The differentiation of germ cells and gonads during development of carp (Cyprinus carpio L.). A study with anti-carp sperm monoclonal antibodies

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SUMMARY
Gonadal development, germ cell differentiation and the appearance of membrane antigenic determinants, specific for male and female germ cells during gonadogenesis, was studied in larval and juvenile carp (Cyprinus carpio L.) until 25 weeks after fertilization.

Indirect immunofluorescence studies with four monoclonal antibodies raised against carp spermatozoa revealed that monoclonal antibody WCS 29 stained the outer membranes of primordial germ cells in larvae from 3 days after fertilization. The monoclonal antibodies WCS 3 and 17 reacted with the outer membranes of germ cells from 7 weeks after fertilization onwards, simultaneously with the onset of germ cell proliferation. With monoclonal antibody WCS 28 germ cell membranes were clearly stained from 18 weeks after fertilization. Similar reactions were observed in both sexes, however, female germ cells reacted at an earlier developmental stage with the monoclonal antibody WCS 28 than male germ cells.

In the developing testis the monoclonal antibodies stained all types of spermatogenic cells. In the ovary, however, only oogonia and early prophase oocytes showed a positive reaction with the four monoclonal antibodies.

The results indicate that germline-specific antigens are present on the outer membranes of primordial germ cells and their male and female descendants, with the exception of elderly oogenic stages. It is assumed that the appearance and disappearance of these membrane antigens reflect differentiation steps of germ cells during gonadogenesis.

INTRODUCTION
The origin and history of the germ cell line in fish and the development of the gonads, with particular emphasis on the mode of sex differentiation, have been studied in several species. Besides morphological characterization of early germ cells and developing gonads using light microscopy (Wolf, 1931; Dildine, 1936; Johnston, 1951; Yoshikawa & Oguri, 1979; van den Hurk & Slof, 1981; Lebrun,

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Abbreviation: WCS, Wageningen carp sperm antibody.

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Billard & Jalabert, 1982; Bruslé, 1983), electron microscopy has also been used to study the ultrastructure of early germ cells (Satoh, 1974; Bruslé & Bruslé, 1978a; Hogan, 1978; Hamaguchi, 1982) and gonadal differentiation (Satoh, 1974; Bruslé & Bruslé, 1978b) in a number of teleosts. However, relatively little is known as compared with higher vertebrates, partly due to the fact that different non-related species are studied and partly due to the heterogeneous sexuality displayed in fish (Yamamoto, 1969). Few data concerning the physiology of developing gonads and differentiating germ cells are available. Furthermore, it remains to be established which factor or combination of factors is responsible for the sex determination of germ cells and gonads in fish and the means by which sex differentiation is regulated, despite an extensive number of experimental, especially endocrinological, studies (Yamamoto, 1969; Yamazaki, 1983).

Today, it is generally accepted that cell membrane determinants reflect cellular differentiation and play a significant role inducing or regulating intra- and intercellular processes. Therefore, knowledge of successive membrane determinants of germ cells during development of fish may contribute to our understanding of germ cell differentiation in this group of vertebrates. Previously, in a study on the presence of specific membrane antigens on spermatogenic cells in adult carp (Cyprinus carpio L.), in which monoclonal antibodies (WCS) raised against mature carp spermatozoa were used, the presence of common membrane determinants on spermatogenic cells and early female germ cells was reported (Parmentier, Timmermans & Egberts, 1984). The present work deals with the appearance of these determinants on the membranes of differentiating germ cells during development of male and female carp. Using the immunofluorescence technique together with routine histological staining, we intend to define the appearance of germ cell membrane antigenic determinants during gonadogenesis and to study a possible relationship between membrane antigens, germ cell differentiation, gonadal development and sex differentiation. Up till now there exist only a few morphological descriptions of gonadal development and germ cell differentiation in cyprinids. Stromsten (1931) investigated gonadogenesis in the goldfish, and Davies & Takashima (1980) studied gonad development in carp, the latter on monthly sampled material. In the present study a detailed description of carp gonadogenesis is given and discussed.

A summary of the results has been published before (Parmentier & Timmermans, 1984).

MATERIALS AND METHODS

Animals

Eggs and spermatozoa were obtained by stripping adult carp reared at our laboratory. After fertilization, the eggs were placed in well-aerated tanks with a constant flow of copper-free water and incubated at 23°C. At this temperature the eggs hatched on the second day after fertilization. Larvae and fry were fed on Artemia salina nauplii. After two weeks, the fish were fed with commercially prepared Trouvit pellets (Trouw and Co., Putten, The Netherlands).
Preparation of monoclonal antibodies

Mouse anti-carp sperm monoclonal antibodies WCS 3, 17, 28 and 29, respectively, were used. These antibodies recognized common membrane determinants on spermatogenic cells and early female germ cells in gonads of adult fish as determined with indirect immunohistochemistry (Parmentier et al. 1984). Preparation and characteristics of the monoclonal antibodies have been described before. Briefly, the hybridomas were obtained from a fusion between mouse myeloma P3-NS1/1-Ag4 (NSI) cells and splenocytes from Balb/c mice hyperimmunized with carp spermatozoa. The hybridoma lines were subcloned and the secreted mouse immunoglobulins belonged to the IgG-(WCS 3 and 28) or IgM-class (WCS 17 and 29), as determined in an Ouchterlony double diffusion test.

Experimental design

First sampling of fish larvae commenced 72 h after fertilization. Samplings of ten animals each were selected once a week until 25 weeks after fertilization. The sampled individuals were selected from a group of average size. After measuring body length, the animals were fixed in Bouin’s solution, dehydrated and embedded in paraffin. Serial 5 μm cross sections of five or six animals and sagittal sections of two animals, respectively, were mounted on albumin-coated slides. Until the age of 10 weeks after fertilization sections of total specimens were mounted. Later on, next to sagittal sections, only a number of cross sections from anterior, mid- and posterior regions were collected. After deparaffinization the sections were stained by the indirect immunofluorescence test and immediately studied. As the cross sections had been prepared from the whole larval or juvenile fish they could be used to study whether somatic tissues showed fluorescence. Subsequently, the same cross sections, together with the sagittal sections were routinely stained with haemalum and eosin (H/E) or Crossmon. Until ten weeks after fertilization the total number of germ cells per animal was estimated. The diameters of the germ cells and their nuclei were measured within each sampled animal.

Immunohistochemical staining

For indirect immunofluorescence, slides were extensively rinsed with PBS (pH 7.3). They were incubated with hybridoma supernatant in a humidified atmosphere for 45 min and then washed in PBS. Afterwards a 1:40 diluted solution of rabbit anti-mouse immunoglobulin antiserum coupled to fluorescein isothiocyanate (RAM/1g/FITC; Nordic, Tilburg, The Netherlands) was applied for another 45 min at room temperature. After a wash with PBS, the slides were mounted with a solution of PBS-glycerol (1/9; v/v) pH 8-0, containing 1 mg ml⁻¹ p-phenylenediamine to prevent photobleaching of the FITC dye (Johnson & Nogueira Araujo, 1981).

Controls included replacement of the primary antiserum by a non-immune mouse serum, an antigen-negative hybridoma supernatant, and PBS. The slides were observed with a Zeiss fluorescence microscope (illuminator LV/F, HBO 50 mercury lamp).

RESULTS

In the present study, the ages of the developmental stages will be given from fertilization onwards.

I. Morphogenesis of carp gonads (between brackets: mean standard body length)

3 days after fertilization (4 mm)

The PGCs were located as a paired string of beads in an anterior–posterior direction at the dorsal body wall on both sides of the dorsal mesentery. These are the sites of the future gonadal ridges. Germ-cell-free spaces were present between the successive PGCs in the strings. In cross sections mostly one PGC was present...
at each side of the dorsal mesentery (cf. Fig. 2B) but sometimes they were located in groups of two. The germ cells were recognized by their large size (15–25 μm) and their ovoid shape. The large and often irregularly shaped nuclei measured 7–10 μm and contained a loose network of thin chromatin filaments, a nucleolus was rarely observed. Characteristic deeply staining dense bodies were peripherally arranged along the nuclear membrane. From electron microscopical observations (data not shown) these were interpreted as ‘nuage’ material (André & Rouiller, 1957).

The PGCs were each enveloped by one or two somatic cells with small nuclei and little cytoplasm. These cells formed a continuous layer with the dorsal peritoneal cells. The average number of PGCs per animal was estimated to be 23 (Fig. 1).

1 and 2 weeks after fertilization (8–9 mm)

The PGCs were located as described above, their morphology was unaltered. However, the germ cells, together with their surrounding somatic cells, had protruded slightly into the coelomic cavity (Fig. 2B). They were located below the posterior third of the developing swim bladder, at the right side dorsal to the liver, at the left anterior side above the left liver lobe and posteriorly above the gut. No
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3 to 6 weeks after fertilization (11–23 mm)

During this period gonad formation started anteriorly and proceeded in a posterior direction. From rostral to caudal the germ cells, and their enveloping cells which from now on will be named cyst cells, became gradually surrounded by round and deeply staining somatic cells (Fig. 3B). Later on, the gaps between the successive PGCs were filled with similar somatic cells in a rostrocaudal direction. Gradually the gonads increased in size, especially in the anterior region. They were attached to the coelomic wall by a short and thick band of somatic cells in which the formation of blood capillaries was noticed from 6 weeks onwards (Fig. 5). In most animals the left gonad was retarded in growth as compared to the right one.

Until the age of 6 weeks the germ cells hardly increased in number, and after a temporary decline to 16 at the age of three weeks, total numbers remained stable at approximately 20 to 40 germ cells per animal (Fig. 1).

7 to 9 weeks after fertilization (25–28 mm)

At 7 weeks a rapid proliferation of germ cells was noticed (Fig. 1) and two germ cell types were distinguished. The morphology of the first type, the resting PGC, was as described for the former stages. The second type, which we named mitotic PGC, was much smaller (10–15 μm) and had a round shape. Its nucleus contained several nucleoli, more dense chromatin and measured 5 μm in diameter. Regularly, mitotic spindle figures and chromosomes were observed in the PGCs. The latter were located in the anterior part of the gonads, in groups of two or three germ cells (Figs 4B, 5A), whereas the resting PGCs were, though frequently observed anteriorly, predominantly present in the middle and posterior gonadal regions. Gradually, the gonads were filled by stroma cells, but the origin of these stroma cells was unclear, although mitoses were sometimes noticed.

At 7 weeks gonadal tissue had extended from the posterior third of the swim bladder to the hind gut; however, germ cells were observed only in the region below the swim bladder. From 9 weeks onwards germ cells gradually populated the total gonad. Sex differences could not be distinguished. Therefore the gonads were interpreted as of the indifferent type.

10 to 16 weeks after fertilization (32–47 mm)

From 10 weeks onwards three gonadal types were distinguished with respect to their anatomical characteristics and the morphology of the germ cells. The first type (Fig. 5B) was slender, club- or arrow-shaped, attached to the coelomic wall by a rather thick mesogonium, and hanging freely in the coelomic cavity. The gonad was largely filled with stroma cells and in the dorsomedial region of the organ blood vessels were present. The germ cells (15–20 μm) were located
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Fig. 5. Schematic drawings of the indifferent, male and female gonads during development: (A) indifferent gonad at eight weeks; (B) male gonad at fifteen weeks; (C) female gonad at fifteen weeks. bv, blood vessel; cc, cyst cell; epo, early prophase oocytes; it, interstitial tissue; Oo, oogonia; mPGC, mitotic primordial germ cell; sg, spermatogonia.

alone or in small groups in the central or lateral parts of the gonad. Each germ cell was surrounded by one or more cyst cells (Fig. 6B).

The second type (Fig. 5C) had a flattened sickle-shaped form. It became attached at two sides to the coelomic wall, at the mediodorsal side by a thin mesogonium and

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Figs 2-4. (A) Indirect immunohistochemistry with monoclonal antibodies (WCS 29) raised against carp spermatozoa. Bouin's fixative. (B) The same sections, stained with Crossmon. PGC, primordial germ cell; cc, somatic (cysts) cell; pe, peritoneum; sc, stroma cell; mPGC, mitotic primordial germ cells.

Fig. 2. Primordial germ cell (PGC) at one week. ×1000.
Fig. 3. Indifferent gonad at four weeks. ×1000.
Fig. 4. Indifferent gonad at nine weeks. ×830.
laterally with a thin extension, thus forming an enclosed cavity, covered by the peritoneum. This gonad consisted of two regions, a dorsomedial region largely

Figs 6, 7. (A) Indirect immunohistochemistry, Bouin’s fixative. (B) The same sections, stained with Crossmon. sg, spermatogonia; sc, stroma cell; cc, cyst cells.
Fig. 6. First type of gonad, testis-like, at eleven weeks (WCS 29). ×600.
Fig. 7. Testis at seventeen weeks (WCS 17). ×675. The same reaction was perceived with WCS 29 and WCS 3.
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filled with stroma cells and blood vessels and a lateral region in which the germ cells were located. The germ cells were individually or in groups enveloped by cyst cells. This gonadal type contained more germ cells than the first type and also the size of the germ cells was smaller; 10–14 μm (Figs 8B, 9B).

The third gonadal type (Fig. 5A) was still undifferentiated initially, containing resting PGCs next to several mitotic ones, and disappeared gradually during this period.

The differences between the gonadal types were most obvious in the anterior region; posteriorly the gonads remained undifferentiated for a longer period.

17 to 20 weeks after fertilization (52–82 mm)

At these stages male and female gonads could be distinguished clearly. The club-shaped gonad (Type 1, Fig. 5B) gave rise to the testis (Fig. 7B). It gradually became filled with cysts containing germ cells or primary spermatogonia. These were more numerous and larger (20 μm) than the mitotic PGCs and each spermatogonium contained a large nucleus, a distinct nucleolus and a loose network of chromatin. In the mesorchial region and the ventral rim of the organ mainly blood vessels and stroma cells were present. At the age of 20 weeks a few cysts with a group of secondary spermatogonia (10–20 μm) or leptotenic primary spermatocytes (12 μm) were noticed, especially in the anterior regions.

During this period the organ with two lines of attachment to the coelomic wall (Type 2, Fig. 5C) had developed into an ovary (Fig. 10B). It was well vascularized with larger blood vessels in the mesovarial and dorsomedial regions and small capillaries between the germ cells. Bordering the ovarian sac were cysts containing individual primary oogonia (16 μm), groups of secondary oogonia (8–10 μm) or groups of early prophase oocytes (8 μm). Larger oocytes were present too; their enveloping somatic cells were named follicle cells. The oogonia contained a nucleus with a conspicuous nucleolus and a weak-staining cytoplasm. In the follicular oocytes a central nucleolus was absent; their cytoplasm was distinctly basophilic.

21 to 25 weeks after fertilization

During these stages the testis gradually obtained the features of the adult organ (see Parmentier et al. 1984) in that it consisted of irregularly shaped tubuli, separated by interstitial tissue with blood vessels and Leydig cells. As in the adult, each tubule was made up of cysts surrounding a central lumen (Fig. 11C). The cyst cells or Sertoli cells, enveloping the cysts, also partly formed the wall of the central lumina of the tubuli. The cysts contained predominantly the early spermatogenic stages, i.e. primary spermatogonia, each separately enveloped by Sertoli cells, groups of secondary spermatogonia, and primary spermatocytes. Secondary spermatocytes and spermatids were only scarcely present and mature spermatozoa were not observed.

The ovary was distinctly lobulated. It was characterized by ventrodorsal-oriented lamellae containing several oogenic stages. Connective tissue with blood
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vessels formed the core of the lamellae which adjoined in the ventral region, but remained free dorsally. Small groups of secondary oogonia or early prophase oocytes and solitary primary oogonia surrounded by cyst cells were predominantly found along the margins of the lamellae (Fig. 10B). Larger follicular oocytes were located further in on the lamellae.

Hermaphroditism

Though female and male gonads were easily distinguishable from the age of 16 weeks onwards, it was observed in a few fishes (3 out of the 36 investigated specimens) that male germ cells were present in the ovaries between the female germ cells. Moreover, in one male fish, a few follicular oocytes were found.

II. Immunohistochemical staining

With indirect immunofluorescence on cross sections of developing larvae it was observed that monoclonal antibody WCS 29 distinctly stained the surface membranes of primordial germ cells from 3 days after fertilization onwards. Up to the age of 17 weeks all differentiating germ cells in both sexes were stained by this antibody (Figs 2A, 3A, 4A, 6A, 8A). From that age onwards all germ cells in the developing testis gave a positive reaction (Fig. 11B). In the ovary, however, only primary and secondary oogonia and early prophase oocytes remained positive (Fig. 10A). No reaction was observed on larger oocytes in follicles. With the WCS 29 antibody staining of germ cell cytoplasm was also observed, especially in primordial germ cells, and in primary spermatogonia and oogonia, whereas all later spermatogenic cells, secondary oogonia and early prophase oocytes showed no or only a slight cytoplasmic staining.

With the monoclonal antibodies WCS 3 and 17 no staining occurred with the resting PGCs until the age of 6 weeks except for an incidental staining at 5 weeks (Fig. 12C). Both monoclonals gave similar reactions with the differentiating germ cells. At 6 weeks small fluorescing patches in the germ cell cytoplasm and on the outer cell membranes of 50–60% of the animals were observed. From 7 weeks until 10 weeks all mitotic PGCs in the undifferentiated gonads were stained but not the larger resting ones. Indirect immunofluorescence reactions for WCS 29 and WCS 3, carried out on consecutive sections of developing gonads showed that at the age of 5 weeks only WCS 29 stained the germ cell membrane, whereas at 8 weeks both monoclonals gave a positive reaction (Figs 12 and 13). From 11 weeks WCS 3 and 17 gave similar reactions in the type 2 gonad and the later ovary as WCS 29 (Figs 9A, 10A), that is, oogonia and early prophase oocytes were distinctly stained but follicular oocytes were not. In the type 1 and the subsequent male gonads, however, a weaker reaction than in the female gonad was observed.

Figs 8, 9. (A) Indirect immunohistochemistry. Bouin's fixative. (B) The same sections, stained with Crossmon. Oo, oogonia.

Fig. 8. Second type of gonad, ovary-like, at 12 weeks (WCS 29). ×700. The same reaction was perceived with WCS 3 and 17.

Fig. 9. Ovary at 16 weeks (WCS 3). ×800. The same reaction was perceived with WCS 29 and 17.
on germ cells from 12 to 20 weeks. At later developmental stages all spermatogenic cells gave a distinct reaction with the exception of some large probably resting PGCs.

With monoclonal antibody WCS 28 a patchy fluorescence was observed on germ cells of the female gonad from 15 weeks onwards. The patches were present on the outer membranes and in the cytoplasm. At 18 weeks the plasma membranes of secondary oogonia and early prophase oocytes (the latter cells were identified from 16 weeks onwards) were distinctly stained, but primary oogonia showed only small patches (not shown). Larger follicular oocytes did not react with this antibody. In the testis primary spermatogonia showed the patchy reaction from 18 weeks onwards. At 20 weeks and later stages the membranes of secondary spermatogonia and spermatocytes were distinctly stained by this antibody with the exception of primary spermatogonia. Indirect immunofluorescence reactions for WCS 29 and WCS 28, carried out on consecutive sections at the age of 25 weeks, showed that WCS 29 stained the membranes of all germ cells, whereas WCS 28 gave only a patchy reaction with spermatogonia, while staining clearly the membranes of the later spermatogenic stages (Fig. 11A,B).
Fig. 11. Testis at 25 weeks. Indirect immunohistochemistry with WCS 28 (A) and WCS 29 (B). Bouin's fixative. A and B are consecutive sections; C is the same section as B, stained with Crossmon. Note that with WCS 29 all germ cells are stained; with WCS 28 the secondary spermatogonia show a clear membrane fluorescence, the primary spermatogonia only a patchy fluorescence in the cytoplasm. sgl, primary spermatogonia; sgII, secondary spermatogonia; t, tubule; it, interstitial tissue. ×480.
Male germ cells present in hermaphroditic ovaries were also stained with the four antibodies; their staining was similar to that in male gonads.

The immunocytochemical staining was done on material fixed in Bouin’s fluid. For comparison, indirect immunohistochemical reactions were carried out on frozen sections at 25 weeks, showing distinct surface fluorescence with the spermatogenic cells (Fig. 14).

From the age of 3 days onwards, none of the four monoclonal antibodies reacted with somatic tissues from carp, i.e. gut, liver, brain, spleen, muscle or kidney.

No fluorescence was observed in controls, using non-immune mouse serum, an antigen-negative hybridoma supernatant or PBS instead of the first antibody.

**DISCUSSION**

In the present study it was shown that in carp (*Cyprinus carpio* L.) at 3 days after fertilization, i.e. one day after hatching (23°C), primordial germ cells (PGCs) were present at the dorsal wall of the coelomic cavity at the site of the future gonadal ridges. Gonad formation started between 2 and 3 weeks. The onset of PGC proliferation was observed at the age of 7 weeks and differentiation into male or female gonad occurred from 10 weeks onwards. In the female gonad the oogonia entered meiosis at the age of 16 weeks and in the testis meiosis was observed for the first time at 20 weeks.

In our study we followed the terminology proposed by Nieuwkoop & Sutasurya (1979) in that the term ‘germ cell’ was used indiscriminately for all stages in the formation of germ cells. The cells were named primordial germ cell (PGC) after early morphological differentiation, during formation of the gonads and in the indifferent gonads, whereas after differentiation of the gonads into ovary and testis, the germ cells were named oogonia or spermatogonia, respectively.

Descriptions of gonad development revealed that considerable differences exist between different fish species (see reviews from Vivien, 1962, and Yamamoto, 1969). Yamamoto concluded that in gonochoristic species two types of gonad development can be distinguished: an undifferentiated type, in which during development all gonads pass through a female phase containing follicular oocytes; and a differentiated type in which the gonads differentiate directly into a female or a male gonad. Besides these two forms of gonochorism, various types of hermaphroditism were found in fishes (Yamazaki, 1983).

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Figs 12-13. Consecutive sections of indifferent gonads. Bouin’s fixative. Indirect immunohistochemistry with WCS 29 (A) and WCS 3 (C); (B) is stained with Crossmon. PGC, resting primordial germ cell; mPGC, mitotic primordial germ cell. ×680.

Fig. 12. Indifferent gonad at 5 weeks. The PGCs are clearly stained with WCS 29 (A), only a few patches are stained by WCS 3 (C). Also with WCS 17 only a few patches were stained.

Fig. 13. Indifferent gonad at 8 weeks. At this stage the PGCs are clearly stained with WCS 29 (A) and with WCS 3 (C). The same reaction was perceived with WCS 17.
Fig. 14. Testis at 25 weeks, further developed than in Fig. 11A. Indirect immunohistochemistry on frozen sections. (A) WCS 29; (B) WCS 3. ×420. Note the clear reaction of germ cell membranes. The same reaction was perceived with WCS 17.

The present study shows that gonadal development in carp is of the differentiated type. This result is in agreement with that of Stromsten (1931) for the goldfish (*Carassius auratus* L.) and Timmermans & Taverne (1983) for the rosy barb (*Barbus conchonius* L.). However, our results differ from those of Davies & Takashima (1980) who reported that carp gonads develop through an initial female phase. The reason for this difference is not known; it might be due to differences in rearing conditions or differences between studied strains. It is known that in carp hermaphroditism can occur. In the present study we found hermaphroditic gonads in 4 out of 36 fishes with sexually differentiated gonads.

In contrast with other vertebrates in which PGCs migrate to previously formed genital ridges (Nieuwkoop & Sutasurya, 1979), PGCs in carp were present at the site of the gonadal ridges before a gonadal anlage originated. Similar observations were previously reported for goldfish (Stromsten, 1931) and rosy barb (Timmermans & Taverne, 1983). The same phenomenon was also observed in *Xenopus laevis* (Wylie & Heasman, 1976; Wylie, Bancroft & Heasman, 1976).

The initial number of PGCs in carp was low and remained so for a considerable period up to 6 weeks, after which rapid proliferation started (Fig. 1). The rather large standard deviation at 7 weeks and in particular at 9 weeks with respect to the number of germ cells may be explained by the fact that not all larvae start proliferation at the same time, and that a number of larvae are somewhat delayed. Our results are corroborated by those of Stromsten (1931) who did not observe division of PGCs during a certain period in which the number of PGCs remained low, and the results of Timmermans & Taverne (1983) who found a ‘resting’ period of 3 weeks, followed by rapid proliferation too. Hamaguchi (1982) classified PGC proliferation in *Oryzias latipes* and noted mitoses of PGCs already at early
developmental stages, after segregation from the somatic cell lineage and after arrival at the gonadal sites. Lebrun et al. (1982) estimated germ cell numbers in post-hatched trout; however, they did not count PGCs but restricted their estimations to 'gonocytes', i.e. mitotic PGCs and their descendants. They and other authors (Wolf, 1931; Satoh & Egami, 1972; Quirck & Hamilton, 1973) found rather small numbers of early germ cells until sexual differentiation, and also no significant differences in numbers between males and females. It was suggested by Hamaguchi (1982) that different rates of germ cell proliferation in *Oryzias latipes* at the time of hatching might be the first sexual difference between males and females. Furthermore, female germ cells might mature earlier as stem cells of gonadogenesis than male germ cells, the latter being arrested in mitosis while female cells immediately entered meiosis. Similar observations had been made for a salmonid (Johnston, 1951) and a cichlid (Yoshikawa & Oguri, 1978). In carp, we were not able to distinguish sex differences at 7 to 9 weeks, when germ cell numbers were increasing; however, meiosis occured earlier in female than in male carp.

In a former paper (Parmentier et al. 1984) we reported that monoclonal antibodies, raised against carp spermatozoa, reacted with the surface membrane of individual spermatozoa and of precursor sperm cells in frozen sections of adult testis. Four monoclonal antibodies stained the surface membrane of early germ cells in sections of the ovary too. In the present study it was shown that these four monoclonal antibodies stained cell membranes of germ cells in frozen or Bouin's fixed sections of developing gonads. Moreover they stained the cell membranes of isolated spermatogenic cells (unpublished data). Only the PGCs, the spermatogonia and oogonia showed also some cytoplasmic fluorescence. This might be due to synthesis of membrane antigen but this has to be elucidated by further study. None of the monoclonal antibodies reacted with somatic tissues.

Immunohistochemistry revealed that monoclonal antibody WCS 29 stained selectively the PGCs as early as 3 days after fertilization, before gonad formation had started. Earlier stages were not studied in detail, but preliminary observations at 1 and 2 days after fertilization showed that somatic tissues were stained too, thereby obscuring possible PGCs. This might be due to fixation, for frozen sections of these stages did not react at all (unpublished data). However, it could not be concluded that the PGCs were not reactive at these stages as we were unable to distinguish them in frozen sections, probably due to their small number.

Though it remains to be elucidated at what developmental stage precisely the PGCs will start to react, we obtained evidence that a germ-cell-specific surface marker that is lacking on somatic cells is already present on early PGCs, before onset of gonad formation. To our knowledge this is the first report of such a phenomenon in lower vertebrates. In mouse it was demonstrated by Millette (1979) and Millette & Bellvé (1977) that polyclonal anti-mouse spermatogonia antisera, absorbed with mouse somatic cells, could recognize a set of antigens present on all germ cells located in the testicular tubule epithelium. Studies on the presence of spermatogogenic specific antigens of precursor sperm cells during murine
gonadogenesis were performed by O'Brien & Millette (1984) with a polyclonal, and by Bechtol (1984) with a monoclonal, anti-mouse sperm antiserum. These authors demonstrated that spermatogenic-specific surface molecules were detectable from early spermatocytes onwards and remained present up to mature spermatozoa. Our study presents evidence that in carp a spermatogenic-specific surface marker is present from an early PGC stage onwards, which on the male germ line remains present during proliferation, meiosis and spermiogenesis, including mature spermatozoa (Parmentier et al. 1984). Furthermore, our results suggest that a second germ-cell-specific surface marker appears on the outer plasma membrane of PGCs concomitantly with the onset of proliferation. This indicates that the transition from the resting phase to the proliferative phase can be considered as a differentiation step, characterized by the insertion of new molecule(s), or an alteration of existing one(s), in the PGC membrane. However, more evidence is needed to prove this assumption.

Monoclonal antibody WCS 28 reacted with the surface membrane of germ cells shortly before start of meiosis. After a patchy reaction from 15 weeks onwards, WCS 28 stained the whole cell membrane of differentiating germ cells in females at 18 weeks and in males at 20 weeks after fertilization. It is tempting to speculate that this represents the appearance of membrane determinant related to the onset of meiosis. O'Brien & Millette (1984) obtained evidence of a meiosis-associated antigen during spermatogenesis in the mouse. This antigen appeared in the cytoplasm of germ cells at the onset of meiosis but the insertion in the outer plasma membrane was delayed till the pachytene stage. Whether a similar phenomenon accounts for WCS 28 antigenic determinant in carp awaits, however, further clarification.

The four monoclonal antibodies reacted not only with male germ cells, but also with female germ cells. In male carp the demonstrated surface markers remained present on all spermatogenic cells, whereas in females only oogonia and early prophase oocytes were stained, but follicular oocytes were not, as in adult carp (Parmentier et al. 1984). These results suggest that germ-line-specific antigens are present in carp that are lost during the formation of the oocyte.

Several polyclonal and monoclonal antibodies have been raised against embryonal or undifferentiated teratocarcinoma cells recognizing F9, EMA1, SSEA1, M1/22.25 antigens and others on murine PGCs (see review from Eddy & Hahnel, 1984). However, while some antigens remained present on spermatogenic cells (F9), others were absent on PGC descendants (EMA1). Furthermore, during early development and often later on, somatic cells were also positive. Our observations indicate strongly that in carp from the age of 3 days onwards certain antigenic determinants are restricted exclusively to the germ line, both in males and females. Moreover, evidence was obtained by Parmentier et al. (1984), that antigenic determinants, specific for the male germ line, also arise during spermatogenesis.

In several studies on early germ cells of fish, a similarity of ultrastructural features of PGCs, spermatogonia and oogonia have been reported (Satoh, 1974;
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Bruslé & Bruslé, 1978a,b). Furthermore, the distinction between spermatogonia and oogonia in fish generally rests on the somatic gonadal characteristics (Reinboth, 1980). In carp, we observed no differences in the morphology of PGCs of both sexes, using light microscopy. Besides, these cells seem to contain common specific membrane antigenic determinants appearing on comparable differentiation stages in both sexes. These results and the scarce observations of hermaphroditic cells, therefore, are not contradictory to the concept that the undifferentiated PGCs in fish are bipotential (Dildine, 1936; Bruslé, 1983).

Summarizing, it can be concluded that there is evidence for the occurrence of three differentiation-specific antigenic determinants on the germ line in carp. The first is present as soon as the PGCs are located at the presumptive gonadal ridges and it might be present on earlier stages too. The second appears concomitantly with the onset of rapid PGC proliferation, and the third might be related to meiosis. Furthermore, none of these determinants were detected on the larger follicular oocytes nor on somatic tissues. We may conclude that with this set of antibodies it will be possible to determine specific differentiation steps in the carp germ line which should facilitate the study of physiological mechanisms during gonadogenesis. However, the nature of these antigenic determinants remains to be clarified.

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