Journal of Chromatography, 212 (1981) 179–186
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,821

USE OF $F(ab')_2$ ANTIBODY FRAGMENTS IN THE SYNTHESIS OF IMMUNOADSORBENTS FOR PREPARING MONOSPECIFIC ANTIGEN

JOHN F. KENNEDY* and JUNOR A. BARNES

Research Laboratory for Bioactive Carbohydrates and Proteins, Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Great Britain)

(Received March 24th, 1981)

SUMMARY

Class specific $F(ab')_2$ antibody fragments were prepared by pepsin digestion of the labile Fc immunoglobulin fragments in antigen–antibody precipitates. The $F(ab')_2$ fragments were covalently coupled to cyanogen bromide-activated Sepharose® 4B and the resultant immunoadsorbent used to isolate IgG from human serum with a single chromatographic step, in high yield and purity and negligible non-specific interaction. This technique affords a simple method for preparing an enriched source of class specific affinity-purified immunoglobulin antibodies suitable for many immunochemical applications.

INTRODUCTION

The use of affinity-purified antibodies in the preparation of immunoadsorbents should, theoretically, increase the specific binding capacity of the immunoadsorbents and minimize non-specific interactions. However, the conventional method for preparing affinity-purified antibodies involves a number of steps. The antigen–matrix immunoadsorbent is first prepared and used to isolate specific antibodies by suitable elution techniques. After subsequent dialysis, concentration and characterization, the antibodies are used to prepare an antibody–matrix immunoadsorbent. The degree of protein denaturation increases with the number of manipulations.

Pepsin hydrolysis of immunoglobulins has revealed that the $F(ab')_2$ fragment is the major undigested product resulting from the proteolysis of IgG[1,2]. Since this fragment possesses the two antigen binding sites of the parent molecule it is still an effective truncated antibody molecule capable of binding the complementary antigen. The class specific Fc region of the immunoglobulin molecule is the most immunogenic part of the molecule[3]. Consequently, antisera raised to immunoglobulins consist mostly of antibodies of the antigenic determinants in the class specific Fc region. Lachman[4] employed pepsin digestion of antigen–antibody precipitates at optimal proportion to prepare class specific anti-immunoglobulin antibodies as $F(ab')_2$ fragments. The application of this method to other antigens depends, however, on their susceptibility to pepsin digestion.

0021-9673/81/0000–0000/S02.50 © 1981 Elsevier Scientific Publishing Company

The removal of the Fc portion of the immunoglobulin molecule should afford several advantages: (i) it should minimize the contribution to non-specific binding associated with the heterologous antibodies[5,6] and (ii) since complement binds primarily to the Fc region removal of this region should eliminate serum complement binding to solubilized antibody[7].

In this study we report the preparation of covalent conjugates of immunoglobulin $F(ab')_2$ fractions with cyanogen bromide-activated Sepharose® 4B and demonstrate that the $F(ab')_2$-matrix immunoadsorbent is more efficient than the intact antibody–matrix immunoadsorbent in the purification of class specific IgG from pooled, whole human serum. Truncation of the antibody molecule did not impair the capacity of the antibodies to bind reciprocal antigens. In addition, no severe loss in stability and activity of the cyanogen bromide-activated Sepharose 4B-$F(ab')_2$ immunoadsorbent was observed after five serial adsorption–desorption cycles. The antibody (sheep anti-human IgG) used in the present study for preparing the $F(ab')_2$ fraction and in previous studies for preparing immunoadsorbents[5,6] was obtained by ammonium sulphate precipitation of crude immunoglobulin from whole human serum followed by DEAE-cellulose anion-exchange chromatography of the dissolved precipitate. Only a small portion (about 10%) of the resultant IgG fraction is specific antibodies (most of the immunoglobulins are inert or non-specific). Nevertheless, the use of $F(ab')_2$ fragments derived from immune precipitates results in an approximately 10–20 fold purification of the initial antibody fraction.

MATERIALS AND METHODS

Cyanogen bromide-activated Sepharose® 4B and Sephadex® G-150 (40–120 μm beads) were obtained from Pharmacia (Uppsala, Sweden). Sheep anti-human IgG (batch Z511G, 24.7 mg ml^{-1}), human IgG (batch D152, 6.34 mg ml^{-1}) and NIRDL (normal human serum code number BR99) serum standard were purchased from Seward Laboratories (London, Great Britain). Chromatography columns GA 10 × 15 (1.0 × 15 cm) were obtained from Wright Scientific (Kenley, Great Britain). Pepsin was purchased from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.), agarose for immunochemical assays from Fisons (Loughborough, Great Britain). The materials used for preparing buffers and salt solutions were of AnalaR grade.

Preparation of antigen–antibody (human IgG–sheep anti-human IgG) precipitates

The titre (the amount in mg of antigen which reacts with a known amount of antiserum at the equivalence point) of the sheep anti-human IgG antibody was determined by a preliminary precipitin titration followed by manual nephelometric quantitation of the amount of precipitate[8]. The antigen–antibody precipitate was then prepared close to the equivalence point. Human IgG (4 ml, 5.93 mg ml^{-1}) dialysed against normal saline was added to sheep anti-human IgG (10 ml, 24.7 mg ml^{-1}) which was also dialysed against saline and adjusted to 0.1 M with respect to ethylenediaminetetraacetate (EDTA) in order to enhance precipitation. After thorough mixing, the solution was incubated at room temperature for 30 min followed by 5 min at 4°C to complete precipitation. The precipitate was then centrifuged for 10 min at 4°C, 2000 g, and after washing with cold saline it was dissolved in

distilled water (10 ml) acidified with a few drops of acetic acid (0.2 ml). The solution was finally adjusted to pH 3.1 with 0.2 ml acetic acid and the volume made up to 15 ml with distilled water. An aliquot (0.5 ml) of the solution was assayed for protein by the Folin Ciocalteu technique[9] and the remaining 14.5 ml were used for pepsin digestion.

Pepsin digestion of the antigen–antibody complex (human IgG–sheep anti-human IgG)

To the solution (14.5 ml) of the dissolved precipitate, freshly prepared pepsin solution (1 mg ml^{-1} in 0.2 *M* acetic acid) was added to yield a 2% (w/w) pepsin solution which was then incubated at 37°C for 3 h. The enzyme reaction was terminated by adjusting the solution to pH 8.0 with sodium carbonate solution (0.5 *M*), and the solution was incubated further at 37°C for 4 h.

Isolation of $F(ab')_2$ from pepsin digest

Anhydrous sodium sulphate (to 1.5 *M*) was added to the pepsin digest with stirring at 37°C. After standing at room temperature ($\approx$20°C) for 30 min, the mixture was centrifuged at 20°C, 2000 *g*, for 10 min. The precipitate was then redissolved in distilled water (5.0 ml) and an aliquot (0.5 ml) used for protein assay by the Folin Ciocalteu method[9]. The remaining 4.5 ml of the dissolved sodium sulphate precipitate were concentrated to 2.5 ml by negative pressure dialysis. To the 2.5-ml concentrate [11.1 mg $F(ab')_2$ equivalent to 16.7 mg antibody], Bence Jones protein marker (10 mg) was added and the mixture loaded on to a Sephadex G-150 column (2.2 × 81 cm) pre-equilibrated with a solution of sodium chloride (0.5 *M*) buffered at pH 7.2 with tris(hydroxymethyl)aminoethane–HCl (Tris, 0.1 *M*) containing 0.02 *M* sodium azide. Elution was effected with the sodium chloride–Tris buffer (pH 7.2) which allowed separation of $F(ab')_2$ from any soluble aggregates, the p Fc′ fraction and smaller peptide products. The eluted fractions (5 ml) were collected with the aid of an LKB (Selsdon, Surrey, Great Britain) automatic fraction collector at a flow-rate of 18 ml/h and monitored at 280 nm with an LKB Uvicord analyser.

Immunochemical analysis

The fractions for the major protein peaks resulting from the Sephadex G-150 gel filtration were pooled, dialysed against distilled water and concentrated by negative pressure dialysis using 18/32 in. Visking dialysis tubing. The purity of the pooled fractions was assayed by Ouchterlony double immunodiffusion[10] and one-dimensional immunoelectrophoresis[11] (i.e.p.) techniques. Immunoelectrophoresis was performed on glass plates (8 × 8 cm) coated with 1% agarose in barbitone buffer (0.06 *M*, pH 8.6). Electrophoresis was conducted for 80 min with a constant current of 10 mA or about 7 V per cm width (interelectrode distance) of the plate. The troughs were filled with antisera and diffusion allowed to occur while the plate was left in a moist atmosphere for about 12 h. The antigen binding capacity, *C*, of an antibody immunoadsorbent column was calculated as

$$C = (P - p)\,T$$

where P is the amount of antibody added to the actual gel; p the amount coupled and T is the titre of the antibody[5].

Coupling of sheep anti-human IgG $F(ab')_2$ to cyanogen bromide-activated Sepharose 4B

Cyanogen bromide-activated Sepharose 4B (1 g dry weight) was swollen in, and washed with 10^{-3} *M* HCl to remove dextran and lactose stabilizers, filtered on a No. 3 sintered Buchner funnel and equilibrated in sodium citrate buffer (0.2 *M*, pH 6.5). The $F(ab')_2$ solution (4 ml, 3.6 mg) predialysed against sodium citrate buffer was added to the Sepharose gel suspended in sodium citrate buffer (6 ml, approximately twice the volume of the swollen gel) in a universal vial and tumbled end over end for 3 h at room temperature. After filtering, the gel was washed in sodium citrate buffer and then lightly stirred magnetically in excess of freshly prepared aqueous ethanolamine solution (1 *M*, pH 9.5) for 2 h at room temperature to block residual active groups. After filtering, the gel was washed with sodium chloride–Tris buffer (0.5 *M* NaCl, 0.1 *M* Tris, pH 7.2) containing 0.5% (w/v) sodium azide. All the washings and filtrates were retained and pooled and an aliquot of the concentrate was monitored for uncoupled antibody by the reverse Mancini technique, *i.e.*, with the antigen instead of the antibody dispersed in the agarose gel mounted on a glass plate.

Immunoadsorbent column preparation

After degassing, the Sepharose 4B-$F(ab')_2$ immunoadsorbent was packed into a column (1.0 × 15 cm), washed with ammonia solution (0.5 *M*, pH 11.5) and equilibrated with NaCl–Tris buffer until the absorbance reading at 280 nm (1-cm cell) corresponded to that of the ambient buffer. Pooled whole human serum (0.1 ml, 1.12 mg IgG) was loaded onto the column which was then washed with NaCl–Tris buffer (0.5 *M*/0.1 *M*, pH 7.2) until the absorbance of the eluate at 280 nm was negligible. The immuno-adsorbed IgG was then eluted with ammonia solution (0.5 *M*, pH 11.5) in 4-ml fractions until the absorbance of the eluate at 280 nm was negligible (<0.005). The column was re-equilibrated in NaCl–Tris buffer before another adsorption–desorption cycle commenced.

The eluates were monitored on an LKB Uvicord analyser at 280 nm and the fractions collected on an LKB automatic fraction collector. Both the pooled unadsorbed and pooled desorbed IgG fractions were quantitated by the Mancini technique[12] using NIRDL serum standard (11.6 mg IgG per ml) for calibration.

RESULTS

Yield of $F(ab')_2$ antibody fragments

In Fig. 1 is illustrated the gel filtration fractionation of the sodium sulphate precipitate resulting from the pepsin digested antigen–antibody precipitate. The yield of $F(ab')_2$ antibody fragments from the sheep anti-human IgG–human IgG precipitate is summarized in Table I. After Sephadex gel filtration, approximately 6 mg $F(ab')_2$ were obtained from the initial antigen–antibody precipitate (19.5 mg), a 30% yield. However, assuming that 6 mg $F(ab')_2$ are equivalent to 9 mg native antibody[4], some 44% of the immunoglobulin in the original precipitate was recovered. This accords favourably with the results reported by Lachman[4].

Activity of $F(ab')_2$ fragments

The activity of the $F(ab')_2$ fraction obtained by Sephadex gel filtration was

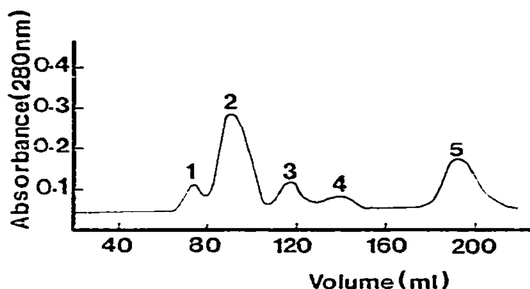

Fig. 1. Elution profile of the dissolved sodium sulphate precipitate from pepsin digested antigen–antibody precipitate. A solution (2.5 ml, 4.4 mg ml^{-1}) of the precipitate was applied to a column of Sephadex G-150 (81 × 2.2 cm) equilibrated with sodium chloride–Tris buffer (pH 7.2). Peaks: 1, soluble complexes; 2, $F(ab')_2$, the only peak to show antibody activity; 3, p Fc′ species (dimers of the C_H3 homology region of the antibody molecule); 4, polypeptides; 5, the Bence Jones protein marker.

TABLE I

SUMMARY OF THE QUANTITATIVE YIELD OF $F(ab')_2$ FRAGMENTS FROM SHEEP ANTI-HUMAN IgG–HUMAN IgG PRECIPITATE AND ITS PEPSIN DIGEST

Antigen–antibody precipitate (mg)	*Protein yield after pepsin digestion (mg)*	*Protein loaded on Sephadex column (mg)*	*$F(ab')_2$ yield (mg)*	*Yield of $F(ab')_2$ from antigen–antibody precipitate (%)*
19.5	12.5	11.1	5.9	30.0

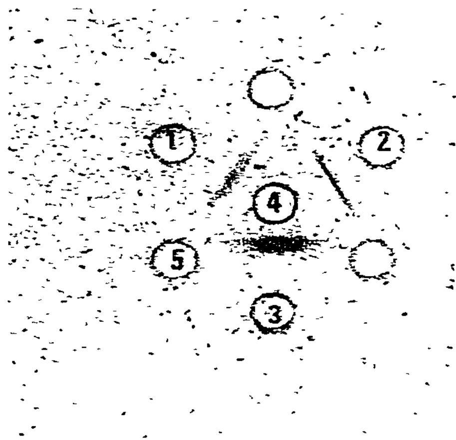

Fig. 2. Ouchterlony double immunodiffusion analysis of sheep anti-human $F(ab')_2$ antibody against human IgG. The central well (4) contained the $F(ab')_2$ fraction (1 μl, 6.3 μg) and wells 1, 2 and 3 contained 6.0, 2.5 and 60 μg human IgG respectively; well 5 contained 6 μg sheep IgG. The wells were punched in a 1% agarose gel on a glass plate (4 × 1 cm).

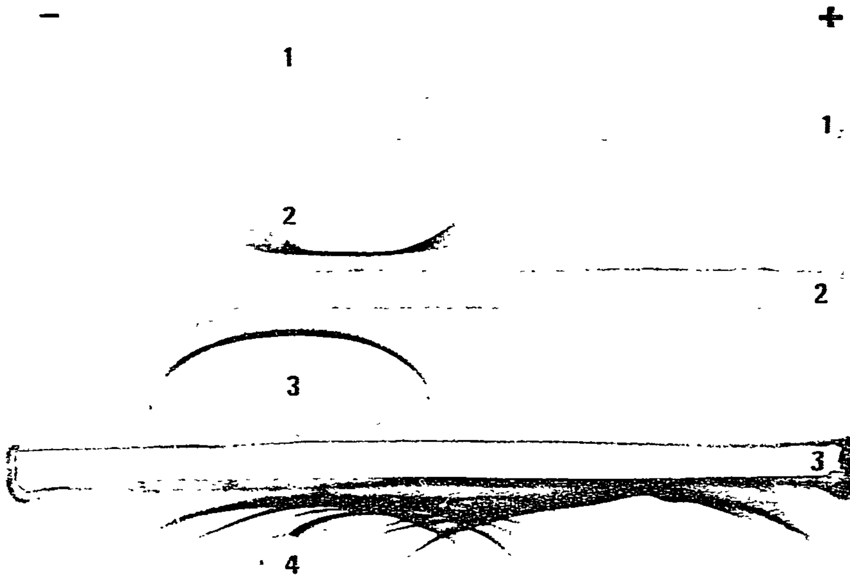

Fig. 3. Immunoelectrophoretic analysis of sheep anti-human $F(ab')_2$. Wells: 1 and 3, 2 μl human IgG, 6.34 mg ml^{-1}; 2 and 4, 1 μl NIRDL standard, 11.6 mg IgG per ml. Troughs: 1, $F(ab')_2$; 2, sheep anti-human IgG; 3, sheep anti-whole human serum.

determined by Ouchterlony double immunodiffusion (Fig. 2) and immunoelectrophoresis (Fig. 3). Pronounced precipitin lines were observed between the $F(ab')_2$ in well 4 and the various antigen concentrations in wells 1, 2 and 3. It is noticeable that the sharpest precipitin line was between wells 2 and 4, indicating optimal antigen–antibody concentrations. There was no precipitin line between sheep IgG in well 5 and $F(ab')_2$ in well 4. In Fig. 2 a distinct precipitin line was obtained between $F(ab')_2$

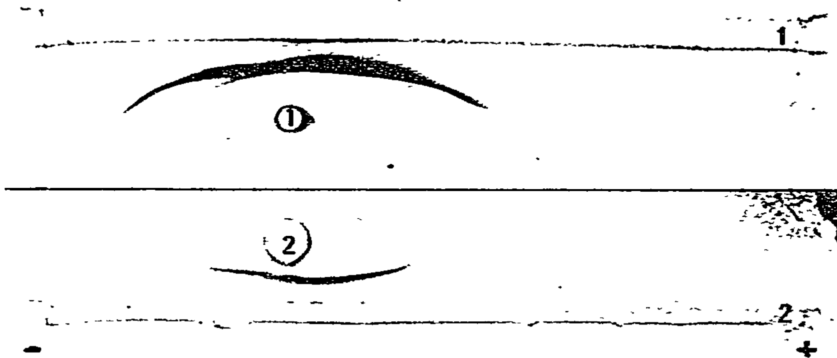

Fig. 4. Immunoelectrophoretic analysis of affinity-purified IgG using the cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG $F(ab')_2$ immunoadsorbent. Wells: 1, 10 μl NIRDL standard; 2, 10 μl eluted IgG, 1 μg. Troughs: 1 and 2, sheep anti-whole human serum.

TABLE II

SUMMARY OF THE QUANTITATIVE PERFORMANCE OF THE CYANOGEN BROMIDE-ACTIVATED SEPHAROSE 4B–SHEEP ANTI-HUMAN $F(ab')_2$ IMMUNOADSORBENT COLUMN

ND = Not determined.

Description	*Cycle*				
	1	*2*	*3*	*4*	*5*
IgG applied (mg)	1.12	1.12	1.12	1.12	1.12
IgG unadsorbed (mg)	0.9	1.1	1.0	0.9	0.6
(%)	(80.4)	(93.7)	(89.3)	(80.4)	(53.6)
IgG adsorbed (mg)	0.22	0.07	0.12	0.22	0.52
(%)	(19.7)	(6.3)	(10.7)	(19.7)	(46.4)
IgG eluted (mg)	0.22	ND	ND	0.22	0.52
(%)	(100.0)			(100.0)	(100.0)
Potential binding capacity (mg IgG)	0.2	0.2	0.2	0.2	0.2
Purity	Very good			Very good	Very good

(trough 1) and human IgG (well 1). The $F(ab')_2$ precipitin line corresponded with similar IgG precipitin lines obtained with the human serum standard (NIRDL) against sheep anti-human IgG (trough 2) and against sheep anti-whole human serum.

Purification of human serum IgG with cyanogen bromide-activated Sepharose 4B-$F(ab')_2$ immunoadsorbent column

The performance of the immunoadsorbent column is summarized in Table II. The yield of IgG was consistently 100% from five serial adsorption–desorption cycles. It is noticeable that the amount of adsorbed IgG corresponded to the binding capacity of the column (0.2 mg IgG) for the first and fourth cycles. Nevertheless, some variation in adsorbed IgG was observed in cycles 2 and 3. The amount of adsorbed IgG in the fifth cycle was approximately twice the value of the potential column binding capacity. This could be attributed to non-specific retention of IgG by the matrix. The purity of the eluted IgG fraction was excellent as determined by immunoelectrophoresis (Fig. 4). The eluted IgG fraction (well 2) gave a single precipitin line against sheep anti-whole human serum. This line corresponded to the IgG precipitin lines formed by human serum standard (NIRDL) in well 1 against sheep anti-whole human serum (trough 2).

DISCUSSION

The separation of affinity-purified $F(ab')_2$ antibodies from immune precipitates by pepsin digestion of the labile Fc region of the immunoglobulin molecule allows the preparation of class specific antibodies. Some pitfalls of the technique are firstly the contamination of $F(ab')_2$ antibody peak with $F(ab')_2$ from the antigen if the antigen is incompletely complexed with antibodies. Since no precipitin line was detected between sheep anti-human IgG and the purified $F(ab')_2$ fraction, this situation did

not occur under the conditions described herein. Secondly, the possible occlusion of antibody binding sites was not a dominant feature in view of the well defined precipitin lines obtained. Although the yield of $F(ab')_2$ antibody is dependent on the pepsin lability of the Fc region, the yield (30%) obtained in this study is quite reasonable for the IgG–anti-IgG system.

The use of cyanogen bromide-activated Sepharose 4B-$F(ab')_2$ immunoadsorbent offers a simple, quicker approach for the purification of class specific antigens. Many conventional intermediate steps are eliminated. High binding capacity immunoadsorbents can be prepared, resulting in negligible non-specific adsorption and requiring minimum antigen loading. After five serial adsorption–desorption cycles there was no drastic change in the column performance with respect to protein yield and purity. This technique has the potential for isolating antigens present in low concentration in plasma, urine and tissue fluid.

ACKNOWLEDGEMENTS

The authors thank the Endowment Fund Medical Research Committee, University of Birmingham, for a research grant to J.A.B., and Dr. J. B. Matthews for helpful suggestions regarding the preparation of this paper.

REFERENCES

1 A. Nisonoff, F. C. Wisler, L. N. Lipman and D. L. Woernely, *Arch. Biochem. Biophys.*, 89 (1960) 230.
2 A. Nisonoff, G. Markus and F. C. Wisler, *Nature (London)*, 189 (1961) 293.
3 E. Hurwitz, R. Maron, R. Arnon and M. Sela, *Cancer Biochem. Biophys.*, 1 (1976) 197.
4 P. J. Lachman, *Immunochemistry*, 8 (1971) 81.
5 J. F. Kennedy and J. A. Barnes, *Int. J. Biol. Macromol.*, 2 (1980) 289.
6 J. A. Barnes and J. F. Kennedy, *Immunochemistry*, (1981) in press.
7 D. R. Stanworth and M. W. Turner, in D. M. Weir (Editor), *Handbook of Experimental Immunology*, Blackwell, Oxford, 3rd ed., 1967, p. 6.1.
8 L. M. Killingsworth and J. Savory, *Clin. Chem.*, 18 (1972) 335.
9 O. H. Lowey, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 O. Ouchterlony, *Progr. Allergy*, 5 (1958) 1.
11 P. Grabar and C. A. Williams, *Biochim. Biophys. Acta*, 10 (1953) 193.
12 G. Mancini, A. D. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.