

Eosinopenia of Acute Infection: *PRODUCTION OF EOSINOPENIA BY CHEMOTACTIC FACTORS OF ACUTE INFLAMMATION*

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J Clin Invest. 1980;65(6):1265-1271. <https://doi.org/10.1172/JCI109789>.

One distinctive aspect of the response to acute inflammation involves a rapid and persistent decrease in the numbers of circulating eosinophils, yet the mechanisms of this eosinopenia are undefined. One possibility is that the abrupt eosinopenia may be the result of release of small amounts of the chemotactic factors of acute inflammation into the circulation. These studies were designed to examine the numbers of circulating eosinophils after an intravenous injection of zymosan-activated serum, partially purified C5a or the synthetic peptide, *N*-formyl-methionyl-leucyl-phenylalanine. Each of these factors caused a virtual disappearance of circulating eosinophils within 1 min, a transient return of eosinophils to ~50% of control levels after 10-90 min, and a subsequent decrease which persisted for 5 h. In contrast, the numbers of circulating heterophils, although dropping transiently, rapidly returned and rose to elevated levels for 6 h after injection. The response was not caused by adrenal mediation as it occurred normally in adrenalectomized rabbits. Two chemotaxins of allergic inflammation, histamine and the tetrapeptide valine-glycine-serine-glutamic acid, did not cause significant eosinopenia.

Circulating granulocytes of patients undergoing hemodialysis, which has been reported to activate complement, demonstrated similar eosinopenic and neutropenic-neutrophilic responses. Thus, in rabbits and in man, intravascular activation or injection of chemotactic factors (C5a or *N*-formyl-methionyl-leucyl-phenylalanine) causes a brief, nonspecific granulocytopenia followed by a prolonged eosinopenic-neutrophilic response analogous to that seen [...]

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Eosinopenia of Acute Infection

PRODUCTION OF EOSINOPENIA BY CHEMOTACTIC FACTORS OF ACUTE INFLAMMATION

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ABSTRACT One distinctive aspect of the response to acute inflammation involves a rapid and persistent decrease in the numbers of circulating eosinophils, yet the mechanisms of this eosinopenia are undefined. One possibility is that the abrupt eosinopenia may be the result of release of small amounts of the chemotactic factors of acute inflammation into the circulation. These studies were designed to examine the numbers of circulating eosinophils after an intravenous injection of zymosan-activated serum, partially purified C5a or the synthetic peptide, *N*-formyl-methionyl-leucyl-phenylalanine. Each of these factors caused a virtual disappearance of circulating eosinophils within 1 min, a transient return of eosinophils to ~50% of control levels after 10–90 min, and a subsequent decrease which persisted for 5 h. In contrast, the numbers of circulating heterophils, although dropping transiently, rapidly returned and rose to elevated levels for 6 h after injection. The response was not caused by adrenal mediation as it occurred normally in adrenalectomized rabbits. Two chemotaxins of allergic inflammation, histamine and the tetrapeptide valine-glycine-serine-glutamic acid, did not cause significant eosinopenia.

Circulating granulocytes of patients undergoing hemodialysis, which has been reported to activate complement, demonstrated similar eosinopenic and neutropenic-neutrophilic responses. Thus, in rabbits and in man, intravascular activation or injection of chemotactic factors (C5a or *N*-formyl-methionyl-leucyl-phenylalanine) causes a brief, nonspecific granulocytopenia followed by a prolonged eosinopenic-neutrophilic response analogous to that seen during acute infection.

Received for publication 16 November 1979 and in revised form 8 February 1980.

INTRODUCTION

Eosinopenia and neutrophilia typically accompany the responses to acute stress or acute infection. These responses are among the most dramatic examples of the differential regulation of the eosinophil and neutrophil leukocyte series, yet the mechanisms are inadequately understood. The eosinopenia of acute stress involves mediation by adrenal glucocorticosteroids and epinephrine (1, 2). The eosinopenia that occurs during acute inflammatory states is not dependent upon these endocrine mechanisms (3), but the causes are not well defined.

In previous studies similar eosinopenic responses followed the induction of acute inflammation by diverse bacterial, viral, and chemical irritant stimuli (4). These studies suggested that the eosinopenia represented a response to the acute inflammatory process itself or to some systemic reaction induced by it. Further studies examined whether substances in the acute inflammatory exudate might mediate an eosinopenic reaction. Acute inflammatory exudate, obtained after pneumococcal infection of a subcutaneous air pouch in mice, was found to contain a material capable of causing eosinopenia when injected intraperitoneally into eosinophilic mice (5). Partial characterization of this substance suggested that it was a large molecular weight glycoprotein. This material produced an eosinopenic response which was apparent between 4 and 20 h after intraperitoneal injection. Eosinopenic activity was detectable in the exudate 10 h after inoculation of pneumococci and reached its highest concentration 20 h after inoculation. Thus, this eosinopenic activity seemed to appear rather late in the inflammatory exudate. It was suggested that this substance may contribute to the maintenance of the eosinopenic state

which normally persists throughout the period of active acute inflammation. However, this material could not explain the initial, abrupt eosinopenia which followed the induction of an acute inflammatory process (4).

The initial eosinopenic response to acute inflammation was interpreted as being the result of a rapid peripheral sequestration of circulating eosinophils (3, 4). The sites of sequestration were uncertain. It was not due to entrapment of eosinophils within the spleen or lymph nodes. Part of the sequestration could be ascribed to migration of eosinophils into the inflammatory site itself, presumably by the chemotactic substances released during acute inflammation. Several of the major chemotactic substances of acute inflammation, including C5a (6), the complex C567 (7), fibrin fragments (8), and *N*-formyl-methionyl peptides (used by many investigators as synthetic analogues of bacterial chemotactic factors) (9) are chemotactic in vitro for eosinophils as well as neutrophils. The presence of such chemotactic factors, in particular C5a, has been detected in the circulation during acute inflammatory states (10, 11). The question arises whether release into the circulation of small amounts of the chemotactic factors of acute inflammation might contribute to the eosinopenia of acute infection. The effects of such substances on circulating neutrophils have recently been studied in some detail. Intravascular activation of complement in rabbits (12) or man (13) or intravenous injection of substances chemotactic for neutrophils (12, 14) in rabbits produce an abrupt neutropenia within a min which is followed by a neutrophilia which occurs after 30–90 min and lasts for several hours. The present studies examined the responses of circulating eosinophils to intravenous injection of such chemotactic factors of acute inflammation.

METHODS

Animals. New Zealand white rabbits, young adult male weighing 2–4 kg, were obtained from Franklin's Rabbitry, Wake Forest, N. C. Adrenalectomized New Zealand white rabbits were obtained from Dutchland Laboratories, Inc., Dutchland Laboratory Animals, Inc., Denver, Pa. Adequacy of the adrenalectomy was determined by lack of eosinopenic responses to mild stress (repeated blood sampling) and to ACTH. In certain experiments rabbits were rendered mildly eosinophilic by infection with trichinosis. Muscle-stage larvae of *Trichinella spiralis* were obtained by acid-pepsin digestion of infected rat musculoskeletal tissues as previously described (15). Rabbits were anesthetized with ketamine (Bristol Laboratories, Syracuse, N. Y.) and xylazine (Haver-Lockhart Labs., Shawnee Mission, Kan.), intubated with a pediatric feeding tube and inoculated orally with 10,000 infective larvae. After 3–4 wk such rabbits were found to have a modest eosinophilia, with a mean eosinophil count of 323/mm³.

Blood sampling and rabbit granulocyte counts. Putative stimuli were injected into an ear vein. Blood samples were drawn at timed intervals from a vein of the opposite ear. Absolute eosinophil counts employed a counting fluid containing 50 g urea and 10 ml 2% eosin/100 ml aqueous solution

(16). Counting chambers were held in moist petri dishes for 30 min, which allowed lysis of the heterophils and bright staining of eosinophils. Total leukocyte counts and differential counts of Wright's stained smears allowed quantitation of the total granulocytes. Preliminary experiments examined the correlation of absolute eosinophil counts obtained with the above counting fluid with eosinophils determined by the less accurate method of a 200-cell differential count times the total leukocyte count. The two methods for determining the number of circulating eosinophils (absolute eosinophil count vs. total cell count × percent of eosinophils) demonstrated a correlation coefficient of 0.94 with a slope of 1.1, *n* = 16.

Stimuli. Zymosan-activated rabbit serum (ZAS)¹ was prepared by incubating 2 ml of fresh rabbit serum with 50 mg twice-washed zymosan (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) at 37°C for 30 min, with removal of the zymosan by centrifugation at 1,000 *g* for 10 min. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP [Andrulis Chemical Corp., Bethesda, Md.]) was dissolved in 0.02 M NaHCO₃ at 1 mg/ml and diluted to desired concentrations in phosphate-buffered saline (PBS). Histamine phosphate (Fisher Scientific Co., Pittsburgh, Pa.) and the eosinophil chemotactic tetrapeptide valine-glycine-serine-glutamic acid (kindly provided by Dr. Ed. Goetzl, Harvard Medical School, Boston, Mass.) were dissolved in PBS and adjusted to pH 7.4.

Preparation of partially purified C5a. 300 ml of fresh human serum was activated by incubation for 30 min with 50 g/liter zymosan in the presence of 1 M ϵ -aminocaproic acid. Preliminary experiments demonstrated that this incubation provided a maximal concentration of C5a (data not shown). C5a was partially purified by a modification of the method of Fernandez and Hugli (17). During purification, the presence of C5a was assayed by the ability of the fraction to stimulate the uptake of [³H]deoxyglucose by normal human neutrophils (18). The activated serum was adjusted to pH 3.8 with 1 M HCl and EDTA was added to a final concentration of 10 mM. All further procedures were conducted at 4°C. The activated serum was dialyzed in small pore tubing (Spectrapor 1, Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 0.1 M ammonium formate at pH 5.0 containing 0.5 mM EDTA until the concentration of ϵ -aminocaproic acid was <1 mM. The dialyzed serum was applied to 6 × 7-cm column of CM-cellulose; the column was washed with 0.1 M formate, pH 5.0, and eluted with 350 ml of 0.5 M ammonium formate at pH 5.0. The eluted material was then concentrated by ultrafiltration (UM-10, Amicon Corp., Lexington, Mass.) to a 28-ml volume. The material was fractionated on a 2.6 × 95-cm Sephadex G-100 column equilibrated and eluted with 0.1 M ammonium formate at pH 5.0 and a flow rate of 12 ml/h. 6-ml fractions were collected. The active fractions were pooled and the C5a activity confirmed by stimulation of chemotaxis in the chemotaxis-under-agarose assay modified as described previously (19). The pooled fractions (termed "crude C5a") contained six bands by polyacrylamide gel electrophoresis. The concentration of the preparation was adjusted to provide bioactivity (stimulation of chemotaxis and deoxyglucose uptake) equal to that of ZAS. A low molecular weight fraction, beyond the resolution of the Sephadex G-100 column and lacking activity in the [³H]deoxyglucose assay, was used as a control preparation.

Circulating granulocytes in patients during hemodialysis. Patients with chronic renal failure, on maintenance hemo-

¹Abbreviations used in this paper: ECF-A, eosinophil chemotactic factor of anaphylaxis; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PBS, phosphate-buffered saline; ZAS, zymosan-activated serum.

dialysis using a Cordis-Dow artificial kidney (hollow cellulose fiber), and not taking corticosteroid or immunosuppressive agents, were examined. Just before, and timed at intervals after the onset of dialysis, blood samples were drawn from the afferent blood line of the dialysis circuit. Absolute eosinophil counts were performed by Discombe's method (20). Total leukocyte counts and differential counts by Wright's stain were used to determine the numbers of circulating neutrophils.

Statistical analysis. The variability of the eosinophil counts at the beginning of the experiments necessitated expression of the circulating eosinophil and heterophil counts as percent of control, the count at the beginning of the experiments. Each experiment was repeated three to eight times with similar results. Significance was examined by the unpaired Student's *t* test.

RESULTS

Effect of ZAS. Injection of ZAS produced an abrupt drop in the number of circulating eosinophils (Fig. 1). Within 1 min after injection, the eosinophils had fallen to zero. Between 20 and 60 min later the eosinophils again appeared in the circulation only to decrease in number with a biphasic response which was observed in every animal examined. The timing of this response varied among animals such that the peak occurred between 20 and 90 min after injection. This is the reason for the large standard errors observed during this phase of the response. Because of this variability, attempts to average all of the responses resulted in a blurring of observations which, although they occurred at slightly different times after stimulation, were consistently observed. For this reason the remainder of the data is presented in the form of representative experiments rather than means of observations. It should be emphasized that the observations reported were seen in every animal injected.

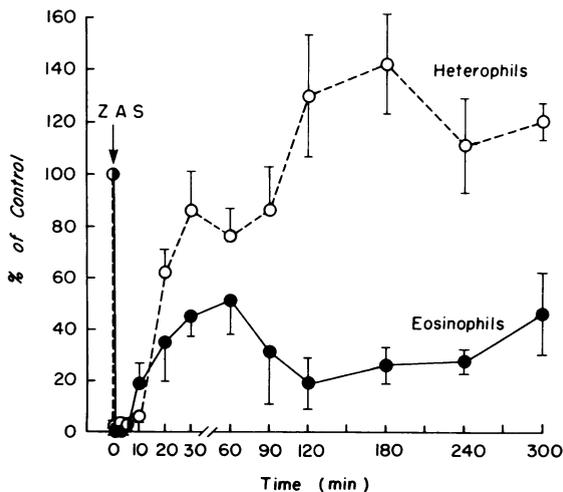


FIGURE 1 Effect of an intravenous injection of ZAS on the numbers of circulating eosinophils and heterophils. Similar responses occurred after injection of 1–8 ml ZAS. Mean \pm SE of five experiments.

Whereas the eosinophils demonstrated a biphasic response with a persistent eosinopenia lasting for 3–5 h, the heterophils, although dropping immediately after injection, rose to control levels within 20–60 min and then proceeded to significant elevations (range of 122–181% of control after 3 h) (Fig. 1).

The eosinopenic response to ZAS was not mediated by stimulation of release of corticosteroids. A 2-mg hydrocortisone injection did not produce a significant eosinopenia until 2–4 h after injection, and at that time, produced an eosinopenia with a mean of 48%. This is comparable to the eosinopenic response reported previously in other animals and man (1, 2). Moreover, the responses to ZAS were if anything more striking in rabbits which had been adrenalectomized (data not shown).

Dose-response curves are shown in Fig. 2. A significant decrease in the number of circulating eosinophils and heterophils occurred 1 min after a 0.5-ml injection of ZAS. There was no significant difference in the sensitivity of the eosinophils or heterophils to induction of this initial abrupt response to ZAS.

Partially purified human C5a produced a similar rapid drop in the number of circulating eosinophils and heterophils (Fig. 3). Fractions from the same column lacking C5a activity did not produce a significant drop in either eosinophils or heterophils.

Production of eosinopenia in man during hemodialysis. It has been reported that hemodialysis is associated with activation of complement by the dialysis membrane and that this is associated with a neutropenia-neutrophilia response similar to the heterophil responses in rabbits after a ZAS injection (13). Fig. 4 shows the mean of observations on eight patients in whom we observed the reported changes in neutrophils and found that this was accompanied by a similar degree of eosinopenia. Moreover, the eosinophil counts followed a course similar to that observed in

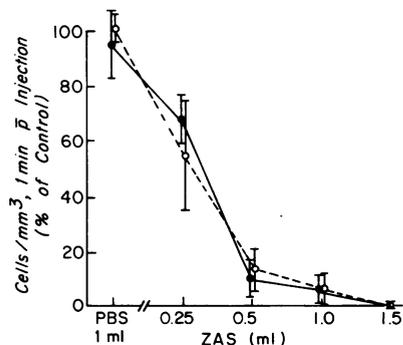


FIGURE 2 Effect of varying volumes of ZAS or of 1 ml PBS on the number of circulating eosinophils (●) and heterophils (○) 1 min after injection. Mean \pm SE of three experiments at each point.

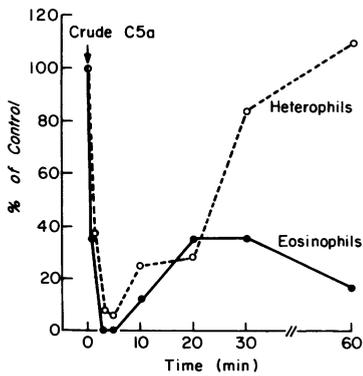


FIGURE 3 Effect of an intravenous injection of partially purified C5a on the numbers of circulating eosinophils and heterophils. As judged by the stimulation of [³H]deoxyglucose uptake, the C5a injected was equivalent to the amount present in 2 ml ZAS. Similar responses were observed in three experiments.

the rabbits after injection of ZAS. Whereas the neutrophils rose to elevated levels between 60 and 300 min (the end of dialysis), the eosinophils remained reduced in numbers throughout the dialysis period.

The eosinopenic effect of fMLP. fMLP is chemotactic for neutrophils in the range of 10^{-4} – 10^{-6} mg/ml. Injection of 10 μ g of fMLP into rabbits with roughly 100 ml blood volume reduced the eosinophil count to zero within 1 min (Fig. 5). Moreover the eosinophils did not begin to appear until after 3 h and remained markedly depressed for the entire 5-h of observation. A 1- μ g fMLP injection produced a biphasic eosinopenic response very similar to that observed after a ZAS injection (Fig. 5). The heterophil response to this dose of fMLP was very brief and rapidly proceeded

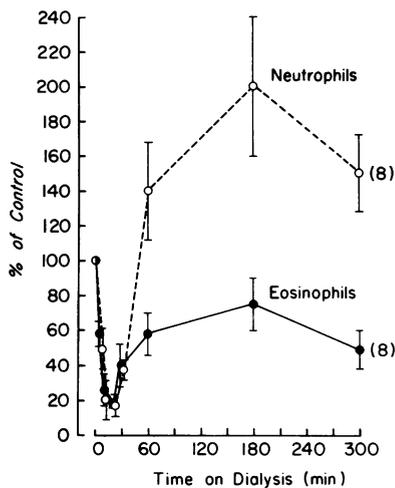


FIGURE 4 Variation in the number of circulating eosinophils and neutrophils in patients during hemodialysis. Mean \pm SE of eight patients.

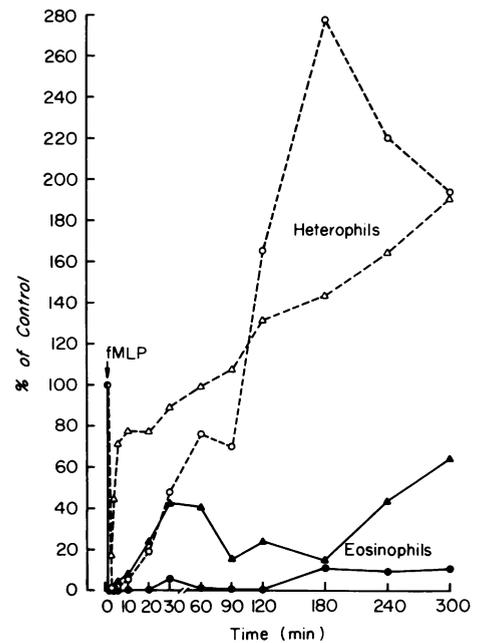


FIGURE 5 Responses of circulating eosinophils and heterophils to intravenous injection of 10 μ g (○) or 1 μ g (Δ) fMLP after adrenalectomy. Similar responses were observed in three experiments.

to elevated levels. A dose-response curve is shown in Fig. 6; significant eosinopenia occurred after a 0.1- μ g fMLP injection.

Effects of histamine, Eosinophil chemotactic factor of anaphylaxis (ECF-A), PBS or injection of Trichinella larvae into immune animals. The following experiments were performed to determine whether the eosinopenic responses to ZAS and fMLP might represent a secondary manifestation of mast cell or

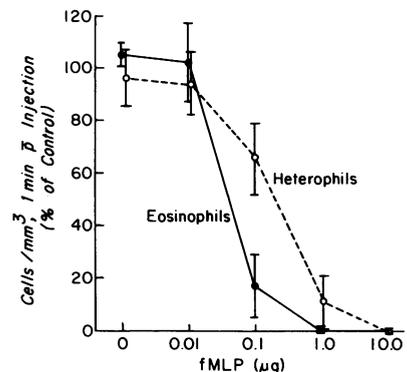


FIGURE 6 Effect of varying amounts of fMLP on the number of circulating eosinophils and heterophils of adrenalectomized rabbits, determined 1 min after injection. All injections were in a 1-ml volume of PBS. Mean \pm SE of three to five experiments at each point.

basophil degranulation induced by the original stimulant. Injections of PBS into normal rabbits was found to produce a modest and transient drop in the number of circulating eosinophils (Fig. 7). 1-ml injections of 1–0.01 mM histamine did not produce a greater eosinopenic response, even though the highest dose of histamine was sufficient to cause significant discomfort to the animal as manifested by wheezing and rubbing of the nose. The ECF-A peptide Val-Gly-Ser-Glu similarly did not produce any greater eosinopenia than that observed after a PBS injection. Animals with trichinosis have elevated levels of immunoglobulin (Ig)E and positive passive cutaneous anaphylactic responses and thus should have mast cell degranulation after secondary exposure to trichinella antigen; nevertheless, injection of 10,000 trichinella larvae into immune rabbits, which caused moderate bronchoconstriction (wheezing) within 10–30 min and vigorous inflammatory responses along with eosinophilia occurring 4–7 d later (data not shown), did not produce a significant eosinopenic response. Further experiments demonstrated that the eosinopenia of PBS could be inhibited by prior injection with 0.4 mg/kg of the β -blocker, propranolol. Moreover, this also inhibited the mild eosinopenia of ECF-A but did not inhibit the eosinopenia of ZAS (data not shown). As the eosinopenic effect of epinephrine may also be blocked by pretreatment with propranolol (21, 22), it is probable that the transient response to PBS was caused by stimulation of the sympathetic system during the intravenous injection.

DISCUSSION

Of the numerous chemotactic substances released during acute inflammation, the active substance in ZAS (presumably C5a [6]) and the peptide fMLP have been extensively employed to examine the diverse biologic effects of the chemotactic factors of acute inflammation. The present studies demonstrated that injection of small quantities of such chemotactic factors into rabbits produced an eosinopenic response that was immediate, that was biphasic, and that produced eosinopenia which persisted for 5 h after a single injection of stimulant. Partially purified C5a also produced an eosinopenic response. Finally, study of patients undergoing hemodialysis, which also causes complement activation (13), revealed a similar eosinopenic response.

Eosinophils disappeared from the circulation within 1 min. This strongly suggests a direct effect on the eosinophils and was probably caused by diffuse margination or sequestration of the cells within an organ such as the lung. For example, such sequestration of neutrophils within the lung has been reported to follow intravascular complement activation or injection of activated serum (13, 14). However, the distinctive responses of eosinophils and heterophils occurred after the initial phase of granulocyte sequestration. Both granulocytes reappeared in the blood 10–90 min after chemotaxin injection but, whereas circulating heterophiles continued to increase and remained elevated for several hours, the eosinophils again decreased in number and remained so for the 5-h observation period. As these observations were made *in vivo*, it is impossible to determine whether this second phase of the eosinopenic response was the result of a direct effect of the chemotaxin injection on the eosinophils. Two other mechanisms also deserve consideration. These include the release of eosinopenia-producing substances by neutrophils or mast cells. Exposure of surface-adherent or cytochalasin B-treated neutrophils to chemotactic factors *in vitro* causes stimulation of oxidative metabolic responses (23, 24) and degranulation (25, 26). Such responses *in vivo* (e.g., during pulmonary sequestration) could cause release of substances chemotactic for eosinophils which might then cause local margination of eosinophils and thereby cause continued eosinopenia. In particular, the possible release of neutrophil-derived eosinophil chemotactic factor (27) could be involved. This hypothesis remains to be tested.

Injection of this stimulatory material might also cause mast cell or basophil degranulation, again with resultant local sequestration of eosinophils. Either C5a (28) or fMLP (29) can cause degranulation of human basophils. However, relatively high concentrations, especially of fMLP, are required. For example,

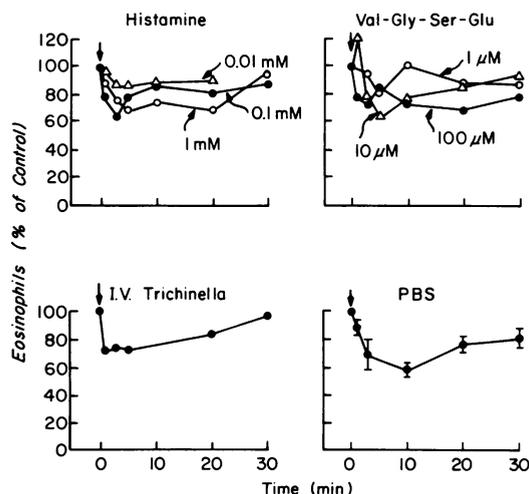


FIGURE 7 Effects of a 1-ml PBS injection or varying concentrations of histamine or the ECF-A peptide, Val-Gly-Ser-Glu and of the injection of 10,000 trichinella larvae into rabbits with trichinosis of 4–6-wk, duration. Histamine, Val-Gly-Ser-Glu, and PBS are means of three experiments; response to trichinella is the mean of two closely agreeing experiments.

0.1 mM fMLP is required to cause mast cell degranulation. Yet 2 nM fMLP causes stimulation of neutrophil or eosinophil motility² and a 0.2-nmol fMLP injection into rabbits with blood volumes of roughly 100 ml caused eosinopenia. Thus the concentration of fMLP which caused eosinopenia was roughly 10⁻⁵ times that reported to cause basophil degranulation. Nevertheless, it must be noted that a brief, mild eosinopenia has been reported after antigen stimulation of allergic patients (30, 31). The mechanisms of these in vivo responses are unknown but could involve release of eosinophil chemotaxins such as ECF-A (32) or histamine (33). Also injections of large doses of histamine into dogs (34) or horses (35) has been reported to cause eosinopenia. Nevertheless, injection of physiologic doses of histamine or the ECF-A peptide, Val-Gly-Ser-Glu, did not produce an eosinopenic response which was any greater than that observed after injection of PBS. These data strongly suggest that the eosinopenic responses observed were not caused by mast cell degranulation.

The relevance of these eosinopenic responses to the eosinopenia of acute inflammation is obviously somewhat conjectural and requires further definition. Nevertheless, the rapid and prolonged eosinopenia produced by these chemotaxins of acute infection suggest that such reactions may contribute to development of the initial rapid eosinopenia that accompanies acute inflammation.

ACKNOWLEDGMENTS

We thank Dr. Samuel Love for his help in purifying C5a and Ms. Vickie Cox for preparation of the manuscript.

This work was supported by National Institutes of Health grants AI 14929 and HL 16769 and a grant from The National Foundation March of Dimes.

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² Unpublished observations.

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