

Chemotaxis of eosinophils and neutrophils by aggregated immunoglobulins

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Human myeloma proteins of all recognized immunoglobulin classes, including IgE, were purified and chemically aggregated. The chemotactic properties of these aggregated immunoglobulins for enriched preparations of human eosinophils and neutrophils were studied in vitro with the use of a modification of the Boyden chamber. Significant chemotactic effects were exerted on eosinophils and neutrophils by IgG and IgM in the presence of fresh human serum. IgE did not attract eosinophils either alone or in the presence of fresh serum. Inhibition of chemotaxis was observed when fresh human serum was heated at 56° C. for 30 minutes and when the same concentration of chemotactic agents was placed in both compartments of the chambers. The results indicate that aggregated IgG and IgM, in the presence of fresh human serum, generate chemotactic factor(s) for eosinophils and neutrophils and that heat-labile serum factors are essential for chemotaxis of these cells. These results are discussed in terms of the eosinophil and neutrophil chemotactic factors derived from the complement system, ECF-C and NCF-C, and the eosinophil chemotactic factor of anaphylaxis, ECF-A, associated with immediate-type hypersensitivity reactions.

The presence of eosinophils in blood and tissues has long been associated with allergic reactions. Studies^{1, 2} in intact experimental animals have shown that eosinophils accumulated in tissues following injections of antigens and at the sites of antigen-antibody deposition. In the guinea pig, preformed antigen-antibody complexes of both the IgG₁ and IgG₂ classes induced eosinophil infiltration, whereas following passive cutaneous anaphylaxis only homocytotropic IgG₁ antibody promoted local accumulation of eosinophils.³ In human beings, blood eosinophilia provoked by penicillin reactions was associated with skin-sensitizing antibody, presumably IgE, and not with IgM and IgG antibodies.⁴ Finally, antigen-antibody complexes containing rabbit IgG in the presence of fresh serum attracted human eosinophils.⁵

Because of the prominence of eosinophils in allergic reactions associated with IgE antibodies, we have analyzed the chemotactic properties of aggregated immunoglobulins of all known classes, including IgE, for human eosinophils and neutrophils. The results indicate that in the presence of fresh serum only those immunoglobulins able to fix complement attract eosinophils and neutrophils.

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MATERIALS AND METHODS

Preparation of leukocytes

Human eosinophils were obtained from the peripheral blood of 2 patients who had eosinophilia of greater than 50 per cent in the peripheral blood and another patient with eosinophilia of 45 per cent. One patient had a diagnosis of chronic eosinophilic pneumonia, another had eosinophilia associated with episodic edema and pretibial nodules, while the third patient had bronchial asthma. Venous blood was drawn in a heparinized, plastic syringe which was placed in a vertical position with the plunger serving as the base. Sedimentation of the erythrocytes usually occurred within one to 2 hours at room temperature. The leukocyte-rich plasma was collected in plastic centrifuge tubes with Medium 199 (Grand Island Biological Company, Grand Island, N. Y.). The cell suspension was mixed, washed twice by centrifugation at low speed (500 g for 3 minutes), and the leukocyte pellet resuspended in 10 per cent heat-inactivated (56° C. for 30 minutes) autologous serum in Medium 199 (M199). One volume of 2 per cent Tween- 80 was added to nine volumes of the cell suspension during the centrifugation procedure to prevent clumping. Neutrophil leukocytes were obtained from patients with various inflammatory states (acute pancreatitis, intestinal obstruction, urinary tract infection, cellulitis, and acute myocardial infarction) who had greater than 90 per cent neutrophils in the peripheral blood.* Neutrophils were collected and processed by the same methods described for eosinophils. Total cell counts on the final suspension for both types of cells ranged from 4.5 to 5.8×10^6 cells per milliliter, 75 to 92 per cent eosinophils and 93 to 99 per cent neutrophils. None of these cell preparations was less than 80 per cent viable as tested by trypan blue exclusion.

Aggregation of immunoglobulins

Myeloma proteins representing the five major immunoglobulin classes, as well as the IgG₁, IgG₂, and IgG₃ subclasses,† were purified by various combinations of starch-block preparative electrophoresis, ion-exchange chromatography on diethylaminoethyl cellulose, and gel filtration with Sephadex G-200. Purified myeloma proteins were aggregated by treatment with bis-diazotized benzidine as described by Ishizaka and co-workers.⁶ After aggregation, the immunoglobulins were dialyzed at 4° C. against 0.15M NaCl and 0.01M K₂HPO₄-KH₂PO₄ (PBS), pH 7.4, and shown to be free from other immunoglobulins and proteins by immunodiffusion analysis against antisera specific for each of the five classes of immunoglobulins and against antiserum to whole human serum. The concentration in micrograms of nitrogen per milliliter of the immunoglobulin preparations during immunodiffusion analysis were: IgG₁, 285; IgG₂, 280; IgG₃, 88; IgA, 292; IgD, 302; IgE, 336; and IgM, 66. The concentration of the immunoglobulins was determined by their absorbance at 280 nm.

For the IgG subclasses, the extinction coefficient of normal IgG, $E_{1\text{cm.}}^{1\%} = 14.1$, was employed, and for IgE a coefficient of $E_{1\text{cm.}}^{1\%} = 13.5$ was assumed. In the case of the other proteins, extinction coefficients were determined by the absorbance of known concentrations determined by prior Kjeldahl analyses, assuming a nitrogen content of 16 per cent. The coefficients were: IgA, $E_{1\text{cm.}}^{1\%} = 14.2$; IgM, $E_{1\text{cm.}}^{1\%} = 13.3$; and IgD, $E_{1\text{cm.}}^{1\%} = 17.0$. The aggregated proteins were stored at -60° C. until the day of the experiment. After thawing these preparations were free from bacterial contamination as shown by appropriate cultures.

Fresh normal human serum (NHS)

Fresh normal human serum was used to supply serum factor(s), presumably complement components, necessary for chemotaxis. The same donor with a complement level of 63 units

*In preliminary experiments neutrophils from one normal subject migrated in response to aggregated IgG₁ and fresh normal human serum.

†The IgG subclass of these myeloma proteins was determined by Dr. W. D. Terry.

TABLE I. Chemotaxis of eosinophils by aggregated immunoglobulins

Materials tested*	Chemotactic activity†				
	1	2	3	4	5
IgG ₁ + NHS	791	512	1,407	497	284
IgG ₂ + NHS	633	446	972	72	119
IgG ₃ + NHS	166	872	301	222	314
IgA + NHS	65	54	38	19	12
IgD + NHS	29	39	45	NT	61
IgE + NHS	112	74	89	72	8
IgM + NHS	664	447	516	506	131
IgG ₁	107	46	54	21	6
IgE	43	34	44	10	6
IgM	NT	NT	NT	4	NT
NHS	60	43	36	39	26
M199	16	18	20	58	5

NT = Not tested.

*Immunoglobulins were tested at quantities between 32 to 47 μ g of N per bottom chamber.†Ten areas of the membrane were examined under light microscopy with a 10 $\times$ ocular and a 45 $\times$ objective, and cells which had migrated completely through the membrane were counted.

(normal 41 to 90) was used as the source of serum for all experiments, and only serum obtained on the day of an experiment was used.

Chemotaxis

Sykes-Moore chambers (Belleo Glass Company, Vineland, N. J.) were used in all experiments to analyze chemotaxis. The two compartments of the chamber were separated by a Millipore filter, Type SSWP 025 SS (Millipore Filter Corporation, Bedford, Mass.), with a 3 μ pore size. This size was selected in accordance with experiments performed by Keller and Sorkin⁷ on rabbit leukocytes and preliminary experiments performed by us with human neutrophils. The upper compartments of the chamber were charged with 1.0 ml. of cell suspension (4.5 to 5.8×10^6 cells per milliliter) in 10 per cent heat-inactivated autologous serum in M199, while the lower compartments were charged with aggregated immunoglobulins, NHS, M199, or combinations of these in a volume of 0.5 ml. After incubation for 4 hours at 37° C., the filters were removed, stained, and cleared in xylene as described by Boyden.⁸ The filters were placed on glass slides, overlaid with cover glasses, and mounted with Permunt (Fisher Scientific Company, Chicago, Ill.). Only cells which had migrated completely through the filter were counted.

Electron microscopy

Portions of filters from chambers containing eosinophil preparations were fixed in buffered glutaraldehyde, postfixed in osmium tetroxide, and prepared in the usual manner for electron microscopic examination. Thin sections were double stained with uranyl acetate and lead acetate and examined with an RCA EMU 3E electron microscope.

RESULTS

Experiments to test the chemotactic effects of aggregated immunoglobulins on eosinophils were performed on five different occasions over a period of several months with the use of cell preparations from 3 patients. Results of these experiments are shown in Table I. Although some variability in the results for immunoglobulins of the same class was noted in the different experiments, it is clear that in the presence of NHS both IgG and IgM were chemotactic.

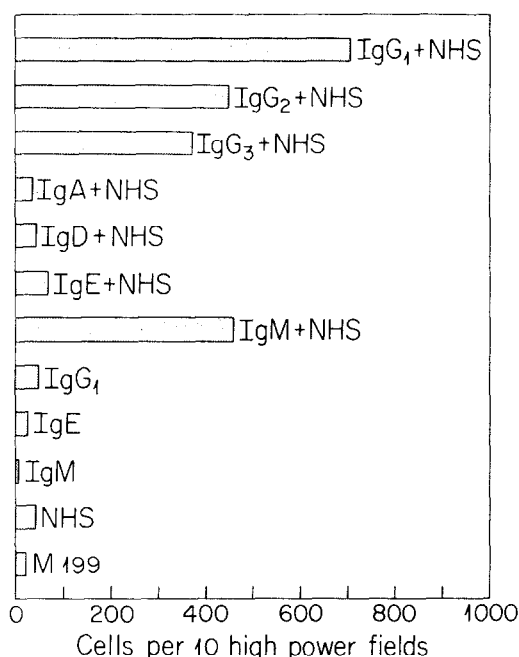


FIG. 1. Chemotaxis of eosinophils by aggregated immunoglobulins. Results represent mean values from the separate experiments listed in Table I.

Also, all three IgG subclasses were active in these experiments. IgA, IgD, and IgE were not active either alone or in the presence of fresh NHS. In Fig. 1 the mean cell counts from the five different experiments are illustrated. Because the preparations of eosinophils were contaminated with small numbers of other cell types, chiefly neutrophils (up to 17 per cent) and lymphocytes (up to 12 per cent), and because our staining procedure did not permit differentiation of eosinophils and neutrophils,⁵ we examined the filters by electron microscopy in one experiment to determine whether eosinophils were the responding cells. All the cells identified in experiments with IgG₁ and IgM had the typical fine structure of eosinophils with bilobed nuclei and large electron-dense granules. In one experiment, a marked diminution of chemotaxis (72 per cent) was seen when IgG₁ was incubated with fresh human serum heated at 56° C. for 30 minutes.

Chemotaxis of neutrophils by the various immunoglobulin classes was also studied. Neutrophils appeared to migrate more actively than did eosinophils, and as shown in Table II our initial results suggested that all the immunoglobulin classes were active in the presence of NHS. IgG₁ was especially active in these experiments, resulting in a five-to-tenfold increase in cell migration. Because of the marked chemotaxis by IgG₁, we analyzed the various immunoglobulin preparations by a very sensitive immunodiffusion technique with the use of a plastic matrix⁹ and found that the preparations of IgA, IgD, and IgE contained trace amounts of IgG. To determine whether these trace quantities of IgG might be responsible for the chemotaxis observed with

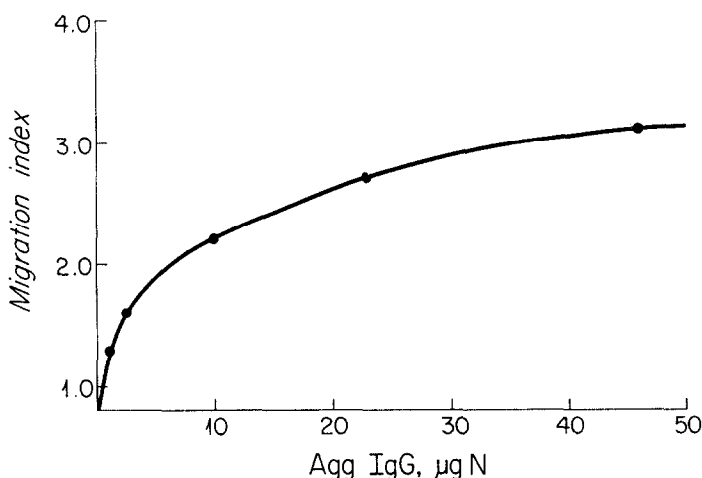


FIG. 2. Chemotaxis of neutrophils by aggregated IgG₁. The migration index equals migrating cells in test preparations divided by migrating cells in the NHS control.

TABLE II. Chemotaxis of neutrophils by aggregated immunoglobulins

Materials tested*	Chemotactic activity†		
	1	2	3
IgG ₁ + NHS	338	1,622	2,308
IgG ₂ + NHS	207	NT	NT
IgG ₃ + NHS	365	NT	NT
IgA + NHS	60	916	389
IgD + NHS	312	965	577
IgE + NHS	293	NT	NT
IgM + NHS	139	922	526
IgG ₁	49	NT	NT
IgE	65	NT	NT
IgM	NT	NT	NT
NHS	68	351	206
M199	30	67	159

NT = Not tested.

*Quantities of aggregated immunoglobulins varied from 32 to 47 μg of N per bottom chamber.

†Sells per 10 high-power fields.

IgA, IgD, and IgE, a dose response curve using IgG₁ was obtained to establish the minimum amount of IgG necessary to produce an increase in chemotactic activity. As shown in Fig. 2, as little as 1 to 2 μg of N aggregated IgG₁ produced a significant chemotactic effect on neutrophils. These findings suggested that the trace quantities of IgG present as a contaminant in the IgD, IgA, and IgE aggregates may have resulted in their chemotactic activity. To determine whether aggregated IgA free from IgG could promote chemotaxis, IgA was purified from myeloma serum by starch-block preparative electrophoresis, diethylaminoethyl cellulose ion-exchange chromatography, and Sephadex G-200 gel filtration. Following each purification step, only those peak fractions with the highest concentrations of IgA were pooled. Analysis of this IgA preparation by the same immunodiffusion technique as above did not reveal the presence of

TABLE III. Chemotaxis of neutrophils by purified IgA

Materials tested	Chemotactic activity*		
	1	2	3
IgG ₁ + NHS	5,167	6,394	2,261
IgA + NHS	220	1,014	383
IgG ₁	1,661	2,036	394
IgA	114	750	302
NHS	1,515	1,270	394
M199	946	716	269

*Cells per 10 high-power fields.

IgG. As shown in Table III, highly purified aggregated IgA was not chemotactic for neutrophils. An experiment to determine whether heat-labile serum factor(s) were necessary for chemotaxis revealed that heating of fresh serum at 56° C. for 30 minutes destroyed its ability (virtually 100 per cent inhibition) to mediate chemotaxis in the presence of aggregated IgG₁. The necessity for a gradient during chemotaxis was shown in several experiments; when IgG₁ and fresh human serum were placed in both the upper and lower compartments of the same chamber, cell migration was decreased by 70 to 90 per cent.

DISCUSSION

The development of an in vitro technique for studying chemotaxis of cells by Boyden⁸ and the observation by Ishizaka and associates⁶ that chemically aggregated immunoglobulins possessed biologic activities similar to antigen-antibody complexes made available tools to investigate the role of the various immunoglobulins in chemotaxis of leukocytes. More recently, the availability of myeloma proteins of the IgE class have made possible experiments to determine whether IgE is involved in eosinophilotaxis. Our experiments with human eosinophils indicate that only those aggregated immunoglobulins, such as IgG and IgM, which have the capacity to fix complement exert a clear-cut chemotactic effect. Similar results using a different testing system were obtained by Ishikawa and associates.¹⁰ Although we did not characterize the chemotactic factors themselves, the need for fresh serum, the inactivation of serum by heating, and the lack of activity of non-complement-fixing aggregated immunoglobulins suggest that the chemotactic factors were derived from the complement system. The prior experiments of Ward and associates^{5, 15, 16} and Lachman and co-workers¹¹ have shown that both high and low molecular weight factors, C567, C3a, and C5a, are chemotactically active. In the case of neutrophils, aggregated immunoglobulins of the IgG and IgM classes clearly exerted a chemotactic effect. The interpretation of the results with those from the other immunoglobulins is complicated by the presence of small amounts of IgG contaminating these preparations. As shown in Fig. 2, quantities of IgG of the order of 1 to 2 µg of N exerted a significant chemotactic effect in the presence of fresh NHS. When IgA was highly purified, it was devoid of chemotactic activity, and we believe the activity seen with the IgD and IgE preparations is explicable on the basis of contamination with

IgG. In these experiments, neutrophils appeared to migrate more actively than did eosinophils, and, because of their marked response to IgG₁, it is evident that only highly purified preparations of the other Ig classes can be used to test with neutrophils.

Our results indicate that IgE does not promote chemotaxis of eosinophils in the presence of serum factors and thus are at odds with the known association of reaginic antibodies and eosinophils.⁴ Recently Kay and associates¹² described an eosinophilotactic factor associated with anaphylaxis in the guinea pig. In their experiments, chopped lung sensitized with homocytotropic IgG₁ antibodies (but not IgG₂ antibodies) and exposed to the homologous antigen released a chemotactic factor along with the slow-reacting substances of anaphylaxis (SRS-A) and histamine. Serum factors did not appear to be necessary for the release of this chemotactic factor, and it has been termed the eosinophil chemotactic factor of anaphylaxis (ECF-A) to distinguish its activity from these factors derived from complement components, ECF-C.* Kay and associates¹³ have also described the release of ECF-A from human lung sensitized with serum from subjects allergic to ragweed pollen. In preliminary studies with the skin window technique, we have found that eosinophils appear in larger numbers when aggregated IgE is applied to skin sites than when IgG₁ or PBS were applied, a result in keeping with the findings of Kay and associates mentioned above. Finally, another eosinophilotactic factor derived from sensitized lymphocytes has been described by Cohen and Ward¹⁴ and may be of biologic importance in inflammatory or immunologic reactions.

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*Using the same terminology, NCF-C, for neutrophil chemotactic factor, refers to materials derived from the complement system (12).

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