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The mediation of tissue eosinophilia in hypersensitivity reaction

I. ISOLATION OF TWO DIFFERENT CHEMOTACTIC FACTORS FROM DNP–ASCARIS EXTRACT-INDUCED SKIN LESION IN GUINEA-PIG

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Summary. In an active cutaneous anaphylaxis induced by DNP–*ascaris* extract in guinea-pig, tissue eosinophilia manifested two phases; the early and mild phase became maximal in about 6 h, while the delayed and intense phase in 18–24 h. Skin extracts from the lesions exhibited chemotactic activities for eosinophils, respectively comparable to the intensity of tissue eosinophilia in each phase; and two different chemotactic factors for eosinophils of skin extracts were separated by gel filtration on Sephadex G-100. The mediation of the early phase seemed to be associated with a thermostable factor with a molecular weight of less than 1400; this factor seemed to be related to mast cell degranulation. The mediation of the delayed phase appeared to be associated with a thermolabile factor with a molecular weight of about 70,000, probably independent of mast cell degranulation; the factor was considered to be more significant than the thermostable factor, because the delayed tissue eosinophilia was more intense than the early tissue eosinophilia.

INTRODUCTION

The isolation and characterization of chemotactic factors present in inflammatory tissues are essential to clarify the mediation of inflammatory leucotaxis, though there remains the difficult problem of how to obtain sufficient chemotactic factors from tissues. As previously reviewed (Hayashi, Yoshinaga and Yamamoto, 1974), a chemotactic factor (leucoegresin) specific for neutrophils has been isolated from inflamed sites and characterized as a natural mediator for tissue neutrophilia.

As is well known, eosinophils are associated with certain allergic tissue reactions, particularly immediate-type hypersensitivity. The cells are also found in the nasopharynx and bronchi of patients with allergic conditions such as hay fever or asthma, and in the intestinal tract as consequence of parasitic infestations. In these disorders, the only clear correlate with eosinophilia is the allergic status of patients. Various mediators of an eosinophilia have been proposed including histamine (Kline, Cohen and Rudolph, 1932; Archer, 1963), antigen–antibody complexes (Litt, 1964) and sensitized tissue treated with antigen (Samter, Kofoed and Piper, 1953; Parish and Coombs, 1968; Kay, 1970b; Kay, Stechschulte and Austen, 1971). However, present knowledge on the natural mediators for tissue eosinophilia in an allergic reaction still seems to be incomplete.

The purpose of the present communication is to describe the isolation and characterization of two

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different chemotactic factors for eosinophils from allergic tissue lesions induced by DNP-ascaris extract in guinea-pigs.

MATERIALS AND METHODS

Animals

Male albino guinea-pigs of Hartley strain weighing 400 ± 50 g were, as a rule, used. For separation of eosinophils from peritoneal exudate, animals weighing 600–1000 g were used.

Preparation of antigen (DNP–ascaris extract) (DNP–As)

Extracts from *Ascaris suum* were prepared by the method of Strejan and Campbell (1967). Live *Ascaris suum*, obtained from the intestine of pigs, were washed several times with physiological saline, homogenized in a Waring blender, and extracted with a five-fold volume of saline in the cold (2°) for 24 h. After centrifugation, the turbid supernatant fluid was dialysed against borate-buffered saline (pH 8·0) for 24 h. The clear supernatant fluid was separated by centrifugation at 15,000 r.p.m. for 30 min and stored at −20°.

Dinitrophenyl (DNP) groups were introduced to the ascaris extracts according to the method of Eisen, Belman and Carsten (1953), and Tada and Okumura (1971). Two hundred milligrams of 2,4-dinitrophenyl sulphonic acid (recrystallized twice) were dissolved in a small quantity of 1·0 M Na_2CO_3 and mixed with 100 mg of ascaris extracts. The mixture was rotated at room temperature for 24 h, dialysed against distilled water for 24–48 h and then against physiological saline for 6 h, and stored at −20°; and $2{\cdot}1 \times 10^{-4}$ mols of DNP were found in 1·0 mg of DNP-coupled ascaris extracts (DNP–As). Killed *B. pertussis*, 1×10^{10}/ml, was a gift from the Kumamoto Sero-Chemotherapy Institute, Kumamoto, Japan. Protein concentration was measured by the method of Lowry, Rosebrough, Farr and Randall (1951).

Induction of active cutaneous anaphylaxis

Immunization of animals was done by a modification of the method of Margni and Hajos (1973). The animals were injected intramuscularly in both hind legs with 0·5 ml of DNP–As (5 mg/ml in physiological saline) with an equal volume of Freund's complete adjuvant (Difco, Detroit, Michigan), with simultaneous intraperitoneal injection of 1·0 ml of killed *B. pertussis* (1×10^{10}/ml in physiological saline). Seven days later, the same injections were repeated.

Twelve days after the first immunizing injection, the eighteen immunized animals of experimental group were intracutaneously injected with 0·1 ml of DNP–As (5 mg/ml in physiological saline) at eight sites over the body, and the treated sites were observed for 48 h. Control animals were divided into two groups; seven animals of the first group were not sensitized but injected with 0·1 ml of DNP–As (5 mg/ml); six animals of the second group were similarly sensitized but not challenged with DNP–As.

The intensity of the inflammatory reaction was graded as follows (Hayashi, Miyoshi, Nitta and Udaka, 1962): ±, mild oedema and erythema in lesions up to 1·0 cm in diameter; +, mild oedema and erythema for at least 24 h; + +, mild to moderate oedema and erythema; the lesion being more than 2·0 cm in diameter, and its haemorrhagic centre up to 0·5 cm in diameter; + + +, moderate oedema; the erythematous lesion was 3–4 cm in diameter; the haemorrhagic and brownish discolouration 0·5–1·5 cm in diameter; + + + +, severe oedema; the markedly erythematous lesion was more than 4·0 cm in diameter with haemorrhage more than 1·5 cm in diameter and sloughing of the test site.

Passive cutaneous anaphylaxis (PCA) reaction

This was performed essentially by the method of Ovary and Bier (1953), and Dobson, Morseth and Soulsby (1971). Twelve days after the first immunization, guinea-pig serum was obtained from individual bleeding. Serial dilutions of the serum were injected intradermally in 0·1-ml quantities into the shaved backs of normal guinea-pigs in duplicate. After 4 h or 6 days, the animals were challenged by intravenous injections of 0·5 ml of DNP–As (2 mg/ml) and 0·5 ml of pontamine sky blue (60 mg/kg). After 30 min, they were killed, the skin was reflected and the extent of extravasation of the dye was determined. Spots below diameter of 0·5 cm were considered dubious reactions, while the ones 0·5–1·0 cm, 1·0–1·5 cm, 1·5–2·0 cm and more than 2·0 cm were considered as positive)+), (+ +), (+ + +) and (+ + + +), respectively.

PCA reaction in 4-h latent period was positive (graded +) in a 2000-fold dilution of the sera, and that in 6-day latent period was positive (graded +)

in a 500-fold dilution of the sera. It was therefore indicated that these animals immunized with DNP–As were useful for the present experiment.

Quantification of tissue leucocytosis

At various intervals following challenge with antigen, the animals were killed, and the injection sites, previously marked, were immediately biopsied using a punch (1 × 1 cm). The biopsy included all layers of the skin including the panniculus carnosus muscle. Following fixation in formal saline, or Susa's medium, sections were taken from the sites and approximately 3 mm either side of the intradermal injection. The sections were paraffin embedded, cut and stained as usual with haematoxylin and eosin. For eosinophil experiments, Luna's (1968) eosinophilic granule staining using Weigert's iron haematoxylin and Bieblich Scarlet was used. For tissue mast cell experiments, aqueous toluidine blue was used. From each section a total of fifteen random high power fields (10 × 40) were counted in 0·3-mm strips between the junction of the epidermis and dermis and the upper limit of the panniculus carnosus muscle using a previously calibrated graticule. The cell count is expressed as a total of fifteen strips which represents the mean of the three sections counted, i.e. forty-five strips (Kay, 1970a).

Estimation of chemotactic activity

The capacity to promote eosinophil migration was measured *in vitro* by a modification (Yamamoto, Yoshinaga and Hayashi, 1971) of Boyden's method (1962) using Millipore filters (SSWPO 1300, pore size 3 μm; SMWP 04700, pore size 5 μm; SCWP 29300, pore size 8 μm; Millipore filter Company, Bedford, Massachusetts) and a stainless steel chamber containing two 1-ml compartments. Test samples, in 0·067 M phosphate buffer (pH 7·4), were placed in the lower compartment, the filter was placed on it, and eosinophil-rich cell suspension was poured into the upper compartment. The chambers were incubated for various durations at 37° using a 5 per cent CO_2 atmosphere. The cells that had migrated through the filter to the lower surface of the filter were stained with Carazzi's haematoxylin and Chromotrope 2R (Kay, 1970b). The chemotactic counts were expressed as the mean count of migrated eosinophils in twenty high power fields (10 × 40) randomly selected.

Guinea-pig eosinophils were obtained by peritoneal lavage from animals which had received weekly multiple (eight to twelve) injections of 2 ml of horse serum (Litt, 1960). Peritoneal cells, containing 55–75 per cent of eosinophils at the 5th day after the last injection of horse serum, were centrifuged and suspended in Hanks's balanced salt solution containing 0·5 per cent ovalbumin at a concentration of 1×10^6 cells/ml.

Preparation of skin extract

Extracts were made by the method of Hayashi *et al.* (1962). The skin sites were excised immediately after animals of experimental and control groups were killed by bleeding from the carotid arteries. The skin (8–12 g per animal) was cut with scissors into several pieces and frozen at −80°. The pieces of frozen skin were cut into slices about 50 μm thick with a freezing microtome, the slices being dehydrated with three changes of cold acetone and powdered. Powdered skin (3–4 g per animal) was extracted with a ten-fold volume of 0·067 M phosphate buffer (pH 7·4) in the cold for 4 h. After centrifugation, the clear supernatant fluid was concentrated by ultrafiltration using Diaflo membrane, UM-05 (Amicon Company, Lexington) to give an absorbancy 40 at 280 nm/ml.

Gel filtration on Sephadex G-100

The clear skin extract (7 ml, absorbancy 40 at 280 nm/ml) was applied to column (3 × 50 cm) of Sephadex G-100, fine (Pharmacia, Uppsala, Sweden) equilibrated with 0·067 M phosphate buffer (pH 7·4) (Porath and Flodin, 1959). The flow rate was 24 ml/h and 3-g effluent fractions were collected for assay of chemotactic activity.

Estimation of molecular weight

This was performed by gel filtration on Sephadex G-200 column (2·4 × 50 cm) equilibrated with 0·067 M phosphate buffer (pH 7·4) (Andrews, 1965). The flow rate was 12 ml/h and 2·4 g effluent fractions were collected. Cytochrome *c* (12,400 molecular weight in monomer) (Sigma, St Louis, Missouri), bovine serum albumin (67,000 molecular weight in monomer; 134,000 molecular weight in dimer) (Armour, Kankakee, Illinois) and blue dextran (2,000,000 molecular weight) (Pharmacia, Uppsala, Sweden) were used as standard substances.

RESULTS

Time-course of leucocyte reaction in active cutaneous anaphylaxis

Intradermal injection of 0·1 ml of DNP–As (5 mg/ml) into sensitized animals of experimental group

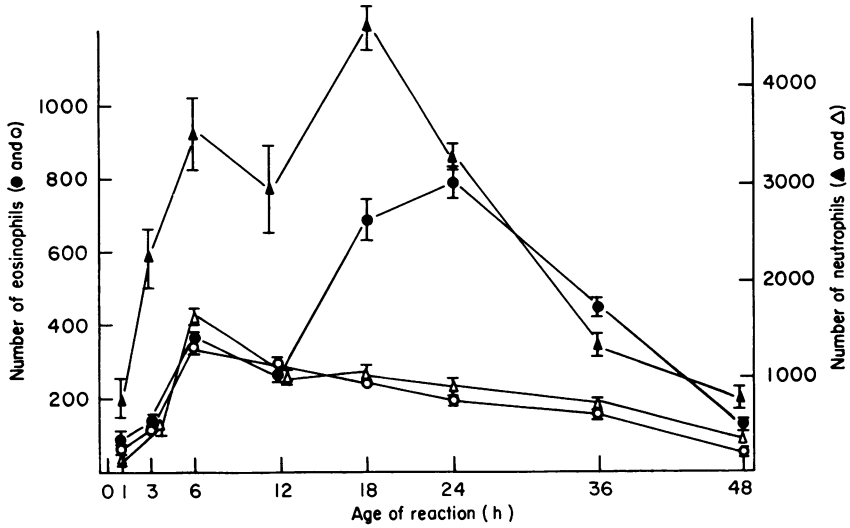

Figure 1. Time-course of tissue eosinophilia and neutrophilia following intradermal injection of DNP–As in guinea-pig. (●) Eosinophils in sensitized animal; (○) eosinophils in non-sensitized animal; (▲) neutrophils in sensitized animal; (△) neutrophils in non-sensitized animal.

induced inflammatory reactions in every instance. The bulla due to the injection itself disappeared in 30 min. In all instances, however, diffuse oedema and erythema appeared in lesions at 1 h, becoming maximal in about 18 h, and persisting to some extent for 48–60 h. In maximal reactions (graded + + to + + +), moderate haemorrhage and sometimes mild tissue sloughing occurred in the lesion centres. The animals showing severe haemorrhage or tissue sloughing were not used in the present experiment.

Tissue leucocytosis in the reaction was histologically examined. The time-course of tissue eosinophilia and neutrophilia in the inflamed sites was found to be biphasic. As shown in Fig. 1, the early phase of tissue eosinophilia was mild and reached its peak in about 6 h, while the delayed phase was clearly more intense and became maximal in 18–24 h. In the early phase (6 h), eosinophils were largely found in and around the small blood vessels immediately above the panniculus carnosus and the dermis. In the delayed phase (18 h), eosinophil infiltration became more intense, and the cells were found in all layers of the skin, including loose connective tissue, diffusely spread and perivascular. The cells decreased gradually in number after the peak (24 h) of tissue eosinophilia.

In contrast, intradermal injection of DNP–As (5 mg/ml) into non-sensitized animals of control group also induced the early and mild tissue eosinophilia reaching its maximum in about 6 h, and its intensity was almost comparable to that of tissue eosinophilia, in the early phase in sensitized animals, as seen in Fig. 1. No delayed phase of tissue eosinophilia was observed in these non-sensitized animals.

As shown in Fig. 1, tissue neutrophilia, induced by intradermal injection of DNP-As in sensitized animals of experimental group, also occurred in two phases, though the difference in the intensity between the responses was very shallow. The early phase of tissue neutrophilia was less obvious, becoming maximal in about 6 h, and the delayed phase was intense, reaching its peak in about 18 h. Injection of antigen into non-sensitized animals of control group also elicited tissue neutrophilia, reaching its peak in about 6 h, but its intensity was clearly weaker than that of tissue neutrophilia in the early phase in sensitized animals, as seen in Fig. 1. No delayed phase of tissue neutrophilia was found in these non-sensitized animals.

As is shown in Fig. 2, tissue mast cells were found to decrease in number rapidly after intradermal injection of antigen into sensitized animals of experimental group; the decrease of the cells seemed to reach its maximum in about 6 h, persisting for further 6 h. However, the cells began to increase gradually in number in about 18 h, as seen in Fig. 2. Injection of antigen into non-sensitized animals of control group also induced a similar tendency to a

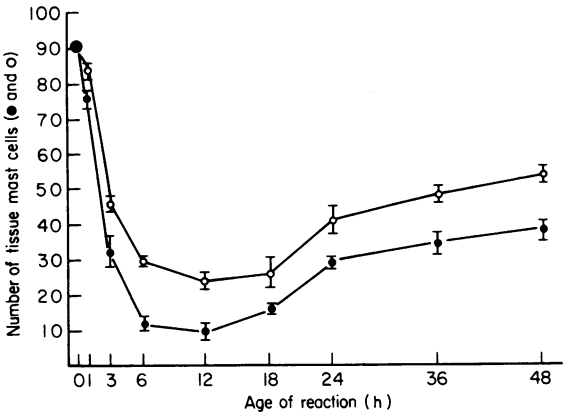

Figure 2. Time-course of tissue mast cell reaction following intradermal injection of DNP–As in guinea-pig. (●) Tissue mast cells in sensitized animal; (○) tissue mast cell in non-sensitized animal.

decrease of tissue mast cells in number; the decrease was slightly less marked when compared with that of tissue mast cells in sensitized animals, as shown in Fig. 2.

Isolation of chemotactic factors for eosinophils

Chemotactic activity of skin extract

First, in order to establish the satisfactory conditions for chemotactic assay, the relationship between the incubation duration of chambers and pore size of Millipore filters was tested on skin extract from 24-h-old lesions of experimental group animals. As illustrated in Fig. 3, 3-h incubation of the chambers in

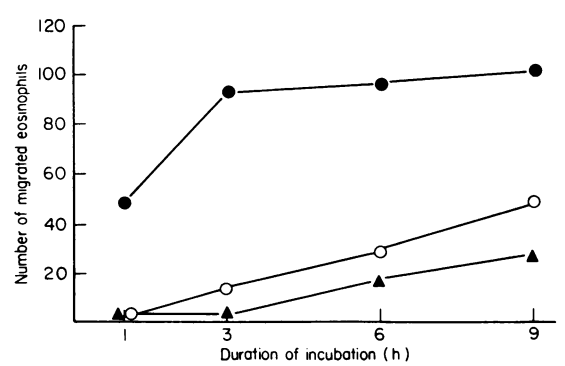

Figure 3. Effects of duration of incubation and pore size of Millipore filters on eosinophil chemotaxis. Extracts from 24-h-old lesion (of animals sensitized and challenged) at the same concentration (absorbancy 10 at 280 nm/ml) were tested at 37°. (●) Pore size 8 μm; (○) pore size 5 μm; (▲) pore size 3 μm.

the use of Millipore filter of pore size 8 μm was found to be most favourable for chemotactic assay for eosinophils; and migration of eosinophils through the filter became almost maximal in 3-h incubation at 37°, persisting for further 3 and 6 h. By contrast, in the use of 3-μm and 5-μm pore size filters, eosinophils seemed to have difficulty in migrating through the filters. Therefore, chemotactic assays for eosinophils were made for 3 h using 8-μm pore size filters in chambers incubated at 37°.

Table 1. Chemotactic activity of skin extracts for eosinophils

Samples tested	Concentration at 280 nm/ml	Mean number of migrated eosinophils¶
Sensitized skin extract* (6-h-old)	10·0	47
Sensitized skin extract* (24-h-old)	10·0	140
Non-sensitized skin extract† (6-h-old)	10·0	46
Non-sensitized skin extract† (24-h-old)	10·0	24
Skin extract, sensitized but not challenged	10·0	40
Normal skin extract	10·0	41
Medium solution‡		3
Buffer§		0

* From animals sensitized and challenged with DNP–As.
† From animals not sensitized but injected with DNP–As.
‡ Hanks's balanced salt solution containing 0·5 per cent ovalbumin (pH 7·2).
§ 0·067 M phosphate buffer (pH 7·4).
¶ Mean value of five assays.

As summarized in Table 1, extract from 24-h-old skin lesions of the animals of experimental group was most potent for eosinophil chemotaxis, reasonably corresponding to the intensity of tissue eosinophilia exhibiting a maximal infiltration of the cells in about 24 h. In contrast, extract from 6-h-old skin lesions of the animals of the same group was apparently less active for eosinophil chemotaxis, corresponding to less severe tissue eosinophilia in 6 h; and its chemotactic activity seemed to be almost comparable to that of skin extract from the control animals sensitized but not challenged with DNP–As, and the non-treated normal animals (Table 1). Similar chemotactic activity was also revealed in extract from 6-h-old skin lesions of the control animals not sensitized but injected with DNP–As (Table 1). However, extract from 24-h-old skin

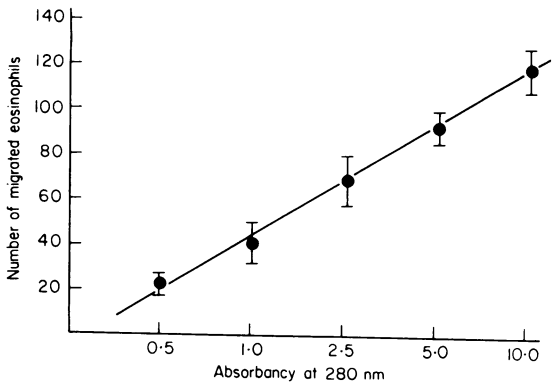

Figure 4. Dose–response curve of skin extract to eosinophil chemotaxis. Chemotactic assay was performed by the use of 8 μm-Millipore filter for 3 h at 37°. Skin extract from 24-h-old lesion (from animals sensitized and challenged) was used.

lesions of the animals of the same group was less active for eosinophil chemotaxis (Table 1).

The response to the chemotactic activity for eosinophils of the skin extracts from 24-h-old lesions of experimental animals was tested at various concentrations (absorbancies 0·5, 1·0, 2·5, 5·0 and 10·0 at 280 nm/ml). As seen in Fig. 4, an almost linear dose–response curve was obtained, indicating a practical use of this dose–response curve at such concentrations of skin extracts.

Separation of different chemotactic factors from skin extracts

Skin extracts from 6- and 24-h-old lesions of experimental group animals were concentrated by Diaflo membrane, UM-05, to give an absorbancy of 40 at 280 nm/ml. Seven millilitres of the extracts were eluted on Sephadex G-100. As illustrated in Fig. 5, there were obtained two chromatographic components after gel filtration of 6-h skin extract. The total yield, measured as the absorbancy at 280 nm, was about 97 per cent of the applied sample; the first comprised 47 per cent and the second 44 per cent. The chemotactic activity for eosinophils was predominantly concentrated in the second component, and that of the first component was weak.

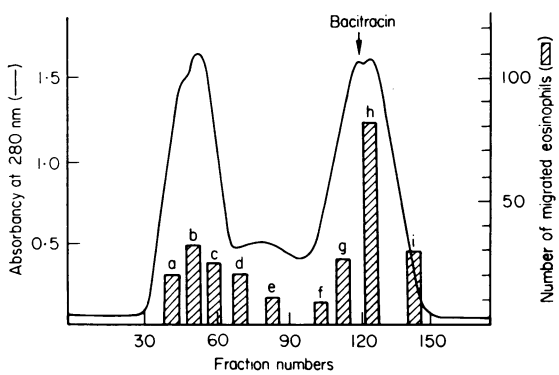

Figure 5. Demonstration of chemotactic activity in chromatographic components, extracted from 6-h-old skin lesion (of animals sensitized and challenged) and eluted on Sephadex G-100. The eluant was 0·067 M phosphate buffer (pH 7·4); flow rate was 24 ml/h; fractions collected every 3 g. Chemotactic activities were assayed at the following concentrations at 280 nm/ml: (a) 0·8; (b) 1·3; (c) 0·5; (d) 0·3; (e) 0·3; (f) 0·4; (g) 1·6; (h) 1·1; (i) 0·4. Bacitracin (1460 molecular weight) was eluted; the elution volume was about 360 ml.

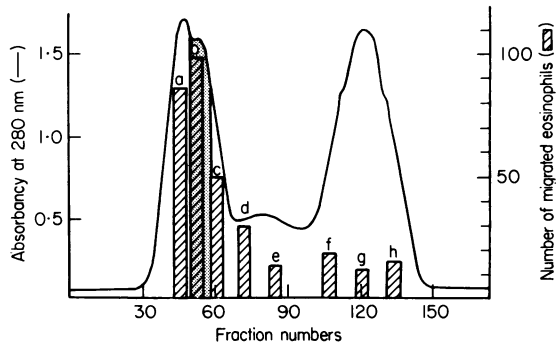

Figure 6. Demonstration of chemotactic activity in chromatographic components, extracted from 24-h-old skin lesion (of animals sensitized and challenged) and eluted on Sephadex G-100. The eluant was 0·067 M phosphate buffer, pH 7·4; flow rate was 24 ml/h; fractions collected every 3 g. Chemotactic assay was assayed at the following concentrations (absorbency at 280 nm/ml): (a) 1·0; (b) 1·0; (c) 0·7; (d) 0·3; (d) 0·3; (f) 0·5; (g) 1·9; (h) 0·5. The first component (under shadow) was used for chromatography on Sephadex G-200.

As can be seen in Fig. 6, two chromatographic components were also obtained after gel filtration of 24-h-skin extract. The total yield, measured as the absorbancy at 280 nm, was about 98 per cent of the applied sample; the first comprised 40 per cent and the second 50 per cent. The chemotactic activity for eosinophils was predominantly concentrated in the first component; and its effect was clearly more potent than that of the first component from 6-h-old skin lesions. Little chemotactic activity was revealed in the second component.

Effects of heat and dialysis on chemotactic factors

As summarized in Table 2, the chemotactic substance of the first component, extracted from 24-h-old skin lesions of experimental group animals and eluted from Sephadex G-100, seemed to be thermolabile,

and was non-diffusible when dialysed against 0·067 M phosphate buffer (pH 7·4) for 12 h in the cold. On the other hand, the active substance of the second component, extracted from 6-h-old skin lesions of experimental group animals and eluted on Sephadex G-100, seemed to be thermostable, and was diffusible when dialysed against 0·067 M phosphate buffer

Table 2. Effect of heat on chemotactic factors*

Samples tested	Remaining chemotactic activity (per cent)	Mean number of eosinophils migrated†
Chemotactic factor from 24-h-old lesion‡		
Non-heated	100	125
Heated at 60° for 30 min	49·6	62
Heated at 80° for 15 min	3·2	4
Chemotactic factor from 6-h-old lesion§		
Non-heated	100	55
Heated at 60° for 30 min	96·4	53
Heated at 80° for 15 min	72·7	40

* Tested at the same concentration (absorbancy 1·0 at 280 nm/ml) of each component.

† Mean value of three assays.

‡ The first component, extracted from 24-h-old lesion and eluted on Sephadex G-100.

§ The second component, extracted from 6-h-old lesion and eluted on Sephadex G-100.

(pH 7·4) for 20 h in the cold. These findings suggested that the early phase of tissue eosinophilia was associated with a thermostable chemotactic factor, while the delayed phase with a thermolabile chemotactic factor.

Estimation of molecular weight of chemotactic factors

The first component (under shadow in Fig. 6) from 24-h-old skin lesions of experimental group animals was eluted on a Sephadex G-200 column equilibrated with 0·067 M phosphate buffer (pH 7·4). As seen in Fig. 7, the elution profile of the component showed a symmetrical homogeneous pattern, and the chemotactic activity of the component paralleled the absorbency at 280 nm. Elution volumes of the standard substances on this column were as follows: 218·4 ml for cytochrome *c* monomer; 152·4 ml for bovine serum albumin monomer; 124·8 ml for bovine serum albumin dimer; 100 ml for blue dextran; 150 ml for the chemotactic factor. The elution volumes were plotted against the logarithmic scale of molecular weight and the linear relationship between the elution volume and logarithmic value of molecular weight was recorded and the molecular weight of this thermolabile chemotactic factor, which was associated with the mediation of the delayed phase

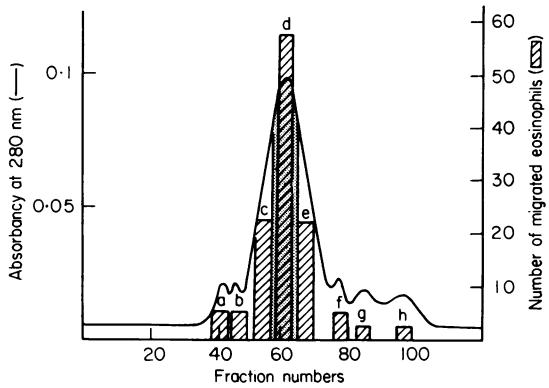

Figure 7. Chromatography of the first component (under shadow in Fig. 6), extracted from 24-h-old skin lesion (of animals sensitized and challenged) and eluted on Sephadex G-100, through Sephadex G-200 column. The eluant was 0·067 M phosphate buffer, pH 7·4; flow rate was 12 ml/h; fractions collected every 2·4 g. Chemotactic activities for eosinophils were assayed at the following concentrations at 280 nm/ml: (a) 0·1; (b) 0·1; (c) 0·3; (d) 0·5; (e) 0·3; (f) 0·1; (g) 0·1; (h) 0·1.

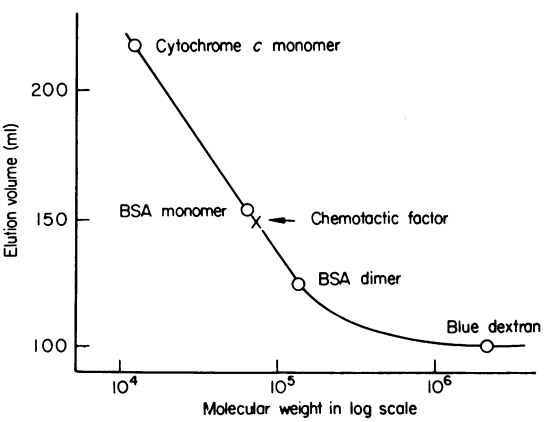

Figure 8. Estimation of molecular weight of a thermolabile chemotactic factor (under shadow in Fig. 7) on Sephadex G-200 column. The eluant was 0·067 M phosphate buffer, pH 7·4; flow rate was 12 ml/h; fractions were collected every 2·4 g. Elution volumes were plotted against logarithmic values of molecular weight. The molecular weight of a thermolabile chemotactic factor was determined 70,000± 7000 from its elution volume (150 ml).

of tissue eosinophilia, was estimated 70,000 ± 7000, as seen in Fig. 8.

On the other hand, the chemotactic peak of the second component from skin extract of 6-hour-old lesions of experimental group animals appeared after the molecular marker 'Bacitracin' (1460 molecular weight) (Sigma, St Louis, Missouri) in the gel-filtration on Sephadex G-100, as seen in Fig. 5; and its molecular weight was assumed to be less than 1400.

DISCUSSION

The observations described here demonstrated that tissue eosinophilia in an active cutaneous anaphylaxis, induced by DNP–ascaris extract in guinea-pigs, manifested two phases, i.e. the early and mild phase becoming maximal in about 6 h, and the delayed and intense phase reaching its peak in 18–24 h (Fig. 1). Similar biphasic tissue eosinophilia has been described in an active cutaneous anaphylaxis induced by egg albumin in guinea pigs (Muller and Healy, 1973).

The mediators, which may be concerned with the tissue eosinophilia, were respectively found in the skin extracts from inflamed lesions 6- and 24-h-old. The chemotactic activity for eosinophils of the 24-h extract was apparently more potent than that of the 6-h extract when assayed at the same concentration (Table 1), reasonably corresponding to the time-course of tissue eosinophilia observed. The chemotactic assay was satisfactorily performed by the use of 8-μm pore size Millipore filters and in the 3-h incubation of the chambers at 37° (Fig. 3), and a linear dose–response curve of extract plotted against chemotactic activity was achieved (Fig. 4).

After gel filtration on Sephadex G-100, two chromatographic components were obtained from the 6-h extract; the chemotactic activity of the first component was weak, while that of the second component was relatively strong (Fig. 5). The chemotactic factor of the second component was thermostable and diffusible (Table 2); its molecular weight was assumed to be less than 1400, because the chemotactic peak appeared after the molecular marker Bacitracin on Sephadex G-100 (Fig. 5). This active substance seemed to be released from tissue mast cells, because there was observed a decrease in tissue mast cells in number (Fig. 2), probably due to a disruption of the cells; and a similar chemotactic activity in extract from 6-h-old skin lesions of the animals not sensitized but injected with DNP-As (Table 1) and similar decrease in tissue mast cells in the lesions (Fig. 2) were observed. Since a similar chemotactic activity was also revealed in the skin extract of the animals immunized but not challenged with DNP–As or of the non-treated normal animals (Table 1), it was suggested that the chemotactic factor present in tissue mast cells was released by the antigenic stimulation or extraction procedure described above. This chemotactic factor released seemed to disappear gradually from the tissue or to be inactivated, because its activity became less marked in 24-h-old skin lesions of animals not sensitized but injected with DNP–As (Table 1). It was of interest to note that the activity of this chemotactic factor was very evident in the early tissue eosinophilia, but that in the delayed tissue eosinophilia was weak, suggesting that the chemotactic factor may be involved only in the mediation of the early tissue eosinophilia.

The chemotactic factor observed above seemed to be different from histamine because of its *in vitro* negative chemotactic effect on eosinophils (Parish, 1970). Thus far, this substance appears to resemble some chemotactic substances with a small molecular weight, previously described by Parish and Coombs (1968), and Kay *et al.* (1971). The ECF-A, which was so termed by Kay *etal.* (1971), was released *in vitro* from anaphylactic guinea-pig lung concomitantly with histamine and SRS-A; and its release was related to a disruption of tissue mast cells. It was independent of the complement system, different from an eosinophilotactic factor (ECF-C), which is complement-dependent, i.e. C5a (Kay, 1970b). A chemotactic factor similar to ECF-A was also described by Parish (1974).

After gel filtration on Sephadex G-100, two chromatographic components were obtained from the 24-h extract; the chemotactic activity of the first component was strong, while that of the second component was weak. As described above, such weak activity of the second component seemed to be due to a gradual disappearance or inactivation of the active substance in the 24-h-old lesions. The active substance of the first component was thermolabile and non-diffusible (Table 2). Its molecular weight was approximately 70,000 when measured by gel filtration on Sephadex G-200 (Fig. 8). This substance differs from leucoegresin for neutrophils in the molecular size (140,000) (Yoshinaga, Yoshida, Tashiro and Hayashi, 1971) and the negative

chemotactic effect on eosinophils (Ogata, 1971), and differs from ECF-C in the molecular size (15,000) (Kay, 1970b). Its formation seemed to be independent of tissue mast cell degranulation, because there was observed a gradual recovery of the cells in number during the delayed phase (Fig. 2). It was of value to note that the activity of this thermolabile chemotactic factor was strong during the delayed tissue eosinophilia, while that during the early tissue eosinophilia was weak, indicating that this active substance may play a significant part during the delayed tissue eosinophilia. At the present step of purification, this thermolabile chemotactic substance seemed to be difficult to compare with previously described chemotactic factors, which have been comprehensively reviewed by Parish (1974). The nature of those chemotactic substances also has not yet been clarified. Since chemotactic assays in the present experiment were performed against eosinophils only, the problem of whether the present chemotactic factors may be selective for eosinophils remained to be ascertained.

As previously described, leucoegresin for neutrophils shared common antigenic sites with serum IgG (Yamamoto *et al.*, 1971), and was produced from IgG by neutral SH-dependent protease isolated from inflamed sites *in vitro* (Yoshinaga, Yamamoto, Maeda and Hayashi, 1971; Yamamoto, Nishiura and Matsumura, 1974) and *in vivo* (Hayashi, 1967; Hayashi, Koono, Yoshinaga and Muto, 1969; Nishiura, Yamamoto and Hayashi, 1974). Its production and effect were independent of the complement system (Nishiura *et al.*, 1974). Furthermore, a chemotactic factor with a molecular weight of about 14,000 for lymphocytes was produced *in vitro* by the protease of neutrophils from serum immunoglobulins, especially IgM (Higuchi, Honda and Hayashi, 1975). The effect of neutral SH-dependent protease has been described on various types of tissue cells, for instance, tissue histiocytes, neutrophils, and mononuclear cells including lymphoid cells, as previously reviewed by Hayashi (1975). Recently, it was also suggested that a neutral SH-independent protease of neutrophils converted *in vitro* serum IgG to a chemotactic factor for peritoneal macrophages (Honda, Higuchi and Hayashi, unpublished results). The problem of whether the production of the present thermolabile chemotactic factor for eosinophils may be related to immunoglobulins would be interesting to investigate.

Muller and Healy (1973) have suggested that the delayed tissue neutrophilia specific for anaphylaxis in egg albumin-induced skin lesion in guinea-pigs may be mediated by leucoegresin, while the delayed tissue eosinophilia may be mediated by mast cell products. However, the present results did not show that the above thermolabile chemotactic factor itself was released from tissue mast cells. Since the peak of the delayed tissue neutrophilia somewhat preceded that of the delayed tissue eosinophilia in DNP–As induced skin lesion (Fig. 1), some relationship was assumed between the chemotaxis of these cells. An extracellular release of the above neutral proteases by antigen–antibody complexes from neutrophils has been demonstrated (Kouno, 1971; Hayashi, 1975).

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