Human Granulocyte/Pollen-binding Protein

Recognition and Identification as Transferrin

Suzanne P. Sass-Kuhn, R. Moqbel, Judith A. Mackay, O. Cromwell, and A. B. Kay

Department of Allergy and Clinical Immunology, Cardiothoracic Institute, Brompton Hospital, London, United Kingdom

Abstract. Normal human serum was found to contain a heat-stable protein which promoted the binding of granulocytes to timothy grass pollen (granulocyte/pollen-binding protein [GPBP]). GPBP was purified by gel filtration, anion exchange, and affinity chromatography. Virtually all of the granulocyte/pollen-binding activity was associated with a β-1-protein having a molecular mass of ~77,000 D and an isoelectric point of between 5.5 and 6.1. By immunoelectrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein was identified as transferrin. Monospecific antisera raised against either GPBP or transferrin removed biological activity from GPBP preparations, and GPBP and transferrin gave lines of identity with these two antisera. The apparent heterogeneity in the molecular size and charge of GPBP observed during progressive purification was minimal when GPBP was saturated with ferric ions before the separation procedures. These experiments indicate that granulocyte/pollen binding is a hitherto unrecognized property of transferrin which appears to be unrelated to iron transport and raises the possibility that transferrin might have a physiological role in the removal of certain organic matter.

Introduction

During the course of experiments designed to determine whether various allergens activate the alternative pathway of complement, we made the unexpected observation that normal human serum, either heated or unheated, promoted firm, prolonged binding of granulocyte to timothy grass pollen (TGP).[1] We had undertaken these studies in the expectation that sera from certain susceptible, i.e., "atopic", individuals might facilitate adherence of neutrophils and/or eosinophils to allergens via IgG or IgE or that normal serum might produce binding through alternative pathway complement activation as was previously shown for helminthic larvae (1, 2). The finding that heated serum from a number of healthy normal individuals promoted granulocyte binding (irrespective of whether they were skin [prick] test positive or negative to the TGP extract) made it unlikely that either IgG, IgE, or complement were involved. Accordingly, we progressively purified this granulocyte/pollen-binding protein (GPBP) and found it to be identical to serum transferrin.

Methods

Materials

Materials were obtained as follows. TGP (*Phleum pratense*) (a gift from Dr. David Moran, Beecham Pharmaceuticals, Epsom, England); transferrin, IgG, vitamin B_{12}, human serum albumin, ferric chloride (Sigma, Poole, England); lactoferrin and antilactoferrin (a gift from Dr. A. Segal, University College Hospital, London); antitransferrin (Dako, Copenhagen, Denmark); anti-whole normal human serum (Dako); anti-β-2-glycoprotein 1, anti-Factor B, antiplasminogen (Behring, Hounslow, England); fibrinogen, thrombin, plasmin, aprotinin (a gift from Dr. P. Gaffney, National Institute of Biological Sciences, Hampstead, London). The plasmin and thrombin were World Health Organization International Reference preparations and fibrinogen was a Kabi Grade L preparation. Sephadex G-200, Sephadex G-75, Blue Sepharose CL-6B, DEAE-Sephacel, protein A-Sepharose CL-4B, CNBr-activated Sepharose 4B, lysine-Sepharose 4B, Blue Dextran 2000, and high-molecular markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Pharmacia, Uppsala, Sweden); Iscove's medium free of bovine serum albumin, human transferrin, and soya bean lecithin, pH 7.35 (Flow Laboratories, Irvine, Scotland); penicillin and streptomycin (Glaxo, Greenford, England).

Methods

LEUKOCYTE POLLEN ADHERENCE ASSAY Leukocyte-rich plasma was obtained by sedimentation with 20% Dextran in the presence of

Address all correspondence to Dr. Kay.

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1. *Abbreviations used in this paper:* CIE, crossed immunoelectrophoresis; GPBP, granulocyte/pollen-binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGP, timothy grass pollen.

preservative-free heparin (1 U/ml). The leukocyte-rich plasma was applied to a density gradient of 9% Ficoll solution and sodium diacrizoate (*d* 1.140) in the proportion 2.4:1. After centrifugation at 200 *g* for 30 min at 20°C, the cell-rich pellet was obtained after lysis of the erythrocytes with 0.83% ammonium chloride. Granulocytes were washed twice with Iscove's medium and resuspended to 2×10^6/ml in the same medium. TGP grains were suspended in Iscove's medium to give ~1,000 pollen grains/ml. Equal volumes (50 μl) of leukocytes, pollen grains, and normal human serum, or various chromatographic fractions as described in the text, were mixed together in flat-bottomed microtiter plates (Nunc/Gibco, Paisley, Scotland) and incubated for 18 h at 37°C in an atmosphere of 95% air and 5% CO_2. In all experiments, the Iscove's contained 100 μg/ml penicillin and 100 μg/ml streptomycin. The number of pollens bearing adherence cells and the degree of adherence was estimated by either bright field or direct interference contrast microscopy (Nomarski Optics). In some experiments, visualization of adherent leukocytes to pollen grains was achieved by the addition of 0.1% aqueous toluidine blue. The percentage of pollen grains having four or more adherent granulocytes were assessed by examining 50 pollen grains. The degree of adherence was recorded according to the following arbitrary scale. (±) represents 4–7 cells adherent to pollen surface; (+) represents pollen surface completely surrounded with one layer of adherent cells; (++) represents pollen surface covered with 2–3 layers of adherent cells; (+++) represents pollen surface covered with 3–4 layers of adherent cells; (++++) represents pollen surface covered with more than four layers of adherent cells. The tests were performed in duplicate and the variation between pair in terms of percent adherence was usually <5%. Accuracy was not improved by increasing the volume of the reaction mixture to enable >50 pollen grains to be counted. The ratio of 2,000 leukocytes to 1 pollen grain was optimal for visualization of the degree and the percent adherence. In most experiments, the results were recorded by two independent observers. Observer's variation was <10%.

Chromatography

GEL FILTRATION 3 ml of human serum were applied to an 80 × 2.5-cm column previously equilibrated with phosphate-buffered saline (PBS) (0.01 M NaH_2PO_4 $2H_2O$, 0.037 M Na_2HPO_4 $2H_2O$, and 0.1 M NaCl, pH 7.35). Chromatography was undertaken at 4°C with a flow rate of 20 ml/h and 2-ml fractions were collected. Alternate fractions were tested for granulocyte/pollen adherence. The column was equilibrated with molecular weight markers (Blue Dextran [2,000,000], IgG [150,000], human serum albumin [67,000], and vitamin B_{12} [1,350]). The major peak of activity was pooled as indicated, dialyzed against distilled water, and lyophilized. The material was resuspended in PBS to a volume of 2 ml and applied to a column of Sephadex G-75 (80 × 1.7 cm). The Sephadex G-75 chromatography was performed in PBS at 4°C with a flow rate of 20 ml/h. 4-ml fractions were collected and alternate fractions tested for granulocyte/pollen adherence as indicated. Molecular weight markers were Blue Dextran, human serum albumin, and vitamin B_{12} (as for G-200 chromatography). The fractions were pooled as indicated, dialyzed against distilled water, and lyophilized.

AFFINITY. *Blue Sepharose.* The lyophilized material prepared by G-200 and G-75 chromatography (Fig. 2) was resuspended in 4 ml of 0.05 M Tris/HCl, pH 7.0, plus 0.1 M KCl, and applied to a Blue Sepharose CL-6B column previously equilibrated with the same buffer. The flow rate was adjusted to 60 ml/h and 2-ml fractions were collected. The column size was 10 × 1.25 cm. After elution of the first major protein peak, 0.05 M Tris/HCl, pH 7, plus 1.5 M KCl, was applied as indicated. The fractions were dialyzed for 18 h at 4°C against PBS and 50-μl samples tested for granulocyte/protein-binding activity. Samples containing activity were pooled as indicated, dialyzed against distilled water for 18 h, and lyophilized.

Protein A-Sepharose CL-4B. Material obtained from Blue Sepharose was reconstituted in 4 ml of 0.1 M phosphate buffer, pH 7.0, and applied to the protein A column (13 × 1 cm) previously equilibrated with the same buffer. The experiment was performed at 22°C and the flow rate was adjusted to 40 ml/h; 2-ml fractions were collected. 1 M acetic acid was applied to the column after the major peak of protein as indicated. Alternate fractions were tested for GPBP activity following dialysis against PBS at 4°C for 18 h and the active fractions were pooled, dialyzed, and lyophilized as indicated.

ANION EXCHANGE A column of DEAE-Sephacel (70 × 4 cm) was equilibrated with 0.02 M sodium phosphate buffer, 0.06 M NaCl, pH 7.8. 100 ml of normal human serum was applied and the experiment was performed at 4°C by using a flow rate of 30 ml/h. 10-ml fractions were collected. After the emergence of the first protein peak, a linear salt gradient of 600 ml, up to 0.5 M NaCl, was applied. Alternate fractions were assayed for GPBP activity following dialysis against PBS at 4°C for 18 h.

SDS-PAGE

The discontinuous Tris-glycine buffer system of Laemmli (3) was used for 1-mm slab gels by using 12.5% acrylamide. A 3% acrylamide stacking gel was added to improve banding of proteins. The samples, previously lyophilized, were analyzed either in the presence or absence of mercaptoethanol, i.e., 3% sodium dodecyl sulfate with or without 5% mercaptoethanol. The samples were heated for 10 min at 60°C and colored with 0.01% bromophenol blue. To each gel, 40 μg of protein contained in 20 μl was added. The samples were electrophoresed at 18 mA until the protein band reached the lower 12.5% gel, at which time the current was increased to 30 mA for another 5 h (approximately). After electrophoresis, the gels were fixed for 45 min in 25% isopropanol/10% acetic acid and stained overnight in 0.1% Coomassie blue R250. The gels were destained in 8% acetic acid. The high molecular weight protein standard was supplied by a kit from Pharmacia Fine Chemicals (Piscataway, NJ).

Isoelectric focusing

This was performed using LKB Ampholine R PAG plates, pH 4–6.5 (LKB Instruments Ltd., South Croydon, England). 20 μl of protein were focused. A pH gradient was determined by placing a pH electrode at 1-cm intervals across the gel at the conclusion of the experiment. The plates were stained for protein with 0.1% Coomassie blue R250. In preliminary experiments, a range of pH 3.5–9.5 was employed. Routinely, a pH range 4.0–6.5 was used.

Crossed immunoelectrophoresis (CIE)

CIE was performed by the method of Lowenstein (4) with the following modifications. The plates were prepared by using 8 ml of multone 1% agarose in 25% veronal buffer, pH 8.0, with an 8 × 8-cm glass slide. Normal serum, with or without GPBP, was electrophoresed at 5 V/cm for 80 min in the first dimension and at 1.4 V/cm for 20 h in the second dimension. The 5 × 3.5-cm "window" contained appropriate dilutions of anti-normal human serum. The plates were washed for 25 h in 0.9% NaCl, dried under filter paper, and stained with 0.5% Coomassie blue R250.

Immunoelectrophoresis

The immunoelectrophoresis was performed by the method described by Lowenstein (4) with the following modifications. 8 ml of multone 1% agarose in 25% veronal buffer, pH 8.6, was layered on to an 8 × 8-

cm glass slide. The antigen was electrophoresed at 5 V/cm for 80 min. A trough was cut (5 × 0.1 cm) and antiserum added. The plate was left overnight in a humidity chamber to diffuse before washing. The plates were stained with 0.5% Coomassie blue R250.

Single radial immunodiffusion

These were performed in 1% agarose, as for CIE, or with commercial "LM-Partigen" plates (Behring, Hounslow, England).

Immunoadsorption with anti-GPBP and antitransferrin

Antisera against GPBP which gave a single line on SDS-PAGE was raised in New Zealand white rabbits according to the following schedule. Rabbits received 650 μg of protein in complete Freund's adjuvant on day 1 by multiple subcutaneous injections. 5 wk later, the rabbits were boosted with 150 μg GPBP in saline in incomplete Freund's adjuvant intramuscularly. This procedure was repeated with 70 μg GPBP at three weekly intervals for 24 wk. The IgG fraction of the rabbit antisera was prepared by ammonium sulphate precipitation and DEAE-Sephadex chromatography as follows. Saturated ammonium sulphate (30 ml) in 0.02 M Tris HCl containing 1 mM EDTA, pH 8.0, was slowly added to 50 ml of the rabbit antisera and gently mixed at room temperature for 30 min. After centrifugation at 4000 *g* for 45 min at 4°C, the pellet was redissolved in 50 ml of 0.02 M Tris HCl, 1 mM EDTA, pH 8.0. This process was repeated until the final precipitation was white, i.e., macroscopically free of other serum proteins. This precipitation was redissolved in 2 ml 0.02 M sodium phosphate buffer, pH 7.8, and dialyzed against this buffer with several changes over 18 h. The partially purified IgG was then applied to a DEAE-Sephacel column by using the same conditions as described above, and the first protein peak was pooled and dialyzed for 48 h against several changes of distilled water before lyophilization. The lyophilized material was coupled to CNBr-activated Sepharose after the following procedure. 28 mg of the partially purified rabbit antisera were added to 1.0 g of CNBr-activated Sepharose 4B. The Sepharose was previously washed in 1 mM HCl and resuspended in 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.6 (coupling buffer). The mixture was rotated at room temperature for 2 h and treated with 1 M ethanolamine for 2 h at room temperature, washed three times with sodium acetate buffer (0.1 M, pH 4.0, containing 0.5 sodium chloride), and alternated with a borate buffer (0.1 M, pH 8.0, also containing 0.5 M NaCl). The material was then washed with the coupling buffer followed by 0.1 M glycine buffer, pH 8.0, followed by the coupling buffer, followed by PBS; then, it was placed in a 5-ml syringe, plugged with glass wool, and equilibrated with PBS at room temperature.

Absorption with lysine-Sepharose

100 ml of normal human serum heated at 60°C for 1 h was applied to a lysine-Sepharose 4B column (20 × 2 cm) previously equilibrated with 0.05 M phosphate buffer, pH 7.5, at 4°C by using a flow rate of 20 ml/h. The serum was assayed for plasminogen on LM-Partigen immunodiffusion plates.

Fibrinogen and fibrin digestion products

3 mg of fibrinogen contained in 1 ml of distilled water was incubated with 0.037 ml of plasmin (80 IU/ml) at 18°C and the reaction stopped at 0, ½, 1, 4, and 24 h with 10 μl of aprotinin (20,000 KI units/ml). The samples were dialyzed against PBS and 50 μl fractions were tested for GPBP activity. For fibrin, the same quantities of fibrinogen were used but thrombin (250 μl containing 5.0 IU/ml with 40 mmol $CaCl_2$) was added for 2 min before mixing with plasminogen.

Results

Fresh serum and serum heated at 56°C for 1 h, from nine normal healthy individuals, were tested for their ability to promote adherence of granulocytes to pollen grains (Fig. 1). A concentration-dependent increase of adherence of neutrophils to TGP was observed. There were no appreciable differences between the heated and unheated sera. In other experiments, it was found that, in general, there were no differences between an autologous system, i.e., serum and granulocytes from the same donor, or experiments in which cells and serum from different individuals were used.

When normal serum was applied to a column of Sephadex G-200, the major peak of granulocyte/pollen adherence eluted together with proteins having a molecular mass of ~70,000 D (Fig. 2 *A*). A smaller peak of activity was also observed at the void volume (V_0), although the degree of adherence as well as the percentage adherence was considerably less than that ob-

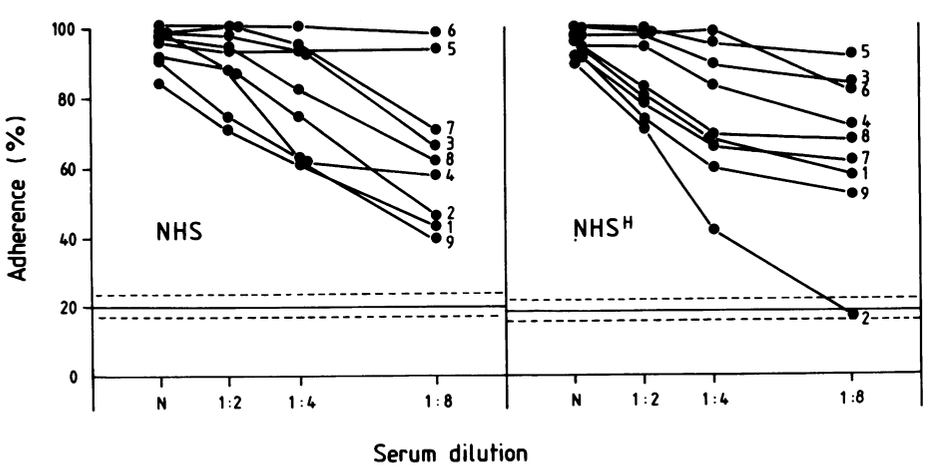

Figure 1. Adherence of granulocytes to TGP by heated and unheated normal sera from healthy individuals. NHS, normal human serum; NHS^H = serum heated at 56°C for 1 h. Granulocytes and sera were obtained from the same donor.

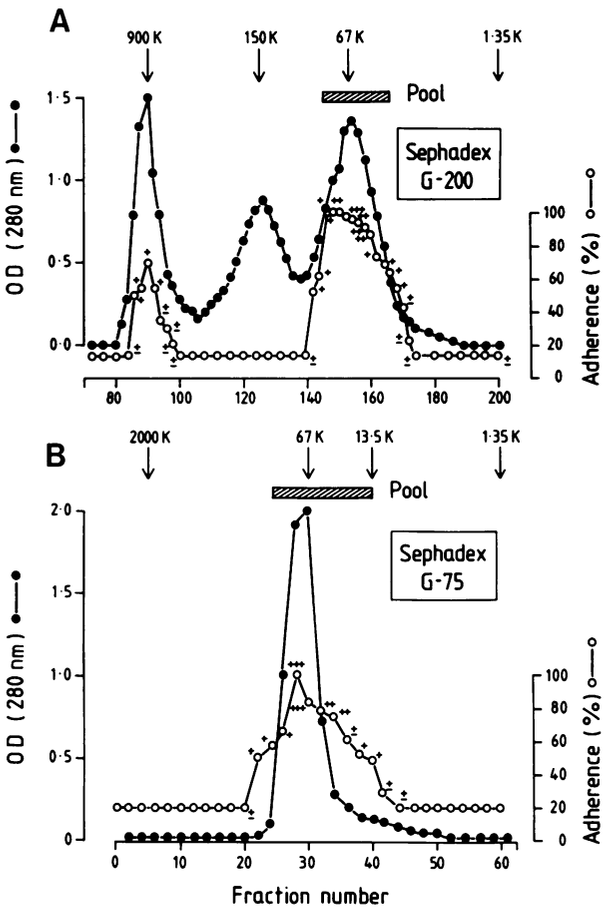

Figure 2. Gel filtration chromatography. (*A*) Sephadex G-200: the experiment was performed 12 times, i.e., NHS (6) and NHS^H (6). The example shown is NHS^H. (*B*) Sephadex G-75: the experiment was performed 12 times (as in Fig. 2 *A*).

NHS, normal human serum; NHS^H, serum heated at 56°C for 1 h; OD, optical density.

served with the peak eluting in the albumin region. The experiment was performed on 12 occasions (six with fresh serum, six with heated serum). The results were virtually identical in all instances. The 70,000-D peak was then pooled as indicated, concentrated, and applied to a column of Sephadex G-75 (Fig. 2 *B*). A single peak of granulocyte/pollen adherence was observed which eluted with a single peak of protein. By SDS-PAGE, this material was shown to contain albumin and other proteins in the 60,000–80,000 mol wt region together with traces of IgG. This experiment was also performed 12 times (six with fresh serum and six with heated serum) and gave virtually identical results on each occasion.

Material prepared by Sephadex G-200 and G-75 chromatography was applied to a column of Blue Sepharose. GPBP was associated with the protein peak that did not bind. The protein that bound to Blue Sepharose (identified as albumin by immunoelectrophoresis against a monospecific antialbumin) and which eluted with 0.05 M Tris/HCl, pH 7.0, plus 1.5 M KCl contained no pollen-binding activity.

Protein-containing GPBP activity, which did not bind to Blue Sepharose, gave a number of faint bands on SDS-PAGE, including IgG. Accordingly, this material was pooled, dialyzed, lyophilized, and reconstituted, and applied to a column of protein A-Sepharose. Virtually all the protein present, which also contained GPBP activity, failed to bind to protein A-Sepharose. A small peak of IgG was eluted with 1 M acetic acid. This did not contain GPBP activity.

The characteristics of GPBP on anion exchange chromatography were studied by using DEAE-Sephacel (Fig. 3). Two broad peaks of GPBP activity were observed in the material which did not bind to DEAE-Sephacel with 0.02 M phosphate buffer and 0.06 M NaCl, pH 7.8. Almost all of the GPBP activity was detected in the second peak which eluted immediately after IgG. Only a weak degree of adherence was observed with the first peak. The second peak was pooled as indicated and applied to a column of G-200 (Fig. 4). GPBP activity was clearly separable from the single major protein peak which consisted mostly of IgG. When a salt gradient was applied, no further GPBP activity was observed in the other proteins eluting from the column.

Further attempts to purify the first peak of GPBP activity (Fig. 3) by Sephadex G-200 gave inconclusive results since several peaks of weak GPBP activity were observed at several bed volumes.

GPBP was then progressively purified by using a combination of DEAE-Sephacel, Sephadex G-200, Blue Sepharose, and protein A-Sepharose affinity chromatography in sequence. In these further studies, the normal human serum was first applied to a column of lysine-Sepharose. This initial procedure resulted in no loss of GPBP activity, but it depleted the unfractionated serum of plasminogen by >90% as assessed by single radial immunodiffusion using a monospecific antiplasminogen. This progressive purification was performed on five occasions. In each instance, the final material contained a protein which gave a broad band on SDS-PAGE (Fig. 5). The molecular weights of these preparations were all between 67,000 and 82,000, and had an isoelectric point (pI) between 5.5 and 6.1 (Fig. 6).

These purified preparations, which had GPBP activity in the concentration range of 10–50 μg/ml, were tested for the presence of a number of recognized serum proteins. These included the C3b inactivator, β-2-glycoprotein 1, Factor B, C4 binding protein, fibronectin, and serum amyloid P component. None of these proteins were detected in purified GPBP. The C4 binding protein, serum amyloid P component, and fibronectin were measured by Dr. M. B. Pepys (Royal Postgraduate Medical School, Hammersmith Hospital, London), by using electroimmunoassay and monospecific antisera.

In further separate studies, fibrin or fibrinogen were digested with plasmin at time intervals and the fibrinogen/fibrin digestion products were tested for GBPB activity. The digestion was

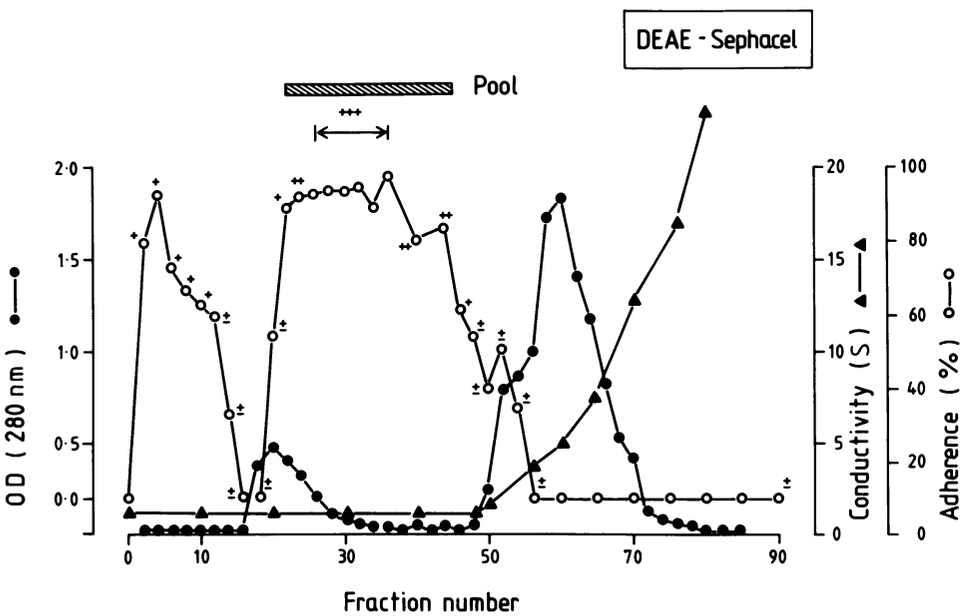

Figure 3. GPBP activity in normal serum separated by DEAE-Sephacel anion exchange chromatography. The experiment was performed five times (3× NHS^H; 2× NHS). OD, optical density.

stopped at 30 min, 1, 4, and 24 h by the addition of aprotinin. No GPBP activity was observed in any of the samples tested. Purified lactoferrin, GPBP, and transferrin were tested for granulocyte/pollen-binding activity at 12.5, 30, 125, and 300 μg/ml on four occasions. No activity was observed with lactoferrin at any of the concentrations tested and no lactoferrin was present in the GPBP or transferrin preparations, as assessed by immunoelectrophoresis. In contrast, GPBP and transferrin gave a dose-dependent increase in granulocyte/pollen binding with 70% (++)–80% (++) adherence with the highest doses.

Crossed-over immunoelectrophoresis was used to identify GPBP. A sample of GPBP was mixed with normal human serum and after electrophoresis in two dimensions, was shown to substantially increase a protein peak in the β-1 region, which was identified as transferrin (Fig. 7). Furthermore, a monospecific antiserum which was raised against GPBP gave a line of identity with transferrin, and antitransferrin gave a line of identity with GPBP and transferrin (Fig. 8). The anti-GPBP was then absorbed on to CNBr-activated Sepharose particles and an immunoabsorption experiment performed as shown in Fig. 9 *A*. A purified

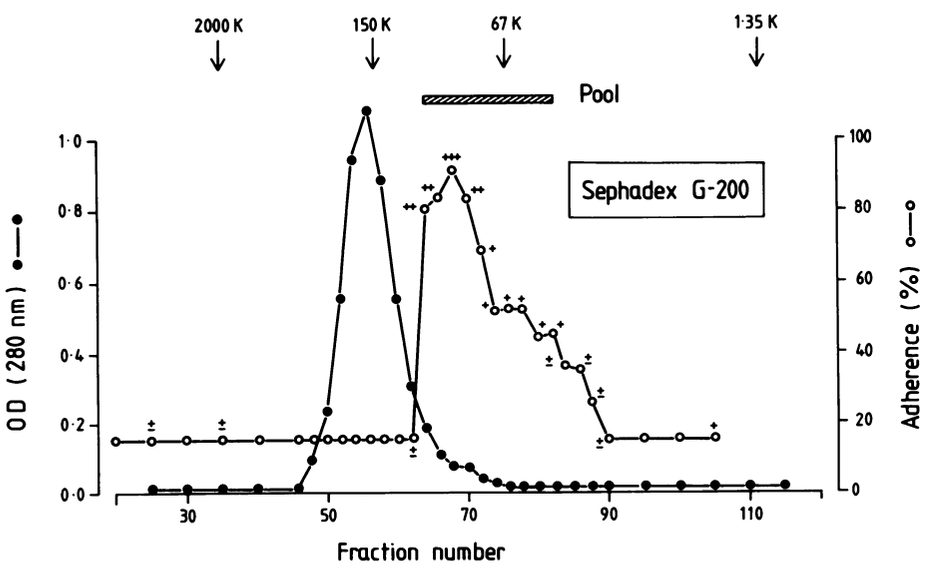

Figure 4. Sephadex G-200 chromatography of GPBP purified by DEAE-Sephacel. The material from DEAE-Sephacel (Fig. 4) was pooled, dialyzed, and lyophilized as indicated, and resuspended in PBS, pH 7.35, and applied to a Sephadex G-200 (85 × 5 cm) column with a flow rate of 30 ml/h. 10-ml fractions were collected and the experiment was performed at 4°C. Alternate fractions were tested for GPBP activity as indicated. The experiment was repeated four times. OD, optical density.

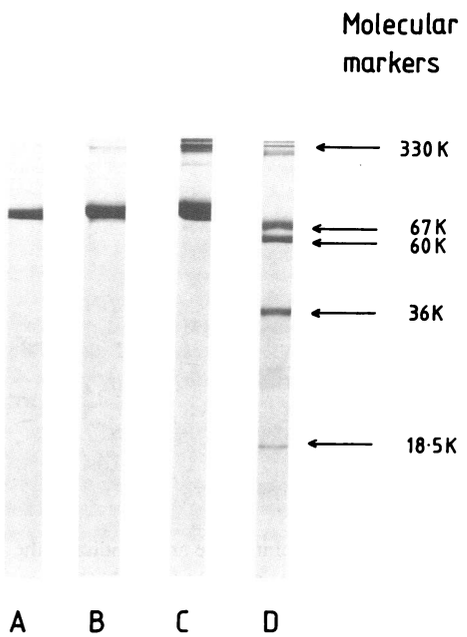

Figure 5. SDS-PAGE of GPBP and transferrin. *A*, GPBP purified by DEAE-Sephacel, Sephadex G-200 and Sephadex G-75, Blue Sepharose, and protein A-Sepharose (+ ME); *B*, as A (without ME); *C*, commercial transferrin (+ ME); *D*, molecular markers (+ ME). ME, mercaptoethanol.

preparation of GPBP, which gave a single band of SDS-PAGE, was applied to the column. There was no protein or biological activity in the material which did not absorb to the column. A single protein eluted from the Sepharose with 0.1 M acetic acid, and this was shown to contain a single protein peak containing a single peak of granulocyte/pollen-binding activity. The same experiment was performed with antitransferrin coupled to Sepharose 4B and the same results were obtained, although with this procedure a very small protein peak with minimal biological activity was observed in material which did not adsorb to the column (Fig. 9 *B*).

The material which adsorbed to and eluted from anti-GPBP gave a single line on immunoelectrophoresis against anti-normal human serum in the beta-1 region and against anti-GPBP and antitransferrin. The material which did not adsorb to anti-GPBP did not give a line against anti-GPBP, whereas GPBP and transferrin gave identical lines with antitransferrin and anti-GPBP.

Having established that GPBP and transferrin were identical, normal human serum was saturated with ferric chloride before chromatography on DEAE-Sephacel. On this occasion, only a single broad peak of biological activity was observed which eluted after IgG, i.e., the weak peak of activity which was observed when the same experiment was performed without iron saturation was not detected (Fig. 3). As before (Fig. 3), no further proteins having GPBP activity were observed after application of the salt gradient. The same experiments were performed in which the 0.06 M NaCl was omitted from the starting buffer. Transferrin and GPBP eluted together after the application of the salt gradient (0.02–0.035 M NaCl). In the two instances where it was performed, the percent recoveries of transferrin, as estimated by gel diffusion, were 81.3 and 84.7%. Virtually the same result was obtained when commercial transferrin, saturated with iron, was separated by DEAE-Sephacel.

When either normal human serum saturated with iron, or commercial transferrin saturated with iron, from DEAE-Sephacel were applied to Sephadex G-200, single peaks of activity were observed which eluted with a major protein peak. In turn, these cochromatographed with the single peak of transferrin as assayed by rocket immunoelectrophoresis. Unlike the results in Fig. 2 *A* in which normal human serum was chromatographed without prior saturation with ferric chloride, the smaller weaker peak of activity previously observed at V_0 was not observed.

Purified iron-saturated GPBP gave a dose-dependent increase in both the percent adherence and the degree of adherence. Concentrations as low as 1.25 μg/ml gave significantly more binding than the diluent control, whereas at 300 μg/ml, there was ~85% binding and (+++) adherence.

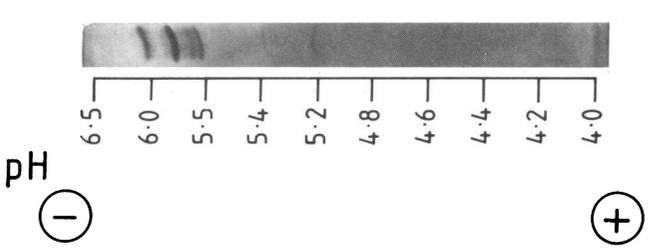

Figure 6. Isoelectric focusing of GPBP. The preparation was prepared as in (Fig. 5 *A*) with the exception of protein A-Sepharose. The experiment was performed three times.

Discussion

The assay used in the present study is simple, reliable, and reproducible. It is essentially a rosette technique but is considerably easier to quantify than erythrocytes/leukocyte rosettes because of the ease of visualization of the pollen/leukocyte complex. With strong reactions, there was multilayer adherence of granulocytes to pollen grains. This was reminiscent of the large numbers of adherent erythrocytes observed in certain erythrocytes/leukocyte rosettes and is possibly due to alterations in the net membrane charge of the innermost granulocytes binding to the pollen grain which, in turn, renders these cells more "sticky" and leads to agglutination with other leukocytes.

The initial observations with GPBP indicated that it was a heat-stable protein, and therefore, unlikely to be IgE or to be generated by complement activation. It is yet to be shown with certainty that serum from allergic individuals who have elevated concentrations of TGP-specific IgE and IgG antibodies necessarily promote a greater degree of pollen/granulocyte binding.

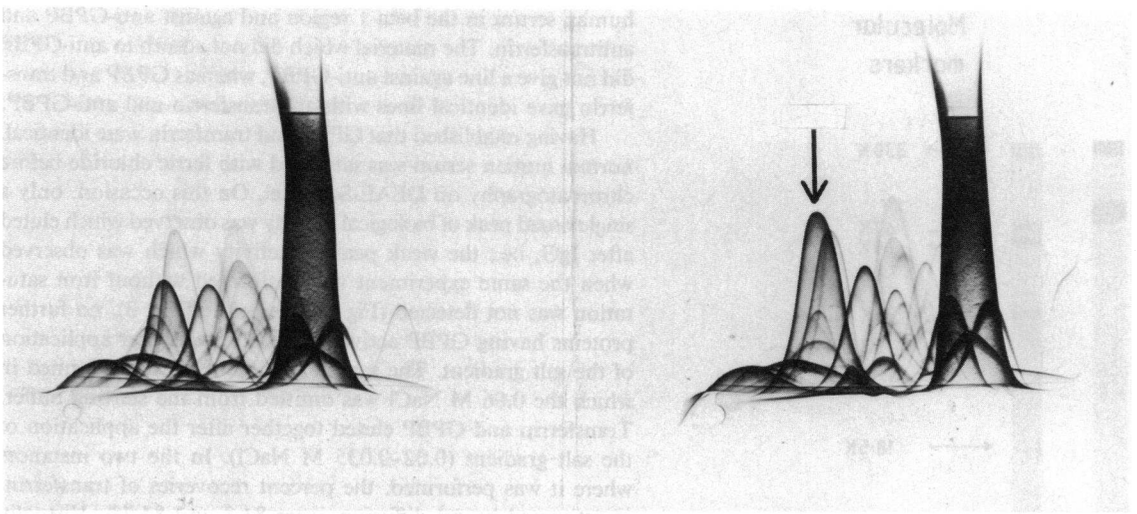

Figure 7. The effect of GPBP on the profile of crossed immunoelectrophoresis of normal human serum. The point of the arrow indicates the transferrin peak. Normal human serum is on the left side, normal human serum plus GPBP (prepared as in Fig. 5*A*) is on the right side.

In fact, in preliminary experiments (Sass-Kuhn, S. P., R. Moqbel, and A. B. Kay, unpublished observations), it was shown that sera from patients with seasonal allergic rhinitis and high TGP-specific IgE had no more granulocyte/pollen-binding activity than normal serum. The gel filtration experiments suggested that the biological activity was associated with albumin (Figs. 2 *A* and *B*). However, it was possible to separate GPBP from albumin by Blue Sepharose affinity chromatography. The protein A-Sepharose studies indicated that IgG was unlikely to be involved and therefore, this affinity step, when considered together with the fact that atopic and nonatopic sera gave similar results (Fig. 1), makes it unlikely that anti-TGP antibodies play a role in this adherence reaction. It was possible to separate GPBP from the majority of plasma proteins by DEAE-Sephacel and this served as a useful initial purification step for further studies (Fig. 3). Thus, by the combination of anion exchange (Fig. 3), gel filtration (Fig. 4), and affinity chromatography, together with immunoelectrophoresis and SDS-PAGE, GPBP was shown to be a 67,000–82,000 mol wt β-1 protein (Fig. 5) with a pI of between 5.5 and 6.1 (Fig. 6) and a component of normal serum. Purified preparations of GPBP were free of detectable amounts of albumin, C3b inactivator, β_2-glycoprotein 1, Factor B, C4 binding protein, fibronectin fragments, serum amyloid P component, and lactoferrin.

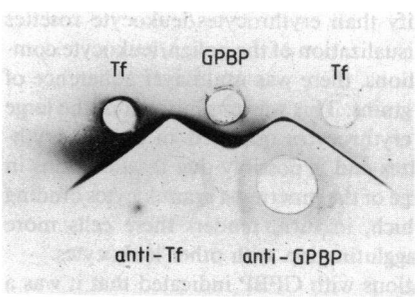

Figure 8. Single radial immunodiffusion of GPBP and transferrin with antitransferrin and anti-GPB. 5 μl containing 10 μg of transferrin and 5 μl of 25 μg of GPBP were used as indicated. The antisera were used at a volume of 75 μl. The antitransferrin was diluted 1 in 2, and this anti-GPBP was used undiluted.

By crossed-over immunoelectrophoresis with whole human serum, GPBP accentuated markedly the height of the transferrin peak but had no effect on the height or intensity of any other peak of plasma protein, including hemopexin, which has a similar molecular weight and charge to transferrin (Fig. 7). With the use of monospecific antisera to both GPBP and transferrin, it was possible to show, by single radial immunodiffusion (Fig. 8) and immuno-affinity chromatography (Fig. 9), not only that GPBP and transferrin were identical but that GPBP activity coeluted with this iron-binding protein.

With the knowledge that transferrin and GPBP were apparently the same protein, further purification procedures were undertaken with prior iron-saturation. This gave single peaks of GPBP activity, i.e., activity in the high molecular weight region of Sephadex G-200 (Fig. 2) was no longer observed, and neither was the earlier peak on DEAE-Sephacel (Fig. 3). We interpret these findings as being a consequence of size and charge heterogeneity of transferrin resulting from poor iron saturation since it was previously shown that the transferrin molecule is unstable in terms of its behavior on DEAE-Sephadex and Sephadex G-200 without prior saturation with iron (5). We also observed that, with iron-saturated starting material, fractions containing GPBP activity had a salmon pink color.

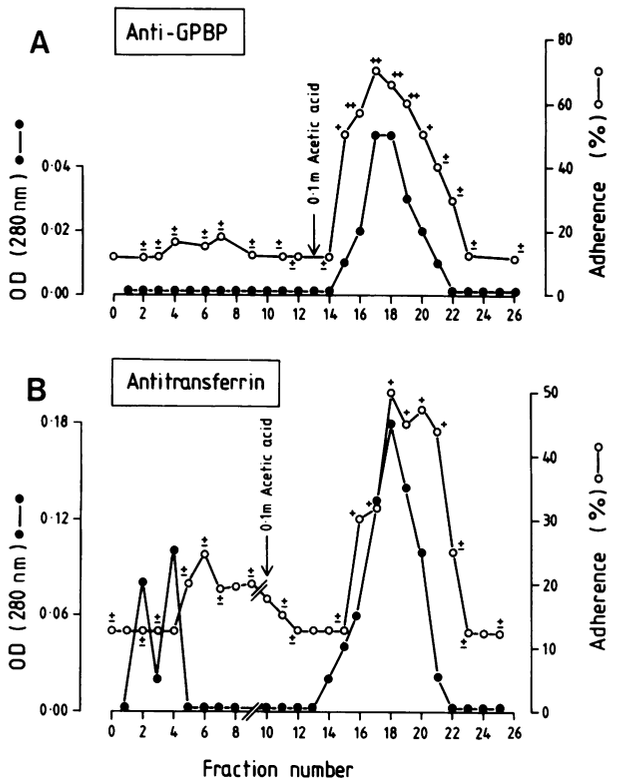

Figure 9. Absorption of GPBP by anti-GPBP and antitransferrin. (*A*) Anti-GPBP. The GPBP applied to the column was prepared as in *A* (Fig. 5). 1 mg of GPBP in 1 ml was applied to the column and 1-ml fractions were collected by gravity. The protein was eluted with 0.1 M acetic acid as indicated in Fig. 10 and alternate fractions were tested for biological activity following dialysis against PBS at 4°C for 18 h. The experiment was performed four times. A representative example is shown. (*B*) Antitransferrin. The GPBP applied to the column was as in *A* (Fig. 5). The antitransferrin-Sepharose 4B column was prepared in an identical fashion with the exception that the coupling buffer was 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl. The column size of the antitransferrin was 22 × 1 cm and the flow rate was 60 ml/h. 2-ml fractions were collected and 2 ml of 1 mg/ml was applied. Other conditions were as for anti-GPBP.

Transferrin (siderophilin) is a β-1-glycoprotein with a molecular weight variously estimated to be between 68,000 and 90,000 (6–8). The pI of iron-saturated transferrin is ~5.4 but this is higher (5.8) in iron-free buffer (which were the conditions used in Fig. 7). It is formed mainly in the liver but possibly also in the reticuloendothelial system. Two atoms of iron in the ferric form attach to one molecule of transferrin but the protein also binds other metals, although much less firmly than iron (9, 10). Normal plasma contains 240–280 mg of transferrin/100 ml but as much as 50–60% of exchangable transferrin is present in extravascular fluid including tracheo-bronchial secretions, saliva, tears, cerebrospinal fluid, and urine (11–13). The reason for the variation in molecular size is unclear. In the present study, the different preparations of GPBP had differing molecular sizes (Fig. 2). Lactoferrin, which is found mainly in breast milk and other secretions, shares many properties with transferrin in terms of iron-binding properties, molecular size, and charge. However, in the present study, we were able to show that purified lactoferrin did not have GPBP activity and that our purified GPBP preparations were not contaminated with lactoferrin.

A number of transferrin variants have been described which show characteristic peptide maps suggestive of differences in primary structure (5, 14). At least 20 variants of transferrin are known in man. We are yet to determine whether these variants differ in GPBP activity. It seems unlikely that GPBP activity is directly related to the iron-binding capacity of transferrin. For instance, apotransferrin was as effective as iron-saturated transferrin in promoting granulocyte/pollen binding (Kay, A. B., S. P. Sass-Kuhn, R. Moqbel, and J. MacKay, unpublished observations). It has been suggested that the two iron-binding sites on the transferrin molecule differ in their iron-binding properties, and the possibility that they also have distinctive roles in iron transport and metabolism points to the complexity of the uptake and release of iron and other metals by this protein (15, 16).

The existence of specific receptors for transferrin on the surface of human reticulocytes (17) and other cells and tissues including lymphocytes (18) and the placenta has been established (19). However, it seems unlikely that this conventional transferrin receptor is involved in the transferrin-induced enhancement of granulocyte/pollen adhesion described in the present study, since the reaction was not inhibitable by a monoclonal antitransferrin receptor (OKT9) antibody (Kay, A. B., S. P. Sass-Kuhn, R. Moqbel, and J. MacKay, unpublished observations).

Although the primary function of transferrin in mammalian metabolism is the transport of iron from sites of absorption and storage to sites of utilization, such as bone marrow of normal animals and the placenta of pregnant animals, it is also considered to have antimicrobial properties resulting from the avid capacity of this protein to bind iron and compete for this essential nutrient required for the growth of most bacteria, fungi, and viruses. The present findings indicate that enhancement of granulocyte/pollen binding is a further function of transferrin that is unrelated to iron transport or to its antimicrobial properties. The widespread extracellular distribution of transferrin may be relevant to the role of this protein in the removal of certain organic matter, including pollen grains.

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