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Characterisation of monoclonal antibodies against heavy and light chains of trout immunoglobulin

C. SÁNCHEZ*, P. LÓPEZ-FIERRO†, A. ZAPATA‡ AND J. DOMINGUEZ*§

**Departamento de Sanidad Animal, INIA, Embajadores 68, 28012 Madrid, Spain, †Departamento de Biología Celular y Anatomía, Facultad de Biología, Universidad de León, 24073 León, Spain and ‡Departamento de Biología Celular, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain*

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Fourteen monoclonal antibodies which recognise antigenic determinants on heavy and light chains of trout immunoglobulins have been characterised. Experiments on immunoprecipitation and immunostaining of lymphoid cells carried out with these monoclonal antibodies suggest the existence of antigenic variants within trout immunoglobulin. Most of these variants seem to be isotypes, since they were present in each of 30 sera from individual trout tested. Analysis of the immunoprecipitates by SDS–PAGE showed two light chains of different molecular weight. Cross reactivity patterns of these monoclonal antibodies with sera from four species and one subspecies of salmonid indicate a high conservation of the epitopes recognised, although four monoclonal antibodies show a more restricted reactivity.

Key words: rainbow trout, immunoglobulin, isotypes, monoclonal antibodies.

I. Introduction

Previous studies on the phylogeny of immunoglobulins suggested that fish had only one class of Ig, that resembled the mammalian IgM (Nisonoff *et al.*, 1975). However, studies carried in the last 10 years have revealed the existence of a higher structural and antigenic heterogeneity in fish Igs. Two isotypes of heavy (H) chain have been described in elasmobranchs (Kobayashi *et al.*, 1984; Kobayashi & Tomonaga, 1988) and in agnatha (Hanley *et al.*, 1990), and the use of monoclonal antibodies (MAbs) has permitted the definition of two isotypes of light (L) chains and three isotypes of H chains in the channel catfish (*Ictalurus punctatus*, Teleostei) (Lobb *et al.*, 1984; Lobb & Olson, 1988).

Immunoglobulin gene organisation shows striking differences between elasmobranchs and teleosts. Whereas the H chain genes in elasmobranchs are arranged in a repeating multicluster pattern $(V_H\text{-}D\text{-}J_H\text{-}C_H)_n$ (Kokubu *et al.*, 1989*a*,*b*), in teleosts, as in mammals, only a single genomic copy of the C_H genes is present and different V_H genes are expressed with the same C region gene (Ghaffari & Lobb, 1989; Amemiya & Litman, 1990). Genomic analyses in catfish

§Author to whom correspondence should be addressed.

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suggest the existence of, at least, two distinct C_H genes, and about 100 V_H genes distributed in five different families (Ghaffari & Lobb, 1989, 1991). These V_H families could account for some of the H chain isotypes defined with the MAbs.

In salmonids, only one class of serum Ig has been described, that has a tetrameric IgM-like structure and a molecular weight around 750 kDa. However, using MAbs, Killie *et al.* (1991) have recently suggested the existence of isotypic variants in the H chains of Atlantic salmon (*Salmo salar*, L.), and we have identified two structurally and antigenically different L chains in rainbow trout (Sánchez & Domínguez, 1991).

We have developed a panel of MAbs against trout Ig to use in the analysis of trout humoral immune responses and as B cell markers. This paper describes the characteristics of 14 of them. Data from molecular and cellular analyses carried out with these MAbs suggest the existence of antigenic variants for the H and L chains of the trout Ig.

II. Material and Methods

FISH

Rainbow trout (*Oncorhynchus mykiss*), approximately 20 cm in length were obtained from a local fish farm. Blood was collected by caudal vein puncture. Serum was separated by centrifugation and stored at −20° C until used.

TROUT IMMUNOGLOBULIN PURIFICATION

Trout Ig was purified by a combination of anion-exchange and gel filtration chromatographies, as previously described (Sánchez *et al.*, 1989). Fractions were assayed for Ig activity by immunodiffusion and by enzyme linked immunoabsorbant assay (ELISA) using sheep anti-trout Ig polyclonal antibodies (Seromed, Germany). Purity was tested by SDS–PAGE under reducing conditions (Laemmli, 1970).

ISOLATION OF IMMUNOGLOBULIN HEAVY (H) AND LIGHT (L) CHAINS

One milligram of trout Ig in 0·5 ml of 0·55 M Tris–HCl pH 8·1, was reduced with 0·02 M 1,4-dithiothreitol (DTT) for 30 min at room temperature, followed by alkylation with 0·12 M iodoacetamide for 1 h. The sample was gel filtered on Sephacryl S-300 (1·5 × 96 cm) in 1 M acetic acid, at a flow rate of 18 ml h^{-1}. Fractions containing H or L chains were adjusted to pH 7·0 with 5 N NaOH and their purity analysed by SDS–PAGE.

Reactivity of MAbs against H or L chains was analysed by an indirect ELISA. M-129 B plates were coated with 0·2 μg/well of isolated H, L chains or trout Ig (assumed $E^{1\%}_{280\,nm} = 1{\cdot}4$), diluted in PBS by overnight incubation at 4° C. The ELISA was performed as described below.

PRODUCTION OF MONOCLONAL ANTIBODIES

Female BALB/c mice were injected intraperitoneally (i.p.) with 20 μg of trout Ig emulsified in complete Freund's adjuvant. Twenty-five days later, a second

dose of 100 μg of trout Ig emulsified in incomplete Freund's adjuvant was given i.p. Three days prior to fusion, made on day 35, the mice received daily by i.p. or intravenous injections, 60 μg of trout Ig, according to the method of Stahli *et al.* (1983). The spleen cells were fused with the non-secreting X63 Ag8.653 myeloma cell line, according to the method of Kohler & Milstein (1975). Culture supernatants from the wells with growing colonies were assayed by an indirect ELISA as described below. The hybridomas selected were cloned by limiting dilution at least twice. The class and subclass of the monoclonal antibodies were determined by an indirect ELISA using rabbit antisera specific for mouse heavy and light chains and a peroxidase conjugated goat-anti-rabbit Ig (Bio-Rad, U.S.A.).

Ascites were produced by injecting $0{\cdot}5–2 \times 10^6$ cells intraperitoneally into pristane-primed BALB/c mice and the ascitic fluid was withdrawn after 10–14 days. Monoclonal antibodies were purified from ascites by 40% saturated ammonium sulphate precipitation or by affinity chromatography on Protein A Sepharose CL-4B (Pharmacia, Sweden) as described by Ey *et al.* (1978).

Protein A-purified MAbs were biotinylated as described previously (Domínguez *et al.*, 1990).

ELISA FOR DETECTION OF MAb SECRETING HYBRIDOMAS

M-129 B ELISA plates (Dynatech, Germany) were coated with 2 μg/well of trout Ig diluted in PBS and incubated overnight at 4° C. Residual binding sites were blocked by incubating the plates with 0·2 ml/well of 1% bovine serum albumin (BSA) in PBS for 1 h at 37° C. Culture supernatants, diluted in PBS containing 1% BSA, 0·2% Tween 20 and 0·01% merthiolate (dilution buffer), were added and incubated for 1 h at room temperature. The plates were washed five times with tap water containing 0·1% Tween 20 (washing solution). Subsequently, 100 μl of rabbit anti-mouse Igs conjugated to peroxidase (Sigma, U.S.A.), diluted at 1/1000 in dilution buffer, were added and incubated for 1 h at room temperature. Finally, after five washes in washing solution, 50 μl of the substrate containing 1,2 *o*-phenylendiamine (2 mg ml^{-1}) in 0·1 M citrate buffer pH 5·0, and 0·03% H_2O_2 were added. Colour development was stopped by adding 50 μl of 3 N H_2SO_4 after 15 min. The plates were read on a Titertek Multiskan (Flow, Scotland) at 492 nm.

To determine whether the MAbs were specific for carbohydrate antigenic determinants, plates coated with trout Ig were treated with 20 mM periodate according to the protocol described by Woodward *et al.* (1985); and the assay was followed as above described.

DOUBLE ANTIBODY (SANDWICH) ELISA

The reactivity of the MAbs with Ig from other salmonid species was analysed using a double antibody sandwich ELISA. Plates coated with 1 μg/well of protein A-purified MAbs were incubated for 1 h at room temperature with a 20-fold dilution of serum from rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), coho salmon (*O. kisutch*), chinook salmon (*O. tshawytscha*), chum salmon (*O. nerka*) and sockeye salmon (*O. nerka*). After five washes with

washing solution, the plates were incubated for 1 h at room temperature with 100 μl/well of a 400-fold dilution of the biotin-labelled MAb 1G7. The reactivity of this MAb with Ig from several salmonid species has been previously demonstrated (Sánchez *et al.*, 1991). After five more washes, the plates were incubated with 100 μl/well of avidin-peroxidase (Dako, Denmark) diluted at 1/2000 for 40 min at room temperature. Plates were washed five times and the enzymatic reaction developed as described above.

COMPETITION ASSAYS FOR EPITOPE DETERMINATION

Using the ELISA technique, competitive binding assays were performed with the biotin-labelled MAbs against unlabelled MAbs.

Plates were coated with 0·2 μg/well of trout Ig. Then, plates were incubated for 60 min at room temperature with 200 μl of a 1/1000 dilution of biotinylated antibodies in dilution buffer, containing different concentrations of purified MAbs (0·8–100 μg ml^{-1}). After five washes, the plates were incubated with 100 μl/well of avidin peroxidase (Dako, Denmark) diluted to 1/2000 in dilution buffer, for 40 min at room temperature. Plates were washed five times, and the enzymatic reaction was developed as described for indirect ELISA. The results were expressed as percentages of inhibition using the following formula:

(optical density (O.D.) in the absence of unlabelled antibody $-$ O.D. in the presence of unlabelled antibody) $\times$ 100/O.D. in the absence of unlabelled antibody.

IMMUNOPRECIPITATION ANALYSIS

Purified trout Ig from a pool of 20 individual sera was radioiodinated by the modified chloramine T method (Johnstone & Thorpe, 1982). The amount of trout Ig recognised by each MAb was determined by immunoprecipitation as previously described (Sánchez & Domínguez, 1991). Immunoprecipitated radioactive samples were run on 17–20% gradient SDS–PAGE. Gels were fixed, dried onto Whatman filter paper, and exposed to X-ray film (AGFA Curix RP-2).

FLOW CYTOFLUOROMETRY ANALYSIS

Thymus, spleen and pronephros cell suspensions were prepared as previously described (Carballo *et al.*, 1992). Peripheral blood lymphocytes (PBL) were obtained by centrifugation over Lymphoprep (Nyergaard, Norway), as described by Blaxhall (1981).

Some 2×10^6 cells were incubated for 1 h at 4° C with MAb ascites diluted 1/20 in PBS. An irrelevant MAb (against IPN virus) was used as negative control. After three washes in PBS with 0·1% sodium azide, the cells were incubated for 45 min at 4° C with FITC-conjugated rabbit anti-mouse Ig (Dako, Denmark) diluted 1/50 in PBS–0·1% sodium azide. Cells were washed four times in PBS–0·1% sodium azide and fixed in 0·3% paraformaldehyde prior to the cytofluorographical analysis on a FACSTAR PLUS apparatus (Becton Dickinson, U.S.A.).

Table 1. Characterisation of monoclonal antibodies specific for trout Ig

MAb	Isotype	Titre*	%Trout Ig precipitated	Carbohydrate reactivity	Allotypic determinant
1A6	IgG_1, k	1×10^6	18·4	—	—
1B4	IgG_1, k	1×10^6	17	—	+
1A3	IgG_{2a}, k	1×10^6	12·8	—	+
1C6	IgG_3, k	1×10^6	13	—	—
1G7	IgG_1, k	1×10^6	85	—	—
1H2	IgG_1, k	1×10^6	15·1	—	—
2A1	IgG_1, k	1×10^6	13·1	—	—
2H9	IgG_1, k	1×10^6	20	—	—
2D12	IgM, k	1×10^4	11·3	—	—
3F7	IgG_1, k	1×10^6	17·6	—	—
5G7	IgG_{2b}, k	1×10^6	18	—	+
4D11	IgG_1, k	1×10^6	97	—	—
3E4	IgG_{2a}, k	1×10^6	93	—	—
3B10	IgG_{2b}, k	1×10^6	95	—	—

*Titres were determined by ELISA as the reciprocal of the highest dilution of a solution of purified MAb (1 mg ml $^{-1}$) giving absorbance values greater than twice the background.

IMMUNOHISTOCHEMICAL STUDIES

Immunoperoxidase staining with the MAbs was carried out on pronephros and spleen cryosections according to a protocol previously described (Razquín *et al.*, 1990).

III. Results

A total of 40 stable hybrids specific for trout Ig were obtained from three independent fusions. Fourteen hybrids were selected for further characterisation. Their isotype, titre, carbohydrate reactivity and the percentage of total trout Ig recognised by each of them in the immunoprecipitation experiments are summarised in Table 1. Eight MAbs were IgG_1, two IgG_{2a}, two IgG_{2b}, one IgG_3 and one IgM. All of them have kappa light chains.

The MAbs titres, determined by ELISA starting with a concentration of purified antibodies of 1 mg ml^{-1}, ranged from 10^4 to 10^6.

The binding of these MAbs was not affected by the periodate oxidation of the trout Ig, which suggest that no carbohydrate residues were involved in the epitopes recognised by these MAbs.

MAbs 1G7, 4D11, 3E4 and 3B10 precipitated almost 100% of trout Ig (85–97%) from a pool of 20 sera. The remaining MAbs precipitated low percentages, between 11% for 2D12 and 20% for 3F7. To rule out that these MAbs were recognising allotypic variants, we tested their reactivity with a panel of 30 individual serum samples in a sandwich ELISA using a polyclonal anti-trout Ig as capture antibody. All the MAbs, except 1A3, 1B4 and 5G7, reacted with each of 30 serum samples tested, indicating that they recognise isotypic variants

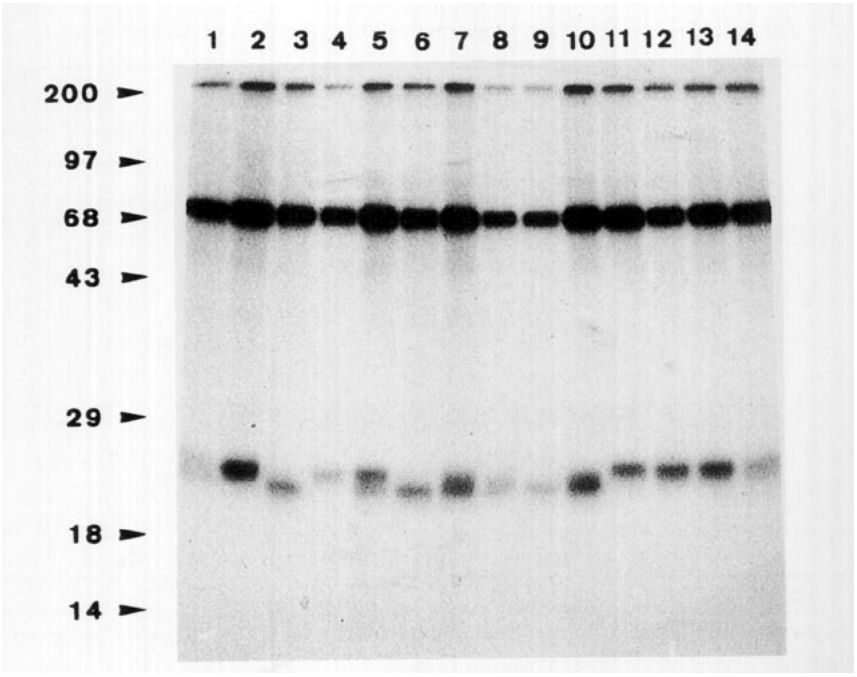


Fig. 1. Autoradiography of SDS–PAGE (17–20% acrylamide gradient) of reduced ^{125}I-trout Ig selected in the immunoprecipitation assays with MAbs 1A6 (1), 1B4 (2), 1A3 (3), 1H2 (4), 1G7 (5), 2A1 (6), 3B10 (7), 3E4 (8), 1C6 (9), 4D11 (10), 2H9 (11), 3F7 (12), 2D12 (13) and 5G7 (14). Numbers on the left correspond to the molecular weight markers in kDa.

of trout Ig. MAbs 1A3, 1B4 and 5G7 failed to react with some of the serum samples, although different for each MAb, and so might be recognising allotypic determinants.

When analysed by SDS–PAGE under reducing conditions, the trout Ig immunoprecipitated by these MAbs showed differences in the number and molecular weight of bands corresponding to the L chains (Fig. 1). This L chain heterogeneity was first defined with MAbs 2A1 and 2H9, and has been the subject of a previous report (Sánchez & Domínguez, 1991). Trout Ig bound by MAbs 2H9, 1A6, 1B4, 1H2, 2D12, 3F7 and 5G7 possessed L chains = 26 kDa, whereas trout Ig precipitated by MAbs 1A3, 1C6 and 2A1 contained only L chains = 24 kDa. Both sizes of L chains were present in the trout Ig immunoprecipitated by MAbs 1G7, 4D11, 3E4 and 3B10.

HEAVY OR LIGHT CHAIN SPECIFICITY OF MAbs

The polypeptide chain specificity of MAbs was determined by ELISA using plates coated with the H and L chains isolated by gel filtration after mild reduction and alkylation of Ig molecules. Three MAbs (1G7, 3F7, and 4D11) reacted against H chains and six (1A3, 1C6, 2A1, 2D12, 2H9 and 3E4) reacted with L chains (Fig. 2). The specificity of the five remaining MAbs could not be clearly resolved by this assay. MAb 3B10 reacted weakly with H chains, whereas MAbs

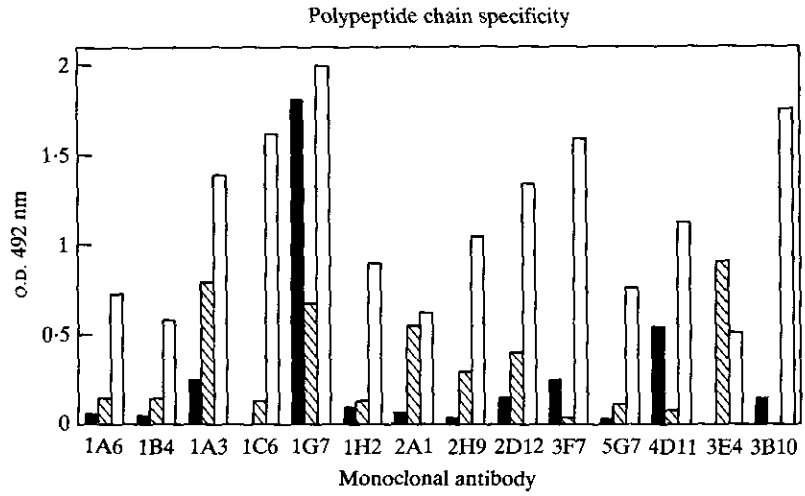


Fig. 2. Reactivity of MAbs to isolated H and L chains were assayed by ELISA. Plates, coated with 0·2 μg/well of purified Ig, H chains or L chains were incubated with a 1/20 dilution of the MAbs (ascites fluid), and the protein–MAb complexes revealed with rabbit anti-mouse Igs conjugated with peroxidase. Blank values, determined with a irrelevant MAb directed against IPN virus, were 0·10 for trout Ig and 0·01 for H and L chains respectively. (■), H chain; (▨), L chain; (□), Ig.

1A6, 1B4, 1H2 and 5G7 appeared to bind L chains, showing values slightly higher than negative control.

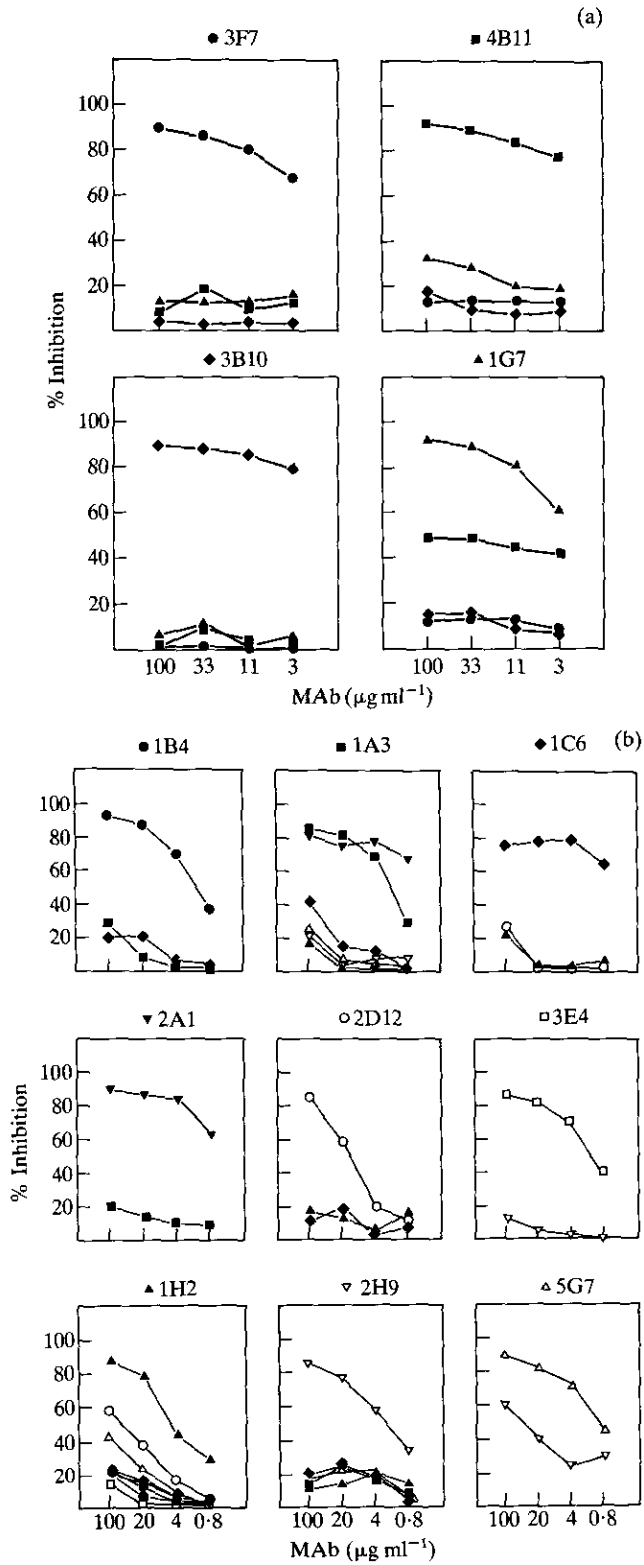
The reactivity of MAb 3B10 with H chains was clearly demonstrated by Western blotting (data not shown). This assay confirmed the binding of MAb 1G7 and 4D11 to H chains, and the binding of 3E4 to L chains. MAbs 1A6, 1B4, 1H2 and 5G7 failed also to react in this assay; however, for subsequent analysis, they were classified as L chain-specific since they only precipitated one of the two L chains bands (Fig. 1).

EPITOPE ANALYSIS

The epitopes recognised by the H and L chain-specific MAbs were analysed by competition assays as described in Materials and Methods section. Representative graphs of competition profiles are shown in Fig. 3. MAbs 1G7 and 4D11 were partially inhibited by each other. On L chains, MAbs 2D12 and 5G7 competed with the binding of biotin-labelled 1H2, whereas MAb 2A1 and 2H9 competed with the binding of biotin labelled 1A3 and 5G7, respectively. No reciprocal inhibition was observed in any case. The remaining MAbs were only competed by themselves, indicating that they recognised different epitopes.

REACTIVITY WITH LYMPHOID CELLS

The percentages of cells bearing surface Ig (sIg) in suspensions of lymphocytes from spleen, pronephros, thymus and peripheral blood, detected by these MAbs, were determined by cytofluorometry using a FACS analyser (Table 2). MAbs 1G7, 4D11 and 3B10 stained percentages close to those obtained with MAb 1.14.


(a)
● 3F7
■ 4B11
◆ 3B10
▲ 1G7
% Inhibition
100
80
60
40
20
100 33 11 3
MAb (μg ml⁻¹)
(b)
● 1B4
■ 1A3
◆ 1C6
▼ 2A1
○ 2D12
□ 3E4
▲ 1H2
▽ 2H9
△ 5G7
% Inhibition
100 20 4 0·8
MAb (μg ml⁻¹)

MAb 3E4 stained similar percentages in pronephros and spleen, but reacted with a low number of peripheral blood lymphocytes. The remaining MAbs stained lower percentages. All the MAbs stained less than 2% of thymocytes, except MAb 4D11 that reacted with a 28% of thymocytes.

Cells containing Ig can also be detected in frozen sections of pronephros and spleen using these MAbs. Figure 4 shows pronephros sections stained with MAbs 1G7 and 2H9. Surface and cytoplasmic immunostaining was observed on scattered cells throughout the tissue sections. As expected, a higher percentage was detected with MAb 1G7 than with MAb 2H9.

REACTIVITY OF MAbs FOR Ig OF DIFFERENT SALMONID SPECIES

The reactivity of the MAbs to serum proteins of various salmonid species and subspecies was tested by a sandwich ELISA. Results are summarised in Table 3.

Five of the L chain-specific MAbs (2H9, 1B4, 2A1, 1A6, 2D12) and three H chain-specific MAbs (3B10, 4D11 and 1G7) reacted with sera from all the salmonid species tested. However, notable differences were found between these species, Chinook and sockeye salmon showing the weakest reactivities. The remaining MAbs reacted only with sera from some of the species tested. MAb 3F7 reacted with sera from all the species except sockeye. MAb 5G7 and 3E4 failed to bind Chinook, sockeye or chum salmon sera. MAb 3E4 also failed to react with Coho salmon. MAb 1H2 did not recognise Chinook or sockeye salmon sera. Reactivity of MAb 1C6 with these salmonid sera was not determined because it failed to react in sandwich ELISA with the rainbow trout Ig.

IV. Discussion

We have characterised a new panel of 14 MAbs against the H and L chains of trout Ig. Immunoprecipitation experiments with these MAbs showed that only four (1G7, 3E4, 3B10 and 4D11) reacted with 85–97% of serum Ig. The rest bound only small fractions of serum Ig. The low percentages of trout Ig precipitated by these MAbs did not appear to be due to damage of specific epitopes by the iodination procedure or to a low affinity of these MAbs, since similar yields were obtained when some of them were used in affinity chromatography experiments, and since any increase in the amount of bound Ig was not obtained when the precleared supernatant was subjected to a second round of immunoprecipitation with the same MAb. These results raised the question of the possible

Fig. 3. Competition profiles of the anti-H chains (a) and anti-L chains (b) MAbs. The binding of biotin-labelled MAbs to plates coated with trout Ig was competed with different amounts of the unlabelled MAbs, purified by affinity chromatography on Protein A Sepharose CL-4B. MAbs that gave less than 10% of inhibition were not represented in order to make the graph more intelligible. (a) (—●—), MAbs 3F7; (—■—), 4D11; (—◆—), 3B10 and (—▲—), 1G7. (b) (—●—), MAbs 1B4; (—■—), 1A3; (—◆—), 1C6; (—★—), 2A1; (—○—), 2D12; (—□—), 3E4; (—▲—), 1H2; (—☆—), 2H9 and (—△—), 5G7.

Table 2. Reactivity of anti-trout Ig MAbs with different cell types determined by flow cytofluorometry

MAb	Pronephros	Spleen	Thymus	PBL
1·14	18·1±4·6	25·8±10	0·6±0·1	46·5±11
1G7	10·7±4·7	27·4±13	0·85±0·2	35·4±7
4D11	9·6* (13)	17* (15·2)	28·1±5·1	N.D.
3F7	3·2* (17)	6·1* (36)	1·4±0·8	13·5±11
3B10	N.D.	N.D.	N.D.	41* (45)
3E4	9·7* (13)	22* (15·2)	N.D.	20±0·8
2H9	6·3±3·2	8·2±2·7	0·85±0·8	14·6±1
2A1	12·2±4·4	18·5±6·5	1·1±1	24·8±2
1B4	N.D.	N.D.	N.D.	22* (45)
1A3	N.D.	N.D.	N.D.	9·2* (45)
1A6	N.D.	N.D.	N.D.	5·7* (45)
5G7	N.D.	N.D.	N.D.	7·8* (45)

Values are expressed as percentages and represent the mean ± S.D. of five experiments for pronephros, three experiments for spleen and peripheral blood lymphocytes (PBL) and two experiments for thymus.

*A single experiment was performed. Number between brackets represent percentage of cells stained with MAb 1.14 in the same experiment.

N.D. Not done.

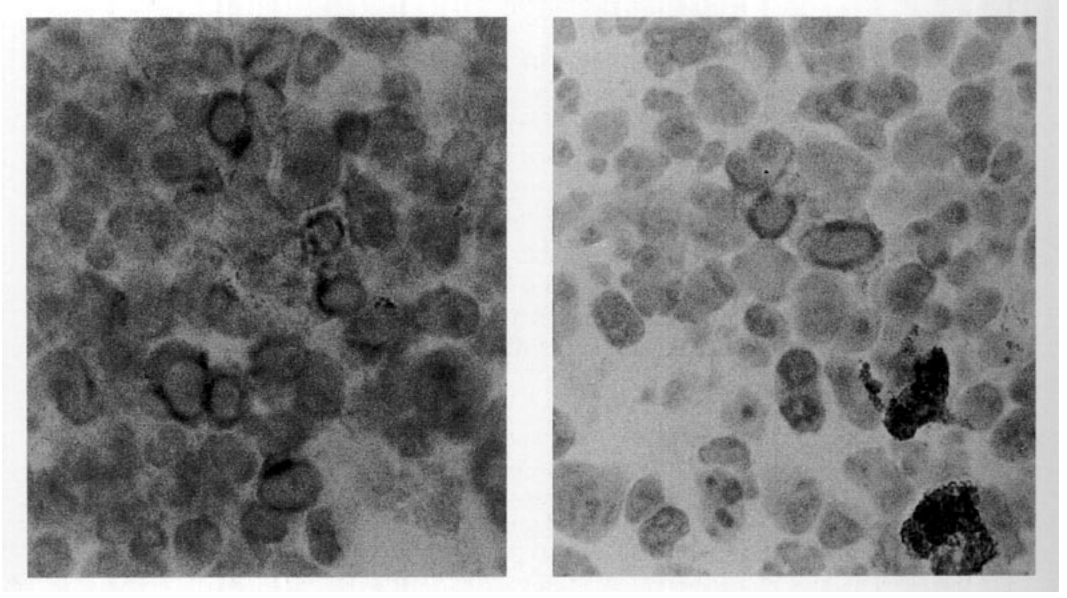


Fig. 4. Immunoperoxidase staining of 7μm frozen cryostat sections of trout pronephros with MAb 1G7 (a) or MAb 2H9 (b). Note the higher percentage of cells stained with MAb 1G7 in comparison with MAb 2H9 (magnification × 1300).

Table 3. Reactivity of anti-rainbow trout Ig monoclonal antibodies to sera of different salmonid species

Species	2H9	1B4	1A3	1H2	2A1	1A6	2D12	3F7	5G7	4D11	3E4	3B10
Rainbow trout	1·47	1·51	1·27	1·07	1·21	0·82	1·07	0·93	1·01	0·96	0·21	1·24
Brown trout	1·64	1·70	1·41	1·24	1·39	1·12	1·20	1·22	1·17	1·00	0·28	1.36
Chinook salmon	0·73	0·90	0·06	0·11	0·49	0·38	0·58	0·46	0·07	0·40	0	0·66
Coho salmon	1·08	1·06	0·72	0·02	0·80	0·41	0·60	0·49	0·54	0·50	0·02	0·67
Sockeye salmon	0·84	0·20	0·03	0·30	0·59	0·32	0·51	0·04	0·01	0·42	0	0·64
Chum salmon	1·21	1·08	0·27	0·50	0·96	0·52	0·65	0·60	0·0	0·65	0·10	0·86

Values represent O.D. at 492 nm after subtraction of the blanks.

existence of antigenic variants within the trout Ig. Such variants have been demonstrated in the L and H chains of catfish (Lobb *et al.*, 1984; Lobb & Olson, 1988), and have recently been suggested for H chains of the Atlantic salmon (Killie *et al.*, 1991). Consistent with this hypothesis also are the results from immunofluorescence and immunohistochemical studies of trout lymphoid organs, which have showed that these MAbs only stained a portion of the Ig-positive cells. In addition, the analysis of the immunoprecipitates by SDS–PAGE revealed differences in the Ig L chains. MAbs 2A1, 1A3 and 1C6 precipitated trout Ig which possessed L chains of 24 kDa, whereas MAbs 2H9, 1A6, 1B4, 1H2, 3F7 and 5G7 precipitated trout Ig with L chains of 26 kDa. MAbs 1G7, 4D11, 3B10 and 3E4, which bound 85–97% of serum Ig, brought down both L chains.

Experiments of simultaneous binding to trout Ig of pairs of MAbs as well as immunoprecipitations with combinations of different MAbs (unpublished results and Sánchez & Domínguez, 1991) indicate that the Ig subpopulations containing 26-kDa L chains do not overlap with the Igs subpopulations that possess 24-kDa L chains. Furthermore, while partial or total overlapping was seen in these experiments among the trout Ig subpopulations with 24-kDa L chains identified by MAbs 2A1, 1A3 and 1C6, the Ig subpopulations containing 26-kDa L chains seemed more heterogeneous, since MAb 1B4 and 3F7 apparently bound a different Ig subpopulation than that identified by MAbs 1A6, 1H2, 2H9 and 5G7. MAb 2D12 bound both subpopulations of Ig with 26-kDa L chains.

The 24-kDa and 26-kDa L chains showed differences at the polypeptide level, as has been demonstrated by recent analyses carried out with Ig bound by MAbs 2A1 and 2H9 (Sánchez & Domínguez, 1991). In these studies, no changes in the size of either L chains were observed after deglycosylation with trifluoromethane sulphonic acid (TFMS), whereas clear peptide differences were revealed by *Staphylococcus* V8 protease digestion.

MAbs 1G7, 4D11, 3B10 and 3F7 reacted with H chains in Western blots and/or ELISAs using isolated H and L chains; MAbs 3E4, 2A1, 2H9, 2D12, 1C6 and 1A3 seemed to recognise L chains in these assays. MAbs 1A6, 1B4, 1H2 and 5G7 failed to bind isolated H or L chains, but probably reacted with L chains, since they only precipitated one of the two bands of L chains detected by SDS–PAGE. However, MAb 3F7, which bound isolated H chains by ELISA, also precipitated

Ig containing only 26-kDa L chains. Although we do not yet have a clear explanation for this finding, it is possible that this MAb recognises an H chain variant which associates preferentially with 26-kDa L chains. Preferential association between isotypes and/or allotypes of H and L chains has been observed in mammals (Natvig & Kunkel, 1973; Naessens *et al.*, 1979) and in amphibians (Hadji-Azimi, 1975; Hsu & Du Pasquier, 1984). Another possibility is that this MAb reacts with a conformational epitope resulting from the association of H chains with the 26-kDa L chains, and it was detecting trace amounts of H–L pairs present in the H chain preparation used in the ELISA, that went unnoticed by SDS–PAGE.

Competition between MAbs indicated that MAbs 1G7, 3B10 and 3F7 recognise distinct epitopes on H chains. MAb 4D11 showed a partial competition with 1G7, and may be recognising an epitope that overlaps with the one bound by 1G7. With regard to L chains, the one-way inhibition observed between MAbs 2A1 and 1A3 (both reacted with 24-kDa L chains) could be explained by differences in affinity, since 2A1 inhibited the binding of biotin-labelled 1A3 even more effectively than unlabelled 1A3. However, this explanation seems unlikely in the inhibitions observed with 2D12 and 5G7, or 2H9 on the binding of biotin-labelled 1H2 or 5G7 respectively, since the concentration of these MAbs required for 50% inhibition were higher than the homologous MAb. Probably, these MAbs reacted with different but structurally close epitopes on 26-kDa L chains.

Most of the determinants recognised by these MAbs seem to be isotypic, since they were present in every trout serum tested. However, it is not yet resolved if they lie in the C or V regions of the H or L chains, and therefore, if the Ig variants identified by these MAbs represent true isotypes or rather are subgroups, products of consensus sequences in the framework regions, shared by the members of a V gene family. Although information on the L chain genes in teleost is lacking, recent analysis of H chain genes in three different species indicate the presence of multiple families of V_H genes (Amemiya & Litman, 1990; Wilson *et al.*, 1991; Ghaffari & Lobb, 1991). On the other hand, the L chains in elasmobranchs are encoded by multiple C_L genes, and appear to be closely related to mammalian λ chains (Schluter *et al.*, 1989), which are also encoded by multiple C_λ genes in some species, and where different isotypes have been chemically and serologically identified (Solomon, 1986). Nevertheless, the finding that MAb 3E4, which is specific for L chains, binds 93% of trout Ig and precipitates both 26-kDa and 24-kDa L chains, indicates that these L chains share common epitopes and probably represent variants of a single type.

The failure of MAbs 1A3, 1B4 and 5G7 to react in the sandwich ELISA with some of the individual trout sera tested, does not seem be due to a low concentration of Ig in the sera, since these sera were strongly recognised by other MAbs which also precipitate low Ig percentages. Furthermore, these MAbs reacted strongly with other sera tested, which excludes a loss of activity of the biotinylated MAbs, or a possible steric hindrance as the cause for the lack of reactivity. It is possible that these MAbs are detecting allotypic determinants, although additional studies are required to confirm this hypothesis.

Analysis of cross-reactivity with this panel of MAbs indicates that most epitopes are highly conserved between the different species of salmonids. However,

epitopes recognised by MAbs 3F7, 5G7, 3E4, 1H2 and 1A3 show a polymorphic distribution, being present only in some species. The failure of MAb 5G7 and 1A3 to bind Chinook, sockeye and chum salmon (only the 5G7) could be explained by their possible allotypic reactivity, as only one serum of each species was tested. No differences were found between brown trout and rainbow trout, in contrast to that reported by Kobayashi *et al.* (1982), who found only a partial identity between Igs of these species, using a polyclonal antisera to chum salmon Ig. In this study, the rainbow trout Ig was antigenically similar to the Ig from other fishes of the genus *Oncorhynchus*.

Anti-Ig polyclonal sera cannot be used for detection of B cells in fish, because of their anti-carbohydrate specificities that cross-react with non-Ig components of the cell surface (Yamaga *et al.*, 1978). MAbs 1G7, 3B10, 4D11 and 3E4, which bound almost 100% of trout serum Ig, can be used as B cell-markers. None of these MAbs appear to react with carbohydrate determinants, since all of them bind to periodate-treated trout Ig. The percentages of sIg-bearing cells in blood, pronephros and spleen detected by MAbs 1G7, 3B10 and 4D11 are similar to that reported by DeLuca *et al.* (1983) and Thuvander *et al.* (1990), although variation was observed from one animal to another. Unexpectedly, the anti-L chain MAb 3E4 stained a much lower percentage of PBL than MAbs 1G7, 3B10 or 1.14. Although this result is difficult to explain, it might reflect the presence in peripheral blood of lymphocytes with H chains on their surface, non-associated to L chains. The surface expression of H chains associated to proteins distinct of L chains (λ/5 and Vpre-B) has been seen in pre-B cells of mammals (Pillai & Baltimore, 1988; Cherayil & Pillai, 1991).

MAb 4D11 also reacts with a subpopulation of thymocytes. This reactivity remains to be investigated, however it has also been observed with some anti-Ig MAbs in carp (Secombes *et al.*, 1983).

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