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The Natural Mediator for PMN Emigration in Inflammation

III. *IN VITRO* PRODUCTION OF A CHEMOTACTIC FACTOR BY INFLAMMATORY SH-DEPENDENT PROTEASE FROM SERUM IMMUNOGLOBULIN G

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Summary. In earlier work, a chemotactic factor (leucoegresin) specific for polymorphonuclear (PMN) leucocytes had been isolated from the sites of Arthus reactions or cutaneous burns. The substance shared antigenic sites with IgG.

The possible existence precursor of the chemotactic factor in the γ_2-globulin fraction of normal rabbit sera is suggested since the protein fraction on incubation with a purified neutral SH-dependent protease from inflammatory tissue became strongly chemotactic.

INTRODUCTION

Previous studies (Hayashi, Koono, Yoshinaga and Muto, 1969; Muto, 1969) have suggested that the *in vivo* production of leucoegresin may be associated with the action of a specific neutral SH-dependent protease which is activated at the inflamed site. Since increased vascular permeability precedes PMN leucocyte emigration in an Arthus reaction or burn (Hayashi, 1967), it was suggested that a precursor of this chemotactic factor might be contained in the exuded serum proteins. The present communication demonstrates that a possible precursor of chemotactic factor is an immunoglobulin G of normal rabbit serum, and that the chemotactic factor can be generated *in vitro* from fractionated immunoglobulin G by treatment with an inflammatory SH-dependent protease.

MATERIALS AND METHODS

Estimation of chemotactic activity

Chemotactic activity for PMN leucocytes was demonstrated by *in vitro* assay according to a modification (Yamamoto, Yoshinaga and Hayashi, 1971) of Boyden's (1962) method using Millipore filters (DAWPO 13, pore size 0·65 μ; Millipore Filter Co., Bedford, Mass., U.S.A.) and a modified chamber containing two 1-ml compartments. The test samples, in M/15 phosphate buffer (pH 7·4), were placed in the lower compartment; the Millipore filter was placed on it, and rabbit PMN leucocytes, collected from the peritoneal cavity by Hirsch's (1956) method and suspended in Gey's buffered solution containing 2 per cent of human serum albumin, pH 7·4, were poured into the upper compartment. 1 ml of cell suspension contained approximately $1{\cdot}5 \times 10^6$ cells.

After incubation at 37° for 3 hours, PMN leucocytes that had passed through the filter were stained with haematoxylin and counted over five microscopic fields (10 × 40) randomly selected; the total counts were recorded.

Isolation of inflammatory SH-dependent protease

Following a previously described method (Koono, Muto and Hayashi, 1968), the inflammatory SH-dependent protease was extracted in the euglobulin fraction of 12-hour-old Arthus lesions and then purified by chromatography using DEAE-Sephadex and GE-cellulose; it behaved as a homogeneous substance on electrophoresis. The optimum pH of the protease was 7·1. Concentrations of the protease solution were read at absorbancy 280 mμ. Before use, the SH-dependent protease was activated by adding cysteine to a final concentration of 10^{-3}M.

Estimation of protease activity

Activity was measured in the presence of 10^{-3}M cysteine by a modification (Hayashi, Miyoshi, Nitta and Udaka 1962a; Hayashi, Udaka, Miyoshi and Kudo, 1965) of the casein digestion method of Kunitz (1947). The pH of the reaction was 7·1 and the ionic strength was 0·25. After incubation at 37° for 60 minutes, an equal volume of 6 per cent trichloroacetic acid was added to the reaction mixture and filtered when precipitation was completed. The extinction at 276 mμ of the filtrate was measured and the arbitrary unit of proteolytic activity was defined as the amount of enzyme which would cause an increase of 0·001 unit of extinction per minute of digestion (Koono and Hayashi, 1969). Accordingly, one proteolytic unit represents 0·06 absorbance units at 276 mμ. Enzyme samples with proteolytic activity of 0·66 units/ml were used.

Preparation of immunoglobulin G (IgG)

Rabbit IgG fraction was separated in the cold from normal rabbit serum according to the method of Kapusta and Halberstam (1964), using sodium sulphate precipitation and DEAE-cellulose column chromatography; the isolated IgG was homogeneous as judged by immunoelectrophoresis with goat antiserum against rabbit serum (Yamamoto, Yoshinaga and Hayashi, 1971). Human IgG fraction was isolated from normal human serum by the same method.

Human myeloma protein was harvested from a patient whose serum contained 5500 mg IgG/100 ml when measured by a single radial immunodiffusion technique (Fahey and Lawrence, 1963) using an immunoplate (Hyland Laboratories, Los Angeles, California, U.S.A.). The serum showed monoclonal IgG pattern by a microzone electrophoresis (Mullan, Hancock and Neil, 1962). From the serum, IgG fraction was isolated by the method of Kapusta and Halberstam (1964). The IgG fraction was eluted through a Sephadex G-200 column to eliminate any aggregated IgG. The fractionated IgG was homogeneous as judged by immunoelectrophoresis with rabbit antiserum against human serum. The molecular weight of the IgG was approximately 160,000. Each IgG fraction was dissolved in M/15 phosphate buffer, pH 7·4 at concentrations of 100 and 200 μg.

Preparative zone electrophoresis

This was performed essentially according to the method of Müller-Eberhard (1960); 2·0 ml of fresh rabbit serum was electrophoresed through a column (3·5 × 40 cm) of Pevikon C-870 (Superfosfat, Stockholm, Sweden), which had previously been equilibrated with M/20 veronal buffer, pH 8·6, at a current of 15 mA for 36 hours. Protein contents of effluent fractions were measured colorimetrically by the method of Lowry, Rosebrough, Farr and Randall (1957). Before use, effluent fractions were dialysed against M/15 phosphate buffer, pH 7·4, for 16 hours.

RESULTS

GENERATION OF CHEMOTACTIC ACTIVITY FROM RABBIT SERUM PROTEIN BY INFLAMMATORY SH-DEPENDENT PROTEASE

By preparative zone electrophoresis on Pevikon C-870, six protein fractions were separated from normal rabbit serum; they respectively contained albumin, α_1–α_2, α_2–β_1, β_1–γ_1, $\gamma_1 \sim \gamma_2$, and γ_2 globulin fractions. Equal volumes (0·5 ml) of the protein fraction (concentration 0·2–1·4 at $E_{280\ m\mu}$ in M/15 phosphate buffer, pH 7·4) and the SH-dependent protease solution (0·66 units/ml in M/15 phosphate buffer, pH 7·4) were mixed and incubated at 37° for 60 minutes. 1 ml of the mixture was tested for chemotactic activity.

As shown in Fig. 1, the γ_2-globulin fraction had no chemotactic activity, but became strongly chemotactic in the presence of active SH-dependent protease. However, other protein fractions showed no chemotactic effect under the same conditions.

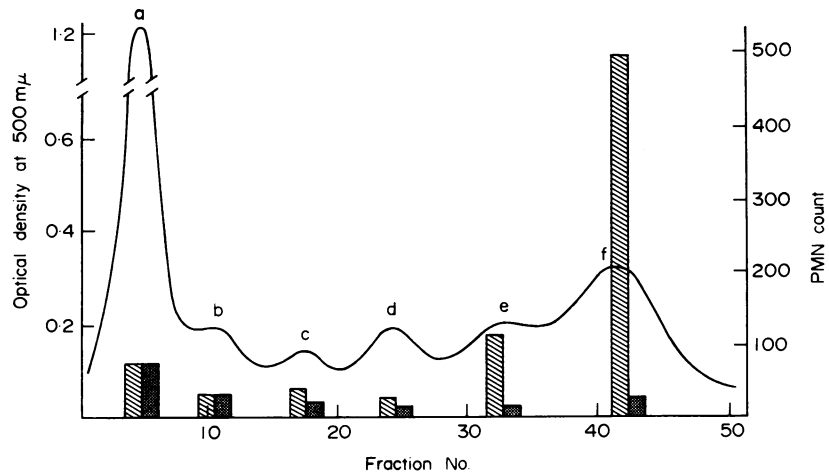

FIG. 1. *In vitro* generation of chemotactic activity in serum protein fraction by inflammatory SH-dependent protease (inflammatory protease). The mixture of each protein fraction (0·5 ml), obtained from normal rabbit serum by block-electrophoresis on a 'Pevikon C-870' column, and inflammatory protease (0·5 ml) was incubated at 37° for 60 minutes, and then tested for chemotactic activity. The activity of inflammatory protease was 0·66 units/ml. See text. Protein concentrations at 280 mμ of each fraction were as follows: (a) albumin, 1·40; (b) $\alpha_1 \sim \alpha_2$-globulin, 0·28; (c) $\alpha_2 \sim \beta_1$-globulin, 0·24; (d) $\beta_1 \sim \gamma_1$-globulin, 0·30; (e) $\gamma_1 \sim \gamma_2$-globulin, 0·28; (f) γ_2-globulin, 0·54. Stippled columns, chemotactic potency before treatment with inflammatory protease activated by 10^{-3} M cysteine. Hatched columns: chemotactic potency after treatment with the activated inflammatory protease.

The $\alpha_1 \sim \alpha_2$-globulin fraction has been found to contain an inhibitor of the SH-dependent protease (Tokaji, 1971); the inhibitor of the protein fraction (0·5 ml) was able to inhibit 0·4 units of SH-dependent protease. Accordingly, the protein fraction (0·5 ml) was treated with a higher activity (2·0 units) of the SH-dependent protease (0·5 ml). However, no chemotactic activity was detected. None of the other protein fractions showed an inhibitory effect on the SH-dependent protease (Tokaji, 1971).

As shown in Table 1, rabbit IgG had no chemotactic activity on PMN leucocytes, but became strongly chemotactic when treated with the SH-dependent protease activated in the presence of cysteine. The SH-dependent protease itself was ineffective for PMN leucocytes.

TABLE 1

In vitro GENERATION OF CHEMOTACTIC ACTIVITY FROM RABBIT IgG BY INFLAMMATORY SH-DEPENDENT PROTEASE (INFLAMMATORY PROTEASE)

Samples in lower compartment			No. of PMNs migrating (per 5 fields)
Rabbit IgG (μg)	Inflammatory protease (proteolytic units)	Cysteine (final conc.)	
200	0·33	10^{-3} M	449
100	0·33	10^{-3} M	215
200	Phosphate buffer	10^{-3} M	24
200	0·33	Phosphate buffer	28
200	Phosphate buffer	Phosphate buffer	18
Phosphate buffer	0·33	10^{-3} M	25
Phosphate buffer			14

The mixture of each sample (0·5 ml) was previously incubated at 37° for 60 minutes, and placed in the lower compartment for assay of chemotactic potency. The proteolytic activity of inflammatory protease added was 0·66 units/ml. See text.

TABLE 2

In vitro GENERATION OF CHEMOTACTIC ACTIVITY FROM HUMAN IgG BY INFLAMMATORY SH-DEPENDENT PROTEASE (INFLAMMATORY PROTEASE)

Samples in lower compartment			No. of PMNs migrating (per 5 fields)
Rabbit IgG (μg)	Inflammatory protease (proteolytic units)	Cysteine (final conc.)	
200	0·33	10^{-3} M	320
100	0·33	10^{-3} M	182
200	Phosphate buffer	10^{-3} M	22
200	0·33	Phosphate buffer	26
200	Phosphate buffer	Phosphate buffer	18
Phosphate buffer	0·33	10^{-3} M	19
Phosphate buffer			12

The mixture of each sample (0·5 ml) was previously incubated at 37° for 60 minutes and placed in the lower compartment. The protease activity was 0·66 units/ml. See text.

GENERATION OF CHEMOTACTIC ACTIVITY FROM HUMAN IgG BY INFLAMMATORY SH-DEPENDENT PROTEASE

Human IgG fraction (100–200 μg) was similarly treated with the SH-dependent protease (0·33 units) in the presence of cysteine. As summarized in Table 2, human IgG fraction showed no chemotactic effect on PMN leucocytes, but it became strongly chemotactic after treatment with the SH-dependent protease.

GENERATION OF CHEMOTACTIC ACTIVITY FROM HUMAN MYELOMA PROTEIN BY INFLAMMATORY SH-DEPENDENT PROTEASE

Human myeloma IgG (100 and 200 μg) was incubated with the SH-dependent protease (0·33 units) in the presence of cysteine.

As shown in Table 3, myeloma IgG showed no chemotactic potency on PMN leucocytes, but it became strongly chemotactic by treatment with SH-dependent protease; the intensity of chemotactic generation of myeloma IgG was similar to that of normal

TABLE 3

In vitro GENERATION OF CHEMOTACTIC ACTIVITY FROM HUMAN MYELOMA PROTEIN BY INFLAMMATORY SH-DEPENDENT PROTEASE (INFLAMMATORY PROTEASE)

Samples in lower compartment			No. of PMNs migrating (per 5 fields)
Myeloma IgG (μg)	Inflammatory protease (proteolytic units)	Cysteine (final conc.)	
200	0·33	10^{-3} M	471
100	0·33	10^{-3} M	249
200	Phosphate buffer	10^{-3} M	20
200	0·33	Phosphate buffer	23
200	Phosphate buffer	Phosphate buffer	20
Phosphate buffer	0·33	10^{-3} M	18
Phosphate buffer			15

The mixture of each sample (0·5 ml) was incubated at 37° for 60 minutes, and placed in the lower compartment. The activity of inflammatory protease added was 0·66 units/ml. See text.

human IgG. Some samples of the IgG fractions, separated from the sera stored during some weeks or separated at room temperature, occasionally showed a slight chemotactic effect on PMN leucocytes before treatment with the SH-dependent protease. Accordingly, all the IgG samples were carefully separated in the cold from fresh serum. Such IgG samples showed no chemotactic activity.

DISCUSSION

As previously described (Hayashi, 1955, 1967, 1968; Hayashi *et al.*, 1958, 1962a, b, 1965, 1969; Udaka, 1963; Koono, Muto and Hayashi, 1968; Koono and Hayashi, 1969), the SH-dependent protease has been shown to satisfy many of the criteria necessary for an inflammatory agent in an Arthus reation or burn: (a) The protease is locally available. (b) Protease activities parallel the time-course of the reactions. (c) The protease can produce morphologic change similar to those of Arthus reactions when injected in concentrations comparable to those detected in sites of inflammation. (d) The protease, when injected locally, can produce chemical mediators which are associated with individual inflammatory manifestations such as vascular permeability changes or leucocyte emigration in the reactions. (e) The protease is inhibited by the specific antagonist. (f) The inflammatory reactions are suppressed by specifically antagonistic substance (Hayashi *et al.*, 1969).

The intradermal injection of the SH-dependent protease into normal rabbits induced increased vascular permeability to plasma proteins followed by pronounced PMN emigration; from the protease-induced skin lesions, chemotactic factor (leucoegresin) was isolated (Hayashi *et al.*, 1969; Muto, 1969). These observations suggested the production of chemotactic factor by SH-dependent protease action on the exuded proteins. At the site of an Arthus reaction or a burn, exudation of plasma proteins preceded PMN leucocyte emigration (Hayashi, 1967). As shown in Fig. 1, a possible precursor of chemotactic factor was located in the γ_2-globulin fraction of normal rabbit serum.The activity of the SH-dependent protease added was 0·33 units, reasonably comparable to that of the enzyme detected at an early stage of PMN leucocyte emigration in an Arthus reaction (Hayashi *et al.*, 1969; Muto, 1969). Since $\alpha_1 \sim \alpha_2$-globulin fraction contained an inhibitor of the

SH-dependent protease (Itoh, 1960; Tokaji, 1971), the protein fraction was treated with larger concentration of the enzyme, but it did not show any chemotactic activity.

The observations on purified IgG fractions suggested that a possible precursor of chemotactic factor in the γ_2-globulin fraction may be associated with IgG; and IgG gives rise to a chemotactic factor by such enzymatic treatment. The previous observations showing that rabbit chemotactic factor (leucoegresin) had antigenic sites in common with rabbit IgG (Yamamoto *et al.*, 1971; Yoshinaga *et al.*, 1970) seemed to suggest IgG as a possible precursor for chemotactic factor. More recently, Wilkinson, Borel, Stecher-Levin and Sorkin (1969) have found that a chemotactic factor of serum, activated by antigen–antibody complex, may be related to a large molecular substance similar to IgG, but the mechanism of such a chemotactic generation remains to be elucidated.

The molecular size (about 140,000) and sedimentation coefficient (about 6·58S) of rabbit chemotactic factor (leucoegresin) were closely similar to those of rabbit IgG, but the chemotactic factor was characterized by a lower isoelectric point (about 5·0). The chemical change in the conversion of IgG to chemotactic factor awaits further study. Thus, besides the well-known specific role as antibody, it was suggested that the IgG molecule may play a role as the precursor of a chemotactic factor in inflammation.

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