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Separation of polysaccharide-specific human immunoglobulin G subclasses using a Protein A Superose column with a pH gradient elution system

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ABSTRACT

Protein A Superose was employed to separate affinity-purified anticarbohydrate antibodies according to immunoglobulin G (IgG) subclass. Separation was achieved with a novel buffer system (disodium phosphate–sodium acetate–sodium chloride–glycine), which allowed the generation of a linear pH gradient from pH 8 to 3. Protein A-bound anti-carbohydrate antibodies were eluted as three peaks, two of them mainly containing IgG2 and one consisting of highly enriched IgG1. The enriched antibody preparations retained their functional activity. This separation procedure can be considered as an alternative to the preparation of IgG subclasses with subclass-specific monoclonal antibodies and could be employed whenever contamination with immune complexes has to be avoided.

INTRODUCTION

Antibody preparations enriched in immunoglobulin G (IgG) subclasses constitute an important tool for studying the biological role of the various IgG isotypes. Several methods for the separation of human IgG subclasses have been developed, mainly utilizing immunoaffinity chromatography with monoclonal antibodies [1–3] or based on differences in the isoelectric points of IgG subclasses [4,5]. The aim of this study was to obtain an IgG preparation enriched in polysaccharide-specific IgG2 without the use of monoclonal antibodies. To achieve this aim we employed Protein A Superose, a new column chromatographic material that allows high flow-rates and is therefore especially applicable for fast protein liquid chromatography (FPLC). By using a novel buffer system that led to the generation of a linear gradient from pH 8 to 3, separation of *Haemophilus* capsular polysaccharide-specific and pneumococcal polysaccharide-specific human IgG into its subclasses could be achieved. The separated IgG fractions retained their functional activity.

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EXPERIMENTAL

Materials

Pneumococcal polysaccharide serotype 3 was obtained from American Type Culture Collection (Rockville, MD, USA). *Haemophilus influenzae* type b capsular polysaccharide polyribosyl ribitol phosphate (HIB-PRP) was prepared by Dr. A. Mitterer (Immuno, Vienna, Austria). Hyperimmunoglobulins specific for pneumococcal polysaccharides and for *Haemophilus influenzae* type b polysaccharide (HIB-PRP) were obtained from Immuno. EAH-Sepharose 4B was obtained from Pharmacia–LKB (Uppsala, Sweden). All other reagents were from Merck (Darmstadt, Germany). The Protein A Superose HR 10/2 column was obtained from Pharmacia–LKB and was connected to a Pharmacia FPLC system (consisting of an LCC-500 control unit, two P-500 pumps and a Frac-100 fraction collector). The column effluent was monitored for absorbance at 280 mm with a 2-mm flow-through cell and a UV-1 monitor (Pharmacia–LKB), and for pH with a flow-through cell connected to a pH monitor (Pharmacia–LKB). Buffer solutions for the FPLC runs were sterile-filtered and degassed.

Affinity purification of antigen-specific antibodies

Haemophilus influenzae type b capsular polysaccharide (HIB-PRP) and pneumococcal polysaccharide serotype 3 were coupled to EAH-Sepharose 4B according to the method of Munson *et al.* [6]. Gel materials were packed into low-pressure columns.

HIB-PRP and pneumococcal hyperimmunoglobulins were dissolved in PBS and loaded on to the respective affinity columns equilibrated with the same buffer. Antigen-specific antibodies were eluted with 3.5 *M* magnesium chloride in phosphate-buffered saline (PBS). Eluted antibodies were dialysed against PBS and stored at −20°C.

Protein A Superose chromatography

Affinity-purified antibodies were dialysed against buffer A [0.1 *M* disodium phosphate–0.1 *M* sodium acetate–0.1 *M* glycine–0.15 *M* NaCl, (pH 8.1)]. Chromatography was performed at room temperature by the following procedure: 5–10 mg of antigen-specific antibodies were loaded on to a Protein A Superose HR10/2 column connected to an FPLC system. Unbound material was removed by washing with 16 ml of buffer A. Bound IgG was eluted with a linear gradient (40 ml) starting with buffer A and ending with a 0.1 *M* sodium acetate–0.1 *M* glycine–0.15 *M* NaCl buffer (pH 2.8) (buffer B). The column was then washed with an additional 24 ml of pure buffer B before regeneration with buffer A. A flow-rate of 1 ml/min was maintained throughout sample application, washing and elution. The fractions from the peak maxima (2–3 ml each) were collected, neutralized immediately and then dialysed against PBS. Up to six runs with each of the antibody preparations were performed, and the corresponding peak fractions were pooled for protein analysis. For chromatography of serum samples, the various sera were diluted at least sixfold with buffer A, and material corresponding to 5–10 mg of IgG, as determined by radial immunodiffusion, was applied to the column and treated as described above.

Myeloma proteins were purified from patients' sera by ethanol precipitation [7] and chromatography on DEAE Affi Gel Blue (Bio-Rad, Richmond, CA, USA) [8]. The proteins were obtained as 20–50 mg/ml solutions and were diluted to 0.4–0.6 mg/ml with buffer A. Aliquots of 2 ml were loaded on to the column and pH gradient elution was performed as described.

Protein analysis

Protein concentration was determined by the use of the IgG extinction coefficient $E_{280}^{1\%} = 13.5$ [9] or by the bicinchoninic acid method (BCA-Protein Assay Kit; Pierce, Oud-Beijerland, Netherlands) using bovine γ-globulin (Bio-Rad) as a standard.

To determine total IgG, commercial radial immunodiffusion (RID) plates (EP-RID; Immuno) and a human standard serum (Immuno) were used. IgG subclass concentrations were determined by radial immunodiffusion with poly-

clonal RID kits (BINDARID; Binding Site, Birmingham, UK).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with reduced samples using ExcelGel SDS gradient 8–18 ready-to-use gels (Pharmacia–LKB) according to the supplier's instructions.

Determination of antigen-specific antibody titres by ELISA

Pneumococcal polysaccharide and HIB-PRP were coupled to tyramine as described by Barra *et al.* [10]. Enzyme-linked immunosorbent assay (ELISA) plates (NUNC, Kamstrup, Denmark) were coated with the coupled material (1 μg per well) by incubation at 4°C for 16 h. After blocking residual binding sites with 2% bovine serum albumin (4 h at 37°C), sample and standard dilutions were applied and incubated overnight at 4°C. A human serum pool (PL30; Immuno) with 10 000 arbitrary units per ml was used as a standard. Bound IgG was detected with biotinylated anti-human IgG (Vector, Burlingame, CA, USA) (diluted 1:1000; 2 h at 37°C) and with the Vectastain ABC kit [avidin-biotinylated peroxidase complex (Vector), 40 min at 25°C] according to the supplier's instructions. Colour reaction was performed using ABTS tablets (Boehringer, Mannheim, Germany) and was measured at 405 nm with an Immuno Reader (NUNC).

RESULTS

Separation of polysaccharide-specific immunoglobulin preparations into IgG subclasses

Anti-Haemophilus influenzae PRP antibodies. An anti-*Haemophilus* hyperimmunoglobulin was affinity-purified using Sepharose-immobilized *Haemophilus* PRP. This purification step resulted in both an increase in the IgG2 content and a marked increase in the ELISA titre per μg protein (Table I). IgG3 and IgG4 were not detectable or comprised less than 1% of the total IgG in this isolate. This affinity-purified material was loaded on to the Protein A Superose column. Bound IgG was eluted with a gradient that started with a phosphate–acetate–glycine–NaCl buffer (pH 8.1) and ended with an acetate–glycine–NaCl buffer (pH 2.8). By using this buffer system, a linear pH gradient from pH 8 to 3 was generated, which allowed the separation of the polysaccharide-specific IgG into three peaks with maxima differing by 0.5–0.7 pH units (Fig. 1A and Table I). Two of the peaks (peaks 1 and 3) consisted primarily of IgG2, whereas in peak 2 IgG1 was predominant. The lowest anti-PRP titre per μg of protein was seen in the IgG1 material of peak 2. The IgG2 eluting at the lower pH (peak 3) had a higher anti-PRP titre than the IgG2 eluting at the higher pH (peak 1). When the three isolated peak fractions were subsequently rechromatographed on Protein A

TABLE I

FRACTIONATION OF ANTI-HIB-PRP ANTIBODIES

Amounts of 5–10 mg of affinity-purified antibodies obtained from hyperimmunoglobulin preparations were loaded on to a Protein A Superose HR 10/2 column and eluted with a linear pH gradient as described under Experimental. Fractions (2–3 ml) were collected at the peak maxima, neutralized and dialysed against PBS. Six runs were performed, and the corresponding peak fractions were pooled and used for further analysis.

	Protein (mg/ml)	IgG1 (%)	IgG2 (%)	Titre (units/μg protein)	Elution pH
Hyperimmunoglobulin	80.02	63	37	7	
Affinity-purified antibodies	10.78	45	55	694	
Protein A, peak 1	0.40	14	86	812	4.8
Protein A, peak 2	0.51	75	25	411	4.1
Protein A, peak 3	0.30	28	72	1501	3.5

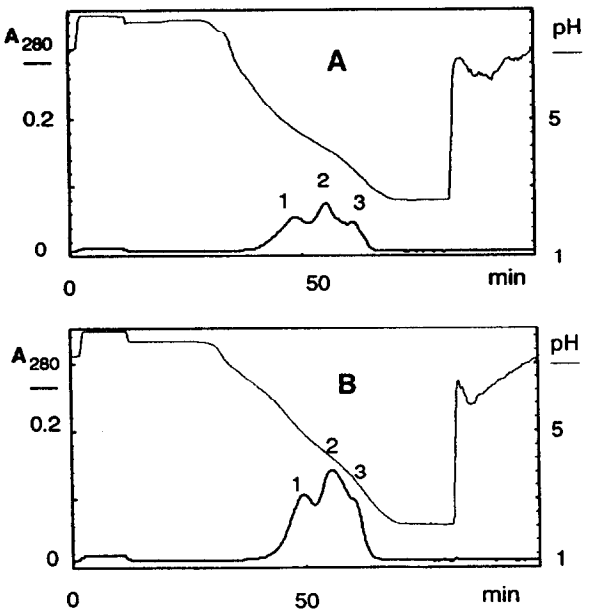

Fig. 1. Fractionation of anti-polysaccharide immunoglobulin preparations into IgG subclasses. (A) Affinity-purified anti-*Haemophilus* PRP antibodies or (B) anti-pneumococcal polysaccharide antibodies were loaded on to a Protein A Superose HR 10/2 column and eluted at 1 ml/min with a pH gradient as described under Experimental. Absorbance was measured at 280 nm.

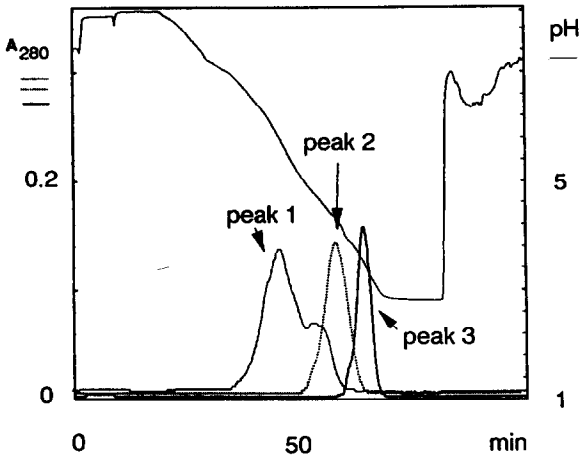

Fig. 2. Rechromatography of the isolated peak fractions of Fig. 1A on Protein A Superose The pooled peak fractions obtained with Protein A Superose chromatography of affinity-purified anti-PRP antibodies (shown in Fig. 1A) were rechromatographed separately on Protein A Superose. The elution patterns of the three runs have been superimposed graphically (peak 1, thin line; peak 2, broken line; peak 3, thick line). Absorbance was read at 280 nm.

Superose, single prominent peaks and, for peak 1, an additional small peak due to the respective impurities from the neighbouring peak were observed (Fig. 2). Their location within the pH gradient was identical with that of their predecessors, indicating that IgG2 can in fact be separated according to differences in the affinity to Protein A Superose. When analysed in reduced form by SDS-PAGE, the three IgG subclass preparations presented the same band pattern as did the hyperimmunoglobulin starting material (data not shown).

Anti-pneumococcal antibodies. As described for the isolation of anti-*Haemophilus* PRP antibodies, anti-pneumococcal antibodies were affinity-purified from an anti-pneumococcal hyperimmunoglobulin (containing 56% IgG1 and 43% IgG2) using immobilized pneumococcal polysaccharide serotype 3. The affinity-isolated material consisted of 52% IgG1 and 48% IgG2, with IgG3 and IgG4 contents of less than 1%. The affinity-purified material was separated on the Protein A Superose column with a pattern (Fig. 1B) nearly identical with that for the separation of anti-*Haemophilus* antibodies, *i.e.*, the three protein peaks obtained had similar IgG subclass distributions (peak 1, 19% IgG1, 81% IgG2; peak 2, 82% IgG1, 18% IgG2; peak 3, 35% IgG1, 65% IgG2). These data show that the separation of IgG into two different IgG2 peaks was not a property of anti-*Haemophilus* PRP antibodies.

Separation of IgG subclasses in normal human sera

When normal human serum from individual donors was passed over the Protein A Superose column and eluted with the pH gradient described above, the bound material was resolved into three peaks (Fig. 3). The first peak (1) consisted of nearly (94%) pure IgG2, the second peak (2) contained 94% IgG1 and 6% IgG2, and the third peak (3) consisted of approximately equal amounts of IgG1 and IgG2. This demonstrates that a biphasic elution pattern is a common property of polyclonal IgG2. IgG3 could not be detected in the eluted peaks, as protein A does not bind IgG3 [11]. IgG4 levels in the protein peak fractions were below the detection limit of our assay system (0.02 mg/ml). Similar

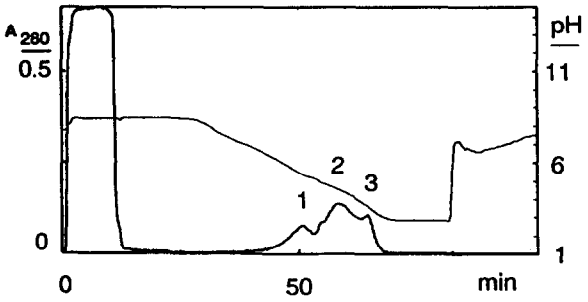

Fig. 3. Separation of human IgG subclasses in normal human serum. A 1-ml volume of human serum from a single donor was diluted with column buffer, loaded on to a Protein A Superose HR 10/2 column and eluted with the pH gradient described.

results were obtained with sera from two other donors (data not shown).

Elution of monoclonal IgG2 as a single peak

In contrast to polyclonal IgG2, three different purified myeloma IgG2 proteins eluted from Protein A Superose as single peaks at distinct pH values (4.9–5.1); the elution profile of one of them is shown in Fig. 4.

DISCUSSION

In pursuing the goal of isolating human IgG2 antibodies against carbohydrate antigens without the use of immunoaffinity chromatography with IgG2-specific monoclonal antibodies, the recently developed Protein A Superose column was employed. A simple buffer system consisting of only two components was developed to generate a linear pH gradient between pH 8 and 3. This allowed the separation of affinity-purified anti-*Haemophilus* capsular polysaccharide antibodies (HIB-PRP) into fractions with highly enriched IgG subclass contents.

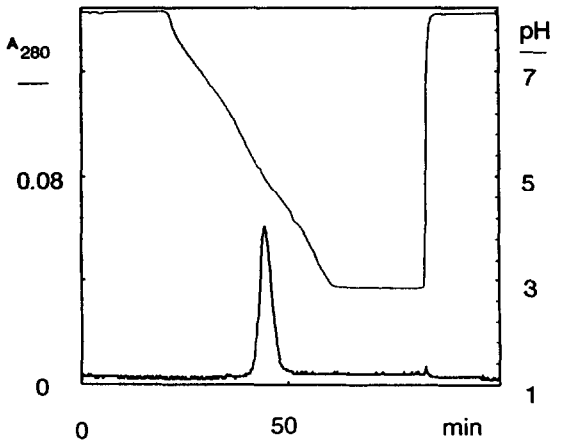

Fig. 4. Elution of purified IgG2 myeloma proteins from Protein A Superose as single peaks. Purified IgG2 myeloma protein diluted in column buffer A was loaded on to a Protein A Superose HR 10/2 column and eluted with the pH gradient described.

The affinity-purified anti-HIB-PRP preparation used as the starting material for IgG subclass purification consisted of nearly equal amounts of IgG1 and IgG2 and only traces of IgG3 and IgG4. This is in contrast to antibody responses to other bacterial polysaccharides, such as group A streptococcal carbohydrates or dextran from Lactobacteriaceae or levan from Corynebacteriaceae, which predominantly result in the production of IgG2 [12]. Our data, however, are consistent with results reported by Goodall *et al.* [3], who also found IgG1- and IgG2-containing anti-HIB fractions following separation of HIB-specific IgG with immobilized mouse monoclonal anti-IgG subclass antibodies. Further, analysis of anti-HIB sera by IgG subclass-specific ELISA also revealed the presence of both HIB-specific IgG1 and IgG2 [13].

When this affinity-purified material was loaded on a Protein A Superose column and eluted with the described buffer system, the IgG preparation was resolved into three peaks. Two of these peaks contained IgG2 and were separated by a central peak consisting of IgG1. This elution pattern was not specific for anti-HIB antibodies, as the separation of anti-pneumococcal antibodies and also of normal serum IgG yielded a corresponding elution pattern and similar IgG subclass distribution of the isolated fractions (see Figs. 1B and 3).

The finding of a second IgG2 peak during elution of polyclonal IgG from the column points to a different protein A affinity for distinct IgG2 moieties and is obviously due to the high resolution of Protein A Superose (even at the relatively high flow-rate of 1 ml/min) in combination with the linear pH elution system. In this respect, the system described is superior to the biphasic pH gradient published by Duhamel *et al.* [14], which resulted in the separation of Protein A Sepharose-bound human serum IgG

into two overlapping peaks composed primarily of IgG1 and IgG2, respectively. The distinctiveness of the two IgG2 peaks obtained by the present procedure was indicated by rechromatography of the IgG subclass fractions, which resulted in the elution of single prominent peaks eluting at exactly the same pH as their predecessors (Fig. 2). The reasons underlying the separation of IgG2 into two fractions with different binding capacities to staphylococcal protein A have yet to be elucidated. The data presented here, however, suggest that the two peaks contain IgG2 specific for different epitopes on the polysaccharide antigen. Monoclonal IgG2 which reacts only with one antigenic epitope elutes from the column as a single peak (Fig. 4).

The novel IgG fractionation procedure described here yielded two functionally active and structurally unmodified IgG2 preparations. Their capacity to bind to their respective antigen demonstrates the presence of a functional antigen-binding region, and both preparations contained largely unmodified IgG molecules, as shown by unimpaired interaction with the second-step antibody. Further, preliminary studies (data not shown) also demonstrated that the complement-binding properties of both IgG2 fractions remained intact.

Although more highly purified IgG subclass preparations can be obtained by monoclonal antibody-immunoaffinity chromatography, the use of Protein A Superose for isolating IgG subclass anti-polysaccharide antibodies has certain advantages. Ready-to-use chromatographic columns are available with comparable lot-to-lot properties. The column can be cleaned with denaturing detergents and even with some organic solvents, making reliable removal of pyrogens possible. Further, the column matrices coupled with monoclonal antibodies against IgG subclasses may leak protein, which will then lead to the formation of immune complexes with sample proteins. These immune complexes may cause unwanted side-effects if present in preparations used for long-term therapy. Even traces of immune complexes present as contaminants have been shown to exert immune-modulating effects which lead to impairment of certain monocyte functions [15–17]. Hence the procedure described here can be employed for the preparation of IgG2 subclass-enriched products in which contamination with immune complexes has to be avoided.

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