

IN VITRO AND IN VIVO ACTIVITY OF A LYMPHO-  
CYTE AND IMMUNE COMPLEX-DEPENDENT  
CHEMOTACTIC FACTOR FOR EOSINOPHILS\*

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Eosinophils are conspicuous components of the inflammatory response in a number of experimental and clinical states. They are present in peritoneal exudates following multiple intraperitoneal injections of foreign protein (1), and in the lymph nodes draining sites of antigen administration (1-3). They may be found in small numbers in guinea pig skin after the injection of antibody-antigen complexes (4), and in larger numbers when antigen alone is injected into a site that has previously developed a delayed hypersensitivity reaction, the so-called "retest reaction" (5). It is well known that eosinophils may be found in the nasopharynx and bronchi of patients with allergic conditions such as hay fever or asthma, and in the intestinal tract as a consequence of parasitic infestations. In these disease states, the only clear correlate with the presence of eosinophils is the allergic status of the patient.

The existence of specific chemotactic factors for eosinophils has not been definitely established. In the horse, it is claimed that histamine will cause the accumulation of eosinophils (6), but this has not been confirmed in other species. In the various studies cited above, the role of antigen-antibody complexes appears crucial, but since these studies all involved intact animals, it is impossible to determine whether the complexes are themselves chemotactic or whether they act in an indirect manner. This latter possibility is quite likely, in that it has been shown in vitro that immune complexes can activate the complement system to produce a factor chemotactic for eosinophils (7). This complement-derived factor is the trimolecular complex C $\bar{5}$ 67 which had previously been shown to be highly chemotactic for neutrophils (8, 9). Therefore, C $\bar{5}$ 67 is not selectively chemotactic for either type of granulocyte. Several other compounds with chemotactic activity for eosinophils, including enzymatic cleavage products of complement components and filtrates from bacterial cultures, all show a greater chemotactic activity with respect to neutrophils (7).

In an elegant series of in vivo experiments, Basten, Boyer, and Beeson (10, 11) have recently found an association between the ability of rats to respond to *Trichinella* infection with a peripheral eosinophilia and the presence of recirculating lymphocytes. They found that this ability could be transferred adoptively by intact cells in diffusion chambers, suggesting the role of a diffusible material. Although the nature of this fac-

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tor was not determined, it was felt to be consistent with a specific kind of immunoglobulin such as homocytotropic antibody.

It is known that when lymphocytes from experimental animals with delayed hypersensitivity are cultured in the presence of specific antigen, a large number of soluble factors is released into the medium, including migratory-inhibition factor (MIF)<sup>1</sup> (12, 13), and factors chemotactic for macrophages and neutrophils (14, 15). Moreover, Arnason and Waksman (5) have shown a relationship between delayed hypersensitivity and eosinophil accumulation in the retest reaction. These observations, taken in conjunction with those of Basten et al. (10, 11) and the studies relating immune complexes to eosinophil responses, all suggested the possibility that a substance chemotactic for eosinophils might be generated by the three-way interaction of a factor derived from activated lymphocytes, antibody, and antigen. The purpose of the present paper is to describe such a factor and to define its *in vitro* and *in vivo* chemotactic activity for eosinophils.

#### *Materials and Methods*

*Eosinophils.*—Peritoneal exudates rich in eosinophils were prepared according to the method of Litt (16). Hartley strain guinea pigs (400–500 g) received a total of 12 intraperitoneal injections of keyhole limpet hemocyanin (KLH) (Calbiochem, Los Angeles, Calif.) in doses of 10 mg per injection. With the exception of the first, each injection was preceded by the intramuscular administration of 5 mg of diphenhydramine hydrochloride (Benadryl) (Parke, Davis & Co., Detroit, Mich.) with an interval of approximately 45 min between injections. Exudates were collected in saline 36 hr after the last injection of KLH with heparinized syringes, washed once, and suspended in Medium 199 (Microbiological Associates, Inc., Bethesda, Md.). The suspensions so obtained were mixtures of neutrophils, eosinophils, and mononuclear cells. The ratio of eosinophils to neutrophils ranged from 20 to 35% in these experiments.

*Lymphocyte Factor.*—Lymphocyte culture fluids were kindly provided by Dr. John David, Harvard Medical School, Boston, Mass. These fluids were obtained by culturing lymphocytes from guinea pigs sensitized to the antigen *o*-chlorobenzoyl-bovine gamma globulin (OCB-BGG). Animals which served as cell donors all developed typical strong delayed-hypersensitivity reactions when skin tested with the antigen. The various lymph node cell suspensions used were divided into two parts, one being cultured in the presence of antigen (stimulated culture), and the other in its absence (unstimulated culture). In the latter culture, antigen was routinely added at the end of the incubation period. The production of MIF in stimulated cultures is a measure of the extent of *in vitro* activation of lymphocytes by antigen, and only those stimulated cultures rich in MIF activity and their unstimulated controls were chosen for the present study. Details of the methodology involved have been previously described (13). A total of four different preparations of culture fluids, with the accompanying controls, were used in these experiments.

*Immune Complexes.*—Guinea pig and rabbit antisera were used. Each antiserum was obtained not less than 3 wk after the intradermal injection of the antigen in complete Freund's

<sup>1</sup> *Abbreviations used in this paper:* BGG, bovine gamma globulin; DNP-GPA, dinitrophenyl-guinea pig albumin; EA, egg albumin; KLH, keyhole limpet hemocyanin; MIF, migratory-inhibition factor; OCB-BGG, *o*-chlorobenzoyl-bovine gamma globulin.

adjuvant. The antigens used included bovine gamma globulin (BGG), egg albumin (EA), dinitrophenyl-guinea pig albumin (DNP-GPA), and KLH. Immune complexes were prepared by reacting antibody with antigen at equivalence ratios in the presence of  $5 \times 10^{-4}$  M ethylenediaminetetraacetate (EDTA). They were washed in 0.01 M phosphate-buffered saline at pH 7.4. As an example of the nomenclature to be used in this paper, immune complexes of bovine gamma globulin and rabbit anti-bovine gamma globulin formed at equivalence will be referred to as RabaBGG-BGG. When a weight is given for the immune complex, it will refer to micrograms of antibody protein contained in that complex.

*Generation of Chemotactic Activity.*—Washed, complement-free immune complexes were incubated with 150  $\mu$ l of the various culture fluids in Medium 199 for 1/2 hr at 37°C. The amount of complexes ranged from 180 to 300  $\mu$ g. In most of the experiments to be described, the mixture of culture fluid and immune complexes was tested for chemotactic activity. However, in some cases the complexes were removed by centrifugation (2200 rpm, 20 min) after the period of incubation, and the supernates alone were tested. Removal or retention of the complexes after the period of incubation did not influence the degree of chemotactic activity generated.

*In Vitro Assay System.*—The method used was the micropore filter assay which has been described in detail (8). In these assays of chemotactic activity, two compartments of a chamber were separated by a micropore filter of 5  $\mu$  pore size. The cell suspension, at a concentration of  $2 \times 10^6$  granulocytes/ml, was made up in Medium 199 with 10% heat-inactivated guinea pig serum. The cell suspension was placed in the upper compartment (1 ml total volume), while the lower compartment received the test material (1 ml total volume). Chemotactic activity was assessed by counting, in five randomly selected high-power fields, the number of cells that had migrated completely through the filter and were on the opposite (lower) side of the filter, after 4 hr of incubation at 37°C. To differentiate between granulocytes, filters were fixed in Hollande's fluid and processed according to Litt's modification of the Dominici stain (17). In each experiment the stimulated and unstimulated culture fluids were tested with and without treatment with immune complexes. All the counts reported are net values and represent the difference between counts obtained from chambers containing stimulated cultures and those from chambers containing unstimulated cultures. This corrects for the various background activities inherent in the assay. Each experiment included chambers with immune complexes alone in the presence of Medium 199, Medium 199 alone, and in some cases antibody or antigen alone. In addition, a chamber in each experiment contained a bacterial filtrate (from *Escherichia coli*) with known chemotactic activity for granulocytes (7). This was a positive control, in order to assess the reactivity of the indicator cells.

*In Vivo Observations.*—The fluids from three active lymphocyte preparations were pooled for this experiment. The lymphocyte culture fluids were incubated with the various immune complexes for 90 min at 37°C and the complexes were then removed by centrifugation at 2200 rpm for 20 min. As controls, the complexes alone were incubated in Medium 199 and then removed in a similar manner. The incubation mixtures contained 210  $\mu$ l of culture fluids and 375  $\mu$ g of complexes. The various supernates were injected intradermally into guinea pig skin according to the following protocol. (a) unstimulated fluid, (b) unstimulated fluid + GPaBGG-BGG, (c) unstimulated fluid + GPaEA-EA, (d) stimulated fluid, (e) stimulated fluid + GPaBGG-BGG, (f) stimulated fluid + GPaEA-EA, (g) GPaBGG-BGG, and (h) GPaEA-EA. Reaction sites were excised at 18 hr, fixed in Hollande's fluid, and stained according to the Litt procedure (17). Paraffin-embedded 5  $\mu$  sections were used. As will be described, the patterns of inflammation within the various subgroups were sufficiently uniform so that total eosinophil counts over a fixed area gave a good quantitative description of the events at the various sites. A total of six sections from each reaction site were studied. A 1 cm segment from the central portion of each section was examined high-power field by high-power field, and the eosinophils were counted without knowledge of the identifying code.

## RESULTS

*Background Activities.*—As already indicated, all of the in vitro assay results are reported as net values, i.e. counts obtained from a preparation which includes stimulated lymphocyte culture fluid minus counts from a similar preparation, but one with unstimulated culture fluid. Although this procedure corrects for “background” activity, it is instructive to enumerate the actual contribution of the various substances to background. The data are shown in Table I. Each value is an average of from 8 to 12 determinations. It is seen that Medium 199 alone results in extremely low counts, with an average of two eosinophils (per five high-power fields). The immune complexes alone, in the absence of lymphocyte culture fluid, similarly give low values. The same holds true for antibody alone and antigen alone, which are not shown in the table.

TABLE I  
*Chemotactic Activities Contributing to Background in the In Vitro Assay System*

Substance	Eosinophil count $\pm$ SE
Unstimulated fluid* (150 $\mu$ l)	12 $\pm$ 5
Immune complexes† (180–300 $\mu$ g)	3 $\pm$ 1
Unstimulated fluid + immune complexes	9 $\pm$ 1
Medium 199	2 $\pm$ 2
Bacterial factor‡	12 $\pm$ 2

\* Obtained from lymphocytes incubated in the absence of antigen. See text for details.

† GPaBGG-BGG complexes prepared at equivalence.

‡ Bacterial factor is included for comparison.

The unstimulated culture fluid, in the absence of immune complexes, gives a count of 12, which is higher than the blank (Medium 199), but this is not increased by the addition of immune complexes. Shown also in the table is the eosinophil response to bacterial factor, which is of the same magnitude as that of the unstimulated culture fluid. This is approximately 20-fold lower than results obtained with circulating human blood eosinophils (7) and suggests that the protein-induced peritoneal eosinophils of the guinea pig behave sluggishly in the chemotactic assay system. This observation underscores the high potency of the eosinophil chemotactic factor to be described.

*Interaction of Lymphocyte Culture Fluids and Immune Complexes.*—A typical experiment is shown in Table II. The stimulated culture fluids, as previously indicated, were prepared from lymphocytes which had been sensitized to OCB-BGG. As most of the systems involving lymphocyte activation and/or proliferation appear to demonstrate carrier specificity similar to that of delayed hypersensitivity, antibody against BGG was utilized to form “specific” immune complexes. In this experiment, only the combination of stimulated fluid and immune complexes led to eosinophil chemotactic activity significantly greater

TABLE II  
*Result of a Typical Experiment Demonstrating the Generation of Eosinophil  
 Chemotactic Activity*

Material tested*	Eosinophil count‡	Eosinophil response§
Unstimulated fluid	1	
Stimulated fluid	8	7
Unstimulated fluid + immune complexes	10	
Stimulated fluid + immune complexes	105	95
Medium 199	0	
Immune complexes	5	
Bacterial factor	15	

\* Dosage and preparation are described in the text. Unstimulated fluid is obtained from lymphocyte cultures incubated without antigen. Stimulated fluid is obtained from cultures incubated with antigen. The immune complexes are GPaBGG-BGG.

‡ Number of eosinophils in five high-power fields on the undersurface of the micropore membrane.

§ Difference in counts between the preparation containing stimulated fluid and the unstimulated controls.

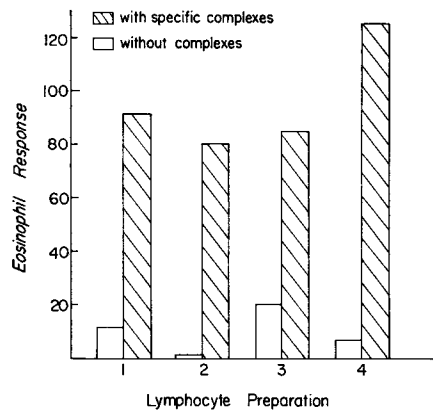


FIG. 1. Eosinophil chemotactic response to culture fluids from four antigen-stimulated lymphocyte preparations, with and without incubation of the fluid with specific immune complexes (GPaBGG-BGG). The eosinophil response is measured as the difference in counts between a chamber containing fluid from stimulated cultures and a corresponding chamber containing fluid from unstimulated cultures.

than the various controls. This activity is approximately six times that of bacterial factor, a substance previously shown to be chemotactic for eosinophils (7).

Fig. 1 shows the eosinophil response, as measured by net eosinophil counts, of four different lymphocyte culture preparations. Each value is the result of

four to five determinations. 150  $\mu$ l of the various culture fluids was utilized for each determination. The immune complexes ranged from 180 to 300  $\mu$ g (antibody protein). No significant variation in response was observed within this dose range, and the results are therefore grouped.

In the absence of specific immune complexes, the culture fluid from antigen-stimulated lymphocytes shows slight activity; the eosinophil responses are 12, 1, 20, and 7. In the presence of immune complexes, the activity is marked, the eosinophil responses being 91, 80, 95, and 125. It should be stressed that the complexes were prepared in the presence of EDTA and were well washed to exclude complement activity. Indeed, complexes alone, prepared in this manner, showed no significant chemotactic activity. In these experiments, the whole mixture of lymphocyte culture fluid and complexes was added to the chambers and tested. In one experiment, following incubation of the two materials, the complexes were removed by centrifugation and only the supernatant was used. This supernate alone was active, with an eosinophil response of 180. In two experiments the antiserum was heat-inactivated at 56°C for 45 min, followed by the addition of antigen at equivalence in the presence of the lymphocyte culture fluid. This procedure also generated chemotactic activity and yielded an eosinophil response of 140. Thus the interaction of stimulated culture fluid, antibody, and antigen generated specific eosinophil chemotactic activity regardless of the sequence of addition of the reactants, and this activity was found in the soluble phase. In this system we found it important to have antibody and antigen at equivalence, since complexes prepared at ten times antigen excess were ineffective in generating chemotactic activity.

*Specificity of Chemotactic Factor.*—The results reported thus far were obtained with immune complexes involving the same antigenic determinants as those to which the lymphocytes had been sensitized. To explore the specificity of the system, three other guinea pig antibodies were obtained. In addition, a rabbit antibody against the specific antigen (BGG), and a rabbit antibody directed against an unrelated antigen were used. The results are shown in Table III, which lists the average results for all experiments involving specific immune complexes made with guinea pig antibody as well. The complexes containing antigens unrelated to the one used in the original lymphocyte culture were ineffective in generating eosinophil chemotactic activity in the stimulated culture fluid. Surprisingly, related but heterologous complexes prepared with rabbit antiserum (RabaBGG-BGG) were likewise ineffective.

*Chemotaxis of Neutrophils.*—Since the peritoneal exudates contained mixtures of eosinophils and neutrophils, it was possible to compare the behavior of these two cell types under identical conditions in the in vitro assay system. In the present study, stimulated culture fluid alone was not effective in causing chemotaxis of neutrophils. This is discordant with previous results (14, 15) but in all likelihood can be explained by the relatively small number of neutrophils used

in the cell suspensions employed in these experiments, and by the suboptimal methods (multiple intraperitoneal injections of protein) used for the preparation of these cells. In the previous experiments, neutrophils were obtained from the peritoneal cavity within 3–4 hr after the intraperitoneal injection of glycogen. This explanation for the relative unresponsiveness of the neutrophils is substantiated by the observed effect of bacterial factor on these cells; this factor gave a mean neutrophil response of 70, which is markedly lower than the previously reported values (18). In spite of this, when specific immune complexes were added to the stimulated culture fluid, significant neutrophil chemotactic activity was generated. A mean neutrophil response of 39 was obtained.

*Dose-Response Relationships.*—Most of the experiments were performed

TABLE III  
*Eosinophil Chemotactic Response to Culture Fluids from Antigen-Stimulated Lymphocytes:  
Effect of Treatment with Various Immune Complexes*

Immune complex*	Eosinophil response $\pm$ SE†
None	10 $\pm$ 3
GPaBGG-BGG	94 $\pm$ 10
GPaEA-EA	<0
RabaEA-EA	3 $\pm$ 3
GPaKLH-KLH	<0
GPaDNP-DNP	13 $\pm$ 8
RabaBGG-BGG	3 $\pm$ 4

\* Lymphocytes were stimulated with OCB-BGG. Resultant culture fluids were incubated at 37°C for 30 min with immune complexes made at antigen equivalence. See Materials and Methods for doses and details.

† The eosinophil response is the difference in counts between the chambers containing stimulated fluid and the corresponding unstimulated control. A value < 0 means that the control had more counts.

using 150  $\mu$ l of the various lymphocyte culture fluids treated with immune complexes ranging in amount from 180 to 300  $\mu$ g. In this range there was no significant difference in the amount of chemotactic activity generated. This probably represents a plateau range, since stimulated culture fluid treated with 50  $\mu$ g of complexes gave an eosinophil response of 20. Using varying amounts of stimulated culture fluid treated with immune complexes in the 180–300  $\mu$ g range, the chemotactic response of eosinophils was a direct function of the volume of fluid tested. In Fig. 2, the results are shown as a semilogarithmic plot of the eosinophil response as a function of the amount of stimulated lymphocyte culture fluid. The response is clearly dose-dependent.

*In Vivo Observations.*—Samples of various preparations which had been studied in the in vitro assay system were injected intradermally into guinea pig skin as described under Materials and Methods. For this purpose, the immune

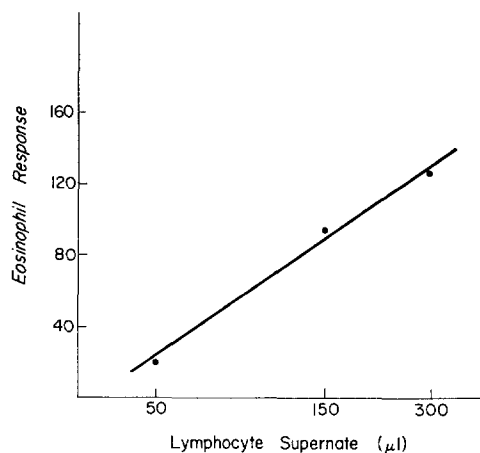


FIG. 2. Eosinophil response as a function of the volume ( $\mu$ l) of lymphocyte culture fluid. The response is dose-dependent over the range of values studied.

TABLE IV  
*Eosinophil Response in Guinea Pig Skin to Lymphocyte Culture Fluids Incubated with Various Immune Complexes\**

Culture fluid	Immune complex†	Reaction size (mm)	Eosinophil count $\pm$ SE
Unstimulated§	None	8	5 $\pm$ 1
Unstimulated	GPaBGG-BGG	11	81 $\pm$ 9
Unstimulated	GPaEA-EA	9	14 $\pm$ 5
Stimulated	None	15	18 $\pm$ 3
Stimulated	GPaBGG-BGG	16	209 $\pm$ 14
Stimulated	GPaEA-EA	14	28 $\pm$ 6
None	GPaBGG-BGG	0	1 $\pm$ 0.5
None	GPaEA-EA	2	5 $\pm$ 2

\* Fluids were incubated with complexes at 37°C for 90 min. The complexes were then removed by centrifugation, and the supernates injected intradermally in a volume of 0.2 ml.

† Immune complexes were formed at equivalence. Each contained 375  $\mu$ g of antibody protein.

§ Lymphocytes cultured in the absence of antigen.

|| Lymphocytes cultured in the presence of antigen.

complexes and lymphocyte culture fluids were incubated at 37°C for 90 min, and the complexes were then removed by centrifugation. This was necessary since complexes alone will produce skin reactions in guinea pigs (4). The experimental results are summarized in Table IV which lists the preparations injected, the size of the reactions produced, and the eosinophil response at each site. In agreement with the work of Bennett and Bloom (19), the stimulated culture



fluids produced a mixed inflammatory reaction with a large mononuclear cell component, similar to a delayed hypersensitivity reaction. Most of the inflammatory cells were located either high in the dermis near the epidermis, or deep and adjacent to the muscle layer. The intervening area showed fewer infiltrating cells. The unstimulated fluids produced a similar, but milder reaction, while the supernates from the complexes which were incubated without lympho-

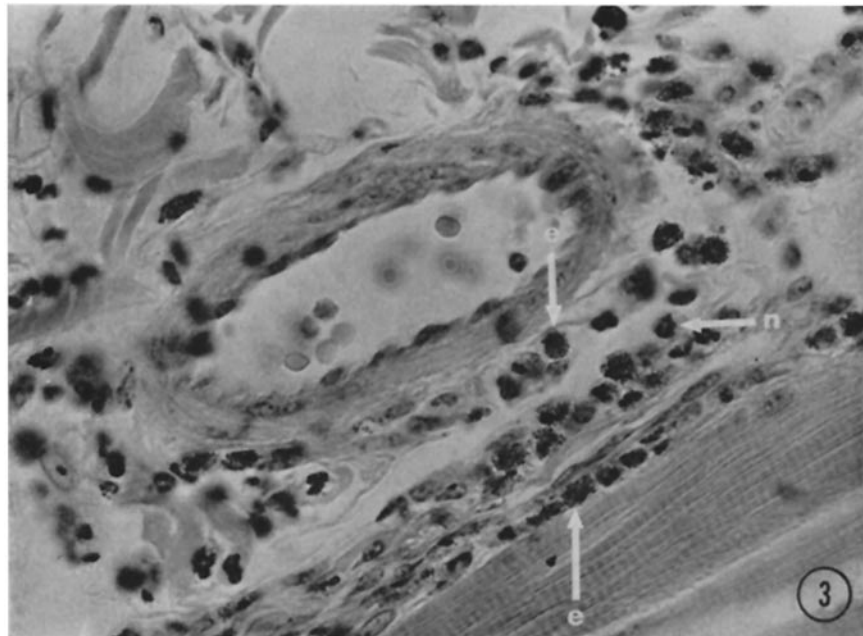


FIG. 3. Cutaneous response to the injection of stimulated lymphocyte culture fluid treated with specific immune complexes (GP $\alpha$ BGG-BGG). A marked perivascular inflammatory reaction is seen near the muscle layer. The majority of the infiltrating cells in this field are neutrophils (*n*). Two eosinophils (*e*) and one neutrophil (*n*) are indicated by arrows.  $\times 560$  (modified Dominici).

cyte fluid produced no skin reactions at all. The addition of either the GP $\alpha$ BGG-BGG or GP $\alpha$ EA-EA complexes to either of the culture fluids had no effect on the intensity of the skin response as measured grossly, or in terms of the numbers of neutrophils and mononuclear cells present. There were, however, marked differences in the eosinophil response at the various sites. In agreement with the *in vitro* observations, the stimulated fluid alone showed slight activity, and this activity was markedly enhanced by treatment with specific immune complexes. Unlike the *in vitro* situation, however, the unstimulated cultures generated significant activity on incubation with specific complexes, suggesting

that there was sufficient activation of the lymphocytes *in vivo*, before culture to generate some of the factor involved in this response when placed in culture. This event was not detected in the chamber assay technique, *in vitro*.

Figs. 3 and 4 demonstrate the eosinophil response to stimulated culture fluids treated with specific immune complexes. The eosinophil response was most marked at the sites at which the inflammatory response was most apparent, namely near the epidermis and near the muscle. In any given field, however,

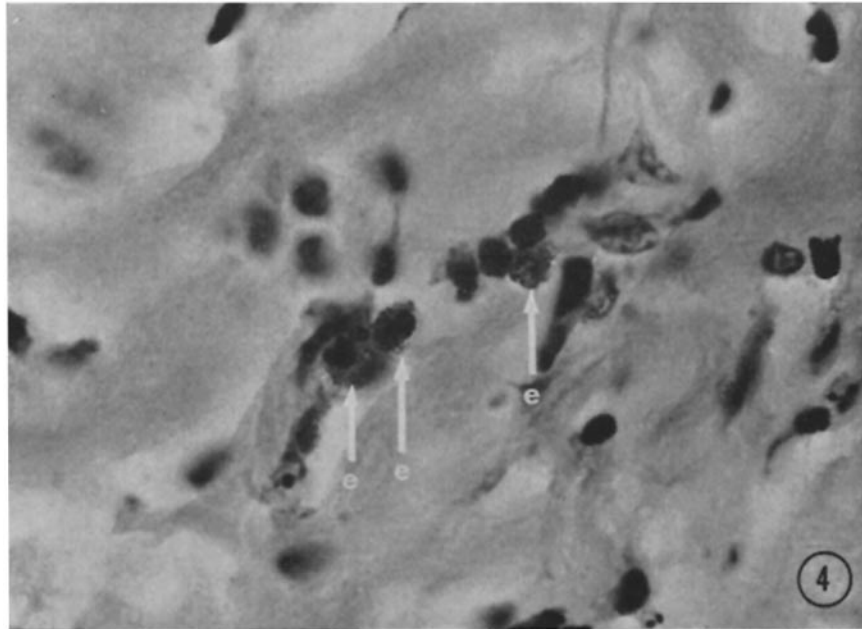


FIG. 4. Eosinophils (*e*) in the upper dermis. Same preparation as that seen in Fig. 3.  $\times 990$  (modified Dominici).

there was no correlation between the number of eosinophils and the number of other cell types.

#### DISCUSSION

The results presented demonstrate the existence of a potent chemotactic factor for eosinophils. This material is unique when compared with previously described factors chemotactic for other inflammatory cells, since its generation is dependent not only upon the interaction of antibody with antigen, but also with a substance elaborated by lymphocytes previously sensitized to the same antigen. The nature of this substance is unknown; it could be related to one of

the previously defined substances such as migration-inhibitory factor, macrophage chemotactic factor, or blastogenic factor, or it could represent an unusual and previously undetected immunoglobulin. In either case, the factor demonstrates immunologic specificity in that complexes prepared with unrelated antigens are ineffective in bringing about generation of the chemotactic factor. There may be some analogy with the situation for MIF, where Bennett and Bloom (20), Svejcar et al. (21), and Amos and Lachmann (22) have found evidence for the requirement of specific antigen in the expression of MIF activity. The last authors, for example, used antigen in insoluble form to stimulate lymphocyte cultures. The resulting antigen-free supernatants required the readdition of (soluble) antigen for full MIF activity.

The apparent species specificity seen here (RabABGG-BGG is ineffective) is puzzling; it is conceivable that a specific immunoglobulin type is required to participate in the formation of the immune complexes, and that this immunoglobulin was present in the guinea pig but absent in the rabbit preparations used. Resolution of this point will await the examination of a large number of well-characterized antisera. If species specificity is confirmed, a possible explanation is that the recognition site on the lymphocyte-derived factor is directed toward some determinant formed by the union of antibody and antigen. Since this specificity had been generated *in vivo* during the course of active sensitization, the antibody involved was the guinea pig's own, and thus guinea pig antibody would be required in the *in vitro* system. There may be some analogies here with delayed hypersensitivity, where antigen-antibody complexes are potent sensitizing agents (23), and where antigen-antibody complexes may readily elicit the reaction (24). Unfortunately, little work has been done to explore the specificity relationships in these situations.

For ease of experimental manipulations, in most experiments the complexes and the lymphocyte factor were not separated after incubation, before *in vitro* testing. However, in a small number of experiments, it has been established that (a) the continued presence of the complex following incubation is not necessary for chemotactic activity, and (b) preformed complexes are not necessary to generate the eosinophil chemotactic factor, i.e., antibody and antigen can react in the presence of the lymphocyte culture fluid. Because washed complexes, formed and reacted with the culture fluid in the presence of EDTA were used, a role for the products of sequential complement activation can be excluded in this system. It appears essential, however, that the antibody and antigen be present in equivalence ratios; in experiments not reported here, immune complexes made in antigen excess have been ineffective in generating the eosinophil chemotactic factor.

Under the experimental conditions described, neutrophils also react chemotactically. There is no evidence that this is due to the existence of a second factor, since the activity always paralleled the eosinophil chemotactic activity.

The eosinophil activity is clearly primary in that the maximal eosinophil response was two and one half times that of the neutrophil response, in spite of the fact that the indicator cell system contained from two to four times as many neutrophils as eosinophils. Moreover, bacterial factor, which was used as a positive control, was approximately six times more effective in attracting neutrophils than eosinophils, so that although the neutrophils were weakly reactive as compared to glycogen-stimulated cells, as previously mentioned, the eosinophils enjoyed no preferential advantage in this regard.

The *in vivo* activity observed suggests the biological effectiveness of the factor described, and provides an explanation for the retest phenomenon as observed by Arnason and Waksman (5). In this situation, retest at the site of a previous delayed hypersensitivity reaction leads to the accumulation of eosinophils at that site. Although the authors found no detectable circulating antibody, they suggested the possibility of local antibody production that was undetectable by their method (neutralization of diphtheria toxin). Treatment of their animals with anti-lymphocyte serum abolished the retest reaction. Our results would suggest, in contrast with those of Arnason and Waksman, that a skin test at a virgin site in an animal making antibody as well as delayed hypersensitivity should contain eosinophils as well. Preliminary studies support this contention. Another prediction currently being tested is that elicitation of a delayed reaction with immune complexes, rather than antigen, should evoke an eosinophil response.

The studies reported here are relevant also to the observations of Basten, Boyer, and Beeson (10, 11) who found that procedures which deplete or inactivate the pool of recirculating lymphocytes in rats (such as thymectomy, anti-lymphocyte serum administration, or thoracic duct drainage) all cause a diminution in the eosinophil response in trichinosis. Possibly as a consequence of parasitic infestation, the rats generate a lymphocyte-derived, antigen- and antibody-dependent chemotactic factor *in vivo* which is similar to that described in the present study.

The results presented establish one mechanism for the accumulation of eosinophils. This mechanism is based upon the generation of chemotactic activity by the interaction of a soluble factor produced by antigen-activated lymphocytes with immune complexes. As indicated, the resulting chemotactic factor's effectiveness *in vivo* as well as *in vitro* suggests that it plays a role in biological reactions in the intact animal. Doubtless, many other mechanisms may be operative in this situation as well. Little is known about the ultimate function of eosinophils. However, as they are highly phagocytic for antigen (3) and for antigen-antibody complexes (1), their accumulation in lymphoid tissue and at sites of peripheral immunologic reactions suggests a possible regulatory role in the immune response.

## SUMMARY

When cultured in the presence of specific antigen, lymphocytes from delayed-hypersensitive guinea pigs release a number of biologically active substances into the culture medium. Such active supernatants can react with immune complexes *in vitro* to generate a factor which is chemotactic for eosinophils. The factor involved is unique, since previously described chemotactic factors for other cell types require for their generation either immune complexes or substances released into lymphocyte culture, but not both. In the case of the eosinophil chemotactic factor, the interaction between the substance elaborated by the lymphocytes and the immune complexes appears to be specific in that the immune complexes must contain the same antigen as that used to activate the lymphocyte cultures. Although this factor was generated in an *in vitro* system, it has been shown to possess *in vivo* as well as *in vitro* activity. It is therefore possible that this factor may be of biological significance in situations where eosinophils are participants in inflammatory or immunologic reactions.

## BIBLIOGRAPHY

1. Litt, M. 1964. Eosinophilia and antigen-antibody reactions. *Ann. N.Y. Acad. Sci.* **116**:964.
2. Cohen, S. G., T. M. Sapp, and A. R. Gallia. 1963. Experimental eosinophilia. V. Specificity of regional lymph node responses to antigen-antibody systems. *Proc. Soc. Exp. Biol. Med.* **113**:29.
3. Cohen, S., P. Vassalli, B. Benacerraf, and R. T. McCluskey. 1966. The distribution of antigenic and nonantigenic compounds within draining lymph nodes. *Lab. Invest.* **15**:1143.
4. Cohen, S. G., and T. M. Sapp. 1965. Experimental eosinophilia. VIII. Cellular responses to altered globulins within cutaneous tissue. *J. Allergy.* **36**:415.
5. Arnason, B. G., and B. H. Waksman. 1963. The retest reaction in delayed sensitivity. *Lab. Invest.* **12**:737.
6. Archer, R. K. 1959. Eosinophil leucocytes and their reactions to histamine and 5-hydroxytryptamine. *J. Pathol. Bacteriol.* **78**:95.
7. Ward, P. A. 1969. Chemotaxis of human eosinophils. *Amer. J. Pathol.* **54**:121.
8. Ward, P. A., C. G. Cochrane, and H. J. Müller-Eberhard. 1965. The role of serum complement in chemotaxis of leukocytes *in vitro*. *J. Exp. Med.* **122**:327.
9. Ward, P. A., C. G. Cochrane, and H. J. Müller-Eberhard. 1966. Further studies on the chemotactic factor of complement and its formation *in vivo*. *Immunology.* **11**:141.
10. Basten, A., M. H. Boyer, and P. B. Beeson. 1970. Mechanism of eosinophilia. I. Factors affecting the eosinophil response of rats to *Trichinella spiralis*. *J. Exp. Med.* **131**:1271.
11. Basten, A., and P. B. Beeson. 1970. Mechanism of eosinophilia. II. Role of the lymphocytes. *J. Exp. Med.* **131**:1288.
12. Bloom, B. R., and B. R. Bennett. 1966. Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science (Washington)*. **153**:80.

13. David, J. R. 1966. Delayed hypersensitivity *in vitro*: Its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc. Nat. Acad. Sci. U. S. A.* **56**:72.
14. Ward, P. A., H. G. Remold, and J. R. David. 1969. Leukotactic factor produced by sensitized lymphocytes. *Science (Washington)*. **163**:1079.
15. Ward, P. A., H. G. Remold, and J. R. David. 1970. The production by antigen-stimulated lymphocytes of a leukotactic factor distinct from migration inhibitory factor. *Cell. Immunol.* **2**:162.
16. Litt, M. 1960. Studies in experimental eosinophilia. I. Repeated quantitation of peritoneal eosinophilia by a method of peritoneal lavage. *Blood J. Hematol.* **16**:1318.
17. Litt, M. 1963. Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigen stimulation. *Amer. J. Pathol.* **42**:529.
18. Ward, P. A., I. H. Lepow, and L. J. Newman. 1968. Bacterial factors chemotactic for polymorphonuclear leukocytes. *Amer. J. Pathol.* **52**:725.
19. Bennett, B., and B. R. Bloom. 1968. Reactions *in vivo* and *in vitro* produced by a soluble substance associated with delayed-type hypersensitivity. *Proc. Nat. Acad. Sci. U. S. A.* **59**:756.
20. Bennett, B., and B. R. Bloom. 1967. Studies on the migration inhibitory factor associated with delayed-type hypersensitivity: cytodynamics and specificity. *Transplantation.* **5**:996.
21. Svejcar, J., J. Pekarek, and J. Johanovsky. 1968. Studies on production of biologically active substances which inhibit cell migration in supernatants and extracts of hypersensitive lymphoid cells incubated with specific antigen *in vitro*. *Immunology.* **15**:1.
22. Amos, H. E., and P. J. Lachmann. 1970. The immunological specificity of a macrophage inhibition factor. *Immunology.* **18**:269.
23. Uhr, J. W., S. B. Salvin, and A. M. Pappenheimer, Jr. 1957. Delayed hypersensitivity. II. Induction of hypersensitivity in guinea pigs by means of antigen-antibody complexes. *J. Exp. Med.* **105**:11.
24. Uhr, J. W., and A. M. Pappenheimer, Jr. 1958. Delayed hypersensitivity. III. Specific desensitization of guinea pigs sensitized to protein antigens. *J. Exp. Med.* **108**:891.