Experimental Eosinophilia X. Relation of Antigen-Antibody Complex Size and Protein Molecular Weight to Cell Responses.*† (31916)

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Mechanisms to explain the development of eosinophilia associated with hypersensitivity states and tissue reactions to foreign proteins are incompletely understood. In previous reports we suggested a relationship between the appearance of eosinophil leukocyte infiltrates and the state of molecular aggregation of injected serum proteins(1,2). This in turn may relate to the phagocytic functions of eosinophils for products of antigen-antibody union (3,4). Serum gamma globulins when complexed either by reaction with specific antigen or by non-specific bonding through physicochemical forces evoked eosinophil cell responses in varying degrees. In the present study we report the eosinotactic effects of specific proteins and their corresponding antigen-antibody complexes. This spectrum of experimental agents was selected for differences in physicochemical character represented by molecular weights and sizes of gamma globulin aggregates.

Materials and methods. Procedures for effecting lymph node models of eosinophilia have been previously described(1,5). Guinea pigs weighing 375 to 500 g were given 5 mg of: (1) one purified protein, and (2) corresponding soluble antigen-antibody complexes; each in 0.3 ml volume of 0.15 M saline intracutaneously into opposite hind foot pads. The specific proteins and antigen-antibody systems employed are given in Table I. Antibody components of sera taken from rabbits immunized with the specific protein in Freund's complete adjuvant were of predominantly 7S character. Soluble antigen-antibody complexes (An:Ab) were prepared from precipitates formed *in vitro* in the equivalence zone (Table I) and subsequently dissolved through addition of the antigen in quantities of 3 times excess.

A second experiment of similar design differed only in that the dosages of selected proteins and their corresponding soluble An:Ab complexes were given in equimolar concentrations based upon that of 5 mg for the hemocyanin system (Table III).

Prior to the administration of foreign species protein blood samples were taken and sera studied to rule out the possible presence of corresponding or cross-reactive preformed circulating serum antibody. Popliteal lymph nodes were removed 6 hours after injections and prepared for histologic study by methods of formalin fixation and staining with hematoxylin and eosin. Eosinophil leukocyte infiltrations were identified within multiple sections taken from each node and cells quantitated per unit area by means of the following determinations:

(1) Total number of cells within the entire surface in a single uniform plane of the section (t).

(2) Number of cross fields (transverse trips) required to cover the entire section at $\times$ 470 magnification (n).

(3) The width (w) and length (l) of each section.

Variations found in widths and lengths required elimination of size bias. A figure considered directly proportional to the node area was obtained by the calculation $\frac{t}{\frac{w}{l}n^2}$.‡

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‡ The mean width (w) and length (l) of a section may be defined as $w = \bar{m}\,b$ and $l = n\,b$, where b is the width of the field, $\bar{m}$ the number of vertical trips, and n the number of cross trips required to cover the entire section. The total area of the section (A_T) is then approximately $A_T \simeq n\,\bar{m}\,b^2 = \frac{w}{l}n^2b^2$. The unbiased parameter $t' = \frac{t\,b^2}{A_T} = \frac{t}{\frac{w}{l}n^2}$ is thus proportional to the count per unit area (details given in reference No. 5).

TABLE I. Purified Proteins of Varying Molecular Weights and Molecular Compositions of Specific Precipitates from Corresponding Rabbit Antisera Utilized in Preparation of Soluble Antigen (An) : Antibody (Ab) Complexes.

Protein (An)	Molecular wt	Molecular ratio, Ab to An (equivalence zone)
Bovine ribonuclease (BRN)*	13,000	1.5
" serum albumin (BSA)†	69,000	4
" gamma globulin (BGG)†	160,000	4.5
Horse spleen ferritin (HSF)*	465,000	7
Bovine thyroglobulin (BTG)*	700,000	7
Hemocyanin (HCN)‡	6,300,000	70
Tobacco mosaic virus (TMV)§	40,700,000	90

Sources:
* Nutritional Biochemicals, Cleveland, Ohio.
† Armour Laboratories, Kankakee, Ill.
‡ Prepared from blood of *Homarus americanus*.
§ Supplied through the courtesy of Dr. C. A. Knight, Virus Research Laboratory, Univ. of California, Berkeley, and Dr. Milton P. Gordon, Dept. of Biochemistry, Univ. of Washington, Seattle.

Results. The pattern of eosinophil leukocyte infiltrations seen within cortical and medullary sinuses of affected lymph nodes was identical to that described for a prototype model(1). Findings in those series of guinea pigs treated with equal weight dosages are summarized in Table II and for those with equimolar concentrations in Table III.

Discussion. Eosinotactic influences similar to that of BGG system(1) demonstrated by other purified foreign proteins and antigen-antibody complexes are reported in this study. Investigation has been limited to immune aggregates standardized at moderate (3 times) antigen excesses in view of anaphylactic(6), skin reactive(7), and cellular inflammatory (1,2) effects established for complexes prepared in these regions. Interaction between antibody globulin molecules has been identified as the responsible factor for induction of such biologic properties(8). Differences in molecular weights and in numbers of antigen combining sites of rabbit antibodies therefore were utilized in preparing a spectrum of gamma globulin complexes of varying size and character (Table I).

The prompt appearance of eosinophils after initial administration of a foreign protein has been attributed to the presenting state of molecular aggregation(1,2). Such chemical alterations may develop spontaneously in *in vitro* solution(8) or even subsequent to *in vivo* administration(9). There are, however, limitations to the extent of

TABLE II. Sampling of Eosinophil Cell Responses Within Sections of Popliteal Lymph Nodes Regional to Foot Pad Sites of Subcutaneous Injections of 5 mg of: (a) Foreign Proteins, and (b) Corresponding Soluble Antigen-Antibody Complexes (An:Ab) 6 Hours After Their Administration.*

Series No.	Protein (An)	Eosinophil index in lymph node sections $\frac{t}{\frac{w}{l}n^2}$† (mean ± S.D.) Foot pad treatment‡ An	An:Ab	An:Ab An
1.	BRN	35.4 ± 22	72.8 ± 26.9	2.5
2.	BSA	9.4 ± 14.7	32.4 ± 40.5	3.4
3.	BGG	17.7 ± 8.6	46 ± 30.3	2.3
4.	HSF	7 ± 9.6	39.4 ± 29.6	5.7
5.	BTG	2.6 ± 3	5.5 ± 4.6	2.3
6.	HCN	12.1 ± 11.6	19 ± 14.1	2
7.	TMV	12.9 ± 9.1	14.8 ± 10	1.7

Eosinophil index in unmanipulated guinea pigs: .2 ± .1 (references 1,5).

Analysis of variance (F tests) indicates significant differences ($p < .01$) among series within: (a) the protein group, and (b) the An:Ab group.

Multiple range tests indicate significant differences ($p < .05$): (a) Protein group—Series No. 1 differs from others. (b) An:Ab group:

Series No. 1 differs from No. 2, 5, 6, 7
" No. 2 " " No. 5, 6, 7
" No. 3 " " No. 6, 7

* Groups of 6 identically treated guinea pigs; An and An:Ab given into opposite hind foot pads.

† t—total number of cells; w—width, and l—length of each section; n—number of cross fields required for coverage of entire cross sectional area of a lymph node. (Cross field—area covered by one cross trip at ×470 microscopic study.)

‡ Protein (An) and soluble An:Ab complex (in antigen excess at 3× equivalence ratio) given in opposite sides.

TABLE III. Sampling of Eosinophil Cell Responses Within Sections of Popliteal Lymph Nodes Regional to Foot Pad Sites of Subcutaneous Injections of: (1) Equimolar Concentrations of Foreign Proteins (An), and (2) Equivalent Concentrations of Corresponding Soluble Antigen-Antibody Complexes (An:Ab) 6 Hours After Their Administration.*

	Dosages (mg)		Mol. ratio	Eosinophil index in lymph node sections $\frac{t}{\frac{w}{l}n^2}$† (mean ± S.D.) Foot pad treatment		An:Ab
Protein	An	An:Ab‡	Ab to An	An	An:Ab	An
BRN	.01	.05	.38	15.1 ± 17.2	28.2 ± 31.7	1.87
BSA	.05	.12	1	22.3 ± 8.5	40 ± 23.6	1.61
BGG	.12	.18	1.12	33.6 ± 35.7	60.4 ± 56.5	1.8
HSF	.35	1.91	1.75	21.8 ± 26.1	52.1 ± 35.9	2.39
HCN	5	5	17.5	56.7 ± 28	81.9 ± 47.9	1.34

Eosinophil index in unmanipulated guinea pigs: .2 ± .1 (references 1,5).

Analysis of variance (at 95% level of confidence): F tests: no significant differences within An and An:Ab groups. Multiple range tests: An groups—no differences; An:Ab groups—differences only in HCN from RNA and BSA.

* Groups of 6 identically treated guinea pigs; An and An:Ab given into opposite hind foot pads.

† See legend given in Table II for explanation of formula.

‡ Soluble antigen-antibody complexes prepared in antigen excesses at 3× molecular ratio in equivalence zone; dosages based upon calculations for $\frac{An_x Ab_y + 3\ An_x}{\text{mol. wts. } An_x Ab_y}$.

molecular aggregation by such non-specific mechanisms(10). This in turn could account for the lesser cellular inflammatory effects of injected gama globulin given in native state than as immune complex in an equal weight dosage.

The seven proteins representing preparations of molecular weights from 13,000 to 40,700,000 and their corresponding soluble immune complexes in antigen excesses demonstrated a uniform pattern of eosinophil effect. The cell responses seen at 6 hours after single treatment with the An:Ab complexes were significantly greater than those following administration of the protein (An) alone ($p<0.01$ by T test). F tests of variance among individual animals indicated significance for each group result ($p<0.05$ for the proteins and $p<0.01$ for An:Ab complexes). Differences were noted between animals grouped according to their treatments with equal weight dosages of: (1) low molecular weight protein—corresponding small sized An:Ab complex (BRN), (2) high molecular weight—large complexes (BTG, HCN, TMV), and (3) those of intermediate classification (BSA, BGG, HSF). The highest values recorded for eosinophil responses were grouped in inverse relationship to the sizes of effecting immune complexes (BRN>BSA, BGG, HSF>BTG, HCN, TMV). Among administered proteins that of the lowest molecular weight (BRN) was also the most effective eosinotactic agent. Eosinophil responses to the larger molecular weight proteins (BTG, HCN, TMV) closely approached those resulting from their corresponding antibody complexes.

The rapid diffusibility of smaller molecules (11) within tissue could be an important factor in their influence on the pathogenesis of cellular inflammation. However, when injected proteins or soluble complexes were given in dosages based upon equivalent numbers of moles, a different picture was found in this same model (Table III). By F test significance could not be attached to any differences that might relate the eosinophil responses to delineated molecular characteristics of those materials studied.

Reports of other investigators suggest that quantitative factors in antigen-antibody complex formation may be important in some animal models of hypersensitivity, *i.e.*, guinea pig anaphylaxis (12) and passive cutaneous anaphylaxis(13,14). Within the limits of our

experiments findings also suggest that the number rather than size of antigen-antibody complexes and molecular aggregates may be one critical factor in the degree of emerging eosinophilia. Specific characteristics of some of these agents however do pose questions for further definition. In the case of injected BRN a possible role for enzymatic activity as an eosinotactic stimulus has been considered. At this stage of investigation the observation that BRN evoked greater eosinophil responses than the other foreign proteins cannot be explained on the basis of size and numbers of given molecules to the total exclusion of possibilities for a specific biologic quality. Inability to prepare bovine lactalbumin free of contaminating BSA or insulin in pure monomer form prevented our additional study of comparable low molecular weight proteins and small size aggregates. The extent of influences created by thyroglobulin heterogeneity, its tendency to spontaneous precipitation(13), and the pH dependent states of association and disassociation of hemocyanin(15,16) requires further study. Eosinophil responses differed in two series of animals each given different lots of hemocyanin preparations in equal dosages. However, in similar experiments results with homogenous systems of RNA, BSA, BGG, and HSF were reproducible within defined ranges.

Reported phagocytic functions for the eosinophil(3,4) prompted our attempts to investigate the fate of these injected protein molecular aggregates and antigen-antibody complexes.§ We were especially interested in tracing possible intracellular localizations by immunohistochemical fluorescent techniques. However, extensive variations in the application of conventional procedures for the removal or minimization of interferent, unwanted fluorescein and rhodamine conjugated materials in labeled preparations were unrewarding. Our inability to effectively bypass the non-specificity of granulocytic leukocyte staining by fluorochromes agrees with the experiences of workers in this field(17,18).

Summary. Eosinophil infiltrations were found in guinea pig popliteal lymph nodes 6 hours after foot pad injections of purified proteins or corresponding soluble antigen-antibody complexes. Quantitated cell responses in this model served to evaluate eosinotactic influences of primary antigen exposure and of antigen-antibody union. Ribonuclease (BRN), serum albumin (BSA), gamma globulin (BGG), and thyroglobulin (BTG) of bovine origin; horse spleen ferritin (HSF), hemocyanin (HCN) from *Homarus americanus,* and tobacco mosaic virus (TMV) were used as representative purified proteins ranging in molecular weights from 13,000 to 40,700,000 and to prepare a spectrum of soluble complexes with rabbit antibody in moderate excesses of antigen. In equal weight dosages the smaller complexes and preparations of lower molecular weight proteins evoked significantly greater eosinophilia (BRN>BSA, BGG, HSF>BTG, HCN, TMV). When dosages of the preparations from specific proteins (BRN, BSA, BGG, BTG, HCN) were based on equivalent numbers of moles, significant differences were not found. It is suggested that the number rather than size of presenting antigen-antibody complexes or protein molecular aggregates may be one critical factor in the degree of emerging eosinophilia of inflammatory cell responses.

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1. Cohen, S. G., Sapp, T. M., Rizzo, A. P., Kostage, S. T., J. Allergy, 1964, v35, 346.

2. ———, ibid., 1965, v36, 415.

3. Sabesin, S. M., Proc. Soc. Exp. Biol. Med., 1963, v112, 667.

4. Archer, G. T., Hirsch, J. G., J. Exp. Med., 1963, v188, 287.

5. Cohen, S. G., Kostage, S. T., Rizzo, A. P., J. Allergy, 1966, in press.

6. Weigle, W. O., Cochrane, C. G., Dixon, F. J., J. Immunol., 1960, v85, 469.

7. Ishizaka, K., Ishizaka, T., Campbell, D. H., J. Exp. Med., 1959, v109, 127.

8. ———, J. Immunol., 1960, v85, 163.

9. Menefee, M. G., Mueller, C. B., Bell, A. L., Myers, J. K., J. Exp. Med., 1964, v120, 1129.

10. Morse, J. H., J. Immunol., 1965, v95, 722.

11. Waksman, B. W., Bocking, D., Proc. Soc. Exp. Biol. Med., 1953, v82, 738.

12. Prusansky, J. J., Feinberg, S. M., Harris, M., Wojcik, N., J. Immunol., 1960, v85, 288.

13. Leskowitz, S., Ovary, Z., Immunology, 1962,

v5, 1.
14. Levine, B. B., J. Immunol., 1965, v94, 121.
15. Bartel, A. H., Campbell, D. H., Arch. Biochem., 1959, v82, 232.
16. Malley, H., Salia, A., Halliday, W. J., J. Immunol., 1965, v95, 141.
17. Mellors, R. C., Analytical Cytology, ed. 2, McGraw-Hill Book Co., N. Y. 1959, p16.
18. Nairn, R. C., Fluorescent Protein Tracing, E. &. S. Livingston, Ltd., Edinburg, 1962, p120.

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Metabolic Inhibitor(s) in Fractions of Orchardgrass (*Dactylis glomerata* L.) Detected by *in vitro* Rumen Fermentation Technique.* (31917)

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The possible presence of a metabolic inhibitor in orchardgrass (*Dactylis-glomerata* L.) hay grown on some Missouri farms was indicated by Hargus(1). Lambs fed either chopped orchardgrass or orchardgrass pellets supplemented with proteins, vitamins and minerals to meet the requirements set forth by the National Research Council failed to grow and showed symptoms of stiffness.

We conducted a series of investigations to find a suitable screening technique for detecting the active factor(s). One of the techniques selected for study was the *in vitro* rumen fermentation technique.

This technique has been used previously to study nonprotein nitrogen utilization(2,3), protein digestion(4), fatty acid production and utilization(5-7), cellulose digestion and factors affecting it (8-14), starch fermentation (15), and roughage evaluation(1,16,17).

This paper describes the technique developed as a screening tool to detect the presence of the metabolic inhibitor(s). Results of the preliminary fractionations are included. The *in vitro* rumen fermentation technique was modified from Garner(18). The inhibition of cellulose digestion by fractions of orchardgrass was considered as an indication of the presence of metabolic inhibitor(s) since the amount of cellulose digested reflects the metabolic activity of rumen microorganisms.

Preparation of nutrient solution and buffers: Nutrient solution was prepared by dissolving 1 g sodium carbonate, 920 mg vitamin free casein, 160 mg ammonium carbonate, 160 mg dextrose, and 160 μg cobalt chloride in 200 ml of distilled water. Equal parts of solutions A and B form a modified Hungate's artificial saliva(19); A was prepared by dissolving 3 g K_2HPO_4 in one L of distilled water; B by dissolving 6 g NaCl, 3 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.6 g $MgSO_4$ and 0.6 g $CaCl_2$ in one L of distilled water. Buffer solution C was prepared by dissolving 10 g $NaHCO_3$ in 100 ml of distilled water.

Preparation of substrate: Alfalfa and orchardgrass were ground through a 1 mm screen in a Wiley mill. Seven hundred mg of original substrate or a residue containing the same amount of cellulose as 700 mg hay was transferred into 50 ml glass tubes referred to as reaction tubes. To each reaction tube 5 ml nutrient solution, 5 ml Hungate's artificial saliva solution, 1 ml buffer solution C and 6 ml distilled water were added. Different levels of extracts of orchardgrass and extractants were added at the expense of distilled water. When organic solvent extractants or extracts were added, they were removed by evaporation over a water bath before other solutions were added. Alkali extract and extractant were neutralized to pH 7.0 before use. Reaction tubes were placed in a water bath at 39°C. Carbon dioxide, pretreated by passing it through nutrient solution, buffers

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