkt tot een deel der (primaire) spermatogonia (WCG 7 reageerde bovendien met vroege secundaire spermatogonia) en in de ovaria tot een deel der oogonia. Na isolatie van de geslachtscellen uit de gonaden waren de door MAbs WCG 12, 15 en 21 herkende antigene determinanten echter niet meer aantoonbaar.

Met MAb WCG 6 werd een antigene determinant aangetoond, die tussen week 2 en week 4 na bevruchting eerst in het cytoplasma van PGCs en vervolgens op het celoppervlak van deze cellen verschijnt. Er is een duidelijke korrelatie tussen het verschijnen van deze determinant en de toename in diameter van PGCs, die werd geinterpreteerd als een voorbereiding op de snelle proliferatie na week 6. Opmerkelijk was dat de WCG-6 expressie op PGCs op willekeurige locaties verscheen en niet in rostro-caudale richting, terwijl de gonade wel in rostro-caudale richting differentiëerde.

In **hoofdstuk 5** is de nieuwe immunogoud-kleuringsmethode (hoofdstuk 2) gekombineerd met scanning electronenmicroscopie om de localisatie van celoppervlakte-antigenen op spermatozoa gedetaileerd te kunnen onderzoeken. Deze methode toonde duidelijk aan dat antigene determinanten herkend door anti-spermatozoa MAbs wél op spermatozoa voorkomen en determinanten herkend door anti-spermatogonia MAbs niet, of vrijwel niet. Hierdoor wordt het verschil tussen beide soorten gedetecteerde antigene determinanten bevestigd. In tegenstelling tot zoogdieren, konden bij karperspermatozoa met de beschikbare MAbs geen localisatie van antigenen in verschillende domeinen worden aangetoond. Alle aangetoonde antigene determinanten waren regelmatig verspreid over kop, middenstuk en staart.

In **hoofdstuk 6** werd een aanvang gemaakt met de biochemische karakterisering van door MAbs herkende antigenen. Er werd uitgegaan van karperspermatozoa, omdat hiervan voldoende eiwitextract bereid kon worden, en van vier geselekteerde MAbs opgewekt tegen karperspermatozoa. Met immunoblotting werd aangetoond dat de vier Mabs een overeenkomstige set van positieve bandjes herkennen, met Mw tussen 30 en >200 kD. De door twee van deze Mabs herkende antigenen zijn waarschijnlijk glycoproteïnen. Uit de diskussie wordt geconcludeerd dat de vier MAbs differentiatie-antigenen karakteriseren. De

functionele betekenis van de antigenen zal nog dienen te worden opgehelderd.

De resultaten van de studies met MAbs naar de differentiatie van geslachtscellen zijn samengevat in tabel 1.

In **hoofdstuk 7** wordt, als aanzet tot onderzoek naar de functionele betekenis van de door de MAbs herkende antigenen, de invloed van externe faktoren, met name van hypofyse-hormonen, op de larvale ontwikkeling van geslachtscellen en gonaden beschreven. Algemeen bekend is de rol van gonadotrope hormonen bij de maturatie van spermatozoa en eicellen. Aan een mogelijke rol tijdens de larvale en juveniele ontwikkeling is relatief weinig aandacht gegeven. Bekend is wel dat in sommige vissoorten de spermatogenese tijdens het juveniele stadium kan worden geïnduceerd door toediening van GTH. In dit hoofdstuk is aangetoond dat bij de karper met homoloog hypofyse-extract de spermatogenese vervroegd kan worden opgewekt in de juveniele testis. Toepassing van dezelfde methode bij karperlarven toonde aan dat toediening van hypofyse-extract leidt tot stimulatie van de larvale gonade-ontwikkeling en vervroegde proliferatie van PGCs. De verkregen evidentie wijst op een funktie van GTH bij deze stimulatie. De vraag of een van de door de MAbs herkende determinanten hierbij betrokken is dient echter nog te worden beantwoord.

Conclusies:

1. Als een van de belangrijkste resultaten van het in dit proefschrift beschreven onderzoek kan worden beschouwd de beschikbaarheid van vijf monoclonale antilichamen (MAbs), opgewekt tegen geïsoleerde en gezuiverde primaire en vroeg-secundaire spermatogonia, die determinanten van differentiatie-antigenen herkennen op voorlopergeslachtscellen. De herkende antigenen zijn niet (of vrijwel niet) aanwezig op spermatozoa. Ze verschillen derhalve van de antigenen die in vroeger onderzoek werden herkend door anti-karper spermatozoa MAbs. Ze markeren differentiatie-stappen in larvale en juveniele geslachtscellen, waarop functioneel onderzoek kan aangrijpen.

2. WCG 6, één van de anti-spermatogonia MAbs, herkent een antigene determinant die in larvale gonaden eerst in het cytoplasma en naderhand op de celmembraan van mitotisch inactieve primordiale geslachtscellen (PGCs) verschijnt. Het verschijnen is gecorreleerd met een toename in diameter van die PGCs en suggereert een functie in relatie tot de start van de snelle proliferatie vanaf week 6 na bevruchting.

3. Vier andere MAbs (WCG 7, 12, 15, 21) herkennen antigene determinanten die in de celmembraan van PGCs verschijnen bij de start van de PGC-proliferatie vanaf week 6. Deze determinanten blijven aanwezig op afstammelingen van die PGCs tot na de start van de spermatogenese en oogenese, en komen dan uitsluitend voor op primaire en vroeg-secundaire spermatagonia en oogonia (WCG 7) of op een beperkt deel van de primaire spermatogonia en oogonia (WCG 12, 15, 21). De functie is niet bekend en dient nog te worden opgehelderd.

4. In geslachtscellen van mannelijke en vrouwelijke karpers verlopen tijdens de larvale en juveniele ontwikkeling overeenkomstige differentiatie-processen. Dit kan worden afgeleid uit het feit dat de antigenen, herkend door MAbs opgewekt tegen karper-spermatozoa (WCS) of -spermatogonia (WCG), voorkomen op voorstadia van geslachtscellen van beide seksen. Geen van de MAbs reageerde uitsluitend met (voorstadia van) mannelijke geslachtscellen.

5. Het somatische gonadeweefsel ontwikkelt zich bij de karper in een rostro-caudale richting. Daarentegen vindt de vroege differentiatie van PGCs, gekenmerkt door het verschijnen van de WCG 6-determinant en de toename in celdiameter, niet in rostro-caudale richting plaats. Dit suggereert dat cel-cel contact tussen PGCs en somatische cellen geen voorwaarde is voor differentiatie van PGCs. In relatie hiermee is het ook interessant dat larvale gonaden zich zonder aanwezigheid van PGCs normaal lijken te ontwikkelen.

6. Een belangrijk resultaat van het onderzoek is dat GTH bevattend (homoloog) hypofyse-

extract de ontwikkeling van larvale gonaden stimuleert en vervroegde proliferatie van PGCs induceert. Bovendien wordt hiermee in juveniele karpers een vervroegde start van de spermatogenese opgewekt. Een invloed van GTH op larvale gonaden en PGCs was nog niet eerder aangetoond. De suggestie dringt zich op, hoewel evidentie nog niet beschikbaar is, dat WCG 6-dragende antigenen mogelijk als receptor fungeren voor factoren die door GTH worden geïnduceerd.

Curriculum vitae

Aart van Winkoop is geboren te Hattem op 11 juni 1956. Na de middelbare school en diensttijd studeerde hij Biologie aan de Vrije Universiteit te Amsterdam. In de kandidaatsfase koos hij voor de richting Biologie en Geneeskunde. Tijdens de doktoraalfase voerde hij leeronderzoeken uit in de hoofdrichting Celbiologie (aan de Vakgroep Celbiologie van de Medische Faculteit van de Vrije Universiteit te Amsterdam en aan het Hubrecht Internationaal Laboratorium voor Embryologie te Utrecht) en de richtingen Immunologie en Histologie aan respectievelijk de Afdeling Immunologie van het Antoni van Leeuwenhoek Research Laboratorium te Amsterdam en de Werkgroep Histologie van de Subfaculteit Biologie van de Vrije Universiteit te Amsterdam. Het doktoraalexamen behaalde hij in oktober 1984.

Aansluitend werd hij aangesteld voor een onderzoek gesubsidieerd door de stichting BION bij het projekt "Differentiatie-processen tijdens de vroege ontwikkeling van vissen" van de vakgroep Experimentele Diermorfologie en Celbiologie, sectie Ontwikkelingsbiologie, aan de Landbouw Universiteit te Wageningen. De resultaten van dit onderzoek, uitgevoerd in de periode 1984 - 1989, zijn in dit proefschrift beschreven.

Vanaf november 1989 volgde hij de opleiding tot systeemontwerper/programmeur via de stichting Promotie Informatica Omscholing Nederland. Sinds juli 1990 is hij werkzaam bij de stichting Samenwerking Automatisering Ziekenhuizen Zuid-Oost Gelderland te Arnhem als applicatie-adviseur; vanaf april 1994 tevens als coördinator van de sectie Applicatie Service.

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