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Source: Zoological Science, 14(2) : 263-270

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.263

ZOOLOGICAL SCIENCE 14: 263–270 (1997)

Characterization of the Effects of Opsonins in Normal Hagfish Serum on the Ingestion of Rabbit Erythrocytes by Hagfish Macrophages

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ABSTRACT—Normal serum from the hagfish *Eptatretus burgeri* strongly enhanced the phagocytic activity of homologous peritoneal macrophages against rabbit erythrocytes (RRBC). Such phagocytosis was effectively but incompletely inhibited by rabbit antibodies against hagfish C3 (HC3). However, when RRBC were pretreated with purified HC3 or normal hagfish serum in the presence of EDTA, little enhancement of ingestion was observed. The results indicate that HC3 binds to RRBC, in collaboration with other serum factor(s), and that bound HC3 functions as an opsonin. Additionally, we obtained evidence for the possible presence of another opsonin (HOP) that was resistant to EDTA in C3-depleted hagfish serum. HOP prepared from plasma had a molecular mass of approximately 1,000 kDa, consisting of various small polypeptides that were able to form aggregates via disulfide bonds and/or noncovalent interactions. The functions of HC3 and HOP might be essential to immunity in hagfish because no clear evidence for the production of antibodies in cyclostomes has been reported to date.

INTRODUCTION

The complement system in lampreys, which are agnathan cyclostomes (jawless fish), and in hagfish, the most primitive extant vertebrates, seems to be a primordial system in which only the so-called alternative pathway functions in opsonization (Nonaka *et al.*, 1984). Recently, an alternative pathway in hagfish (*Eptatretus burgeri*), similar to that in lamprey, was identified on the basis of the ability of zymosan to activate this pathway (Fujii *et al.*, 1992). Moreover, we have succeeded in isolating an intact, two-subunit chain C3 (HC3) from the plasma of hagfish (Fujii *et al.*, 1992), as well as a fragment of HC3 (designated C3b), that has a three-subunit structure (Fujii *et al.*, 1995). Our successful isolation of these molecules enabled us to examine whether two forms of HC3 function in opsonization, as well as the way in which they manifest their respective functions. Even in this most primitive vertebrate, HC3 functions in opsonization against rabbit erythrocytes (RRBC), but only after activation by other hagfish component(s) in hagfish serum. During our functional analysis of HC3, we recognized that hagfish serum contains another serum opsonin (designated HOP), a major glycoprotein that is structurally very different from HC3 (Fujii *et al.*, 1992, 1995). We describe here the purification and characterization of the HOP detected in HC3-depleted hagfish serum (HR3). We also discuss the origin and functional differentiation of opsonins from a phylogenetic viewpoint, in terms of the primitive versions of complement and antibody-like proteins (*cf.* Raison, 1996).

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MATERIALS AND METHODS

Materials

This study was conducted with mature hagfish, *Eptatretus burgeri*, of 40 to 60 cm in body length. Serum and EDTA-plasma (plasma plus 10 mM EDTA) were obtained from hagfish as described previously (Fujii *et al.*, 1992) and stored at -80°C until use.

Rabbit antisera

Rabbit antiserum against HC3 and also against unfractionated hagfish serum was obtained as described previously (Fujii *et al.*, 1992).

To obtain antiserum against HOP, a 2% (v/v) suspension of RRBC was prepared with veronal-buffered saline (pH 7.5) plus 0.1% gelatin and 10 mM EDTA (EDTA-GVB). Thirty-two ml of this suspension were incubated with 16 ml of 20-fold diluted HC3-depleted hagfish serum (HR3), as described below, for 1 hr at 25°C. The HR3-treated RRBC

were washed 5 times with PBS, emulsified in Freund's complete adjuvant (Nacalai Tesque Inc., Kyoto), and injected subcutaneously into a rabbit, 4 times at weekly intervals. The rabbit was bled 7 days after the last injection. The antiserum prepared from the blood was originally used as a crude antiserum against HOP. In later stages of this study, a more specific antiserum was prepared by immunizing a rabbit with the HOP serum protein that had been obtained by the fractionation procedure described below.

The antisera were used for assays of the antigenicity of samples by rocket immunoelectrophoresis, immunoelectrophoresis (*cf.* Fujii *et al.*, 1992) or immunofluorescence analysis.

Rabbit IgG and $F(ab')_2$ fragments were prepared as described elsewhere (Onoue *et al.*, 1964; Utsumi and Karush, 1965).

Purification of HC3, HC3b and HOP from plasma, and preparation of HR3

HC3, as well as the three-subunit-chain HC3b, was purified from pooled EDTA-plasma as described elsewhere (Fujii *et al.*, 1992, 1995).

HOP was purified from pooled EDTA-plasma by a series of chromatographic steps on columns of DEAE-Sepharose CL-6B, Sepharose CL-6B, Sephacryl S-300 and CM-Sepharose CL-6B, as described for the purification of HC3 (Fujii *et al.*, 1992). All the resins used for column chromatography were obtained from Pharmacia (Uppsala, Sweden). All other methods for purification have been described elsewhere (Fujii *et al.*, 1992).

HC3 was removed from normal hagfish serum by affinity chromatography on an anti-HC3 column. Four milliliters of rabbit anti-HC3 serum were passed through a column (1.3 cm i.d. × 3 cm) of protein A-Sepharose CL-4B (Pharmacia) that had been equilibrated with GVB with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (GVB^{2+}) or with EDTA-GVB. After an extensive wash with the appropriate buffer, 1 ml of hagfish serum was applied to the column. The flow-through fractions equivalent to 10 ml were pooled, divided into aliquots, and stored at -80°C until use as 10-fold diluted HR3.

Collection of macrophages

A 16-gauge plastic needle was inserted in the hagfish through a coelomic pore located in the cloaca and tied in place at a position immediately anterior to the cloaca. The hagfish was then injected intraperitoneally through the needle with 20 ml of cold culture medium, pH 7.4, that consisted of Eagle's MEM (10.6 g/L; Gibco), NaCl (3.5 g/L), penicillin (10^5 U/L) and streptomycin (50 mg/L). The injected medium, containing peritoneal cells, was then retrieved after the ventral surface of the hagfish had been massaged for 5 min, and the cell suspension was transferred to a test tube on ice. Aliquots of 3 ml of the suspension of cells harvested from 6 to 8 hagfish were placed in plastic petri dishes (3.3 cm in diameter; Falcon) and incubated at 15°C in an atmosphere of 5% CO_2 in air to allow macrophages to attach to dishes. After incubation for 4 to 6 hr, the petri dishes were washed three times with hagfish PBS (Raftos *et al.*, 1992) to remove non-attached cells. Approximately 70 to 90% of the attached cells were macrophages after washing and, although a number of granulocytes and spindle cells (Mattisson and Fänge, 1977; Tomonaga *et al.*, 1973) still remained, the latter could be distinguished from macrophages under a light microscope.

Assays for phagocytosis and the inhibition of phagocytosis

Phagocytosis of hagfish serum-coated RRBC (ES) by hagfish phagocytes was examined in a similar manner to that described for immune phagocytosis by lamprey macrophages (Nonaka *et al.*, 1984). To prepare ES, we incubated RRBC at 1.0×10^8/ml with an equal volume of diluted hagfish serum at 25°C for 40 min. After washing three times with the incubation buffer (GVB^{2+}), a suspension of cells at 1.0×10^8/ml suspension in culture medium was prepared and used as test erythrocytes. A 2-ml aliquot of each test suspension of ES was layered on macrophages in a culture from which the medium had been removed by decanting. Cultures were maintained overnight at 15°C in an atmosphere of 5% CO_2 in air. The monolayers were rinsed free of excess ES, and remaining ES were lysed by treatment with NH_4Cl-Tris buffer (pH 7.1). After thorough washing, macrophages were fixed and stained with Wright's staining solution and more than 300 macrophages were examined by light microscopy. Cells with three or more ingested ES were scored as positive.

To determine the effect of EDTA on the opsonic activity of hagfish serum, we incubated RRBC in EDTA-GVB with a series of serum dilutions as described above. After washing with the same buffer, the RRBC were resuspended with culture medium, and used for assaying an opsonic activity, resistant to EDTA, in the standard assay conditions described above.

To examine the inhibition of ingestion of ES by anti-HC3, ES were treated with IgG or $F(ab')_2$ fragments of IgG from either rabbit antiserum against HC3 or normal (non-immune) rabbit serum for 1 h at 4°C . After washing three times with GVB^{2+}, the suspension of cells at 1.0×10^8/ml in culture medium was used as test erythrocytes.

The statistical significance of differences between results of various trials was determined by two-tailed Student's *t*-tests. Differences were deemed to be significant when $P < 0.05$.

Characterization of HOP

The molecular weight of HOP was determined by gel filtration with the column of Sepharose CL-6B, as described for the purification of HC3 (Fujii *et al.*, 1992). High molecular weight gel filtration calibration kit (Pharmacia) was used as standards.

The subunit structure of HOP was analyzed by two-dimensional SDS-PAGE by the method of Laemmli (1970), under non-reducing conditions and reducing conditions. This procedure involved re-electrophoresis, in the second dimension, of an entire lane from the first gel, as described in Hynes and Destree (1977) and Hanley *et al.* (1990). MW-SDS-200 and MW-SDS-70L kits (Sigma) were used for calibration of gels.

Purified HOP, at 1 mg/ml in a solution of 100 mM K_2PO_4, 50 mM EDTA (pH 7.0), 0.5% Triton X-100, 0.1% SDS, was incubated at 37°C for 1 hr with endoglycosidase F (Boehringer Mannheim, Mannheim, Germany) at a final concentration of 1 U/mg protein. Digested samples were analyzed by two-dimensional SDS-PAGE.

An aliquot of purified HOP was subjected to amino-terminal amino acid sequence analysis, as described elsewhere (Fujii *et al.*, 1995).

Fluorescence and scanning electron microscopy

To examine the binding of HC3 to RRBC, aliquots of ES were exposed successively first to the anti-HC3 and then to goat IgG against rabbit IgG $F(ab')_2$ conjugated with fluorescein isothiocyanate (FITC) (Cappel, Westchester, USA), for 40 min each. Observations were made under a fluorescence microscope (Nikon XF-EF).

For scanning electron microscopy, monolayers of macrophages before and after ingestion were fixed as described elsewhere (Mattisson and Fänge, 1977; Tomonaga *et al.*, 1973). Specimens were dehydrated in acetone and dried by the critical-point drying method. Observations were made under a scanning electron microscope (JSM T300; JEOL).

RESULTS

Ingestion of ES

Normal (unactivated) hagfish peritoneal macrophages that had attached to plastic dishes resembled flat disks with well expanded, thin, cytoplasmic peripheral veils (Fig. 1a). Macrophages incubated with ES had a prominent elevation in the central region due to ingested RRBC (Fig. 1b). A few ghosts of ES, as well as intact ES, were observed on the surface of the macrophages (Fig. 1b).

The phagocytic activity of normal macrophages with

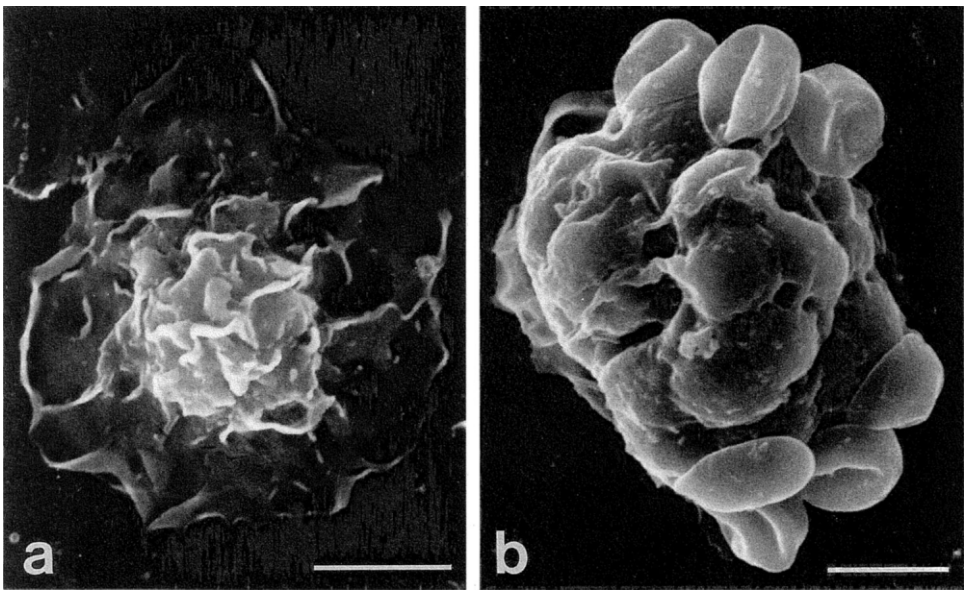

Fig. 1. Scanning electron micrographs of macrophages before (**a**) and after (**b**) incubation with ES, which had been prepared to yield a sub-maximal positive value for ingestion by macrophages. Note the flat disk-like shape with well expanded, thin cytoplasmic veils in (**a**) and the prominent elevation in the central region due to ingested ES in (**b**). Bar = 5 μm.

untreated RRBC was usually low, ranging from 5.7-26%, depending on the batches of RRBC and macrophages used. To detect the opsonic activity in hagfish serum, RRBC that had been treated with a known concentration of hagfish serum (referred to as ES) in GVB^{2+} were cultured with hagfish macrophages. At more than a 20-fold dilution of serum, the phagocytic activity of the macrophages was dependent on the dilution of the opsonizing serum (Fig. 2). The ES prepared with 20-fold diluted serum in GVB^{2+} were ingested by about 73% of macrophages, whereas only 20% of macrophages were positive for ingestion of ES that had been incubated in EDTA-GVB (Fig. 2). It was, thus, evident that the opsonic activity of hagfish serum that we observed was sensitive to EDTA. Moreover, the activity of macrophages increased slightly when the target RRBC had been preincubated with 10-fold diluted serum in EDTA-GVB (Fig. 2, $P < 0.05$ *vs.* unopsonized controls). The opsonic activity of serum that we observed was extremely heat-labile: approximately 70% of the activity was lost after heating of serum at 47°C for 30 min.

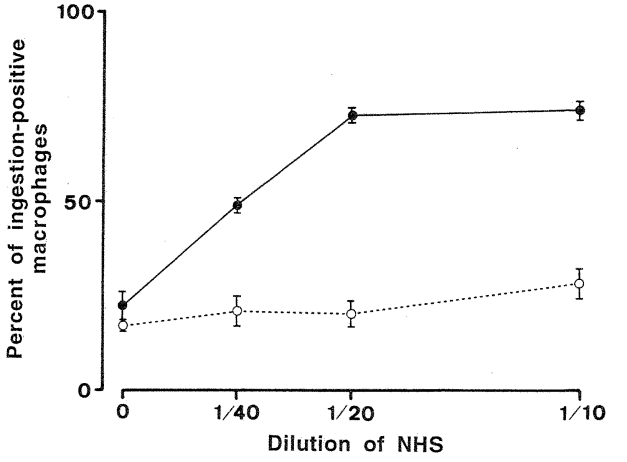

Fig. 2. Ingestion of ES by hagfish macrophages. ES were prepared with various dilutions of normal hagfish serum (NHS) in GVB^{2+} (●) or EDTA-GVB (○). Each point on the curve represents the percentage of ingestion-positive macrophages. Cells with three or more ingested RRBC were scored as positive. Bars: standard errors of means (SEM).

Inhibition of opsonization by anti-HC3 antibodies and detection of HC3 on ES

To determine whether or not HC3 participates in enhancement of the ingestion of RRBC by macrophages, we examined the effects of rabbit anti-HC3 IgG and its $F(ab')_2$ fragments on ingestion (Fig. 3). ES were pretreated with rabbit IgG or $F(ab')_2$ fragments against HC3 for 1 hr at 4°C. ES treated with anti-HC3 IgG at concentrations above 10 mg/ml agglutinated and were, thus, unsuitable for assays. Therefore, the experiments were performed with IgG at concentrations below 5 mg/ml. Under these conditions, the ingestion of cells was effectively, albeit incompletely, suppressed, with the extent of suppression depending on the concentration of IgG (Fig. 3). Normal rabbit IgG had no inhibitory effect on the ingestion of ES by the macrophages, even at 10 mg/ml (Fig. 3).

An attempt was made to demonstrate the presence of HC3 bound to RRBC by immunofluorescence analysis (Fig. 4). HC3-specific fluorescence was observed with virtually 100% of ES prepared with 10-fold diluted serum. Their staining intensity, as well as the percentage of fluorescence-positive ES, was dependent on the concentration of hagfish serum which had been utilized for preparing ES (*cf.* Fig. 4). When

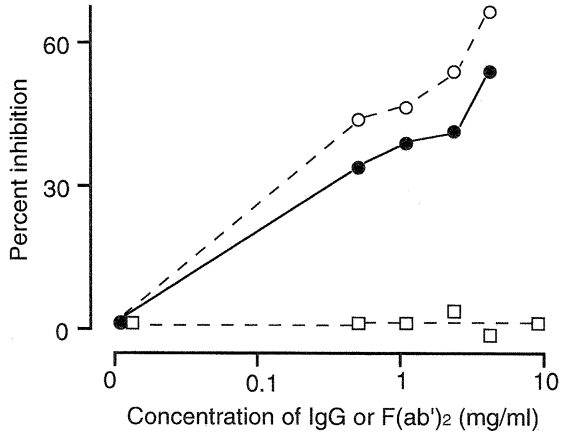

Fig. 3. Inhibitory effects of anti-HC3 antibodies on the ingestion of ES by hagfish macrophages. ES treated with 20-fold-diluted normal hagfish serum were pretreated with anti-HC3 rabbit IgG (●) or $F(ab')_2$ fragments (○) prepared from the same sample of IgG for 1 hr at 4°C . After washing with GVB^{2+}, the ES were added to the macrophages. Each point of the curve represents the percent inhibition. The $F(ab')_2$ fragment (□) from normal rabbit IgG had no inhibitory effect on phagocytosis of ES by the macrophages, even at a concentration of 10 mg/ml.

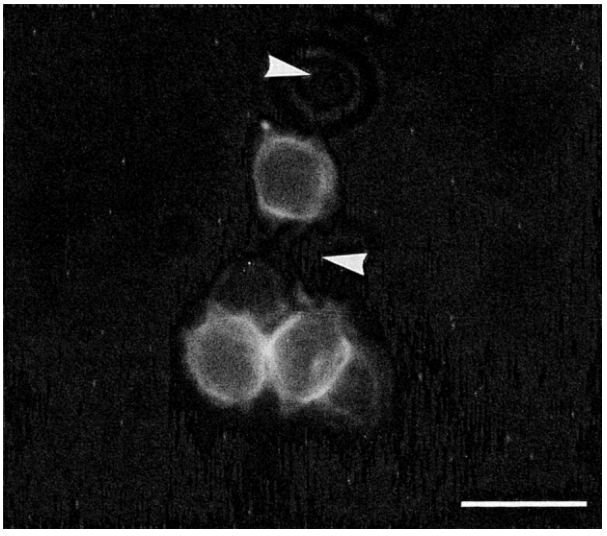

Fig. 4. Fluorescence micrograph showing distribution of HC3 antigens in the membranes of ES. ES prepared with 20-fold-diluted normal hagfish serum were stained with rabbit anti-HC3 followed by goat anti-rabbit IgG $(Fab')_2$-FITC. Several fluorescence-negative ES are also shown (arrowheads). Bar = 10 μm.

ES were incubated with anti-HC3 or anti-rabbit IgG-FITC, no positive fluorescence was observed. The results indicated that HC3 in unfractionated hagfish serum was capable of binding to RRBC.

Opsonization by HC3 and/or HC3-depleted serum (HR3)

In view of a possible analogy to the mammalian complement system and the inability to completely inhibit opsonization with anti-HC3, we anticipated the participation of some other components in HR3 in the ingestion of ES. To examine this issue, we investigated the effect of purified HC3 and/or HC3-depleted serum (HR3) on ingestion of ES (Table 1). When RRBC that had been treated with purified HC3 at 0.05-0.1 mg/ml were used as the target, a similar result was obtained to that obtained with the control E (Table 1). HR3 appeared to lack approximately 60% of the activity needed to enhance the ingestion of ES by macrophages (Table 1). We failed to remedy this insufficiency by adding HC3 (Table 1) or HC3b (data not shown) that had been purified from normal hagfish plasma. Similar results were obtained in another experiment using 10-fold diluted HR3 (data not shown).

However, the ingestion of ES, possibly mediated by an opsonic factor(s) other than HC3, was confirmed by the following experiment, in which RRBC were prepared in EDTA-GVB with HR3 (Table 2). When the RRBC had been treated with 10-fold diluted HR3, a significantly higher number of positive cells (31.9%) was obtained than that in the case of the control E (16.7%), indicating that the residual activity in HR3 was resistent to EDTA (Table 2). Similar results were obtained with detections on 20-fold- and 40-fold-diluted HR3 (Table 2).

Table 1. Inability of HC3 and/or HR3 to induce effective ingestion of ES by hagfish macrophages

Pretreatment of RRBC with[a]	Percent of ingestion-positive macrophages	
GVB^{2+} (E control)	22.8	(0)[b]
NHS[c] (ES control)	68.6	(100)
HC3 (0.1 mg/ml)	25.6	(6.1)
HC3 (0.05 mg/ml)	23.5	(1.5)
HR3[d] (1/20)	40.5	(38.6)
HR3 (1/20) + HC3 (0.1 mg/ml)	37.9	(33.0)
HR3 (1/20) + HC3 (0.05 mg/ml)	42.3	(42.6)

[a] GVB^{2+} was used as the incubation buffer.
[b] Numbers in parentheses are relative percentages in terms of the value obtained when the results for the E and ES controls were taken as 0 and 100%, respectively.
[c] Normal hagfish serum. Twenty-fold-diluted serum was used.
[d] HC3-depleted hagfish serum.

Table 2. Opsonic effect of HR3 on the ingestion of RRBC by hagfish macrophages

Pretreatment of RRBC with[a]	Percent of ingestion-positive macrophages
EDTA-GVB (E control)	16.7 ± 0.9[b]
HR3[c] (1/10)	31.9 ± 5.5
HR3 (1/20)	33.5 ± 3.8
HR3 (1/40)	32.2 ± 4.9

[a] EDTA-GVB was used as the incubation buffer.
[b] Means ± SEM.
[c] HC3-depleted hagfish serum.

Isolation of HOP

An attempt was made to purify and characterize HOP with rabbit anti-HOP serum as a probe. The starting material was pooled plasma (330 ml) that contained 10 mM EDTA. Separate 110-ml aliquots of plasma were dialyzed against the starting buffer and then applied to a column of DEAE-Sepharose CL-6B. Most of the HOP-associated antigenicity was eluted immediately before the major peak of protein (Fig. 5). The corresponding fractions were pooled, concentrated, and applied to a column of Sepharose CL-6B. A large portion of HOP was eluted in the first protein peak . Then HOP was rechromatographed by gel filtration on Sephacryl S-300. HOP antigenicity was recovered as a single peak, with essentially the same elution pattern as that of the protein. The concentrated pool of HOP was finally applied to a column of CM-Sepharose CL-6B. Most of the HOP-associated antigenicity failed to adsorb to the CM-Sepharose. However, a small amount eluted as a trickle after the concentration of NaCl in the eluent began to increase. On immunoelectrophoresis, HOP, eluted in retarded fractions that corresponded to a conductivity of more than 9 mS/cm at 1°C, formed a single precipitin line in the slow β-globulin region when reacted with the rabbit antiserum against unfractionated hagfish serum (Fig. 6). These HOP fractions were pooled, concentrated, dialyzed against PBS, and stored at -80°C. The total weight of the fractionated HOP was 1.9 mg, if we assume that the final preparation was 100% pure. Rocket immunoelectrophoresis, with the isolated HOP used as the standard, led to an estimate of the level of this protein in the original plasma of 14 mg/ml.

Properties of HOP

The final preparation of HOP was recovered as a single peak, after gel filtration on Sepharose CL-6B, in the fractions identical to those of rabbit IgM (data not shown). The results of calibration suggested that the purified HOP had a molecular mass of approximately 1,000 kDa (data not shown).

Analysis by SDS-PAGE of the HOP, under non-reducing conditions, revealed the presence of various polypeptides with apparent molecular masses that ranged from 28.5 to 66 kDa. Under reducing conditions, some of these polypeptides appeared to be dissociated into smaller subunits with molecular masses of 20 to 40 kDa. To confirm this multiplicity of components, we analyzed the subunits by two-dimensional SDS-PAGE. As shown in Fig. 7a, the HOP proteins were dissociated into at least seven major polypeptides and several additional minor ones. Among the corresponding spots, several "vertical pairs" of spots were located in the region that corresponded to molecular masses of 40 to 55 kDa under non-reducing conditions, indicating the occurrence of intermediates that consisted of disulfide-bound subunits.

In order to determine whether or not degradation might have occurred during the purification of HOP, we immunoprecipitated HOP directly from hagfish plasma and analyzed the dissolved precipitate by SDS-PAGE under reducing

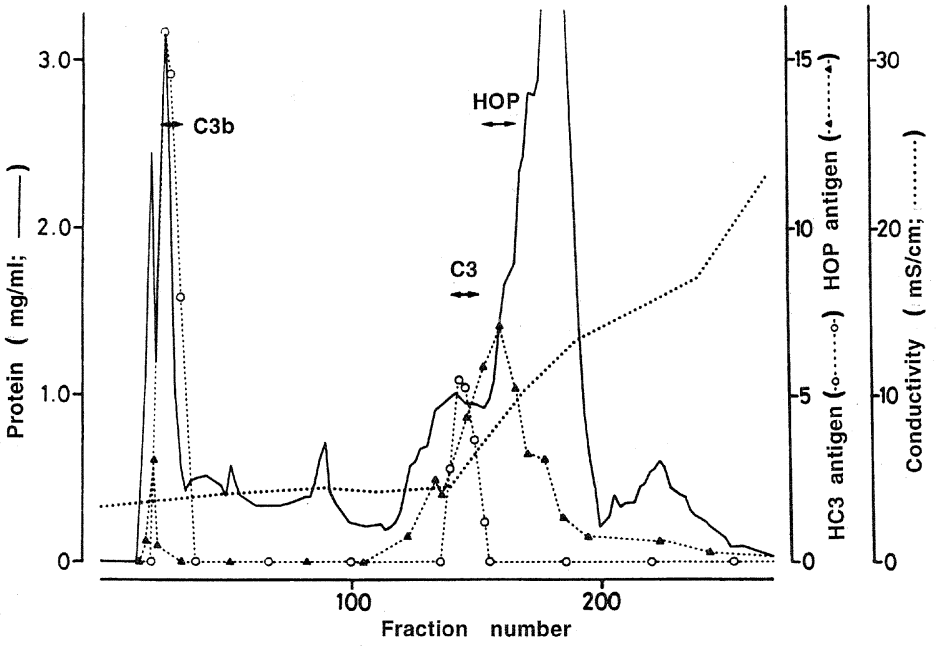

Fig. 5. Elution of hagfish plasma from a column (4 cm i.d. × 40 cm) of DEAE-Sepharose CL-6B. After washing with starting buffer, namely, 25 mM sodium phosphate buffer (pH 7.5) that contained 10 mM EDTA, 50 mM ε-aminocaproic acid and 10 mM benzamidine, the column was eluted with a gradient of 0–0.4 M NaCl in the same buffer. Fractions of 20 ml were collected and determinations were made of protein concentrations and levels of HOP and HC3 antigen. The latter were estimated in terms of the concentration relative to that in whole serum, by rocket immunoelectrophoresis with rabbit antiserum raised against HOP or HC3. The flow rate was 60 ml/hr. Fractions 21–32, fractions 140–152, and fractions 154–171 (indicated by horizontal double-headed arrows) were used for further purification of HC3b, HC3, and HOP, respectively.

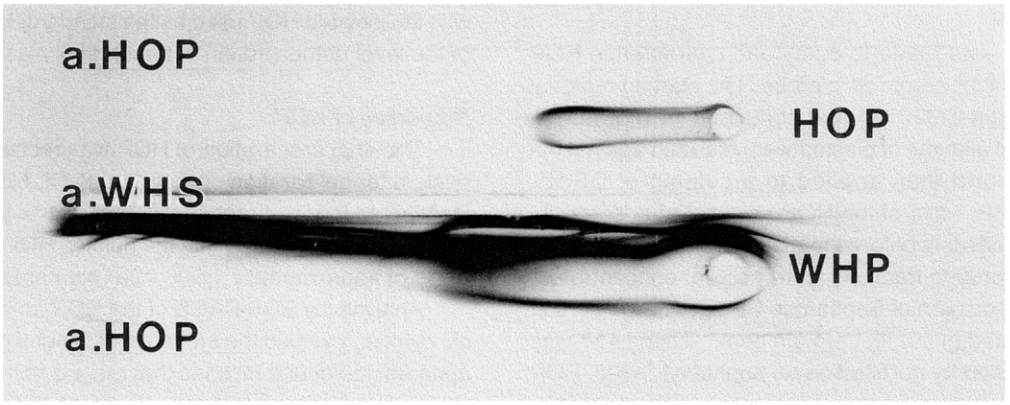

Fig. 6. Immunoelectrophoretic analysis of purified HOP with rabbit antiserum against whole hagfish serum (a.WHS, middle trough) and with antiserum raised against HOP (a.HOP, top and bottom troughs). A single precipitin line in the β-globulin region is visible. WHP, unfractionated whole hagfish plasma. The anode is to the left.

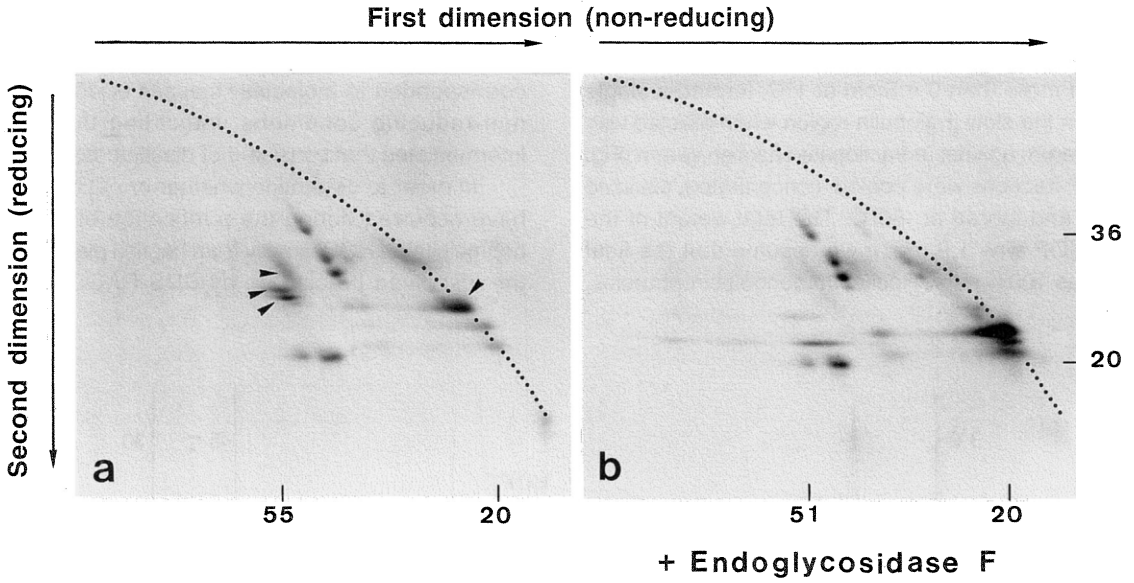

Fig. 7. Analysis by two-dimensional SDS-PAGE of the polypeptide chains of intact and deglycosylated HOP. HOP was incubated for 1 hr with endoglycosidase F at a concentration of 1 U/mg protein. HOP (30 μg/lane) before (**a**) and after (**b**) digestion was subjected to SDS-PAGE in a 10% gel under non-reducing conditions. Without staining, one lane was excised, soaked in reducing buffer and placed horizontally in the preparative well of a 12.5% gel. Electrophoresis was then performed in the second dimension under reducing conditions. Proteins were visualized by staining with Coomassie blue. Arrowheads indicate spots of proteins with increased relative mobility after digestion; numbers indicate molecular masses $\times 10^{-3}$ Daltons; dotted lines indicate positions of proteins that do not consist of disulfide-bonded subunits.

conditions. The antigen that reacted with the antiserum against HOP was found to be a protein that consisted of multiple polypeptides, which were identical to those of the purified HOP (data not shown). The results indicated that the purified HOP had a subunit structure identical to that of the native molecule, assuming no degradation occurs during immunoprecipitation.

The multiplicity of molecular masses of components of HOP was investigated with respect to the possible contribution of differing degrees of glycosylation. Samples of HOP were digested with endoglycosidase F and analyzed in the same way as intact HOP (Fig. 7b). Deglycosylation of HOP apparently increased the relative mobilities of four polypeptides. Three of these polypeptides, migrating in the control profile as proteins with molecular masses of 55 kDa under non-reducing conditions, formed a slender band after deglycosylation. Similarly, a significant increase in relative mobility was observed for the protein that yielded the most intense spot with an original molecular mass of 25.5 kDa, under both non-reducing and reducing conditions (Fig. 7). These results indicate that some but not all of the major polypeptides of HOP, are modified by addition of carbohydrate moieties of varying size.

In order to evaluate the relationships among the polypeptides of HOP, we attempted to determine the amino

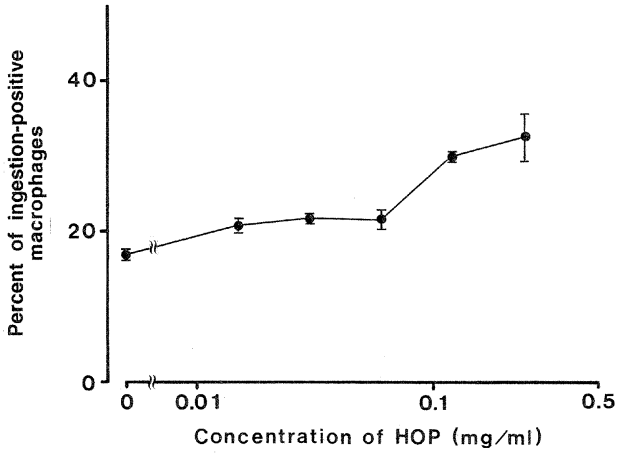

Fig. 8. Opsonic effects of HOP on the ingestion of RRBC by hagfish macrophages. RRBC were pretreated in EDTA-GVB with various concentrations of purified HOP. Each point on the curve represents the percentage of ingestion-positive macrophages. Cells with three or more ingested RRBC were scored as positive. Bars: SEM.

acid sequences of the major polypeptides and their derivatives by Edman degradation. The amino-terminal sequence of the 30-kDa polypeptide, under reducing conditions, was WPDGALKMSK. Furthermore, the amino-terminus of the 25-kDa polypeptide, GLEGGLRMIKYLVFLA, was identical to that of the 24-kDa polypeptide. We also observed that the amino-terminus of the 21-kDa chain, NNXPSP, was the same as that of the amino-terminus of the 20-kDa chain.

To determine whether or not isolated HOP could enhance the ingestion of RRBC by macrophages, we examined the effect of pretreatment of target cells with HOP. When RRBC that had been treated in EDTA-GVB with purified HOP at 0.015-0.24 mg/ml were used as a target, a significantly elevated score (20.6-32.4%) was obtained as compared to that of the E control (16.7%, $P < 0.05$ *vs.* unopsonized controls; Fig. 8). This result indicated that isolated HOP bound to RRBC and acted as an opsonin.

DISCUSSION

During the functional analysis of HC3, we recognized that hagfish serum contains at least two distinct opsonins, namely, activated HC3 and the another major glycoprotein, designated HOP. The binding of HC3 to RRBC was confirmed by an immunofluorescence analysis using the anti-HC3 antibody. The present study suggests, for the first time, that hagfish serum possesses a multicomponent opsonization system which may be a forerunner of the vertebrate complement system (*cf.* Dodds and Day, 1993). Our hypothesis that HC3 functions in opsonization after activation in the presence of RRBC, which can trigger the alternative complement pathway from several vertebrate phyla (Nonaka *et al.*, 1981; Sekizawa *et al.*, 1984), is based on the following functional criteria: (a) the specific IgG against HC3 inhibited phagocytosis of ES (Fig. 3); (b) when RRBC were pretreated with either purified HC3 or HC3b, no enhanced phagocytosis was observed (Table 1); and (c) the binding of HC3 to RRBC was extremely sensitive to divalent cations. Our hypothesis is consistent with our previous observations that the binding of HC3 to zymosan particles (Fujii *et al.*, 1992), a phenomenon which is extremely sensitive to heat and divalent cations, resembles the alternative complement pathway in mammals (Müller-Eberhard and Schreiber, 1980). Recently, we succeeded in remedying the insufficiency due to heating of sera by adding the retarded fractions, eluted immediately after the major peak of protein, from chromatography on DEAE-Sepharose in a similar manner as that shown in Fig. 5 (our unpublished observation). We did not, however, succeed in remedying the insufficiency of HR3 by adding purified HC3 (Table 1). This result might be attributable to the loss and/or inactivation, during preparation of HR3, of serum factor(s) essential to the activation of HC3. In this connection, it is appropriate to mention the fact that lamprey has complement factor B showing an amino acid sequence similarity and the same domain structure with mammalian factor B or C2 (Nonaka *et al.*, 1994). Contrary to our observations, Hanley *et al.* (1992) reported that a complement-like protein, similar to our HC3b and isolated from the Pacific hagfish, bound directly to streptococcal cells and enhanced the phagocytosis of yeast cells by hagfish leukocytes. Further investigations to resolve these discrepancies are essential if we are to elucidate the extent to which other components of the complement cascade system occur and/or function, as well as to establish the way in which HC3 binds targets, such as RRBC and yeast cells. In this context, it is of interest that HC3 has a thioester site, identical to that in mammalian C3 and C4 (Fujii *et al.*, 1995; Hanley *et al.*, 1992). It is possible that this unique site in HC3 might play a role in the covalent binding to targets, as described elsewhere (Fujii *et al.*, 1992).

The present study demonstrates, for the first time, the presence in hagfish of an opsonin that acts independently of the primitive complement system identified in this species. The HOP appears to have a complicated structure, consisting of multiple types of polypeptide that are bound together by non-covalent and covalent linkages. We did not, however, find counterparts of the HOP among defense molecules in the serum components from other vertebrates by homology search (our unpublished observation). Given the possible absence in hagfish of antibodies (Raison, 1996) and the related classical pathway of complement, it seems possible that the function of the HOP might be important in the defense mechanism of hagfish. In order to validate this hypothesis, further analysis of the functional features displayed by the unique molecule in this most primitive of extant vertebrates is essential. An effort also should be made to determine whether our HOP shares functional and molecular features with opsonic factors in the Pacific hagfish, *Eptatretus stouti*, that were reported by Raftos *et al.* (1992).

ACKNOWLEDGMENTS

The authors thank Ms. Y. Ikeda, Ms. K. Ogawa, and Ms. K. Sugiyama for their excellent technical assistance, and Professor Robert L. Raison (University of Technology, Sydney) for his critical reading. This work was supported in part by grants (nos. 02740378 and 03640631) from the Ministry of Education, Science, Sports and Culture of Japan to T.F.

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(Received September 20, 1996 / Accepted December 28, 1996)