Characterization of immunoglobulin from the Pacific hagfish, a primitive vertebrate

(immunophylogeny/radiiodination/polypeptide chains/electrophoresis)

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ABSTRACT Antigodies to group A streptococcal carbohydrate, demonstrable by radioimmunoassay, were elicited in Pacific hagfish, Eptatretus stoutii, after prolonged immunization with whole cell vaccine. This hagfish antibody, isolated by ion-exchange chromatography of immune serum, exhibited a single precipitin line after immunoelectrophoresis against rabbit antiserum to hagfish serum. The isolated antibodies retained high binding activity for the streptococcal carbohydrate, but lacked the natural hemagglutinating activity found in both nonimmune and immune sera. Analysis by gel filtration in neutral and dissociating buffers and by sodium dodecyl sulfate/polyacrylamide gel electrophoresis under nonreducing conditions indicated an intact molecule of molecular weight approximately 160,000 that may be noncovalently associated in a polymer of higher molecular weight. Heavy chains of mobility identical to that of murine μ chains and light chains of mobility significantly slower than murine λ chains were obtained after sodium dodecyl sulfate gel electrophoresis under reducing conditions. Instability of the tertiary structure of the antibody molecule was indicated by partial dissociation in buffers containing sodium dodecyl sulfate and by dissociation at low concentrations of reducing agent. In contrast to its well-developed system of cell-mediated immunity, only a minimal system of circulating antibody production is evident in this primitive fish. No evidence for divergence of cyclostome lymphocytes into separate T- and B-cell systems has yet been discerned.

Studies of antibodies in lower vertebrates indicate an IgM-like protein as the most evolutionarily ancient immunoglobulin (reviewed in refs. 1 and 2). The immune macroglobulin of bony fishes of several species circulates mainly as a 14-16S tetramer (3), but may also occur as monomeric antibody. In the elasmobranchs, an immunoglobulin resembling IgM of mammals exists in 17-19S pentameric and 75 monomeric forms (4, 5). The pentameric form is stabilized by a polypeptide chain analogous to the J chain of human IgM (6). Little is known of the nature of specific immunoglobulins in species more primitive than the elasmobranchs. Conflicting reports have been published on the structure of the immunoglobulin of the sea lamprey. Marchalson and Edelman (7) characterized lamprey antibody to bacteriophage f2 as being IgM-like, existing in 14S and 6.6S forms, but lacking disulfide bonding between heavy (H) and light (L) chains. Litman and coworkers (8) described a high molecular weight immune immunoglobulin in the serum of lampreys immunized with human erythrocytes. This molecule possessed a polypeptide chain structure unlike any previously described immunoglobulin. Immunochemical analysis of serum components from normal hagfish suggested a multimeric immune immunoglobulin consisting mainly of light chain-like subunits (9). However, immunoglobulin characterization in the cyclostomes is complicated by their possession of naturally occurring agglutinins for erythrocytes of many species (7, 10). Thus the distinction between known specific antibodies and other naturally occurring molecules with antibody-like activities has become a critical issue.

The hagfish, an agnathan cyclostome, is possibly the most primitive vertebrate extant and therefore represents an important link in the chain of evolution of the immunoglobulins. Humoral and cellular immune response capabilities have been previously documented for this species (10, 11). Although the immunoglobulin has been described as IgM-like on the basis of molecular weight and immunoelectrophoretic properties (12), detailed structural characterization of hagfish immunoglobulin is lacking. In a recent publication (13) we described the production and specificity of antibodies to group A streptococcal carbohydrate in the Pacific hagfish, Eptatretus stoutii. We now report the purification of the streptococcal antibodies and characterization of this primitive immunoglobulin.

MATERIALS AND METHODS

Hagfish Antibody Production and Detection. Hagfish trapped off Point Dume in southern California were maintained at Pacific Biomarine Laboratories (Venice, CA) in a recirculating sea water system at 17-18°C. They were fed with fresh, homogenized bovine liver at weekly or biweekly intervals (12). Procedures for immunization of hagfish with group A streptococcal vaccine and subsequent detection of antibody to the group A carbohydrate by radioimmmunoassay have been described recently (13). Serum fractions from chromatographic separations were assayed for hemagglutinating activity against sheep erythrocytes by the microtiter method (9, 10). All samples were assayed in buffer containing 0.15 M NaCl, 0.005 M CaCl₂, and 0.05 M Tris-HCl, pH 7.5.

Rabbit Anti-Hagfish Serum. Antiserum to hagfish serum was prepared by intradermal injection of New Zealand white rabbits with 0.5 ml of hagfish serum emulsified in 0.5 ml of complete Freund's adjuvant. The rabbits were boosted four weeks later by intramuscular injection of 0.5 ml of hagfish serum in 0.5 ml of incomplete Freund's adjuvant and the antiserum was taken after a further two weeks.

Chromatographic Techniques. Gel filtration on Sephadex S-200 (Pharmacia, Uppsala, Sweden) of 2 ml of whole serum was done at 5°C on a 1.6 X 200 cm column in buffer containing 0.15 M, NaCl, 0.005 M CaCl₂, and 0.05 M Tris, pH 7.5. The column effluent was continuously monitored at 280 nm, and those fractions containing protein were assayed for both streptococcal binding and for hemagglutinating activity. Fractionation of radioiodinated material on Sephadex G-200

Abbreviations: H and L, heavy and light chains, respectively, of immunoglobulins; NaDODSO₄, sodium dodecyl sulfate.

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(1.6 × 100 cm column) employed the same buffer. Ion-exchange chromatography at 5°C on DEAE-cellulose (DE-52, Whatman, Clifton, NJ) employed linear gradient elution, with a starting buffer of 0.05 M Tris, pH 8.0, and a limiting buffer of 0.05 M Tris, 0.5 M NaCl, pH 8.0. Protein-containing fractions were then assayed for both streptococcal binding and hemagglutinating activity.

**Immunoelectrophoresis.** Immunoelectrophoresis, at pH 8.6, was performed in 1% agarose gels in 0.06 M sodium barbital buffer. After addition of the rabbit antiserum, the precipitin pattern was allowed to develop for 48 hr at 22°C before the gel was dried and stained with amido black.

**Iodination and Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis.** Aliquots (50–100 µg) of purified immunoglobulin were iodinated in the presence of sodium dodecyl sulfate (NaDodSO₄) by using Chloramine T (14). NaDodSO₄/polyacrylamide gel electrophoresis was carried out as described by Atwell and Marchalonis (15). Molecular weight markers included in electrophoresis runs were radiiodinated mouse myeloma proteins MOPC 104E (IgM) and MOPC 21 (IgG).

**RESULTS**

**Serum Fractionation.** Antisera from three animals exhibiting high antigen-binding capacities were pooled and a 2-ml aliquot was fractionated by gel filtration on Sephacryl S-200 (Fig. 1). Streptococcal binding activity was recovered in the high molecular weight material (eluted in the exclusion volume) and in the fractions eluting at the same position as human IgG. Hemagglutinating activity to sheep erythrocytes was also detected in the first two peaks. Neither streptococcal binding nor hemagglutinating activity was detected in the low molecular weight material.

In preparation for fractionation by ion-exchange chromatography on DEAE-cellulose, whole serum was dialyzed exhaustively against 0.05 M Tris buffer, pH 8.0. A pink precipitate formed and was removed by centrifugation. This material was found to correspond to the low molecular weight material eluted on gel filtration of whole serum and appears analogous to the "pink protein" described by Thoenes and Hildemann (12). The supernatant was applied to DEAE-cellulose and a fraction was eluted by the starting buffer prior to the initiation of the gradient (Fig. 2). This fraction, DE-I, exhibited high binding activity for ¹²⁵I-labeled group A carbohydrate, lacked hemagglutinating activity, and gave a single precipitin line after immunoelectrophoresis against rabbit anti-hagfish whole serum (Fig. 3). Further elution of the ion-exchange column by increasing salt concentration yielded several fractions containing both hemagglutinating and streptococcal binding activity. The purified fraction DE-I was tentatively designated as the hagfish immunoglobulin and was further characterized as described below.

**Mass Characteristics of the Hagfish Immunoglobulin.** Gel filtration of ¹²⁵I-labeled DEAE-purified immunoglobulin on Sephacryl G-200 in nondissociating buffer (Fig. 4) indicated two components. A high molecular weight component eluted in the exclusion volume, while a second component eluted slightly ahead of an aldolase marker (Mr 154,000). Rechromatography of the iodinated immunoglobulin in 3 M urea (not shown) resolved the material into a single component of approximately 160,000 Mr.

**FIG. 1.** Sephacryl S-200 filtration (1.6 × 200 cm column) of 2 ml of pooled serum from immunized hagfish exhibiting high streptococcal carbohydrate binding activity. Protein eluted from the column was monitored by absorbance at 280 nm (---). Fractions (4 ml) were collected and assayed for binding activity to ¹²⁵I-labeled group A carbohydrate (••••••) and for hemagglutinating activity (bars).

**FIG. 2.** Chromatography on DEAE-cellulose of 2 ml of pooled serum from immunized hagfish exhibiting high streptococcal carbohydrate-binding activity. The ion-exchange column was equilibrated in 0.05 M Tris buffer, pH 8.0, and the first protein peak eluted in that buffer (---). The remaining serum proteins were eluted by application of a linear gradient of NaCl from 0 to 0.5 M in the starting buffer. Fractions were assayed for binding activity to ¹²⁵I-labeled group A carbohydrate (••••••) and for hemagglutinating activity (bars).

**FIG. 3.** Immunoelectrophoresis at pH 8.6 of whole hagfish serum (upper well) and DE-I (lower well) obtained by ion-exchange chromatography of whole serum (Fig. 2). The trough contained rabbit antiserum to hagfish whole serum. The anode is to the right. The slide was dried and stained with amido black before photographing.

**FIG. 4.** Sephadex G-200 filtration (1.6 X 100 cm column) of $^{125}$I-labeled DEAE-purified hagfish immunoglobulin (DE-I, Fig. 2) in pH 7.5 buffer containing 0.05 M Tris, 0.15 M NaCl, 0.005 M CaCl$_2$. Fractions were monitored for radioactivity in a gamma counter. $V_o$ = exclusion volume of the column as determined by elution of dextran 2000 ($M_t \approx 2 \times 10^6$); 154,000 = aldolase marker, 25,000 = chymotrypsinogen marker.

**FIG. 5.** Electrophoresis of intact radioiodinated hagfish immunoglobulin in NaDodSO$_4$-containing 5% polyacrylamide gel. Mouse IgG (MOPC 21) was run as a marker on a separate gel. The anode is to the right.

**NaDodSO$_4$/Polyacrylamide Gel Electrophoresis of Hagfish Immunoglobulin.** Characterization of the intact and the reduced radiolabeled immunoglobulin was undertaken by analysis on NaDodSO$_4$/polyacrylamide gels. Hagfish immunoglobulin, analyzed under nonreducing conditions on 5% gels, migrated as a single major peak (Fig. 5) with a mobility similar to that of intact murine IgG. Analysis on 10% gels under reducing conditions (0.7 M 2-mercaptoethanol) resolved the immunoglobulin into two peaks (Fig. 6B), one with a mobility corresponding to that of murine $\mu$ chains and one with a mobility slower than that of murine $\lambda$ chains. Under nonreducing conditions on 10% gels (Fig. 6A) the immunoglobulin was resolved as a single component migrating more slowly than $\mu$ chains. In some experiments, evidence of free heavy and light chains was seen on gels run under nonreducing conditions. The susceptibility of the molecule to dissociation into $L$ and $H$ chain-like components was further demonstrated by mild reduction of the intact molecule. Light and heavy chain peaks were clearly observed upon NaDodSO$_4$ electrophoresis after reduction with 0.005 M 2-mercaptoethanol (Fig. 6C).

**FIG. 6.** Electrophoresis in NaDodSO$_4$-containing 10% polyacrylamide gels. (A) Radioiodinated hagfish immunoglobulin under nonreducing conditions. (B) Radioiodinated hagfish immunoglobulin reduced with 0.7 M 2-mercaptoethanol. (C) Radioiodinated hagfish immunoglobulin reduced with 0.005 M 2-mercaptoethanol. Molecular weight markers run on separate gels were MOPC 104E ($\mu$ and $\lambda$) and MOPC 21 ($\gamma$ and $\lambda$). The anode is to the right.
DISCUSSION

Three hagfish from a group of 20 adults immunized with streptococcal vaccine over a period of several months survived long enough to produce high levels of antibody directed against the streptococcal group carbohydrate and particularly against rhamnose-containing determinants in that polysaccharide. Antigen-binding activity could be depleted by absorption of immune serum with streptococcal cells but not with sheep erythrocytes. Sugar inhibition studies indicated that rhamnose was the immunodominant sugar recognized in the A carbohydrate by hagfish antibody (13). This is in contrast to previous identification of N-acetylgalactosamine as the immunodominant sugar recognized by antibodies from mammalian species. Fractionation of pooled serum from responding hagfish yielded a fraction possessing anti-streptococcal carbohydrate activity but lacking the hemagglutinating activity found in both normal and immune serum (Fig. 2). This fraction yielded a single precipitin line upon immunoelectrophoresis against rabbit antisera to hagfish whole serum (Fig. 3) and exhibited λ-region mobility at pH 8.6. The purified antigen-binding molecule is here referred to as hagfish antibody.

In neutral buffer (Fig. 4) the antibody exists in both a low molecular weight form (approximately 160,000) and a higher molecular weight multimer that dissociates to yield only the 160,000 molecular weight species in 3 M urea, indicating the noncovalent nature of the complex. Estimation of the sedimentation coefficient of this complex was not attempted due to lack of sufficient material.

Characterization of the polypeptide chains of hagfish immunoglobulin revealed a heavy chain of electrophoretic mobility quite similar to that of murine μ chains (Fig. 6). The light chains, however, were of significantly slower molecular weight form (approximately 160,000) and a higher molecular weight form (approximately 180,000) and a higher molecular weight species in 3 M urea, indicating the noncovalent nature of the complex. Estimation of the sedimentation coefficient of this complex was not attempted due to lack of sufficient material.

Identification of the polypeptide chains of hagfish immunoglobulin revealed a heavy chain of electrophoretic mobility similar to that of murine μ chains (Fig. 6). The light chains, however, were of significantly slower mobility than murine λ chains. Lability of the tertiary structure of the hagfish immunoglobulin was demonstrated by significant reduction of the molecule to heavy and light chains in 0.005 M 2-mercaptoethanol. In parallel experiments using mouse IgM (MOPC 104E), no dissociation to free heavy and light chains was observed at this concentration of reducing agent. Stability of elasmobranch IgM monomer to reduction with 0.005 M 2-mercaptoethanol has been demonstrated (5), thus suggesting that covalent stabilization of immunoglobulin tertiary structure has been an evolving process.

The hagfish immunoglobulin bears striking resemblance to the immunoglobulin of the lamprey characterized by Marchalonis and Edelman (7). Both molecules are labile in their tertiary structure, suggesting a lack of disulfide bonding of the polypeptide chains. Both possess heavy chains of molecular weight similar to mammalian μ chains, but possess light chains of greater molecular weight than the mammalian counterpart. Cyclophilic light chains could differ substantially from mammalian light chains in amino acid composition, carbohydrate composition, or both. This question obviously invites further study. The apparent lack of tertiary structural stabilization in the immunoglobulins of the cyclostomes may have important implications concerning the humoral response systems of these primitive vertebrates. That a system of circulating antibodies is poorly developed in the cyclostomes is supported by several lines of evidence: (i) the low levels of immunoglobulin in cyclostome serum (ref. 7 and Fig. 2); (ii) the extended period of immunization required to elicit detectable serum antibody even at elevated temperatures (10, 12); (iii) the apparent lack of plasma cells (16); and (iv) the lack of a defined thymus in the hagfish (17). With these observations in mind, we propose that hagfish may lack a separate humoral immune response system equivalent to the B lymphocyte–plasmacyte system of higher vertebrates. Rather, the immunoglobulin described herein may exist primarily in a membrane-bound state functioning as a lymphocyte surface receptor. Membrane association of the molecule would also confer added stability to its tertiary structure. Thus, the presence of this molecule in the serum of immunized hagfish could result from prolonged antigen-induced lymphocyte proliferation followed by shedding rather than active secretion of the cell-bound immunoglobulin. The hagfish immunoglobulin possesses multichain structural features similar to those of certain T cell-associated polypeptides (18, 19), but this could reflect different molecular ancestry from an earlier common precursor. Divergence of the ancestral lymphocytes into separate B and T cell systems (20) has not been demonstrated at the phyleogenetic level of hagfish, but this question must remain open until more decisive evidence is forthcoming.

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