AN EOSINOPHIL LEUKOCYTE CHEMOTACTIC FACTOR OF ANAPHYLAXIS*

BY A. B. KAY, Ph.D., DANIEL J. STECHSCHULTE, M.D., AND K. FRANK AUSTEN, M.D.

(From the Department of Medicine, Harvard Medical School, and the Robert B. Brigham Hospital, Boston, Massachusetts 02130)

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The mechanism by which eosinophil leukocytes migrate into the site of immediate-type hypersensitivity reactions has been difficult to study in vivo because of the large numbers of variables in any experimental design. Certain of these difficulties have been overcome by the use of a modification of the in vitro Millipore technique of Boyden by which the eosinophilotactic properties of substances can be directly measured. Using this technique, it was shown that guinea pig IgG1 or IgG2, as pre-formed antigen-antibody complexes, could both generate from whole serum a factor which was specifically chemotactic for eosinophils (ECF-C) and was apparently identical to C5a (1).

An earlier report (2), using cell counts in guinea pig skin, had shown that both IgG1 and IgG2, as preformed complexes, also prepared the tissue for a subsequent local eosinophilia 12 hr after injection. However, if antibody was first placed in the skin and after a variable latent period the animal was challenged with antigen and Evans blue dye intravenously (as in a usual passive cutaneous anaphylactic reaction), IgG1 but not IgG2 elicited a local eosinophil response 8-12 hr after the initial bluing reaction (2).

It was the purpose of the present study to determine whether tissue, actively sensitized or passively sensitized with IgG1, but in the absence of serum, can

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1 In this presentation, an eosinophil chemotactic factor (ECF) derived from whole guinea pig serum by treatment either with antigen-antibody complexes (1) or with zymosan, which appears to be identical with C5a and therefore a product of the interaction of complement proteins, is termed ECF-C. Material selectively attracting neutrophils, also of complement origin (1) but having a larger molecular size, is termed NCF-C. A factor with eosinophilotactic activity appearing in association with immediate-type hypersensitivity reactions and generated in the apparent absence of a complement requirement is designated ECF-A.

Other abbreviations used in this paper: DNP-BGG, dinitrophenol conjugates of bovine gamma globulin; DNP-BSA, dinitrophenol conjugates of bovine serum albumin; SRS-A, slow reacting substance of anaphylaxis; zym G/P, zymosan-treated guinea pig.
release an eosinophilotactic agent after the addition of specific antigen and whether such an agent is the same or different from that produced from the interaction of serum with preformed antigen–antibody complexes (1) or zymosan. For this purpose, an in vitro model involving the release of chemical mediators from guinea pig lung was used, which has been employed by numerous workers as a model of immediate-type hypersensitivity in the guinea pig (4–7). It has enabled the definition of an immunoglobulin mediating the release of pharmacological agents (6, 7) and allowed the mediators themselves to be quantitated (4, 7). This report, therefore, combines two in vitro systems, namely the Millipore technique for measuring chemotaxis and an in vitro model of anaphylaxis.

Materials and Methods

Materials were obtained as follows: histamine acid phosphate, zymosan, serotonin creatinine sulphate, and succinic acid (Mann Research Labs., Inc., New York); bradykinin triacetate (Sandoz Pharmaceuticals, Basel, Switzerland); prostaglandins PGE1, PGE2, and PGE2a (generously supplied by Dr. John Pike of the Upjohn Co., Kalamazoo, Mich.); maleic acid (Hopkin & Williams, Ltd., Chadwell Heath, Essex, England); blue dextran (Pharmacia Fine Chemicals Inc., New Market, N. J.); glucagon (Nutritional Biochemicals Corp., Cleveland, Ohio); vitamin B12 (Wyeth Laboratories, Philadelphia, Pa.); glycogen (J. T. Baker Chemical Co., Phillipsburg, N. J.); horse serum (Grand Island Biological Co., Grand Island, N. Y.); ovalbumin (Pentex Biochemical, Kankakee, Ill.); and guinea pig serum (Pel-Freez Biologicals, Inc., Rogers, Ark.).

Dinitrophenol conjugates of bovine gamma globulin (DNP-BGG) and bovine serum albumin (DNP-BSA) were prepared according to the method of Benacerraf and Levine (8) and contained 40 and 20 haptenic groups per molecule, respectively. Partially purified cobra venom was prepared as previously described (9). The titer of “late acting” complement components was estimated in microtiter plates using equal volumes of 1 × 10^8 sensitized sheep erythrocytes prepared with the first component of guinea pig complement and the fourth component of human complement (EAC1+4m), 50 effective molecules of guinea pig second component (C2), and doubling dilutions of guinea pig serum as a source of terminal components. The plates were incubated for 30 min each at 30°C and 37°C and centrifuged at 100 rpm for 5 min at 4°C. The 50% lytic endpoint was determined by inspection. The guinea pig serum was obtained by cardiac puncture before and after the intravenous injection of 1 ml of cobra venom factor or Tyrode’s solution.

Guinea pig IgG1 and IgG2 7S immunoglobulin fractions were prepared as follows. Antisera to ovalbumin were prepared in guinea pigs following the same injection schedule as previously described for raising guinea pig antiserum to ferritin (2). The antiserum was fractionated by diethylaminoethyl (DEAE)-cellulose chromatography (1). The functional purity of the IgG1-and IgG2-containing fractions was tested by passive cutaneous anaphylaxis and by passive hemolysis as described by Bloch et al. (10) and by immuneelectrophoresis and gel diffusion using rabbit anti-7S IgG and specific anti-guinea pig IgG1 (2). Passive hemagglutination was performed as previously described (10).

Preparation of Lung Tissue for the In Vitro Release of Mediators.—Whole lung: The heart and lungs from guinea pigs were excised and perfused free of visible blood with Tyrode’s solution as previously described (4). Animals were actively sensitized with DNP-BSA by the

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2 A. B. Kay, Unpublished observations.
method of Benacerraf et al. (11), or with ovalbumin in phenol (5), or by a single injection of 200 μg of ovalbumin in complete Freund’s adjuvant into each hind footpad. The lungs of actively sensitized animals were challenged with 4 mg of antigen (ovalbumin or DNP-BGG) via the pulmonary artery and the perfusate was collected for 30 min after antigen challenge. The perfusates were placed on ice and assayed the same day for histamine and slow reacting substance of anaphylaxis (SRS-A) according to the method of Brocklehurst (4), and for chemotaxis as described below. Some perfusates were tested after a single freezing at −70°C and rapid thawing.

Lung fragments: In experiments using lung fragments the perfused tissue was sliced with fine scissors, washed in Tyrode’s solution, and divided into 300 mg portions using an overhead weighing balance. With actively sensitized lung, the portions were suspended in 2.5 ml of Tyrode’s solution to which was added the antigen suspended in 0.5 ml of Tyrode’s solution. The samples were gently agitated in a water bath at 37°C. For passive sensitization, the lung fragments were suspended in 1.8 ml volumes of fractions containing antibody at 37°C. After the incubation period the fragments were washed twice and resuspended in 2.5 ml of Tyrode’s solution. Antigen was then added as for the actively sensitized lung. After incubation with antigen the diffusates were removed with a Pasteur pipette and assayed for histamine, SRS-A, and chemotaxis. In all studies using lung fragments the values for chemotaxis, histamine release, and SRS-A release represent the mean of duplicate samples. For all three measurements the values obtained with duplicate samples varied by less than 15%.

Some experiments with lung fragments were modified as follows. In experiments with ethylenediaminetetraacetate (EDTA), the samples were washed once either with Ca++- and Mg++-free Tyrode’s, containing 5 mM EDTA, or in the case of the control samples, with Ca++- and Mg++-free Tyrode’s containing EDTA in which Ca++ and Mg++ had been replaced before the addition of antigen. The reconstituted Tyrode’s solutions were adjusted by adding 6.8 mM of Ca++ and 1 mM of Mg++. The effect of succinate or maleate was studied using 5 mM concentrations in Tyrode’s solution adjusted to pH 7.4 with 0.1 N NaOH. Antigen was added to actively or passively sensitized fragments 1 min after incubation of the lung samples with maleate or succinate. The diffusate from the samples challenged with antigen alone was tested for chemotaxis in the presence of 5 mM of succinate or maleate.

Measurement and Fractionation of Eosinophil Chemotactic Activity of the Anaphylactic Reaction Mixture.—

Measurement of chemotaxis: A modification of the Millipore technique of Boyden was used as previously described (1). Guinea pig eosinophils were obtained by peritoneal lavage from animals which had received multiple injections of horse serum (1). Neutrophils were harvested from the peritoneal cavity of animals injected with glycogen 3–6 hr previously. For measuring eosinophil migration an 8.0 μm pore size was used and for neutrophils 1.2 μm, 3.0 μm, and 8.0 μm pore sizes were employed.

In the assay for chemotaxis, Tyrode’s solution containing 0.5% ovalbumin was used in both the cell and the test compartments. Zymosan-treated guinea pig (zym G/P) serum served as a reference pool of material having both eosinophil and neutrophil chemotactic activity. Zymosan which had been boiled and washed twice in distilled water was incubated for 30 min at 37°C with guinea pig serum using 20 mg of zymosan/ml of serum. The zymosan was removed by centrifugation and the serum subsequently heated at 56°C for 30 min. Samples were stored at −70°C until used.

In experiments using the perfusate from whole lung, a value for the total chemotactic activity of the perfusate was expressed as V × C. V represented the volume of the perfusate and C the percentage chemotaxis of 1 ml of perfusate as compared with 0.1 ml of a zymosan-treated reference serum when both were tested against the same suspension of eosinophils.

The chemotactic activity of the diffusate from lung fragments was usually determined using
0.6 ml volumes to which were added 0.1 ml of 5% ovalbumin and 0.3 ml of Tyrode's solution. When the release of SRS-A and histamine was low, 0.9 ml volumes of the diffusate was used. The chemotactic counts were expressed as the mean cell count of five high power fields (1).

Fractionation of chemotactic activity by gel filtration chromatography: A Sephadex G-25 column (40 × 1 cm) was equilibrated using Tyrode's solution as a buffer. In each experiment, 1 ml of lung supernate was applied and 1 ml fractions were collected. The column was standardized by preliminary fractionation of various substances of known molecular weight. The tubes containing the highest concentration of blue dextran and vitamin B₁₂ were read visually. The peak of glucagon was recorded at 280 nm using a DU-2 Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.).

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Relation between the total amount of lung histamine released and the total chemotactic activity, \( V \times C \) (see Methods), of the perfusate from sensitized lung following challenge with antigen. The animals were killed at various time intervals following an initial sensitizing injection. The data on individual animals sensitized with ovalbumin either in complete Freund's adjuvant or phenol are depicted. Correlation coefficient = 0.48; number of samples = 27; 0.02 > \( P > 0.01 \).

### RESULTS

**The Antigen-Induced Release In Vitro of an Eosinophil Chemotactic Factor from Actively Sensitized Guinea Pig Lung.**—When guinea pig lungs from actively sensitized animals were perfused free of visible blood via the pulmonary artery and subsequently challenged with specific antigen, the perfusate contained, in addition to histamine and SRS-A, a factor specifically chemotactic for guinea pig eosinophil leukocytes. This eosinophil chemotactic factor of anaphylaxis has been designated ECF-A. ECF-A was measured in the perfusate from animals sensitized with either ovalbumin or DNP-BGG and challenged with ovalbumin or DNP-RSA, respectively. A single injection of ovalbumin given in
complete Freund's adjuvant was slightly better than ovalbumin in phenol for preparing guinea pigs for the specific antigen-induced release of histamine, SRS-A, and ECF-A. Sensitization for the release of all these mediators was observed 10 days after the administration of ovalbumin in phenol or complete Freund's adjuvant but was not pronounced until 3 wk or later and was satisfactory up to 8 wk, at which time the study was terminated. The relationship between the total quantity of specific antigen-induced release of histamine and ECF-A from the lungs of each animal is shown in Fig. 1. A similar direct relationship was also demonstrated between the total amount of SRS-A released and the total ECF-A activity.

Studies were next carried out to determine whether ECF-A could be released from fragments of actively sensitized lung, and the results are shown in Table I. Addition of specific antigen to the lung fragments induced the release of histamine, SRS-A, and ECF-A, whereas no activities were detected in the absence of antigen. A linear dose-response curve was obtained when dilutions of the anaphylactic diffusate were tested for chemotaxis (Fig. 2). Replicate samples of sensitized lung fragments therefore provided a convenient test system for investigating both the antibody involved and the mechanism of release of ECF-A.

Selective Chemotactic Activity of the Anaphylactic Diffusate for Eosinophils.—Anaphylactic diffusates were examined for eosinophil and neutrophil chemotactic activity using a suspension of cells rich in neutrophils but containing 12% eosinophils. Guinea pig serum treated with zymosan has both neutrophil and eosinophil chemotactic activity (2) but attracted only neutrophils when millipores with a 1.2 μ or 3.0 μ pore size were used. With an 8.0 μ pore size both eosinophils and neutrophils migrate towards zym G/P serum, the ratio of migrating eosinophils to neutrophils being almost the same as in the cell compartment before migration commenced. Chemotactic activity of the lung diffusate was detectable only with an 8.0 μ pore size; 84% of the cells migrating were eosinophils although only 12% were present in the cell compartment (Table II). The experiment was repeated using diffusates from actively or passively sensitized lung challenged with antigen. On these occasions, using a

| TABLE I | Antigen-Induced Release of ECF-A, Histamine, and SRS-A from Fragments of Actively Sensitized Guinea Pig Lung |
| --- | --- | --- |
| | Eosinophil chemotaxis | Histamine release | SRS-A |
| Lung + antigen | 21.5 | 4 | 450 |
| Lung + diluent | 1.6 | 0 | 0 |
| Zym G/P serum | 18.6 | | |

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neutrophil-rich suspension and an 8.0 μ millipore size, 100% of the cells migrating towards the anaphylactic diffusate were eosinophils. Taken together these experiments demonstrated a selective chemotactic property for eosinophils of the anaphylactic lung diffusate.

Fig. 2. Chemotactic dose-response curve following dilutions of lung diffusate containing ECF-A.

**TABLE II**

Selective Chemotactic Property of an Anaphylactic Lung Diffusate for Eosinophil Leukocytes

| Millipore size       | Eosinophil and neutrophil chemotaxis (mean cell count)* |
|----------------------|--------------------------------------------------------|
|                      | 1.2 μ  | 3.0 μ  | 8.0 μ  |
| Anaphylactic lung diffusate | 1.2 + 1.4 | 2.2 + 0.6 | 14.2 + 4.6 |
| Zym G/P serum        | 0 + 12 | 0 + 13 | 6.4 + 26.4 |
| Diluent              | 0      | 0      | 0      |

* Mean cell counts are expressed as (E + N)/T where E is the eosinophil count, N the neutrophil count, and T the total mean cell count. The cell suspension contained 80% neutrophils, 12% eosinophils, and 8% mononuclear cells.

Identification of the Antibody Mediating the Release of ECF-A from Guinea Pig Lung.—Analysis of the sera obtained from the actively sensitized animals shown in Fig. 1 revealed a relationship between the passive cutaneous anaphylaxis (PCA) titer of the sera and the histamine release from the donor animals after antigen challenge. The sera of animals whose lungs released large amounts
of histamine and ECF-A had circulating antibody characteristic of IgG1. The antibody was heat stable and gave a high 4 hr PCA titer which at 48 hr showed a falling titer of one or two doubling dilutions. No evidence was found of an IgE-type antibody.

Direct evidence that IgG1 mediated the release of ECF-A was sought by experiments in which lung fragments were passively sensitized with fractions (Fr) of guinea pig 7S IgG. Biologic evidence for separation of IgG1 and IgG2 is shown in Fig. 3. Fractions 1–5 all agglutinated red cells which were passively sensitized with ovalbumin, but only those cells sensitized with the IgG2 containing fractions (Fr 1, 2, and 3) hemolyzed on the addition of guinea pig complement. PCA titers were given only with the IgG1 containing fractions (Fr 4, 5, and 6). Immunoelectrophoretic and gel diffusion studies with specific antisera to guinea pig 7S IgG1 demonstrated IgG1 only in fractions 4, 5, and 6. Only those fractions which contained IgG1 (Fr 4, 5, and 6) prepared lung fragments for the antigen-induced release of ECF-A, histamine, and SRS-A (Fig. 3). In
these experiments, the dose of antigen, the time of sensitization with antibody, and the time of incubation with antigen were arbitrarily chosen on the basis of a previous study (6).

The heat stability of the antibody mediating the release of ECF-A was also determined and provided further evidence that IgG1 and not an IgE-type antibody mediated its release. Fractions 4 and 5 were heated for 4 hr at 56°C and when compared with the unheated fractions showed no difference in their ability to mediate the release of histamine, SRS-A or ECF-A (Table III).

**The Mechanism of Release of ECF-A.**

*Optimal conditions for the release of ECF-A after passive sensitization of lung fragments:* The optimal conditions required for the release of ECF-A after passive sensitization of normal lung were determined as follows. Using a 2½ hr sensitization period and 200 μg of ovalbumin/300 mg of lung tissue, a 1:50 dilution of an IgG1-containing fraction (Fr 4) was found to release more ECF-A than a 1:5 or a 1:500 dilution. The time course of sensitization of tissue with antibody is shown in Fig. 4. The release of ECF-A, histamine, and SRS-A was detectable after 10 min of sensitization, reached a peak at 1 hr, and was maintained for at least 2½ hr. In further experiments either a 1 hr or a 2½ hr sensitization time was used. With regard to the time course of release, incubation with antigen beyond 15 min did not result in greater release of ECF-A. With a 1 hr sensitization period and a 1:50 dilution of fraction 4 (IgG1) 20 and 200 μg of ovalbumin yielded greater release of ECF-A, histamine, and SRS-A than 2 or 2,000 μg of ovalbumin/300 mg of lung (Fig. 5).

**The effect of decomplementation with cobra venom factor:** Animals were depleted of circulating complement by an intravenous injection of a purified factor from

| Sensitization | Eosinophil chemotaxis | Histamine release | SRS-A |
|---------------|-----------------------|------------------|-------|
| **Fraction 4** |                       |                  |       |
| Unheated      | 12.7                  | 1.5              | 50    |
| Heated        | 14.7                  | 1.5              | 80    |
| **Fraction 5** |                       |                  |       |
| Unheated      | 14.1                  | 1.3              | 80    |
| Heated        | 14.4                  | 1.3              | 70    |

* Fractions were heated for 4 hr at 56°C and diluted 1:50. 0.9 ml volumes of lung effluents were tested for chemotaxis to which was added 0.1 ml of 5% ovalbumin. Control samples sensitized with fraction 4 or 5 but not challenged with antigen gave a background chemotactic count of less than 1.5.
cobrā venom. Less than 5% of the circulating complement level was detectable 10 hr after the administration of 2 units of purified cobra venom factor. Accordingly, actively sensitized animals were sacrificed 10 hr after injection, their lungs removed, perfused, and sliced as described above and portions challenged

![Graph](image1)

**Fig. 4.** Time course of passive sensitization of lung fragments by an IgG1-containing fraction (fraction 4 diluted 1:50) for the antigen-induced release of ECF-A, histamine, and SRS-A.

![Graph](image2)

**Fig. 5.** Effect of antigen concentration on the release of ECF-A, SRS-A, and histamine from guinea pig lung passively sensitized with IgG1 (fraction 4 diluted 1:50).

with specific antigen. Table IV shows that there was no difference in the amounts of ECF-A released from lungs of cobra venom-treated animals and untreated animals. Although absolute histamine release in the actively sensitized animals appeared to be somewhat affected by venom treatment, the per
cent release was similar in the two groups, being in animals one to six 31.0, 37.5, 31.0, 43.2, 36.2, and 35.2 respectively.

An experiment was also performed using normal animals whose circulating complement levels were depleted with cobra venom factor and whose lungs in the same manner as described were then passively sensitized with a 1:50 dilution of fraction 4 (IgG1). Virtually identical results were obtained as with lungs from actively sensitized animals (Table IV).

### TABLE IV

Effect of Complement Depletion In Vivo on the In Vitro Release of ECF-A, Histamine, and SRS-A from Guinea Pig Lung Fragments*

| Animal No. | Treatment                  | Change in hemolytic titer of the terminal complement components | Eosinophil chemotaxis | Histamine release | SRS-A units/g |
|------------|----------------------------|---------------------------------------------------------------|-----------------------|-------------------|--------------|
|            |                            | mean cell count                                              | µg/g                  |                   |              |
|            |                            |                                                                  |                       |                   |              |
| Actively sensitized lung |                           |                                                                  |                       |                   |              |
| 1          | Cobra venom factor         | 5120 → 100                                                     | 26.1                  | 5.5               | 400          |
| 2          | “ “                        | 5120 → 80                                                     | 34.4                  | 7.0               | 520          |
| 3          | “ “                        | 5120 → 100                                                     | 30.3                  | 5.5               | 550          |
| 4          | Tyrode's solution          | 5120 → 5120                                                   | 35.1                  | 9.5               | 1000         |
| 5          | “ “                        | 5120 → 5120                                                   | 29.7                  | 7.1               | 620          |
| 6          | “ “                        | 5120 → 5120                                                   | 34.2                  | 9.5               | 1100         |
| Passively sensitized lung |                           |                                                                  |                       |                   |              |
| 7          | Cobra venom factor         | 10,240 → 640                                                   | 17.4                  | 4.0               | 500          |
| 8          | “ “                        | 10,240 → 640                                                   | 16.1                  | 3.6               | 400          |
| 9          | “ “                        | 10,240 → 640                                                   | 15.5                  | 3.5               | 400          |
| 10         | Tyrode's solution          | 10,240 → 10,240                                                | 16.7                  | 3.3               | 1000         |
| 11         | “ “                        | 10,240 → 10,240                                                | 15.0                  | 3.6               | 750          |

* With animals 1–6, 0.9 ml volumes of lung effluent were tested for chemotaxis and with animals 7–11, 0.6 ml volumes were used. Lung fragments suspended in Tyrode's alone gave background counts of less than 3.4 and 0 in the two experiments, respectively. Lungs were passively sensitized for 21/2 hr with a 1:50 dilution of fraction 4.

### Inhibition and enhancement:

A requirement of divalent cations for the release of ECF-A was determined by experiments in which actively sensitized lung was challenged with antigen in the presence of 5 mM of EDTA (Table V). In the absence of calcium and magnesium ions, there was no release of ECF-A, histamine, or SRS-A. Before testing for chemotaxis, calcium and magnesium ions were added to the diffusates containing EDTA. Tyrode's EDTA in which the divalent cations had been replaced served as a control in that this solution, together with antigen, was used to challenge sensitized lung for ECF-A release and the same solution was used in the chemotactic chamber. Replacement of
cations fully restored antigen-induced release of ECF-A, histamine, and SRS-A. The experiment was repeated with passively sensitized lung and gave the same result.

The capacity of succinate and maleate to enhance the release of histamine and SRS-A (12) was also sought in terms of ECF-A release. When actively sensitized lung was used, the enhancement of ECF-A release in the presence of 5

### TABLE V
*Effect of EDTA on the Antigen-Induced Release of ECF-A, Histamine, and SRS-A from Actively Sensitized Guinea Pig Lung Fragments*

| Suspending medium for lung fragments | Challenge | Eosinophil chemotaxis | Histamine release | SRS-A |
|------------------------------------|-----------|-----------------------|-------------------|-------|
|                                    |           | mean cell count | µg/L | units/g |           |
| 5 mM EDTA in Tyrode's              | Antigen   | 0.4                  | 0    | 0      |
| 5 mM EDTA in Tyrode's with Ca++, Mg++ replaced | Antigen | 20.4                  | 9    | 100    |
| Normal Tyrode's solution            | Antigen   | 24.6                  | 8    | 110    |
| Normal Tyrode's solution            | Tyrode's  | 3.6                   | 0    | 0      |

### TABLE VI
*Effect of Succinate and Maleate on the Antigen-Induced Release of ECF-A and Histamine from Actively Sensitized Guinea Pig Lung Fragments*

| Suspending medium for lung fragments | Eosinophil chemotaxis (mean cell count) | Volume of lung diffusate assayed | Histamine release |
|------------------------------------|----------------------------------------|----------------------------------|-------------------|
|                                    |                                        | 0.6 ml | 0.3 ml | 0.15 ml | µg/L   |
| Antigen with 5 mM succinate in Tyrode's | 30.8                                    | 16.2  | 3.0    |
| Antigen with 5 mM maleate in Tyrode's | 31.8                                    | 26.2  | 3.5    |
| Antigen in Tyrode's (succinate)*    | 26.2                                    | 17.0  | 1.6    |
| Antigen in Tyrode's (maleate)†      | 25.0                                    | 16.2  | 1.6    |
| Tyrode's solution                   | 1.2                                     | 0.0   | 0.0    |

* Succinate (5 mM) added to chemotactic chamber.
† Maleate (5 mM) added to chemotactic chamber.

mm of succinate or maleate approximated the augmentation of histamine release (Table VI). When the usual volume of 0.6 ml of lung diffusate was used, the enhancement was less apparent than when smaller volumes were employed. The experiment was repeated using lung fragments passively sensitized for 2½ hr with a 1:50 dilution of IgG1 (Fr 4); 5 mM succinate or maleate again strikingly enhanced the release of ECF-A and histamine. SRS-A was not measured in these enhancement experiments.

*Differentiation of ECF-A from the Other Pharmacological Mediators of Ana-
### TABLE VII

**Experiments to Show that Histamine, Bradykinin, Serotonin, Prostaglandins E₁, E₂, and F₂α are not Chemotactic for Eosinophils Per Se and are not Responsible for the Release of these Agents**

| Experiment | Eosinophil chemotaxis | mean cell count |
|------------|-----------------------|----------------|
| **Experiment 1** |                       |                |
| Lung + antigen | 27.1                  |                |
| Lung + Tyrode's | 2.6                   |                |
| Lung + histamine (0.5 µg/ml) | 3.0           |                |
| Lung + histamine (5 µg/ml) | 4.4                  |                |
| Histamine (0.5 µg/ml) | 0.0                   |                |
| Histamine (5 µg/ml) | 0.0                   |                |
| **Experiment 2** |                       |                |
| Lung + antigen | 20.4                  |                |
| Lung + Tyrode's | 0.0                   |                |
| Lung + bradykinin (0.01 µg/ml) | 0.0            |                |
| Lung + bradykinin (0.1 µg/ml) | 0.0           |                |
| Lung + bradykinin (1.0 µg/ml) | 0.0            |                |
| Bradykinin (1.0 µg/ml) | 0.0                  |                |
| Lung + serotonin (0.01 µg/ml) | 0.0            |                |
| Lung + serotonin (0.1 µg/ml) | 0.0           |                |
| Lung + serotonin (1.0 µg/ml) | 0.0            |                |
| Serotonin (1.0 µg/ml) | 0.0                  |                |
| **Experiment 3** |                       |                |
| Lung + antigen | 17.0                  |                |
| Lung + Tyrode's | 3.2                   |                |
| Lung + PGE₁ (0.01 µg/ml) | 1.0                  |                |
| Lung + PGE₁ (0.1 µg/ml) | 2.0                  |                |
| Lung + PGE₁ (1.0 µg/ml) | 0.6                  |                |
| PGE₁ (1.0 µg/ml) | 0.0                   |                |
| Lung + PGE₂ (0.01 µg/ml) | 1.0                  |                |
| Lung + PGE₂ (0.1 µg/ml) | 1.0                  |                |
| Lung + PGE₂ (1.0 µg/ml) | 1.4                  |                |
| PGE₂ (1.0 µg/ml) | 0.0                   |                |
| Lung + PGF₂a (0.01 µg/ml) | 1.0                  |                |
| Lung + PGF₂a (0.1 µg/ml) | 1.0                  |                |
| Lung + PGF₂a (1.0 µg/ml) | 3.4                  |                |
| PGF₂a (1.0 µg/ml) | 0.0                   |                |
| **Experiment 4** |                       |                |
| Lung + antigen | 16.6                  |                |
| Lung + Tyrode's | 0.0                   |                |
| Lung + SRS-A* (15 units/ml) | 2.0                  |                |
| Lung + SRS-A (30 units/ml) | 0.6                  |                |
| SRS-A (50 units/ml) | 4.0                   |                |

* The SRS-A was prepared by boiling an SRS-A ethanol extracted preparation in 0.05 N NaOH for 10 min. Before boiling in alkali the preparation had 50 units of SRS-A/ml and an eosinophil chemotactic count of 61.0.

The histamine release in micrograms per milliliter of lung diffusate was 0.5, 0.25, 0.35, and 0.13 in experiments 1, 2, 3, and 4, respectively.
phylaxis.—In order to determine whether ECF-A was in fact an already recognized pharmacological agent, various chemical mediators of anaphylaxis were tested directly for their ability to evoke the migration of eosinophils (Table VII). It was found that histamine, bradykinin, serotonin, and prostaglandins PGE₁, PGE₂, and PGF₃α were not chemotactic for eosinophils per se; and, furthermore, when incubated with sensitized lung in the absence of antigen, these agents did not secondarily affect the release of ECF-A. The 0.5 μg dose of histamine used in experiment 1 was comparable to the amount of histamine released from the same lung by specific antigen.

Fig. 6. Effect on ECF-A and SRS-A activity of boiling in acid or alkaline solutions.

Some crude preparations of SRS-A were chemotactic for eosinophils. These were obtained by extraction of lung diffusates in 80% ethanol, removal of the precipitate by centrifugation, and evaporation to dryness of the supernate under vacuum using a rotary evaporator. The extracted material was reconstituted in distilled water. It was possible, however, to differentiate ECF-A and SRS-A activity by boiling the crude material in alkaline solution. Since it had been previously reported that SRS-A survives boiling in 0.05 N NaOH but not in 0.05 N HCl (13), the behavior of ECF-A under these conditions was investigated. As seen in Fig. 6, ECF-A activity rapidly disappeared after boiling in acid or neutral solution and even more rapidly when boiled in alkali. In these experiments, the preparations were immediately adjusted to neutral pH before testing for SRS-A and chemotaxis. Thus by boiling in alkali, it was possible to destroy all ECF-A without influencing SRS-A activity (Table VII). It was also
possible to separate ECF-A from SRS-A by Sephadex G-25 chromatography (Fig. 7, *vide infra*). The peak of SRS-A activity was usually ill defined but appeared after ECF-A.

*Differentiation of ECF-A from ECF-C.*—ECF-A partially survived ethanol extraction suggesting that its biological activity is not associated with a large molecule; for this reason, a lung diffusate having high chemotactic activity was applied to a column of Sephadex G-25. In each of six experiments, five using material from actively sensitized lung and one from a passively sensitized lung, the peak of eosinophil chemotactic activity eluted after the fractions containing the molecular marker glucagon (mol. wt. 3485) and vitamin B₁₂ (mol. wt. 1357) but before histamine acid phosphate (mol. wt. 310) (Fig. 7). Thus, the molecular weight of ECF-A is thought to be approximately 500–1000.

![Graph](image-url)
In order to calculate the per cent recovery of ECF-A following passage through a Sephadex column, advantage was taken of the linear dose response for chemotaxis when dilutions are made of material containing ECF-A activity (Fig. 2). In this way, it was possible to obtain an arbitrary chemotactic value for an ECF-A preparation in relation to one preparation of target cells. When the same cells were tested against fractions of the ECF-A preparation, it was possible to demonstrate a 100% recovery of ECF-A activity after passage through a column of Sephadex G-25.

It has been shown that ECF-C, produced either by treating whole serum with antigen-antibody complexes (1) or with zymosan,2 has a molecular weight of approximately 15,000. As shown in Fig. 7, ECF-C activity from zymosan-treated serum was excluded by Sephadex G-25.

DISCUSSION

The capacity of actively (Table I) or passively (Fig. 3) sensitized guinea pig lung to react with antigen to release a factor chemotactic for eosinophil leukocytes (ECF-A) has been demonstrated. In all experiments, the release of ECF-A was also accompanied by the release of both histamine and SRS-A. Indeed, the release of all these mediators exhibited a similar response in terms of the time course of passive sensitization (Fig. 4), the effect of antigen dose (Fig. 5), and the time course of release. Furthermore, the release of ECF-A was dependent on divalent cations (Table V) and was strikingly enhanced by the presence of succinate or maleate (Table VI). These features are again characteristic of the release of histamine and SRS-A (12, 14).

It has previously been demonstrated that guinea pig IgG1 can passively sensitize guinea pig lung slices for the antigen-induced release of histamine (6) and SRS-A (7), and this same immunoglobulin has been shown to mediate the release of ECF-A (Fig. 3). The fractions which prepared tissue for ECF-A release (Fr 4 and 5) lost no activity after heating at 56°C for 4 hr (Table III), a property characteristic of IgG1 but not of an IgE-like immunoglobulin. The IgG2-containing fractions, having hemagglutinating activity virtually equal to that of IgG1 fractions, failed to prepare lung fragments for the antigen-induced release of histamine, SRS-A, or ECF-A (Fig. 3).

Since guinea pig IgG1 appears capable of mediating the release of several pharmacologic agents, it was necessary to exclude the possibility that ECF-A was an already recognized chemical mediator or was released secondary to the presence of other reaction products recognized herein. Histamine, SRS-A, and the prostaglandins PGE1, PGE2, and PGF2α were not eosinophilotactic per se; neither was ECF-A detected after the incubation of these agents with sensitized lung in the absence of antigen (Table VII). Studies with sensitized guinea pig lung (15, 16) have shown the release of kallikrein after antigen challenge; this enzyme was not studied herein, but it was demonstrated that the product of its
reaction with kininogen, namely bradykinin, was not itself chemotactic for eosinophils and did not secondarily affect the release of ECF-A (Table VII). Similar findings were observed with serotonin (Table VII).

Since eosinophilotactic activity could still be detected after extraction of anaphylactic lung diffusate in 80% ethanol and evaporation to dryness, the possibility was considered that SRS-A contained chemotactic activity. Reasons for considering ECF-A as separate from SRS-A are that SRS-A survived boiling in alkaline solution for 20 min whereas ECF-A activity was abolished by this procedure (Fig. 6); and in addition SRS-A and ECF-A could be separated by gel filtration (Fig. 7).

The finding that ECF-A generation is a consequence of antigen–antibody interaction and dependent upon the presence of divalent cations raises the question of whether it is identical with the eosinophilotactic factor formed in guinea pig serum following treatment with immune complexes (1) or zymosan.2 ECF-C produced in vitro by antigen–antibody complexes, prepared either with IgG1 or IgG2 and antigen, requires the participation of complement since ECF-C is not generated from serum heated at 56°C for 30 min or from ammonia-treated serum or from serum activated in the presence of 0.01 m EDTA (1). No evidence was found, however, that ECF-A generation was dependent on an intact complement system. Guinea pig lung, perfused free of visible blood and then cut in slices and thoroughly washed was capable of being passively sensitized with an IgG1-containing fraction (even after heating for 4 hr at 56°C) for the subsequent antigen-induced release of ECF-A (Table III, Fig. 3). Furthermore, depleting the donor animals of 95% of the circulating complement level had no effect on the capacity of their isolated lung fragments to yield ECF-A after passive sensitization and antigen challenge (Table IV). Decomplementation was accomplished by the administration of purified cobra venom factor, a procedure which depletes C3 and to a lesser extent the complement components acting thereafter (17, 18). This maneuver also had no effect on the antigen-induced release of ECF-A from lung fragments from actively sensitized animals (Table IV).

ECF-A and ECF-C can be distinguished not only in terms of the complement requirement for their generation but also by their apparent size differences as determined by gel filtration. On Sephadex G-25, ECF-C is excluded as shown by the molecular marker blue dextran (Fig. 7), consistent with its previously reported molecular weight of approximately 15,000 (1). In contrast, ECF-A appeared after the molecular marker vitamin B12 (mol. wt. 1357) but before histamine acid phosphate (mol. wt. 310), suggesting a molecular weight of approximately 500–1000 (Fig. 7). It should further be noted that the complete recovery of ECF-A in the diffusate introduced onto the Sephadex column indicated that the eosinophilotactic activity of the lung diffusate is unlikely to be a mixture of molecules having appreciable size differences.
In a cell suspension containing predominantly neutrophils, only eosinophils migrated towards ECF-A whereas zymosan-treated serum, which contains both ECF-C and NCF-C (1), attracted both neutrophils and eosinophils in the same proportion as was present in the original cell suspension (Table II). Thus, ECF-A represents a hitherto undescribed agent which, like ECF-C, selectively attracts eosinophil leukocytes.

SUMMARY

The capacity of actively or passively sensitized guinea pig lung to react with antigen to release a factor specifically chemotactic for eosinophil leukocytes (ECF-A) has been demonstrated. The release of ECF-A was also accompanied by the elaboration of both histamine and SRS-A and the appearance of all these mediators exhibited a similar response in terms of the time course of passive sensitization, the effect of antigen dose, the time course of release, divalent cation dependence and enhancement by the presence of succinate or maleate. Decomplementation by the administration of purified cobra venom factor had no effect on the antigen-induced release of ECF-A from actively or passively sensitized lung fragments.

When fragments of guinea pig lung were passively sensitized with fractions of guinea pig 7S IgG, only the IgG1-containing fractions prepared tissue for the antigen-induced release of ECF-A. Histamine, SRS-A, bradykinin, serotonin, and the prostaglandins PGE1, PGE2, and PGF2\textalpha were not eosinophilotactic per se; neither was ECF-A detected following the incubation of these agents with sensitized lung in the absence of antigen.

Both eosinophilotactic activity and SRS-A survived extraction in 80% ethanol and evaporation to dryness. SRS-A, however, withstood boiling in alkaline solution for 20 min, whereas ECF-A activity was abolished by this procedure. SRS-A and ECF-A could also be separated by gel filtration. ECF-A activity was completely recovered following its passage through a column of Sephadex G-25 and had an estimated molecular weight of between 500 and 1000. On the basis of size and a formation mechanism independent of the complement system, ECF-A is distinguishable from a previously described complement-dependent eosinophilotactic factor (ECF-C). Thus, ECF-A represents a hitherto undescribed agent which selectively attracts eosinophil leukocytes.

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