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Human eosinophils, acidic tetrapeptides (ECF-A) and histamine

INTERACTIONS *IN VITRO* AND *IN VIVO*

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Summary. The ECF-A acidic tetrapeptides Val-Gly-Ser-Glu, Ala-Gly-Ser-Glu and the analogue Val-Gly-Asp-Glu were selectively chemotactic for human eosinophils over a narrow dose range although eosinophils from different individuals varied in their dose-response pattern. Histamine abrogated the chemotactic properties of the individual tetrapeptides. When Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu were combined in various concentrations the resultant chemotaxis was either negligible or no greater than that produced when each peptide was tested separately.

Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu both promoted eosinophil accumulation when applied to the abraded skin of man or i.d. to the marmoset. Biopsies of marmoset skin revealed that peptide-induced eosinophilia was not associated with mast-cell degranulation.

Histamine, which was chemotactic *in vitro*, did not lead to appreciable eosinophil accumulation *in vivo*, and combinations of histamine and the acidic tetrapeptides evoked little or no cutaneous eosinophil infiltration either in man or the marmoset.

These studies suggest that there is a complex interaction between histamine and the ECF-A tetrapeptides; however, the tetrapeptides alone can promote the recruitment and localization of eosinophils by a mechanism apparently independent of mast-cell degranulation.

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INTRODUCTION

It is now recognized that a number of chemical mediators associated with immediate-type (type I) hypersensitivity reactions can attract selectively human eosinophils *in vitro*. These include the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay, 1969; Kay & Austen, 1971), now identified as at least two acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (Goetzl & Austen, 1975), an analogue, Val-Gly-Asp-Glu (Kay, 1976), histamine (Clark, Gallin & Kaplan, 1975) and one of its major catabolites, imidazole acetic acid (Turnbull & Kay, 1976). In the present report we describe the effect of combining these peptides with histamine in terms of their capacity both to attract selectively the eosinophil in chemotaxis and to evoke eosinophil accumulation following administration to the skin of man and a non-human primate.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: (HCl)-Val-Gly-

Ser-Glu (mol. wt 427), (HCl)-Ala-Gly-Ser-Glu (mol. wt 497) and (HCl)-Val-Gly-Asp-Glu (mol. wt 455) (a gift from Dr R. Camble, ICI Ltd, Pharmaceuticals Division, Alderley Park, Macclesfield); histamine acid phosphate (BDH Chemicals Ltd., Poole); cellulose nitrate filters 8·0 μ (Sartorius-Membrane Filters, 34 Göttingen, W. Germany), Millipore filters 0·2 μ (Millipore Corp., Bedford, Massachusetts); Timothy grass pollen (TGP), lyophilized and defatted (a gift from Beecham Research Laboratories, Betchworth).

Eosinophil chemotaxis

Peripheral blood was obtained from individuals with an eosinophilia of between 10 and 25%. The preparation of eosinophil leucocytes and the chemotactic assay were performed as previously described (Turnbull & Kay, 1976).

Marmoset skin studies

Marmosets (*Callithrix jacchus*) of either sex, with an average weight of 350 g, were maintained on a balanced vegetable diet with added vitamins and salts. Six marmosets received i.d. injections of 0·05 ml of serum from an individual (R.B.) sensitive to TGP. Following a 48-h sensitization period the same sites were challenged i.d. with 1 μg of TGP contained in 0·05 ml of saline. At the same time other sites, all on the abdomen, received 0·05 ml of the following: histamine; Val-Gly-Ser-Glu (valyl-peptide); Ala-Gly-Ser-Glu (alanyl-peptide); valyl-peptide and histamine; alanyl-peptide and histamine; valyl-peptide, alanyl-peptide and histamine; TGP; or saline—all at a final concentration of 10^{-3} mol/l whether given alone or in combination; and TGP (1 mg/ml). Immediately after the injections, 0·5 ml of 1·0% Evans blue in saline was administered i.v. These procedures were carried out on six animals of which three were killed at 12 h and three at 24 h following injection of Evans blue. Immediately after the animals were killed, the full thickness of the skin sites were excised and fixed and stained for eosinophils and mast cells. This was performed on the same sections using a modification of the method of Lendrum (1944) in which Alcian blue and chromotrope 2R were used to identify mast cells and eosinophils respectively (Hogg, R.M. & Banks, D., manuscript in preparation). Cell counts were performed as described (Kay, 1970a). Neutrophil counts were expressed semi-quantitatively on a 'plus' basis.

Human skin studies

'Skin window' studies were performed on four atopic volunteers who had seasonal, allergic rhinitis and an immediate-type skin weal and flare reaction to TGP. The method used was slightly modified from that described by Felarca & Lowell (1968). Skin sites on the forearm were thoroughly cleaned with 70% alcohol, and minimally abraded with a high-speed drill. The penetration of skin was insufficient to cause bleeding. Skin sites were approximately 0·3 cm in diameter and 5 cm apart. Plastic 'hats' (10 mm diameter and 5 mm high) were packed with sterile cotton wool and sealed with a Millipore filter (0·2 μ) using a thermoplastic glue. The cotton wool acted as a reservoir for the test solutions. Between the abrasion and the plastic 'hat' a second 0·2 μ filter was introduced which was removed and replaced with a new filter at time intervals of 2, 4, 6, 8, 12, 16, 20 and 24 h. Fresh filters were dampened with the appropriate test solution and at each change the interior of the plastic 'hats' was moistened by injection of the same solution into the cotton-wool reservoir. The plastic 'hats' were securely attached to the skin with adhesive tape and crepe bandages. The solutions were as follows: Tyrode's diluent; histamine; valyl-peptide; alanyl-peptide; valyl-peptide and histamine; alanyl-peptide and histamine; valyl- and alanyl-peptide; valyl-peptide, alanyl-peptide and histamine—all at a concentration of 10^{-3} mol/l whether given alone or in combination; and TGP (1 mg/ml). The filters, removed at the time intervals described above, were fixed and stained as previously described (Kay, 1970b). Counts were expressed as the total of 10 random high-power fields (×90), per filter.

RESULTS

Eosinophil chemotaxis

The valyl- and alanyl-tetrapeptides, and the analogue Val-Gly-Asp-Glu, were tested for eosinophil chemotaxis over a wide dose range (Fig. 1). Eosinophils from six subjects gave two types of dose-response pattern with the valyl- and alanyl-peptides. Of these, four individuals gave peak activity to the valyl-peptide at 10^{-8} mol/l, with inhibition at higher doses, whereas two patients gave two peaks of activity, one at 10^{-4} mol/l and one at 10^{-7} mol/l. Eosinophils from individuals giving a double peak of activity with the valyl-peptide gave a similar pattern

of response to the alanyl-peptide; however the response at 10^{-4} mol/l was less marked. Similarly the four individuals whose eosinophils gave a single peak at 10^{-8} mol/l with the valyl-peptide gave a comparable response to Ala-Gly-Ser-Glu. Only those subjects who gave two peaks of activity with the valyl- and alanyl-peptides were tested against Val-Gly-Asp-Glu. The eosinophils from these individuals gave a single peak with this analogue which was maximal at 10^{-7} mol/l. The patterns of activity, in terms of the shape of the dose-response curve, remained constant for eosinophils from the same person tested against any of the individual peptides on three or more separate occasions.

Eosinophils from the four individuals who gave a single peak of activity to the valyl- or alanyl-peptides, were tested in chemotaxis against histamine, either alone or in combination with the peptides. With histamine alone these subjects gave a linear dose response with peak activity at the highest concentration. When the valyl- (Fig. 2) or alanyl- (Fig. 3) peptides were tested in combination with histamine at either 10^{-3}, 10^{-5} or 10^{-7} mol/l the resultant chemotaxis was negligible.

When the two peptides were mixed the resultant eosinophil chemotaxis was dependent on their relative concentrations (Fig. 4). Increasing concentrations of the alanyl-peptide were combined with the valyl-peptide at either 10^{-4}, 10^{-6} or 10^{-8} mol/l. Chemotaxis was only observed when the alanyl-peptide, at concentrations from 10^{-5} to 10^{-9} mol/l, was combined with the valyl-peptide at 10^{-8} mol/l.

Further experiments (not shown in the figures) using a variety of dose combinations of both the peptides and histamine again resulted in negative chemotaxis. A similar effect was observed when the analogue Val-Gly-Asp-Glu was combined with histamine.

In all studies neutrophil chemotaxis paralleled the eosinophil response although the counts for the former were considerably lower. Thus the pattern of the various dose responses was virtually identical for both cell types, there being neither neutrophil nor eosinophil chemotaxis using combinations of the peptides and histamine.

Marmoset skin studies

In preliminary experiments it was shown that following passive cutaneous anaphylactic (PCA) reactions in the marmoset a peak of eosinophil accumulation was seen at 12 h, whereas untreated skin examined at 4, 8, 12 and 24 h from animals given Evans blue dye i.v. contained neither eosinophils nor neutrophils. With increasing concentrations of serum from the TGP-sensitive individual (R.B.) there was a concomitant increase in both the 'blueing' reaction and the subsequent infiltration of eosinophils. The PCA reactions therefore served as a positive control enabling a comparison of the eosinophil promoting effects of histamine and the valyl- and alanyl-peptides. Following i.d. injection of these agents either alone or in combination, the resultant eosinophil and neutrophil accumulation was determined at 12 and 24 h (Fig. 5). At 12 h neutrophil infiltration was observed with the controls [antigen alone, antibody alone (not shown in Fig. 5) and saline] but considerably more was seen at the treatment sites and following the PCA reaction. At 24 h, few neutrophils were seen with saline, antigen, antibody or histamine alone but with the other treatments large numbers of neutrophils were still present. Few eosinophils were found at 12 or 24 h with antigen, antibody, saline or histamine alone; however, when these treatments were compared to the alanyl- and valyl-peptides the eosinophil accumulation at 24 h was significantly greater ($P < 0.02$). At 12 h there was relatively little eosinophilia with the peptides. At 24 h, when the valyl-peptide was combined with histamine, the resultant eosinophil infiltration was significantly less than that obtained when the peptide was injected separately ($P < 0.02$). When the alanyl-peptide was combined with histamine the infiltration although much less than the peptide alone was not significantly different. Similarly when all three agents were administered simultaneously inhibition of eosinophil accumulation was observed when this mixture was compared to the alanyl- ($P < 0.02$) or valyl-peptide ($P < 0.05$) alone. None of the differences was significant at 12 h. In the control PCA sites there were more eosinophils at 12 h than at 24 h, whereas the individual peptides gave a greater eosinophil response at 24 h.

In general there was little difference in the number of mast cells present in the test and control sites at 12 h. However at 24 h, there were fewer mast cells in the PCA site, compared to the control and treatment areas of skin. These studies indicate that the marmoset PCA reactions are associated with mast-cell depletion (presumably as a result of

degranulation) but that the acidic tetrapeptides administered either alone, or in combination with histamine, did not affect mast cells as assessed by light microscopy of paraffin sections. The 'blueing' reactions evoked by the peptides in the marmoset skin were usually diffuse and ill-defined and therefore it was not possible to quantify them with accuracy.

Human skin studies

The valyl- and alanyl-peptides and TGP all evoked eosinophil infiltration following application to the abraded skin of atopic individuals (Fig. 6), the TGP positive control giving the greatest response. The pattern of eosinophil infiltration to TGP was biphasic with peaks at 8 h and 20–24 h. A similar, but less marked, response was observed when either the valyl- or alanyl-peptides were administered separately. At 24 h the counts with these peptides were significantly greater than the Tyrode's control ($P < 0.05$). However, even at the concentrations used (10^{-3} mol/l) the resultant eosinophilia was only approximately one-third that of the TGP control. When the two peptides were applied together there was an apparent inhibition of eosinophil accumulation when the values from this combination were compared to the agents administered separately ($P < 0.02$). Applications of histamine led to minimal eosinophil infiltration but this was not significantly different to the Tyrode's control at any of the time intervals studied.

The response to Val-Gly-Ser-Glu or Ala-Gly-Ser-Glu administered in combination with histamine was significantly less ($P < 0.05$ and $P < 0.05$, respectively) than with the peptides alone. A mixture of either of the two peptides and histamine gave a smaller response than the combination of both peptides and histamine although this difference was not significant. Whatever the nature, or combination, of the agents giving a positive eosinophil response the patterns tended to be biphasic. The first peak was between 6 and 8 h and the second, which was always the more marked, between 20 and 24 h. Neither of the peptides, nor combinations of the peptides with histamine, gave an eosinophil response comparable in magnitude to the TGP control. With all treatments, excluding TGP, the predominant cell type was the neutrophil but the numbers were too numerous for accurate quantification.

DISCUSSION

A product of the anaphylactic reaction which selectively attracted eosinophils from a mixed leucocyte population (Kay, 1969) was later identified as the eosinophil chemotactic factor of anaphylaxis (ECF-A) and shown to be a unique mediator of type I hypersensitivity (Kay & Austen, 1971). It now appears that this activity is the property of a family of closely related acidic peptides, two of which were identified as Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu (Goetzl & Austen, 1975). A certain amount of aspartic acid was also present in highly purified ECF-A and it has subsequently been shown that the analogue Val-Gly-Asp-Glu possessed comparable eosinophilotactic activity (Kay, 1976). The observation that histamine (Clark, Gallin & Kaplan, 1975) and one of its major catabolites, imidazole acetic

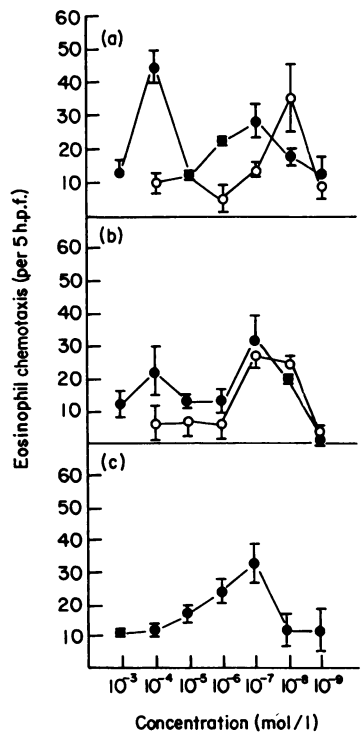

Figure 1. Eosinophil chemotaxis by Val-Gly-Ser-Glu (a), Ala-Gly-Ser-Glu (b) and the analogue Val-Gly-Asp-Glu (c). The two patterns of dose-response were from four individuals (●) with eosinophilia in association with non-Hodgkin's lymphoma, microfilariasis, carcinoma of colon and loa-loa, and two (○) in association with hypersensitivity to mefenamic acid and the 'hypereosinophilic syndrome'. Bars represent ± s.e.m.

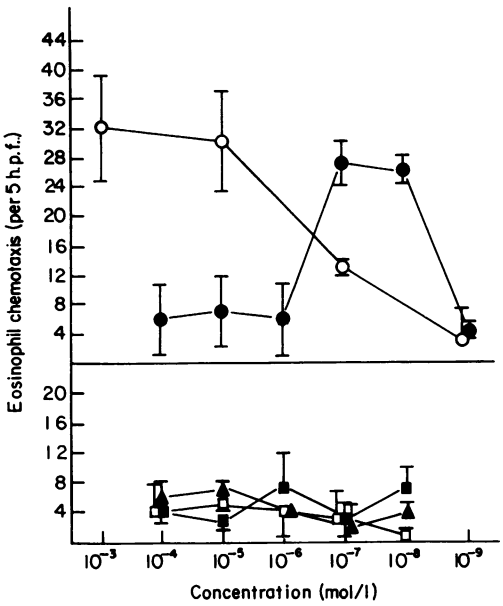

Figure 2. Eosinophil chemotaxis to Ala-Gly-Ser-Glu (●), or histamine (○), or the two agents in combination (■) Ala-Gly-Ser-Glu+histamine 10^{-3} mol/l; (□) Ala-Gly-Ser-Glu+Histamine 10^{-5} mol/l; (▲) Ala-Gly-Ser-Glu+histamine 10^{-7} mol/l.

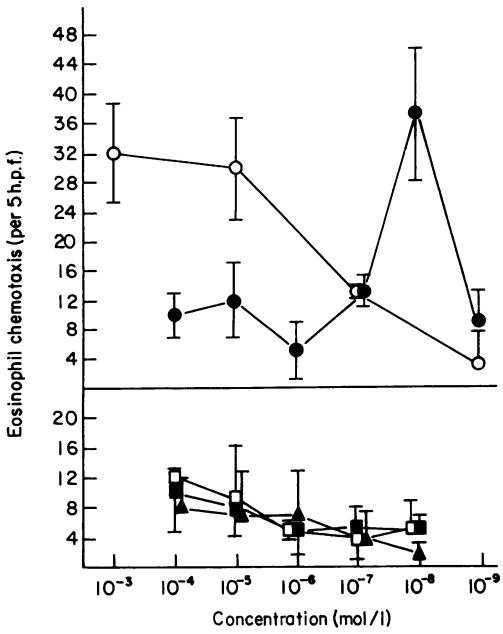

Figure 3. Eosinophil chemotaxis to Val-Gly-Ser-Glu (●), or histamine (○), or the two agents in combination. (■) Val-Gly-Ser-Glu+histamine 10^{-3} mol/l; (□) Val-Gly-Ser-Glu+histamine 10^{-5} mol/l; (▲) Val-Gly-Ser-Glu+histamine 10^{-7} mol/l.

acid (Turnbull & Kay, 1976) are also preferentially chemotactic for eosinophils points to the complexity of anaphylaxis-associated eosinophil chemotactic agents. In previous studies anaphylactic diffusates, in general, gave a linear dose response in chemotaxis, maximal activity being observed with the highest concentrations. In contrast, the peptides, or histamine, gave varying patterns of dose-response activities. Furthermore, different sources of target cells also gave differing chemotactic profiles to these agents. In general the peptides gave a peak of activity between 10^{-6} and 10^{-8} mol/l with apparent inhibition at higher doses (Fig. 1). Eosinophils from some individuals gave an additional peak with the valyl- and alanyl-peptides at 10^{-4} mol/l.

In this report various combinations of the peptides or histamine were tested in chemotaxis in order to ascertain whether the pattern of response was comparable to that of previously published studies employing anaphylactic diffusates. It was found that when histamine was combined with either the valyl- or alanyl-peptides the resultant chemotaxis was negligible (Figs 2 and 3). Thus not only did histamine and the peptides fail to act additively or synergistically but their combination abrogated the chemotactic response suggesting that there may be cross-deactivation between these agents. However we have recently shown that prior incubation of cells with histamine did not affect their response to the peptides and similarly incubation with the peptides did not abrogate the response to histamine (Turnbull & Kay, unpublished observations). In contrast prior incubation with histamine deactivated the cells for chemotaxis towards histamine or imidazole acetic acid (Turnbull & Kay, 1976) and prior incubation with the individual peptides deactivated to the same peptides (Goetzl & Austen, 1975). At the present time we are unable to explain these clearly complex interactions but suggest that *in vivo* there are possibly two processes which may require different relative concentrations and combinations of the various agents. Such events may be, firstly, directional migration of the cells and secondly, stabilization of the eosinophil at the site of allergic reactions. A possible role for histamine in localizing the eosinophil at the site of anaphylactic reactions has previously been suggested (Parish, 1974). It may be that there are also other, as yet unrecognized,

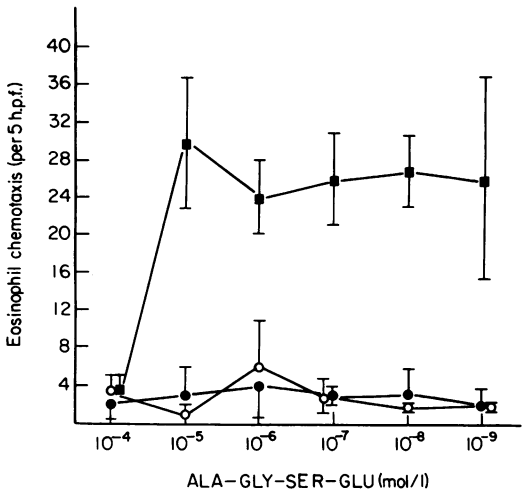

Figure 4. Chemotaxis of eosinophils to varying combinations of Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu. (●) Alanyl+ Val-Gly-Ser-Glu 10^{-4} mol/l; (○) analyl+Val-Gly-Ser-Glu 10^{-6} mol/l; (■) alanyl+Val-Gly-Ser-Glu 10^{-8} mol/l.

chemotactic agents in the anaphylactic diffusate which also contribute to directional migration and/or localization. In addition there may be, *in vivo*, differing pharmacokinetics in terms of histamine and ECF-A diffusion and/or inactivation, all of which may be critical for the observed eosinophil accumulation and localization. Many of these problems will not be solved until the relative

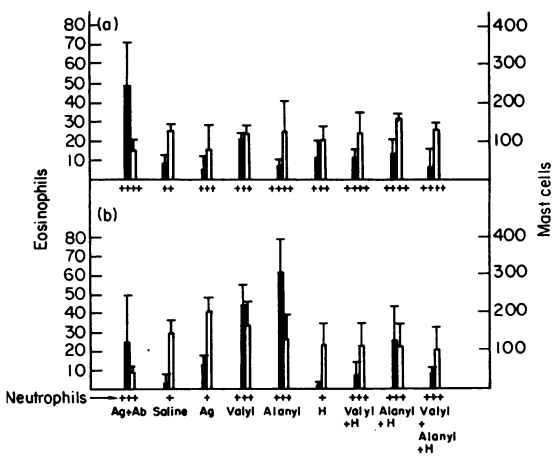

Figure 5. Eosinophil (solid columns) and neutrophil accumulation, and mast-cell counts (open columns) in the skin of marmosets at 12 h (a) and 24 h (b) following the i.d. injection of the acidic tetrapeptides, histamine or following passive cutaneous anaphylaxis (Ag+Ab).

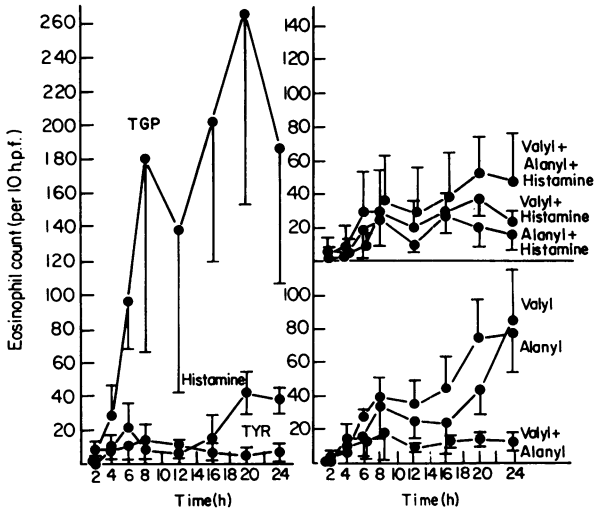

Figure 6. Eosinophil accumulation in human 'skin windows' following the application of the valyl-, the alanyl-, or histamine or these agents in various combinations. Timothy grass pollens (TGP) and Tyrode's (TYR) solution served as the positive and negative controls respectively.

amounts of the acidic peptides, histamine and possibly imidazole acetic acid present in anaphylactic diffusates are known. For this purpose the development of a quantitative assay for the peptides in biological fluids is required.

When the valyl- and alanyl-peptides were combined at low doses in the absence of histamine the resultant chemotaxis was similar to that of each peptide tested alone (Fig. 4). The apparent inhibition of chemotaxis by higher doses of the alanyl-peptide alone could be abrogated when this peptide was mixed with the valyl-peptide at 10^{-8} mol/l.

The studies *in vivo* using the skin of marmosets and humans had essential differences in their experimental design. Experiments in marmoset skin involved full thickness skin biopsies following a single injection of the agents either alone or in combination (Fig. 5). The human skin window studies, however, involved repeated applications of the agents to the skin site at 2- or 4-h intervals over the 24 h study period. These probably explain the major differences between the results obtained in humans and the non-human primate. In marmosets and man both the alanyl- or valyl-peptide could promote infiltration of eosinophils (Fig. 5). Combinations of the peptides with histamine gave lower eosinophil counts in both experimental situations. The exception was when histamine and

the valyl- and alanyl-peptides were combined together in human skin (Fig. 6). In this situation the resultant eosinophilia was comparable to the valyl- or alanyl-peptide alone and only slightly more than histamine alone. These, however, were the observations at 24 h. At 12 h, in human skin, histamine promoted very little eosinophil response whereas the peptides alone showed some activity. It was the impression both with the peptides and the positive control (Timothy grass pollen) that there was a biphasic response with a peak at 8 h, a slightly lower count at 12 h and then rising to a greater peak at 24 h. This dual response was not particularly marked but was a comparable finding to that of Hirashima & Hayashi (1976). These workers found a biphasic response in terms of eosinophil infiltration in active anaphylaxis in the guinea-pig. The first peak was associated with ECF-A-like material whereas the second was related to an eosinophilotactic protein of molecular size approximately 70,000, the nature of which was undetermined. The studies described in this report may be indicative of a comparable phenomenon in humans.

There have been a number of studies relating to capacity of histamine to promote a local eosinophilia in the skin of man. Both Eidinger, Wilkinson & Rose (1964) and Feinberg, Feinberg & Lee (1967) reported that histamine could induce a modest eosinophilia in atopic individuals but this response was far less than that produced by specific antigen. In contrast Felarca & Lowell (1968) found no such eosinophil promoting effect with histamine alone. In general, these studies, and those described in the present report suggest that in man histamine has slight eosinophil-attracting activities *in vivo* although we were unable to show that the response, by this agent, was significantly different from the Tyrode's control. In any event the response to histamine was far less than that of the individual tetrapeptides and these, in turn, evoked less eosinophil infiltration than the specific antigen.

Our demonstration of eosinophil recruitment in the skin of man by acidic tetrapeptides may possibly have clinical significance in terms of an individual's capacity to mobilize this cell type in various disease states associated with an eosinophilia.

ACKNOWLEDGMENTS

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