Monoclonal antibodies against goldfish (Carassius auratus) immunoglobulin: application to the quantification of immunoglobulin and antibody-secreting cells by ELISPOT and seric immunoglobulin and antibody levels by ELISA in carp (Cyprinus carpio)

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Vet Res (1994) 25, 458-467

Original article

Monoclonal antibodies against goldfish *(Carassius auratus)* immunoglobulin: application to the quantification of immunoglobulin and antibody-secreting cells by ELISPOT and seric immunoglobulin and antibody levels by ELISA in carp *(Cyprinus carpio)*

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(Received 15 February 1994; accepted 17 May 1994)

Summary — Monoclonal antibodies (mAbs) raised against heavy and light chains of goldfish immunoglobulin (Ig) were characterized by a Western blot technique. A complete cross-reactivity was observed between carp and goldfish Ig. These mAbs were used for the quantification of carp Ig and anti-*Yersinia ruckeri* antibodies by ELISA. An ELISPOT assay was also developed in carp to quantify Ig-secreting cells (ISC) and antibody-secreting cells (ASC). The number of ASC was maximum on day 18 post-vaccination and decreased to the basal level on day 28. The antibody levels in sera were maximum on day 18 and slowly decreased until day 28.

monoclonal antibody / ELISA / ELISPOT / antibody-secreting cells / *Cyprinus carpio*

Résumé — Utilisation d'anticorps monoclonaux spécifiques de l'immunoglobuline (Ig) de carassin à la quantification de cellules sécrétrices d'Ig et d'anticorps (Ac) par ELISPOT et des taux d'Ig et d'Ac sériques par ELISA chez la carpe. *Des anticorps monoclonaux (AcMc) dirigés contre les chaînes lourde et légère d'immunoglobuline (Ig) de carassin ont été caractérisés par immuno-empreinte. Une réaction croisée avec les chaînes lourde et légère d'Ig de carpe a été observée. Ces AcMc ont été utilisés pour détecter les Ig et les anticorps anti-*Yersinia ruckeri *(Ac) de carpe*

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par ELISA. Par ELISPOT, on a pu quantifier les cellules sécrétrices d'Ig (CSI) et d'Ac (CSA). Le nombre de CSA était maximal 18 j après la vaccination et était revenu au niveau de base au bout de 28 j. La réponse anticorps dans les sérums était maximale 18 j après la vaccination et diminuait lentement par la suite.

***anticorps monoclonal / ELISA / ELISPOT / cellule sécrétrice d'anticorps /* Cyprinus carpio**

INTRODUCTION

Polyclonal antibodies (Ab) raised against fish immunoglobulin (Ig) are not specific enough because they are mainly directed towards the carbohydrate part of the Ig molecule. This is a well-known problem for lower vertebrates in which carbohydrate moieties of Ig molecules are highly immunogenic in the rabbit or mouse. Consequently, monoclonal antibodies (mAbs) were produced against light (L) and heavy (H) chains of the Ig molecule of goldfish (this work) or carp (Secombes *et al,* 1983; Koumans-van Diepen *et al,* 1994) which were specific for the polypeptide part of the Ig molecule. These mAbs were used in the present work to study the immune response of carp.

The humoral response is one part of the immunological reaction of an organism after exposure to a pathogen: phagocytosis, antigen processing, proliferation of lymphocytes and secretion of antibodies are the major steps.

For many years, the haemolytic plaque assay (PFC) was the most widely used test to assess antibody-secreting cells (B lymphocytes) in rainbow trout *(Oncorhynchus mykiss)* (Chiller *et al,* 1968; Anderson *et al,* 1979), or in carp *(Cyprinus carpio)* (Rijkers *et al,* 1980; van Muiswinkel *et al,* 1985; Siwicki *et al,* 1990). Because of the high non-specific background observed in PFC test in carp, due to the natural high haemolytic reaction, the assay was performed with bream *(Aramis brama)* complement (van Muiswinkel *et al,* 1985).

An alternative assay called ELISPOT, based on a solid-phase immunoassay technique, has been developed to quantify antibody-secreting cells (ASC). The original technique of Czerkinsky *et al* (1983) used horseradish peroxidase (HRP)-labelled antibodies and a chromogen substrate for the detection of ASC. Secombes *et al* (1991) adapted this method to dab *(Limanda limanda)*, and Davidson *et al* (1992) adapted it to rainbow trout for quantification of specific ASC to *Aeromonas salmonicida*. We have previously used ELISPOT to quantify ASC in rainbow trout immunized with *Yersinia ruckeri* (Siwicki and Dunier, 1993).

In the present paper, we adapted the ELISPOT assay to quantify Ig-secreting cells (ISC) and ASC in carp using one of the mAbs produced. The same mAb was also used in ELISA to quantify total Ig and specific Ig against *Y ruckeri* in carp sera.

MATERIALS AND METHODS

Animals

Mice

Balb/c mice were provided by the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France) and bred at the Université Pierre-et-Marie-Curie (Paris, France).

Fish

Ig purification

Goldfish *(Carassius auratus)* and carp *(C carpio)* were obtained from commercial suppliers, maintained in tap water (17 ± 2°C) and fed daily with trout pellets (Trouvit, France) at the Université

Pierre-et-Marie-Curie (Paris, France). Sera were used for Ig purification. The Ig were then used to prepare the immunizing antigen (goldfish Ig) and to screen and characterize positive hybridomas (carp and goldfish Ig).

ELISPOT and ELISA applications

Sixty healthy carp weighing 100–150 g were purchased from a local fish farm (La Dombes, Ain, France). They were maintained in 300 l tanks with a continuous flow of dechlorinated water thermoregulated at 23°C. Fish were fed daily with commercial carp pellets at 3% body weight. After 2 weeks acclimatation, 40 fish were immunized by intraperitoneal injection of 0.2 ml *Y ruckeri* vaccine (Yersivax®) at 10^8 bacteria/ml phosphate-buffered saline (PBS) (Rhône-Merieux IFFA Laboratory, Lyon, France). The 20 control fish received PBS only. At days 12, 18, 21 and 28 after vaccination, the fish were anesthetized, the blood collected from the caudal vein and head kidneys sampled (Cossarini-Dunier *et al,* 1987).

Purification of carp and goldfish Ig

Natural anti-(di or tri)nitrophenol (DNP/TNP) fish antibodies were purified by affinity chromatography on a DNP-lysine Sepharose column, as previously described (Vilain *et al,* 1984). Alternatively, Ig were enriched from normal fish sera by extensive dialysis at 4°C for 3 d against several changes of 5 mM Tris-HCl, pH 7.5 (Dighiero *et al,* 1985). The precipitates were washed at 4°C with the dialysis buffer, dissolved in 0.1 M Tris-HCl, pH 8.6, and 0.15 M NaCl and dialyzed against PBS. Ig formed the major component of these euglobulin fractions.

Obtention of mAbs

First fusion (F 14)

An indirect strategy was used to immunize mice. First, goldfish were immunized with Balb/c mice red blood cells (MRBC) twice on days 0 and 4 with 0.1 ml of 20% MRBC in saline and bled on day 15. This schedule was previously shown to elicit high haemagglutinin titres in the goldfish (Desvaux and Charlemagne, 1981). MRBC were then incubated (15 h at 4°C) with a subagglutinating dilution of goldfish anti-MRBC pooled sera. The immune complexes formed between MRBC and fish antibodies were extensively washed in cold PBS. Second, these immune complexes were injected to Balb/c mice with 0.1 ml of 25% MRBC/anti-MRBC goldfish Ig complexes in PBS. Two injections were made at 3 weeks interval. Immunized spleen cells were collected 3 d after the booster injection and fused with myeloma cells. This first fusion allowed the selection of several hybridomas secreting antibodies which strongly precipitated goldfish serum Ig.

Second fusion (F 24)

Immune precipitates were obtained by incubation of twice-diluted ascitic fluid from hybridoma clone 14/35 from fusion F14 with an equal volume of undiluted goldfish serum (15 h at 4°C). Precipitates were extensively washed in cold PBS and used to immunize Balb/c mice. Five weekly injections were made in the presence of complete (1st injection) or incomplete (2nd to 5th injections) Freund adjuvant and fusion F 24 was made 3 d after the last injection. Positive hybridomas were selected by ELISA against pure goldfish Ig and were cloned twice by the limiting dilution method with mouse thymocytes as feeder cells. Positive clones were frozen in liquid nitrogen.

For the production of large amounts of mAbs, 1 to 5 x 10^6 hybridoma cells were injected intraperitoneally into 8-10-week-old Balb/c mice that had been primed 5–20 d before with 0.5 ml pristane (Sigma). Culture supernatants derived from *in vitro* growing hybridoma clones were concentrated 10–20 times by filtration (Minicon B125, Amicon, France) and tested for the presence of various classes of mice Ig by double diffusion in agar in the presence of class-specific antisera (Nordic, Tilbing, The Netherlands).

Applications in performed with ELISA and ELISPOT were unconcentrated culture supernatants of hybridomas named 24–38 and 24–46.

SDS-PAGE and Western blot analysis

The selected mAbs were checked for their properties to recognize determinants associated with heavy (H) or light (L) chains of fish Ig molecules. For this purpose, an immunoblotting technique was used. Briefly, purified fish Ig or normal sera

were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Polypeptides were then electrophoretically transferred onto nitrocellulose membrane (Tranfor, LKB, Sweden). After a saturation step (1 h in 0.5% skim milk), membranes were incubated 2 h with 1/500-diluted mAb (ascite), washed, and incubated with peroxidase-labelled Ab anti-mouse Ig (Dako). The peroxidase activity was revealed by diaminobenzidine (DAB, Sigma) in the presence of H_2O_2.

ELISA

Four different ELISA methods were used in a similar way to those described in Vergnet and Dunier (1993a,b).

Murine IgG concentrations in hybridoma culture supernatants were determined with slight modifications (Vergnet and Dunier, 1993a) of the method of Fleming and Pen (1988) (ELISA-1). Briefly, the coating step (step 1) was performed with goat Ab anti-mouse IgG (GAM-Ig, Zymed), then mAb supernatants (10-fold serial dilutions) and standard pure murine IgG (Sigma) in step 2 and peroxidase-labelled GAM-Ig (HRP-GAM-Ig) in step 3 (1:3 000).

ELISA-2 allowed the screening and the comparison of mAb activity against pure carp Ig. This indirect assay was done with pure carp Ig in step 1 (24 ng/well), mAb supernatants (1:1 000) in step 2 and HRP-GAM-Ig in step 3 (1:3 000). Pure trout Ig (Vergnet and Dunier, 1993a) was used as a negative control antigen (56 ng/well) and mAb 1–14 anti-heavy chain of trout Ig (Deluca *et al,* 1983) as a negative control antibody. Activity was expressed in $OD_{450\ nm}$ for 1 ng mAb per well.

ELISA-3 was designed for application of mAbs to quantify carp Ab (anti-*Y ruckeri*). This was an indirect sandwich assay with *Y ruckeri* in step 1 (Yersivax vaccine 1:400), carp sera (1:80) in step 2, mAb 24–38 in step 3 (1:50 diluted supernatant) and HRP-GAM-Ig in step 4 (1:3 000). MAb 1–14 (Deluca *et al,* 1983) was used as a negative control antibody. Ab levels were expressed in $OD_{450\ nm}$.

ELISA-4 was the application of mAb to the quantification of carp Ig in sera. This test was done with sheep Ab anti-carp Ig (SAC-Ig, Seromed) in step 1 (1:200 000), carp sera (1:4 000) in step 2, mAb 24–38 in step 3 (1:100 diluted supernatant) and HRP-GAM-Ig in step 4 (1:3 000). Standard curves could be obtained with pure carp Ig in step 2 (110–1 200 ng/ml). Ig levels were expressed in $OD_{450\ nm}$.

Isolation of carp leucocytes

Leucocytes from blood were purified on a Lymphoprep gradient (Nycomed) as previously described (Cossarini-Dunier *et al,* 1988). Head kidneys from non-vaccinated and vaccinated carp were removed and single cell suspensions obtained by teasing the tissues in medium through a steel mesh (150 µm). Cell suspensions were separated and prepared as described for carp by Bayne (1985), using a 54% Percoll gradient (Pharmacia).

Viable cells from head kidney and blood were counted by trypan blue exclusion after 3 washings in Leibovitz medium (L15, Gibco). The cells were prepared at 5 x 10^6/ml for head kidney and at 1 x 10^7/ml for blood in L15 with 5% fetal calf serum (FCS, Gibco). Erythrocytes isolated from blood were used as a negative control.

Quantification of ISC and ASC by ELISPOT

ISC ELISPOT assay

Multiscreen-HA 96-well filtration plates (cellulose ester 0.45 µm, Millipore) were coated with 100 µl SAC-Ig (Seromed) at dilution 1:1 000 and incubated overnight at 4°C. After 3 washings with PBS (Nunc-Immuno Wash 12 system, InterMed), the plates were incubated with 200 µl L15 plus 5% FCS for 1 h at 37°C to block the remaining binding sites.

After removal of this blocking medium, 100 µl lymphocyte suspension was added to each well at 5 x 10^5 cells/well for head kidney and 1 x 10^6 cells/well for blood. Assays were repeated 4 times. The cells were incubated 6 h at 22°C. A control was carried out for each cell type and concentration.

After incubation, the plates were washed 3 times in PBS and 8 times with PBS + 0.05% Tween. Then 100 µl mAb 24–38 diluted 1:100 in PBS (optimal concentrations was previously determinated by ELISA-4) were added to each well

and incubated overnight at 4°C. After 3 washings in PBS, 100 µl HRP-GAM-Ig (Zymed), diluted 1:2 000 in PBS-Tween containing 1% FCS, was added to each well. Plates were incubated 6 h at 4°C. After 3 washings in PBS, 100 µl 'TMB membrane peroxidase substrate system' (Kirkegaard and Perry Lab Inc, MD, USA) were added to each well. The plates were incubated 15 min before washing with tap water and allowed to dry. The blue spots were counted using a Leitz microscope at low magnification (x 4) and results expressed as spot-forming cells per 10^6 cells from head kidney or blood.

ASC ELISPOT assay

Multiscreen-HA plates were coated with 1 x 10^7 *Y ruckeri* bacteria per well (100 µl) and incubated overnight at 4°C. After 3 washings in PBS, the cells from head kidney and blood were assayed as previously described above for the non-specific ELISPOT assay.

Statistical analysis

Statistical analysis were performed using ANAVAR. Differences in means were considered statistically significant at $P < 0.05$ with the F values of Snedecor.

RESULTS

Activity of anti-goldfish mAbs

Of the hybridomas produced, only 2 were retained for high specific reaction against goldfish Ig. They were both of the IgG1-kappa isotype.

Molecular specificity

MAbs were analysed by a Western blot technique using goldfish and carp purified Ig submitted to SDS-PAGE and transferred to a nitrocellulose membrane. MAbs 24-46 and 24–38 were specific for the heavy chain and the light chains of goldfish Ig, respectively (fig 1). Moreover, these mAbs show identical specificity against heavy and light chains of carp (fig 1). This cross-reaction between these 2 Cyprinid Ig allowed us to use these antigoldfish Ig mAbs against the carp Ig on which we focused the major part of this work.

MAb activities in ELISA

We compared 5 mAb 24–38 supernatants with 5 mAb 24–46 supernatants in which murine IgG concentrations were determined by ELISA-1. Their mean activities against pure carp Ig (ELISA-2) were 0.834 and 0.299 ($OD_{450\ nm}$ for 1 ng mAb per well), respectively. In ELISA-3, mAb 24–38 was

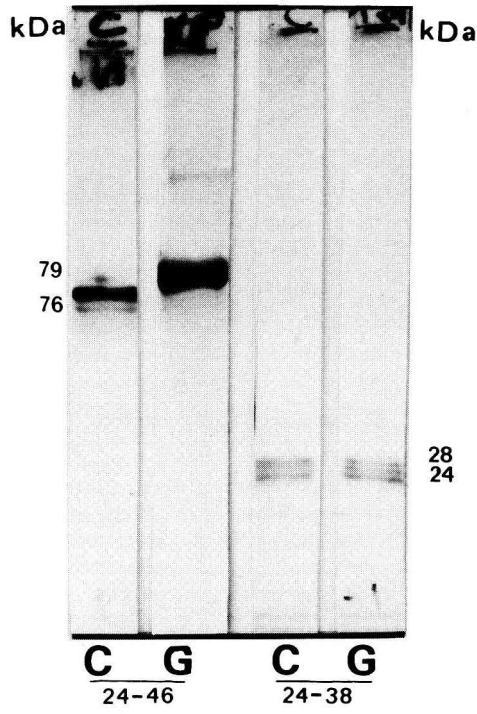

Fig 1. Western blots of goldfish (G) and carp (C) purified immunoglobulins; mAb 24-46 is specific for the heavy chain and mAb 24-38 for the light chain.

successful for measuring anti-*Y ruckeri* Ab in a vaccinated carp serum (1:400) at 0.73 ($OD_{450\ nm}$ for 10 ng mAb per well). The specificity was certified by the absence of reaction with a non-vaccinated carp serum (1:400) at 0.03 (OD_{450nm} for 10 ng mAb per well). Unexpectedly, mAb 24–46 was negative with a vaccinated carp serum in this procedure at 0.01 ($OD_{450\ nm}$ for 10 ng mAb per well).

Ig and Ab kinetics in carp sera (ELISA)

The Ig concentrations (determined by ELISA-4) of vaccinated and control fish sera were similar and constant (fig 2).

The Ab levels (determined by ELISA-3) were different in the same sera. The control group showed no natural anti-*Y ruckeri* Ab as expected in healthy fish. The vaccinated group showed high Ab level on day 18 which slowly decreased on days 21 and 28 (fig 2).

ISC and ASC kinetics in head kidney and blood (ELISPOT)

ISC kinetics

The kinetics for ISC in head kidney and blood leucocytes from non-vaccinated and vaccinated carp are shown in figure 3. On day 12 after vaccination, similar numbers of ISC in vaccinated carp were observed in head kidney and blood (89.5 ± 18.5 and 86.8 ± 9.5 cells per 10^6 leucocytes, respectively). After day 12, the numbers of ISC increased rapidly with the maximum observed 18 days post-vaccination (359.5 ± 32.5 for head kidney and 324.5 ± 21.5 for blood). Decreases in the numbers of ISC were observed on days 21 and 28, which were close to day-12 levels at the end of the kinetics. ISC numbers in control carp were lower and constant during the kinetics, with a statistically significant difference when compared with the vaccinated group.

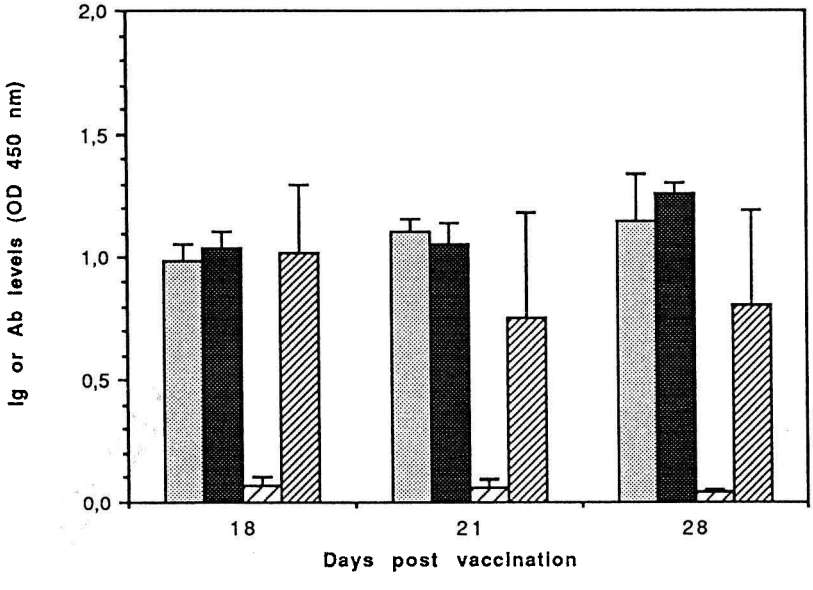

Fig 2. Kinetics of seric Ig and anti-*Y ruckeri* antibody (Ab) levels in vaccinated (vac) and control (con) carp. Mean ± SD, $n = 5$. ⬚ con Ig; ▩ vac Ig; ⧄ con Ab; ▨ vac Ab.

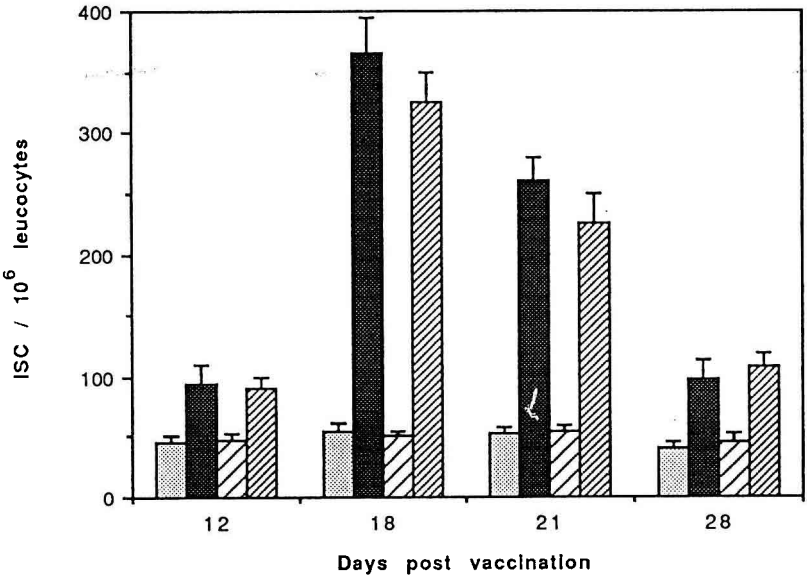

Fig 3. Kinetics of the numbers of the ISC per 10^6 head kidney (Hk) or blood (Bl) leucocytes from carp vaccinated with Yersivax® (vac) or non-vaccinated (con). Mean ± SD, $n = 5$. ☐ con Hk; ▨ vac Hk; ▨ con Bl; ▨ vac Bl.

ASC kinetics

The kinetics for ASC in head kidney and blood from vaccinated and non-vaccinated carp are shown in figure 4. On day 12 post-immunization, the numbers of ASC were higher in head kidney (48.5 ± 3.5) compared with blood (37.9 ± 2.6). ASC in vaccinated fish were statistically significantly higher than that from control fish. The ASC levels greatly increased by day 18 in head kidney and blood (301.8 ± 28.4 and 287.6 ± 33.5, respectively), and began to decrease by day 21 (190.5 ± 18.5 and 165.6 ± 19.2). On day 28, numbers of specific ASC were similar to day 12 in kidney and blood. ASC numbers in control fish were very low in the kinetics.

DISCUSSION

The 2 mAbs against goldfish Ig, specific for the heavy (24–46) and the light (24–38) chains, respectively, cross-reacted with heavy and light chains of carp Ig as was evidenced by Western blot results.

These mAbs gave different activity pattern in ELISA. Firstly, mAb 24–46 gave lower antigen–antibody reaction than mAb 24–38 for the same IgG level against pure coated carp Ig (ELISA-2). Secondly, only mAb 24-38 could detect seric Ab adsorbed on coated *Y ruckeri* antigen (ELISA-3). MAb 24–46 was negative when used at the same IgG levels.

We assumed that this last result could be due to differences in epitope specificity and affinity. In trout, we could use mAb 1–14, an anti-heavy chain as is mAb 24-46 in carp, to measure Ab levels in trout sera by an ELISA similar to the ELISA-3 described in this work (Vergnet and Dunier, 1993b). Comparisons of epitope specificity and affinity of these 2 anti-heavy chain mAbs could help understand their differences in Ab quantification by ELISA.

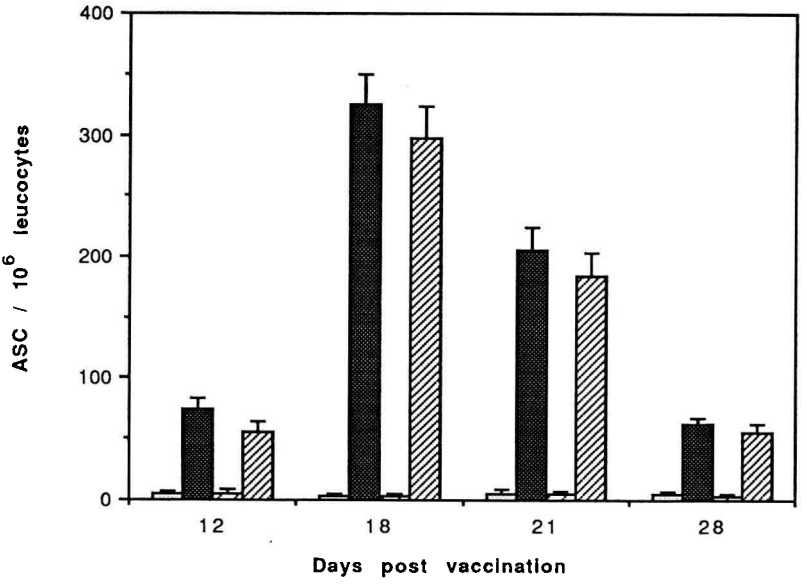

Fig 4. Kinetics of the numbers of anti-*Y ruckeri* ASC per 10^6 head kidney (Hk) or blood (Bl) leucocytes from carp vaccinated with Yersivax® (vac) or non-vaccinated (con). Mean ± SD, $n = 5$. □ con Hk; ▩ vac Hk; ▨ con Bl; ▨ vac Bl.

Applications in seric Ab and Ig quantification by ELISA were developed with mAb 24–38, which was further used to measure ISC and ASC in head kidney and blood by ELISPOT assays. It could be interesting to compare this anti-goldfish Ig mAb with the anti-carp Ig mAbs (Secombes *et al,* 1983) in the same application assays.

ELISPOT assay was more specific and easier to perform than the reference PFC test in carp. It had the particular advantage to avoid the use of bream complement needed in PFC test. Furthermore, the detection of ASC to pathogenic agents was easy, as shown in the present study using *Y ruckeri* as an antigenic model. We adapted the ELISPOT assay developed by Secombes *et al* (1991) from dab to carp. As mAb 24–38 was not labelled, an additional step was needed with HRP-GAM-Ig to visualize spots. The optimum time to incubate head kidney and blood leucocytes was similar in carp and dab (Secombes *et al,* 1991) and the number of ASC correlated with the concentration of *Y ruckeri*. Low doses of antigen (10^6) gave lower ASC levels, when compared with higher doses (10^7 and 10^8). Similar results in rainbow trout were observed by Davidson *et al* (1992) with *A salmonicida*.

We applied these ELISA and ELISPOT assays to study carp immune response after vaccination against *Y ruckeri*.

Ig levels (fig 2) were similar in control and vaccinated groups and kinetics were nearly constant. Ab levels were very low in control group, as expected for healthy fish. Vaccinated carps gave high Ab levels from day 18 to 28. The kinetics of ISC (fig 3) and ASC (fig 4) in the control group were low and constant. The vaccinated group showed very different kinetics. From day 12 to 28, ISC and ASC concentrations increased to a maximum on day 18 and decreased on day 28 to the day 12 level. Comparison of

figures 3 and 4 showed that the major part of ISC after vaccination were specific for *Y ruckeri*.

The kinetics of ASC were not parallel to kinetics of Ig and Ab levels. This was particularly evident for Ig and ISC. No difference between control and vaccinated groups were observed in Ig levels whereas ISC in vaccinated group were higher than in non-vaccinated group. These results meant that the reponse to vaccination could be seen in ISC which were higher, but was not detected in the Ig levels. The kinetics of the Ab levels and ASC parallel more closely, with the same maximum on day 18 but with a higher decrease for ASC than for Ab levels after peak. The modification of ASC induced by the vaccination was a relatively short event, since it was seen during 2 weeks, and after one month; only Ab levels could attest for vaccination.

These results were slightly different than those obtained in trout by Anderson *et al* (1990) which kinetics were done on a larger time scale and with more samplings. Moreover, our data were obtained in special conditions of breeding carp in a 23°C water temperature in order to maximize the efficacity of the vaccination. Extrapolation of these results to the field could be done with precaution until more data are obtained.

ACKNOWLEDGMENT

We thank MA Costa for her diligent technical assistance.

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