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A QUANTITATIVE *IN VITRO* STUDY OF THE CHROMATOGRAPHIC DISTRIBUTION AND IMMUNOGLOBULIN CHARACTERISTICS OF HUMAN BLOCKING ANTIBODY¹

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Two antibodies with markedly different biologic activities are of importance in human allergic disorders. Reaginic antibody, which is in some fashion "fixed" to the target cells and thereby mediates the allergic response, has been extensively studied (1, 2). Recent work by Ishizaka suggests that it is a hitherto unrecognized immunoglobulin, IgE (3). Other workers, however, have reported that reaginic activity is not limited to a single group of immunoglobulins (4).

Quite different in its postulated activity is the so-called "blocking antibody" (5, 6). This antibody, being unable to "fix" to cells, can be defined by its ability to block the allergic response presumably by combining with the antigen in fluid phase. In this fashion it prevents antigen from reaching the cellular antibody and triggering an allergic reaction. Blocking activity was first recognized in the sera of patients who had received immunotherapy with extracts of antigens from pollens to which they were sensitive (6). More recently it has been recognized that the sera of all allergic individuals, whether immunized or not, contain blocking as well as reaginic antibody, although the titer of the former (but not the latter) is markedly increased by immunotherapy (7, 8).

Attempts to isolate and characterize blocking antibody were largely carried out before techniques for the typing of immunoglobulins by their antigenic structure had been developed (9-13). These studies were limited by the lack of precision in skin assays of blocking activity as well as by the use of crude extracts of pollen which are recognized to contain multiple antigens.

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² American College of Physician's Research and Teaching Scholar.

Because of the difficulties involved in the skin assay, several *in vitro* methods of measuring circulating anti-pollen antibodies were developed. These include hemagglutination (14, 15), fluid phase combination with labeled antigens (16) and radioimmuno-electrophoresis (17). With the latter it has been possible to show ragweed binding antibodies in all immunoglobulin classes. The relationship between antibody measurements obtained with these techniques and those obtained in the skin assay is not clear.

Several developments made another attempt to characterize blocking antibody appear reasonable. As already mentioned, the antigenic classification of immunoglobulins is now in general use. In addition, a technique involving the antigenically induced release of histamine from isolated human leukocytes has been described and found applicable to studies of blocking antibody (18). This bioassay provides an increase in precision of more than 10-fold as compared to the skin assay and has been demonstrated to be a pertinent model of human pollinosis (7, 19, 20). Finally, the most potent of the ragweed allergens (antigen E) has been isolated from crude ragweed extract (21, 22). With these tools serum has been fractionated by several chromatographic methods and characterized as to blocking activity and immunoglobulin type. The precision of the *in vitro* bioassay has allowed us to assess quantitatively the recovery of total serum blocking activity in each immunoglobulin class.

MATERIALS AND METHODS

Serum. A large pool of allergic serum was obtained from 20 ragweed-sensitive donors who had been immunized with ragweed antigen E.³

³ The antigen E used for immunotherapy was prepared by Abbott Laboratories for the National Institutes of Health committee on Standardiza-

The patients had received an average total dosage of 0.3 mg given in 15 weekly injections from May to mid-August, 1965 (19). Sera were obtained 2 to 4 weeks after the last immunization, pooled and frozen in aliquots at -20°C without preservative. The inhibiting activity of each serum was determined. In terms of the concentration of serum required to cause a 50% inhibition of histamine release the titers varied over about a fivefold range. The control serum was from a nonallergic donor.

Antigen. Antigen E, a highly purified protein with a molecular weight of about 38,000, was kindly provided by Dr. T. P. King, Rockefeller University. Previous study has shown this to be the most potent allergen in ragweed pollen (21, 22).

Antibody assays. Details of the leukocyte histamine release system and of the methods of antibody assay have been reported (7, 18, 20). For the latter, cells were prepared from a donor whose dose-response curve to antigen E was known. A quantity of antigen adequate to release 70 to 80% of the total cellular histamine in the absence of blocking antibody was mixed with duplicate dilutions of the allergic serum (AHS) or the fractions thereof. Controls were prepared in normal serum (NHS). The serum concentration in all tubes was maintained at 10% through the addition of normal serum. After 30 to 60 min of incubation at 20°C , identical amounts of cell suspension were added to each of the above preparations which were then incubated at 37°C for 60 min. The percentage of the total histamine released (HR) in each tube was determined and the percentage inhibition calculated according to the formula:

$$\frac{\% \text{HR}_{\text{NHS}} - \% \text{HR}_{\text{AHS}}}{\% \text{HR}_{\text{NHS}}}$$

A plot was constructed for serum (or serum fraction) concentration *vs.* percentage inhibition and the concentration required for 50% inhibition of histamine release interpolated. These 50% end points provided a basis for comparisons between the activity of serum and the fractions obtained by chromatography.

Immunoglobulin determinations. Qualitative immunoglobulin determinations were performed by

immunodiffusion in 2% agar in Veronal buffer, pH 8.6, on microscope slides, following the procedure of Ouchterlony (23). The antisera used were obtained from Hyland Laboratories, Los Angeles, Calif. Quantitative immunodiffusion was carried out by a modification of the Preer technique (24).

Column chromatography. Ion exchange chromatography was performed at room temperature. Elution of proteins was monitored at 280 or 230 $\text{m}\mu$ with a Gilford model 2000 Absorbance Meter coupled with a Radiometer CDM-2 Conductivity Meter for continuous measurement of the conductivity of the effluent. Flow cells in the spectrophotometer were maintained at 20°C .

Diethylaminoethyl (DEAE)-Sephadex. Thirty to 50 ml of the pooled human serum were dialyzed at 4°C against 0.03 M potassium phosphate buffer, pH 7.8. The slight precipitate was removed by centrifugation and the supernatant fluid warmed and applied to a 35 by 2.5 cm column of DEAE-Sephadex A-50⁴ equilibrated with the same buffer. Following application of the sample, the column was rinsed with this buffer until all of the nonbinding protein passed through the column. A continuous gradient was then applied, using a variable gradient multi-chambered device⁵ with 400 ml of 0.03 M potassium phosphate, pH 7.8, in the first chamber; 400 ml of 0.15 M potassium phosphate, pH 7.8, in the second chamber; and 400 ml of 0.5 M potassium phosphate, pH 7.8, in the third chamber. Three fractions were pooled and concentrated in Schleicher and Schuell collodion bags under vacuum.

Carboxymethyl (CM)-cellulose. Concentrated fraction II was dialyzed overnight at 4°C against 0.01 M potassium phosphate, pH 5.9. The slight precipitate was removed by centrifugation and the supernatant fluid warmed and applied to a 30 by 2.5 cm column of Whatman CM-52 cellulose equilibrated with the same buffer. The sample was followed by the 0.01 buffer until the nonbinding protein was completely eliminated from the column. A continuous gradient was then applied with 400 ml of 0.01 M potassium phosphate, pH 5.9, in the first chamber and 400 ml of 0.20 M potassium phosphate, pH 5.9, in the

⁴ Pharmacia Corporation, New York, New York.

⁵ Varigrad, Buchler Instrument Company, Fort Lee, New Jersey.

tion of Allergens. Immunization was carried out by Drs. P. S. Norman and W. L. Winkenwerder.

second chamber. Including the breakthrough, three fractions were pooled and concentrated.

Sephadex G 200. Four milliliters of fraction IIb from the CM-cellulose were dialyzed against Tris-NaCl buffer (0.15 M) and applied to a column of Sephadex G 200, 92 by 2.5 cm, equilibrated with the same buffer at 4°C. Elution was with the Tris buffer. IgM was obtained by molecular sieve filtration of 3 to 4 ml of whole human serum on the same column.

DEAE-cellulose. Fraction IIc from the CM-cellulose was dialyzed overnight at 4°C against 0.01 M potassium phosphate, pH 7.8. The slight precipitate was removed by centrifugation and the supernatant was applied to a Whatman DE-52 cellulose column 20 by 1 cm equilibrated with the same buffer. Following elimination of the nonbinding protein, a continuous gradient was started with 100 ml of 0.01 M potassium phosphate, pH 7.8, in the first chamber; 100 ml of 0.10 M potassium phosphate, pH 7.8, in the second chamber; and 100 ml of 0.2 M potassium phosphate, pH 7.8, in the third chamber. Four fractions were collected.

RESULTS

Chromatography of whole serum on DEAE-Sephadex. Continuous gradient chromatography of whole serum on DEAE-Sephadex is shown in Figure 1. Fractions were collected on the basis of absorbance measurements at 280 m μ and concentrated by negative pressure dialysis. From this first chromatographic step 3 fractions were pooled: fraction I, the breakthrough peak; fraction II, the material eluting after the breakthrough and before the albumin peak; and fraction III, the albumin peak. Fraction I consisted of "clean" IgG; we were unable to detect any line but that of IgG by immunodiffusion against specific anti-immunoglobulin sera, and anti-whole human serum gave a single line which fused with that produced by the anti-IgG serum. Quantitative Preer measurements of the IgG and IgA in fraction I indicated that contamination by the latter was less than one part in 1000. Fraction II contained 39% IgA and 61% IgG.⁶ Fraction III was largely albumin with traces of IgG and IgA. IgM could not be detected by immunodiffusion in

⁶ These and subsequent percentage figures refer to the per cent of total immunoglobulin present rather than per cent of total protein.

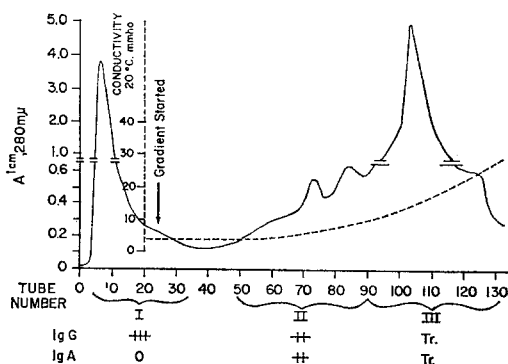


Figure 1. Continuous gradient elution pattern of serum applied to DEAE-Sephadex, K-phosphate, pH 7.8, 25°C. Starting buffer was 0.03 M. The gradient varied from 0.03 to 0.5 M.

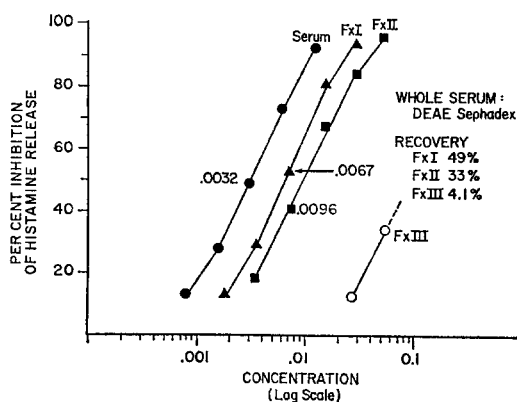


Figure 2. Blocking activity of whole serum and fractions obtained by chromatography on DEAE-Sephadex. The concentration of fractions is based on volume alterations as explained in text. Recovery is relative to the activity of whole serum.

fraction I, II or III even when they were highly concentrated, and we assume that the IgM remained on the column (see below). The table below Figure 1 notes the qualitative distribution of IgG and IgA found on six different chromatograms of pooled serum on DEAE-Sephadex.

Blocking activity of fractions I, II, and III. The blocking activity of serum or serum fractions is demonstrated by the ability to combine with ragweed antigen E in the fluid phase, thereby decreasing the number of antigen molecules able to interact with cell bound antibody to release histamine. Figure 2 shows the inhibition of histamine release (relative to control) which resulted

when allergic serum or the fractions obtained by DEAE-Sephadex chromatography of this serum were assayed on the cells of a single allergic donor. Several dilutions of serum and of each fraction were assayed; this allowed the construction of a plot of inhibition *vs.* concentration and the interpolation of the concentration of serum or serum fraction required for 50% inhibition. The concentrations attributed to the serum fraction are corrected for volume alterations incurred in passing through the column, dialysis, and concentration. For example, if 40 ml of serum were added to the DEAE-Sephadex column and fraction I was collected in 400 ml and concentrated to 20 ml, this material was considered to be twice as concentrated as serum. We assumed no loss of material in the chromatography, dialysis or concentration. Calculations related to percentage recovery were based on 50% inhibition of the control. In the experiment shown, the pooled serum provided 50% inhibition at a concentration of 0.0032, that is at a final dilution of 1:313; it took slightly more than twice as much of fraction I to achieve 50% inhibition so that the recovery in this fraction was 49%. From similar data (Fig. 2) it can be seen that fraction II had 33% of the total blocking activity and fraction III had about 4.0%. In several runs of serum on DEAE-Sephadex the recovery of blocking activity was $70 \pm 10\%$. Somewhat more than half of the blocking activity was contained in fraction I and somewhat less than half in fraction II. Fraction III had 1 to 4% of the total.

Fraction III was subsequently concentrated and rechromatographed on Sephadex G 200. Blocking assays and qualitative immunodiffusion studies were carried out on a tube by tube basis and the antibody activity paralleled the elution of IgG. Since a negligible amount of the total activity was found in this fraction no further analysis was attempted.

Chromatography of fraction II on CM-cellulose. Fraction II, containing both IgG and IgA (but without detectable IgM), was concentrated, dialyzed against 0.01 M phosphate buffer at pH 5.9 and applied to a CM-cellulose column. Three peaks emerged (Fig. 3): the breakthrough, fraction IIa, and two closely spaced later peaks, IIb and IIc. Results of qualitative immunodiffusion on the concentrated fractions are shown in Figure 3. Quantitative Preer analysis showed that fraction IIa was 90% IgA and 10% IgG, IIb was

44% IgA and 56% IgG, and IIc was 10% IgA and 90% IgG. The IgG concentration of fraction IIc was about twice that of IIb.

Blocking activity of fractions IIa, IIb, and IIc. Assays of the fractions obtained by chromatography of fraction II on CM-cellulose were performed as outlined above (Fig. 4). Fraction IIa, which was largely IgA, had well under 1% of the total activity. Fraction IIb had 35% and IIc 65% of the blocking activity recovered from fraction II; this paralleled the elution of IgG, which was twice as concentrated in fraction IIc as in IIb. Recovery of blocking activity on this column was about 60%. It is of interest that the specific blocking activity of fraction IIa was (on a weight basis) less than 1% that of the IgG in fraction I, although the contamination of this IgA-rich fraction with IgG is considerably greater than 1%.

Further analysis of fraction IIb. Fraction IIb, containing about 35% of the blocking activity in fraction II, was first applied to a DEAE-cellulose column; this achieved no further separation of the proteins. The fraction was then applied to a Sephadex G 200 column and the effluent was pooled 2 to 4 tubes at a time. Each fraction was concentrated and analyzed qualitatively by immunodiffusion against specific anti-immunoglobulins. Finally, the blocking activity in each tube was measured. As expected, the IgA emerged somewhat earlier from Sephadex G 200 than the IgG, although there was no clean separation of the two immunoglobulins. In spite of this, it is clear from Figure 5 that blocking activity eluted with the IgG rather than with the IgA.

Further analysis of fraction IIc. The major part of the blocking activity of fraction II, concentrated in fraction IIc, was put on a DEAE-cellulose column (Fig. 6). The IgG was most concentrated in the breakthrough peak and decreased in each succeeding peak, while the IgA concentration was highest in the third peak. The blocking activity once again eluted with the IgG molecules.

Blocking activity of IgM. IgM was obtained by chromatography of whole allergic serum on Sephadex G 200. A preparation containing IgM about half as concentrated as that in the starting serum had no significant blocking activity. These data are of questionable value, however, since a serum factor which enhances histamine release has been found to elute from Biogel P 300 with

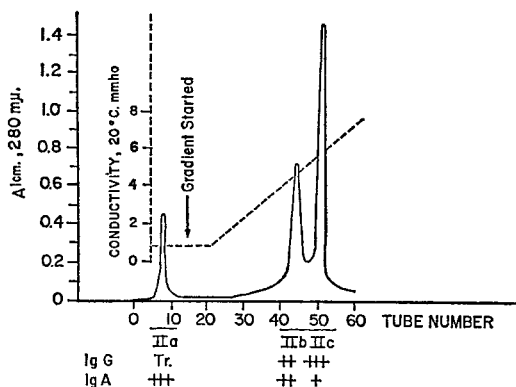


Figure 3. Continuous gradient elution pattern of fraction II applied to CM-cellulose, K-phosphate, pH 5.9, 25°C. Fraction II was obtained from DEAE-Sephadex chromatography of whole serum (Fig. 1). Starting buffer was 0.01 M. Gradient varied from 0.01 to 0.20 M.

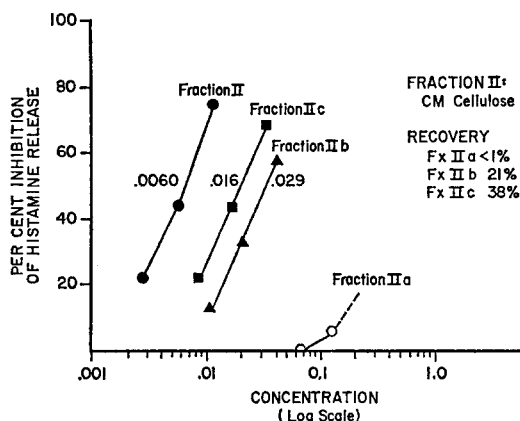


Figure 4. Blocking activity of fraction II and subfractions obtained by chromatography on CM-cellulose. Recovery of subfractions is relative to the activity of fraction II.

the macroglobulins (25). This material is associated with serum lipoprotein and can be removed by ultracentrifugation in a solution of 1.21 density. The procedure was carried out on the above sample with the help of Dr. S. Margolis, and the remaining IgM, dialyzed and concentrated to the original volume, once again demonstrated no appreciable blocking activity. Since the starting serum inhibited 50% at a dilution of greater than 1:300 and an IgM preparation half as concentrated as that in serum did not block, it follows that the IgM contributed under 1% of the total serum blocking activity.

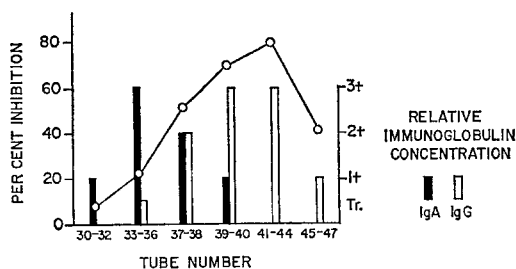


Figure 5. Blocking activity and relative immunoglobulin concentrations of eluates of subfraction IIb after chromatography on Sephadex G 200. Fraction IIb was obtained by chromatography of fraction II on CM-cellulose (Fig. 3).

Rechromatography. To demonstrate that the discrete fractions obtained were not due to overload phenomenon or protein interactions, several fractions were rechromatographed. In each instance the fraction applied was redialyzed against the starting buffer with which the column was equilibrated. Fraction I was rechromatographed on a fresh DEAE-Sephadex column, equilibrated with 0.03 M potassium phosphate, pH 7.8. Eighty-four per cent of the protein applied did not bind to the column. On the other hand, when fraction IIc was applied to a fresh DEAE-Sephadex column equilibrated with the same buffer, all of it was bound. Sixty-five per cent of the protein applied was eluted after the ionic strength was increased.

When fraction IIa was rechromatographed on a fresh column of CM-cellulose equilibrated with 0.01 M potassium phosphate, pH 5.9, 83% was obtained in the breakthrough. On the other hand, none of fraction IIb applied to a fresh CM-cellulose column equilibrated with the same buffer was eluted before the gradient was started. When fraction IIc₂ and IIc₃ (Fig. 6) were each applied to separate DEAE-cellulose columns, equilibrated with 0.01 M potassium phosphate, pH 7.8 and the same gradient applied as described above, each protein eluted at the same ionic strength as occurred with the initial chromatography.

Absorption studies. The pooled serum was absorbed with commercial anti-IgG until the IgG line noted on immunodiffusion was greatly reduced. Inhibition studies with the absorbed and unabsorbed sera (Fig. 7a) showed that the former had lost more than 97% of its blocking activity. A similar absorption was carried out with anti-

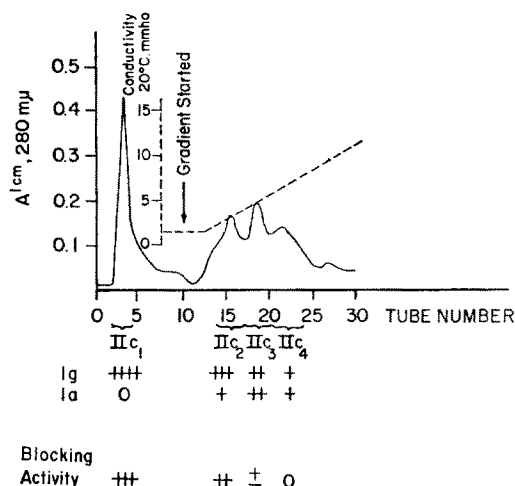


Figure 6. Continuous gradient elution pattern, relative blocking activity and immunoglobulin concentration of subfraction IIc applied to DEAE-cellulose, K-phosphate, pH 7.8, 25°C. Fraction IIc was obtained by chromatography of fraction II on CM-cellulose (Fig. 3).

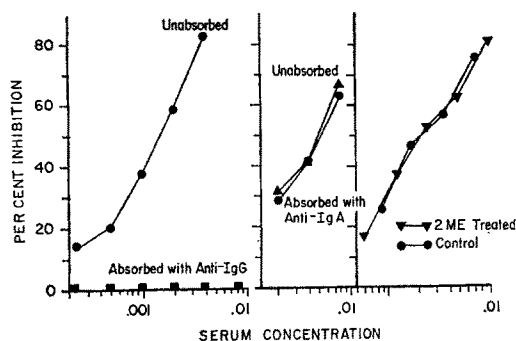


Figure 7. Inhibition of antigen E-induced histamine release: a) by the pooled serum from immunized donors and the same serum absorbed with an anti-IgG serum; b) by the pooled serum from immunized donors and the same serum absorbed with an anti-IgA serum; c) by the pooled serum from immunized donors and the same serum treated with 2-mercaptoethanol.

IgA until no IgA line could be seen by immunodiffusion. No significant loss of blocking activity was noted (Fig. 7b). Although serum absorbed with anti-IgM lost none of its blocking activity, after the dilutions necessary for absorption no IgM line could be seen in the control serum and it was therefore impossible to be sure that IgM had been removed. To test for IgM blocking

antibody the serum was therefore exposed to a 0.2 M concentration of 2-mercaptoethanol for 1 hr at 37°C. After dialysis no loss of blocking activity was observed (Fig. 7c).

DISCUSSION

Although immunotherapy is widely utilized to achieve amelioration of symptoms in clinical pollinosis, it has only recently been demonstrated to be effective (19, 26, 27, but cf. also 28). The results achieved are considered to be related to the production of blocking antibody, but this remains to be definitely established (29). In the present report we have analyzed the chromatographic distribution and immunoglobulin nature of what we have called "blocking antibody." It is recognized that, classically, blocking antibody has been demonstrated by the inhibition of an antigenically induced wheal and flare reaction in the skin (6). The antibody activity described in this report does, however, appear to be very similar if not identical to classical blocking antibody, and it would seem less confusing to assume this name rather than to coin a new one. One reason for confidence in this assumption is that the *in vitro* technique used to assay this antibody is based on a reaction which is a relevant model of clinical allergy. This has been demonstrated by the significant correlation between the sensitivity of a patient's leukocytes to antigen E and the degree of symptomatology that patient suffers during the ragweed season (7).

The characteristics of reaginic antibody have not been considered in this report. The possibility exists that this antibody, when circulating rather than cell-fixed, could combine with antigen and block the allergic reaction. We have observed, however, that the blocking activity of the sera used in this study was increased by immunotherapy several 100-fold above control levels (19). Studies of the same sera showed that the level of skin-sensitizing activity was not increased following immunotherapy (30). Thus, although we cannot preclude the possibility that reaginic antibody has blocking activity, quantitative considerations indicate that this probably amounts to well under 1% of the total serum blocking antibody.

The use of the *in vitro* assay has greatly increased the precision with which blocking antibody can be measured, allowing us to study the distribution of this activity in a quantitative

manner. Our results indicate that blocking activity is essentially a function of IgG molecules. In DEAE-Sephadex chromatography about half of the blocking antibody eluted in the "breakthrough" peak which contained only IgG. The remaining half of the blocking activity was in a preparation containing both IgA and IgG. This material was fractionated on CM-cellulose into three peaks. The first (fraction IIa), which contained the bulk of the IgA, had less than 1% of the blocking activity. IgG molecules were distributed in fractions IIb and IIc in a 1:2 ratio (actually 1:1.86) and blocking activity paralleled this (1:1.81). Fraction IIb was further chromatographed on a Sephadex G 200 column; antibody activity was observed to emerge with the latter eluting IgG. Fraction IIc was applied to a DEAE-cellulose column and, again, elution of the IgG and blocking activity paralleled one another. IgM, which could not be identified following ion exchange chromatography, was isolated on Sephadex G 200; no antibody activity could be demonstrated in this preparation.

Several of the fractions obtained were rechromatographed and found to elute under the same conditions. It is of interest that the IgG molecules did not elute continuously (*cf.* Fig. 6). Moreover, it appears that different segments of the IgG spectrum had different specific blocking activity. This discontinuity and differential activity of the IgG population is under study.

These data do not preclude the possibility that some blocking activity is present on IgA or IgM molecules. Since our recoveries after chromatography, dialysis and concentration were on the order of 70% it is possible that the material lost differed significantly from that recovered. This is unlikely since the inevitable losses attending dialysis and concentration would not be expected to affect immunoglobulins differentially. Moreover, it was observed that on *rechromatography* of several preparations only 70 to 80% of the protein was recovered. Another consideration is that the highly purified preparation of IgA had less than 1% the specific activity of the IgG preparations, while the IgM preparation had less than 1% of the activity of the serum from which it was isolated. Finally, absorption with anti-IgG removed more than 97% of the serum blocking activity while treatment with anti-IgA had no appreciable effect; exposure of the serum to 2-mercaptoethanol likewise pro-

duced no decrease in blocking activity. Taken together, these considerations make it highly likely that the great bulk of serum blocking activity is carried by IgG molecules.

The use of a pooled serum requires some qualification of this conclusion. It is possible that 1 of the 20 individual sera pooled contained IgA or IgM molecules which accounted for about 10% of its blocking activity. That this was the case in more than one individual or amounted to more than 10% of that individual's total activity is rendered unlikely by the data obtained with the purified IgA and IgM preparations. Chromatography of a number of sera sufficient to explore this possibility would be extremely time consuming and did not appear to be warranted.

Our observations are compatible with earlier work suggesting that blocking activity was to a greater or lesser degree associated with the "γ-globulins" (*i.e.*, the proteins migrating most slowly on electrophoresis) (9-11). Our chromatographic findings differ somewhat from those of Perelmutter *et al.* (13), who found practically all of the blocking activity to be in the first protein peak eluted from DEAE-cellulose. This difference is probably due to different chromatographic techniques and the differing degree of precision of the blocking antibody assays utilized. Ragweed binding has been demonstrated in IgA and IgM as well as IgG molecules by radioimmuno-electrophoresis (17). This might be merely a function of the sensitivity of the isotopic method. On the other hand, it is quite possible that some antibody molecules which can bind ragweed are not capable, due to steric or avidity considerations, of also blocking the histamine release reaction.

SUMMARY

This report describes the immunoglobulin and chromatographic characteristics of human blocking antibody. Studies were carried out on a pool of serum from 20 allergic donors who had been immunized with ragweed antigen E as a form of therapy for ragweed hay fever. The serum and serum fractions were subjected to chromatography on DEAE-Sephadex, CM-cellulose, DEAE-cellulose and Sephadex G 200. Blocking activity was assayed by an *in vitro* technique: the inhibition of antigen E induced histamine release from isolated human leukocytes. This technique is more than 10 times as precise as the usual skin assay. Immunoglobulins were determined quali-

tatively by immunodiffusion against specific antisera and quantitatively by the Preer technique. Recovery of blocking activity was about 70%. In each fractionation procedure blocking antibody and IgG eluted together. Purified IgA had less than 1% the activity of IgG preparations and IgM antibody contributed less than 1% to the activity of the whole serum. Absorption of the serum with anti-IgA and treatment with 2-mercaptoethanol did not decrease its blocking titer, while absorption with anti-IgG caused a decrement of more than 97%. Circulating reaginic antibody appeared to account for less than 1% of the total serum activity. It was concluded that blocking activity is essentially a function of IgG molecules.

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