CHARACTERIZATION OF ANTIGENS SPECIFIC FOR SPERMATOZOA AND GERM CELLS DURING EARLY GONADAL DEVELOPMENT OF CARP (*CYPRINUS CARPIO*, CYPRINIDAE, TELEOSTEI) WITH MONOCLONAL ANTIBODIES

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Fishes are suitable models to study early intragonadal germ cell development. In cyprinids like zebrafish or carp, the onset of gonadal development as well as primordial germ cell (PGC) proliferation occurs after hatching in the free-swimming larvae (Timmermans, 1987). 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 macromolecules will be inserted into the surface membrane, and they can be recognized with appropriate reagents such as monoclonal antibodies (Mabs).

MAbs against carp spermatozoa have been raised, which bound exclusively to germ cells but not somatic cells. Surprisingly, some MAbs recognized not only antigenic determinants on spermatozoa but also on (precursor) germ cell stages during development (Fig. 1). MAb WCS 29 bound (primordial) germ cells from hatching onward, whereas from the start of proliferation of PGCs in the indifferent gonad (after 6 weeks) a clear staining with MAbs WCS 3 and 17 was observed. From the onset of gametogenesis, i.e. spermatogenesis after the age of 19 weeks, germ cells were recognized by WCS 28 (Parmentier and Timmermans, 1985). These four MAbs reacted selectively with germ cells in histological sections and with germ cells isolated from gonadal tissue, thereby providing evidence that they recognized antigenic determinants from macromolecules inserted into the surface membranes of germ cells (van Winkoop and Timmermans, 1990).

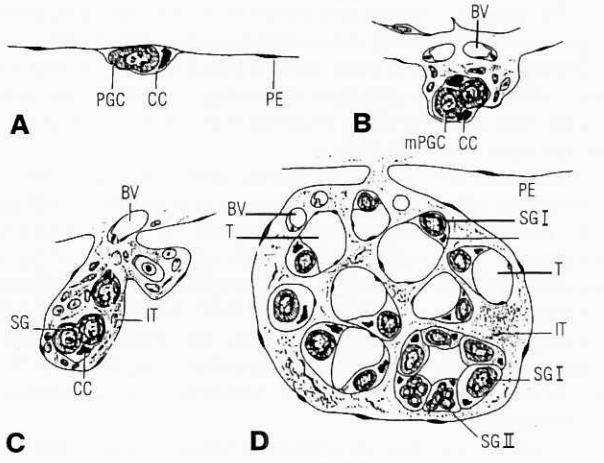

Figure 1. Schematic drawings of developing gonads of the carp (not to scale). A. Genital ridge after hatching showing primordial germ cell (PGC) surrounded by a somatic cell (cc). B. Indifferent gonad at the age of 8 weeks. C. Male gonad at the age of 15 weeks. D. Male gonad at the onset of spermatogenesis within tubules (19 weeks). BV, blood vessel; IT, interstitial tissue; mPGC, mitotic PGC; PE, peritoneum; SG, spermatogonia; T, tubule. (After Parmentier and Timmermans, 1985)

It is the aim of this study to further characterize the differentiation antigens, recognised by the MAbs WCS 3,17, 28 and 29 respectively, which are expressed on carp germ cells from early gonadal development onward, as a step towards unraveling the function(s) of these antigens.

For biochemical characterization a protein extract was prepared from carp spermatozoa according to procedures, developed for the extraction of surface membrane proteins from carp leucocytes (Koumans-van Diepen, 1993). Proteins were separated for Mw by SDS-PAGE. The sensitive immunoluminescence method (ECL-Western blotting, Amersham, England) was used for immunoblot analyses. With this method a set of at least 10 distinct bands was observed with WCS 3 and WCS 28 (Fig. 2). Bands ranged from a Mw of approx. 30 kD to Mw >200kD. With WCS 29 and WCS 17 a slightly different set of 5-10 bands was immunostained, although weaker, within the same range of mW. Moreover, in repeated immunoblottings with WCS 3 and WCS 28, positive bands with Mw of approx. 10-15 kD were intensely stained. A similarly located band was weakly stained by WCS 17. Immunoblots of carp thymocyte membrane lysates, prepared in the same manner as the spermatozoa lysate, served as control. None of the thymocyte immunoblots were stained with the four anti-carp spermatozoa MAbs (Fig.2). This provided further evidence that the recognized macromolecules in the immunoblots of carp spermatozoa extract are specific for carp germ cells, thereby confirming previous studies (Parmentier and Timmermans, 1985). The staining of multiple bands by all four MAbs suggested that multicomplexed forms of the antigenic determinants were isolated by the purification procedure.

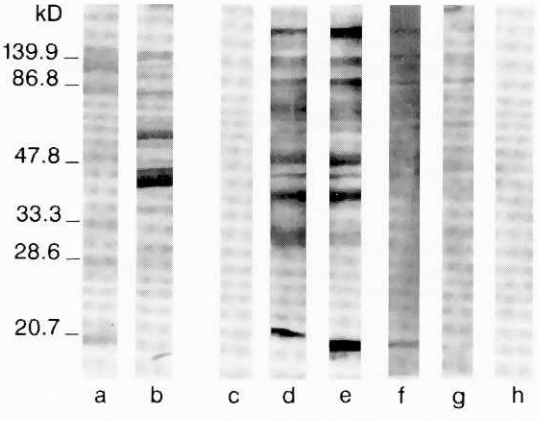

Figure 2. Immunoblotting of carp spermatozoa lysate with anti-carp spermatozoa Mabs WCS 3 (lane d), WCS 28 (lane e), WCS 17 (lane f) and WCS 29 (lane g). Left: numbers indicate Mw values of reference proteins in kD (lane a). Lane b is stained with amido black. Lane c represents an immunoblotting of thymocyte lysate with WCS 3 (a similar blotting was obtained with WCS 28) and lane h an immunoblotting with WCS 29 (similar blotting with WCS 17).

To investigate the possible carbohydrate nature of the recognized epitopes cryosections of frozen carp testes were pre-incubated with periodate before immunohistochemistry with the four MAbs. An anti-desmine MAb was used as a control for the possible protein nature of the recognized epitopes. The results showed that binding of WCS 17 and WCS 29 was inhibited after treatment with 100 mM periodate, whereas WCS 3, WCS 28 and the anti-desmine MAb showed still a distinct although diminished fluorescence. After treatment with lower concentrations periodate immunofluorescence with WCS 17 and WCS 29 was distinctly reduced but not with WCS 3, WCS 28 and the antidesmine Mab. These results indicate that WCS 17 and WCS 29 probably recognize proteins with sugar moieties (Woodward et al., 1985) and that WCS 3 and WCS 28 recognize (lipo-) proteins.

In mammals it is known that spermatozoa and precursor germ cells from the early pachytene stage onward are shielded from the individuals immune apparatus by a blood-testis barrier formed by tight junctions between Sertoli cells. Within these sheltered tubular compartments autoantigens arise on precursor germ cells and spermatozoa, which were recognized by appropiate MAbs from the pachytene stage, or from later spermatogenic stages onward, i.e. after the blood-testis barrier had been established (Eddy, 1988).

In a number of teleosts the presence of a blood-testis barrier has been demonstrated (a.o. Parmentier et al., 1985, in the carp; Lou and Takahashi, 1989, in the Nile tilapia (Oreochromis niloticus)). In these fish species the barrier is also formed by tight junctions between Sertoli cells, but contrary to mammals, only spermatozoa and late spermatids are shielded from the blood by this barrier. Consequently, it may be expected that autoantigens will be present only on these germ cell stages. Induction of autoimmunity in testes of teleosts has indeed been reported by several authors (see Lou and Takahashi, 1991).

The antigens recognized by the four anti-carp spermatozoa MAbs are specific for germ cells and consequently it cannot be excluded that with respect to spermatozoa, they respresent autoantigens or form part of autoantigens. In that case they may share determinants with differentiation antigens appearing on germ cells during early development. To investigate this hypothesis, autoantibodies were induced in carp specimens by injection of allogeneic carp spermatozoa.

Autoantibodies from immunized carp with high antibody titer (11-12) were purified by absorption with autologous carp sperm and after centrifugation eluted from the sperm pellet with potassium phosphate solution, pH 7.0. The autoantibody containing eluate (AAE) was applied on immunoblots from sperm extract prepared by ultrasonication and discontinuous sucrose gradient centrifugation according to Lou and Takahashi (1991), using HRP-labeled conjugates. It was observed that AAE stained a band of approx. 28.6 kD (Fig. 3). This corresponded with a band of similar Mw stained by WCS 3, suggesting that WCS 3 does indeed recognize (part of) an autoantigen. As MAb WCS 28 stained a similar set of bands as WCS 3 (see fig. 2), it very probably also recognizes (part of) autoantigen(s) on carp spermatozoa.

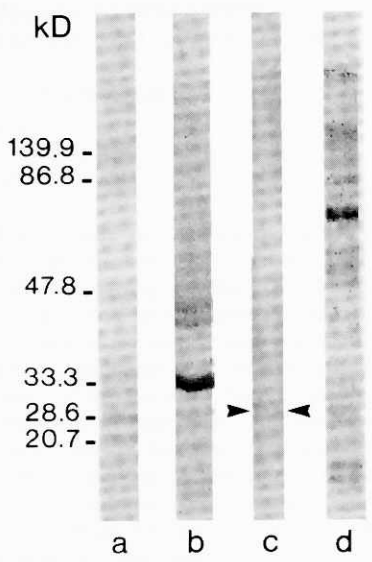

Figure 3. Immunoblotting of carp spermatozoa plasma membrane extract with anti-carp sperm autoantibody (lane c) and with WCS 3 (lane d). Note the positive band with Mw of approx 28.6 kD corresponding with band stained by WCS 3 (arrows). Left: numbers indicate Mw values of reference proteins in kD (lane a). Lane b is stained with amido black.

To our knowledge, in addition to work on carp, only two reports have appeared on anti-spermatozoa (auto)antibodies in a fish species (Lou and Takahashi, 1991; Mochida et al., 1994). Immunoblotting of spermatozoa membrane extract from the Nile tilapia revealed at least 15 constituent protein macromolecules which were recognized as surface antigens by conventional antibodies (Lou and Takahashi, 1991). Six polypeptides, corresponding with the major surface antigens, were identified as autoantigens by autoantibodies purified from the blood of male Nile tilapia specimens which had been previously immunized with allogeneic spermatozoa. Mochida et al. (1994) raised MAbs against Nile tilapia spermatozoa autoantigens, which had been isolated with autoantibodies, prepared according to the method of Lou and Takahashi (1991). Four MAbs were produced, recognizing polypeptides with Mw of approx. 27, 40, 80 and 120 kD, i.e. well within the range of Mw, recognized by the four anti-carp spermatozoa MAbs. Immunostaining of Nile tilapia germ cell surface membranes with the MAbs raised by Mochida et al. (1994) was restricted to late spermatids and spermatozoa, i.e. the germ cell stages shielded from the blood by the blood-testis barrier.

This is in contrast with our results in carp which suggest that WCS 3 and possibly also WCS 28 do recognize at least parts of an autoantigen on the surface of carp spermatozoa. It is unlikely, however, that WCS 3 and 28 recognize autoantigenic epitopes, since these MAbs recognize also determinants on the surface of precursor germ cells from early larval stages onward, i.e. outside the blood-testis barrier. It is hypothised that the latter determinants belong to germ cell specific differentiation antigens (appearing on the surface membrane of germ cells when new differentiation steps occur), which might form a core of future autoantigens. However, the nature of germ cell specific antigens in carp requires further studies.

The available MAbs will allow to isolate and sequence the recognized antigen(s) enabling the investigation of their possible function(s). That will be the object of future studies.

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