A SIMPLE PROCEDURE FOR THE PRODUCTION OF Fab FROM BOVINE IgG AS AN ABSORBENT IN THE PREPARATION OF CLASS-SPECIFIC ANTI-IMMUNOGLOBULIN

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Abstract—A simple and rapid procedure is proposed for the preparation of Fab from bovine IgG which is used as a single absorbent (removal of anti-light chain antibodies) in the production of class specific antisera anti γ (IgG), anti µ (IgM) and anti α (IgA).

The papain digest is chromatographed on DEAE-cellulose equilibrated with 0·01 M PO 4 pH 8·0, then rechromatographed on DEAE-cellulose with 0·01 M Tris-HCl pH 8·6 + 0·05 M NaCl, then precipitated with ammonium sulfate at 60% saturation. The supernatant contains Fab which is free of the Fc piece and of intact IgG.

In order to render anti-immunoglobulin sera class specific the anti-light chain antibodies must be absorbed. The fact that bovine IgG is readily available either from commercial sources or by anion-exchange chromatography makes the absorption of anti-IgA and anti-IgM antiserum relatively easy.

On the other hand, the isolation of bovine IgM or IgA which would be necessary for the absorption of anti-IgG is very time consuming (Messerli and Fey).

The differentiation and measurement of the bovine immunoglobulin classes IgG, IgM and IgA is becoming increasingly important. We therefore tried to produce the Fab fragment from papain-digested bovine IgG in order to use it as an absorbent. It contains the light chains and the Fd-piece of the gammaglobulin molecule and consequently should absorb anti-light chain antibodies from any anti-IgG antiserum. Since it is known that most of the antigenicity of the heavy chain polypeptide lies in the Fe-piece (Porter) and not in the Fd-piece, the latter should not absorb too much of the desired class specific anti-heavy chain activity of a given anti-IgG antiserum.

We did not use the Ig light chains for this purpose for the following reasons:

1. After reduction and alkylation of the immunoglobulins the resulting light chains must be separated from the heavy chains by gelfiltration on Sephadex G 75 in 1 N acetic acid (Fleischman et al., 1962), which is a laborious procedure with a small yield of light chain protein. After dialysis against phosphate buffered saline the heavy chains become unstable. The light chains, however, are less unstable but there is still a considerable loss due to precipitation at a neutral pH.

2. Ion exchange, which we use for our Fab production, is easier and more efficient on a preparative scale than gelfiltration.

3. In the bovine no clinical condition comparable to the human myelomatosis has been found where Bence Jones proteins (free urinary light chains) are present, which would make the production of light chains easier.

In a preliminary experiment with bovine Fab, kindly made available by Dr. Kickholén, it was proved that the class specific absorption of anti-IgG, anti IgA and anti-IgM is indeed practical.

We first followed the procedure of Škvářil (1960 and personal communication) and precipitated papain treated bovine IgG with ammonium sulfate at 50 per cent saturation which eliminates the intact gammaglobulin molecules. The supernatant was then brought to 75 per cent saturation which precipitates the Fab; this Fab, however, was found to be heavily contaminated with Fc.

With the original method of Porter who fractionated papain digested rabbit IgG by ion-exchange on CM-cellulose at pH 5·5 using a molarity gradient, we obtained a double breakthrough peak containing Fab and Fc as well. A combination of both of these methods (ammonium sulfate precipitation followed by cation-exchange) revealed an enriched Fab product which was, however, not pure enough.

Kickholén et al. filtered their mildly papainized IgG (2 mg of mercuripapain per 2 g of IgGS) through Sephadex G 100 and obtained a 3·5 S Fab/Fc-mixture in the second peak. This was further purified by zone electrophoresis on Geon Resin X-427; this procedure, however, depends on an apparatus which is not available everywhere.
The human and bovine Fab behave differently from the rabbit Fab, the rabbit Fab being acid, the human/bovine Fab being alkaline (Hammer, personal communication). Therefore Franklin used CM-cellulose for chromatography with 0.01 M PO_4 at pH 7.6. Under these conditions both Fab and Fc of my papain digested bovine IgG appeared in the breakthrough peak and were not separated.

We therefore tried other combinations of buffer, molarity and pH. Since bovine Fab is alkaline, we used DEAE-cellulose at an alkaline pH, expecting that the alkaline Fab would appear in the first peak. It is likely that the first eluted protein fraction is purer than the subsequently isolated ones which have to run through those parts of the column where the first fraction has already passed.

MATERIALS AND METHODS

Bovine immunoglobulin: IgGs precipitated by Na_2SO_4 from bovine colostral whey according to Kickhofen et al. was used in some experiments, in others the commercially obtainable Bovine Gammaglobulins Fraction II B and later A grade (Lot 201379) from Calbiochem.* In another trial the 7 S fraction from bovine serum globulin chromatographed by Sephadex G 200 was papainized. That means that in most of the experiments we used IgG as a mixture of IgG_1 and IgG_2 (Fig. 1). The gammaglobulin fraction II B grade (Calbiochem) was even slightly contaminated with other proteins. In a final confirming experiment we worked with a practically pure IgG_2 peak from DEAE cellulose equilibrated with 0.01 M PO_4 pH 7.6.

The procedure described by Kickhofen, Hammer and Westphal was applied with the exception that we used papain puriss. 60,000 U/g (Fluka, Buchs) at a concentration of 1 per cent rather than the pure mercaptpapain:

Two grams of IgG in 30 ml of phosphate buffered saline (PBS)pH 7.2 were added to 50 ml of a buffer containing 0.2 M sodium phosphate, 0.02 M 2-mercaptoethanol and 4 mM EDTA (pH 7.0) 20 mg (i.e. 1%) of papain in distilled water were added and the solution brought to a final volume of 100 ml with distilled water. The mixture was kept at 37°C for 2.5 hr after which the reaction was stopped by adding 25 ml of 1.0 M iodoacetamide. maintaining the pH at 7.5. After standing for 1 hr at room temperature the digest was dialysed against PBS for 2 days.

For gelfiltration we used Sephadex G 100 equilibrated with Tris (0.2 M)-Glycine (0.5 M)-buffer at pH 7.6. The second peak which emerged was considered to consist of a mixture of Fab and Fc and was used in the first ion-exchange experiments. Anion-exchange-chromatography was performed with DEAE-cellulose (Whatman Chromedia DE 52 microgranular, Bender and Hobein Zurich). Before application the protein solutions were dialysed over night against the starting buffers. Concentration of protein solutions was done by vacuum dialysis.

Immunoelectrophoresis and gel-diffusion on Ouchterlony plates were carried out in 1", agarose in barbital buffer, pH 8.6. The slides were stained with Coomassie Blue.

Production of antiserum:

100 to 200 µg of protein in 10 ml of PBS were emulsified with 10 ml of Freund's complete adjuvant and four times 0.2 ml each were injected between the toes of each foot of a rabbit on the first and the 25th day. So the rabbits received 50–100 µg of antigen. The bleeding was carried out on the 35th day (von Fellenberg, personal communication).

For the differentiation of Fab and Fc we used antisera of a previous investigation (Fey) namely anti-Fab, which had been produced with pepsinized IgGs = F(ab')_2 and anti-Fc. The latter was prepared by using a papain digest of IgGs as an immunogen (Fab/Fc-mixture), and the resulting antiserum was absorbed with F(ab')_2. Furthermore we isolated the Fc piece from papainized 7S IgG by zone electrophoresis on Geon Resin in barbital buffer 0.05 M pH 8.6. The most anodically moving protein was used as immunogen.

For confirmation some anti-Fc was kindly offered by Dr. Kickhoen, Freiburg i/Br.

RESUMEN

In preliminary experiments the papain digest was chromatographed on Sephadex G 100 and the proteins from peak No. 2 were mixed with DEAE-cellulose, which had been equilibrated with 0.01 M PO_4 pH 8.0 or with 0.01 M Tris–HCl pH 8.6 and eluted by a NaCl molarity gradient or by stepwise increases of the NaCl molarity. With the starting buffer a first peak appeared which contained a high concentration of Fab and traces of Fc. A peak which developed at 0.1 M NaCl
Production of Fab from Bovine IgG

Fig. 2. First step chromatography of papain treated bovine IgG (bovine serum was precipitated twice with ammonium sulfate at 50 per cent saturation, the globulin was passed through Sephadex G 200 and the second peak taken as 7S IgG) on DEAE-cellulose (DE 52) equilibrated with 0.01 M PO 4 pH 8.0. 130.8 mg protein, column 1.5 x 16 cm, fractions 4 ml. The first peak contains Fab, intact IgG and traces of Fc.

Fig. 3. Second step rechromatography of the protein collected with the first peak of the first step, on DEAE-cellulose equilibrated with 0.01 M Tris-HCl pH 8.6 + 0.05 M NaCl. 96 mg protein, column 1.5 x 16.5 cm, fractions 4 ml. The first peak contains Fab and intact IgG, but no Fc.

Fig. 4. Evaluation of our Fab product by immunoelectrophoresis.

1 = IgG Papain digest
anti bovine serum

2 = Fab

1 anti IgG
2

1 anti Fab
2

1 anti Fc
2 no line present

3 = Fc, indicating the two subclasses IgG1 and IgG2
Table 1. Procedure for the preparation of Fab from bovine IgG

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
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<tbody>
<tr>
<td>IgG</td>
<td>(a) 7 S peak from ammonium sulfate precipitated globulin, gel-filtered on Sephadex G 200 (e.g. 500 mg protein for a column of 2.5 x 95 cm)</td>
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<td></td>
<td>or (b) first peak from DEAE-cellulose equilibrated with 0.01 M PO_4 pH 7.6 (e.g. 700 mg protein for a column of 1.5 x 27 cm)</td>
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<td>or (c) Bovine Gammaglobulins Fraction II, B grade (later A grade) (Calbiochem, Lucern)</td>
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<td></td>
<td>or (d) IgG collected by chromatography with DEAE-cellulose from Bovine Gammaglobulins Fraction II (Calbiochem) (e.g. 6000 mg protein for a column of 2.0 x 60 cm).</td>
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<td>Papain treatment = Mixture of Fab Fc and IgG</td>
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<td></td>
<td>Chromatography on DEAE-cellulose (DE 52 Whatman) equilibrated with 0.01 M PO_4 pH 8.0 (e.g. 500 mg protein for a column of 1.5 x 15 cm)</td>
</tr>
<tr>
<td></td>
<td>Rechromatography on DEAE-cellulose equilibrated with 0.01 M Tris-HCl pH 8.6 + 0.05 M NaCl (e.g. 500 mg protein for a column of 1.5 x 15 cm)</td>
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<td></td>
<td>Ammonium sulfate precipitation at 60°C, saturation, pH 7.0</td>
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<td>Supernatant, dialysed against PBS and concentrated by vacuum dialysis = Fab.</td>
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exhibited a mixture of Fab Fc and a third at 0.2 M NaCl only Fc. (Fig. 2).

Later we omitted the preparition of the papain digest on Sephadex G 100. The elution patterns remained the same when we directly applied the digest into the anion-exchange columns, but consequently intact IgG, which had escaped the papainization, appeared in the first peak together with Fab.

Since the concentrated protein solutions of the first peaks eluted from PO_4- or Tris-equilibrated DEAE-cellulose proved to be slightly Fc-contaminated, we rechromatographed the pooled first peaks from the PO_4 columns on DEAE-cellulose, equilibrated with 0.01 M Tris-HCl pH 8.6 + 0.05 M NaCl, and in the first peak obtained a Fab product free from Fc but still containing intact IgG, which became evident when the ultracentrifugal analysis (Prof. v. Tavel, Th.-Kocher-Institut Bern) and gelfiltration on Sephadex G 100 revealed a peak of 7 S and another of 3.5 S (Fig. 3).

We therefore fractionated the IgG-Fab mixture by ammonium sulfate precipitation at pH 7.0 and found that a final concentration of 60 per cent saturation produced a supernatant which no longer contained intact IgG and yielded an immunoelectrophoretically pure Fab (Fig. 4), which, when injected into a rabbit, induced a single line and a faint trace of anti-Fc. In Ouchterlony double diffusion tests (diameter of the wells 4 mm) a strong single line was developed against anti-Fab but no line against anti-Fc. Ultracentrifugation and gelfiltration on Sephadex G 100 revealed a single symmetric peak. Table 1 gives a graphical outline of the whole procedure.

This Fab product was now used as an absorbent of rabbit antibovine immunoglobulin antisera: one ml each of the different sera were mixed with increasing amounts of Fab, incubated at 37°C for 1 hr and then over night at 4°C. For anti-IgG we used 150 µg of Fab, for anti-IgM 300 µg and for anti-IgA 180 µg for the complete absorption of the anti-light chain antibodies. Thus the sera were rendered class specific anti γ- and anti μ- and anti κ-heavy chain. Fab absorbent in excess did not weaken the antibody against the IgG heavy chains. (Fig. 5).

DISCUSSION

The bovine immunoglobulins have not yet been studied as extensively as the human immunoglobulins
but increasing knowledge is accumulating and it is evident that the time has come to introduce the routine differentiation of bovine Ig classes and subclasses into the clinical investigation. This calls for a ready availability of class specific antibodies for the radial diffusion test. We studied the usefulness of Fab from papain treated IgG as an absorbent for all three class antibodies and applied three preparative steps for its purification, i.e. two chromatographies and a precipitation with ammonium sulfate. One of the great advantages of ion-exchange chromatography over gelfiltration is the capacity of relatively small columns to fractionate great amounts of protein, in a couple of hours if the desired protein appears in the breakthrough peak. We generally put about 300–500 mg of the digested protein, dialysed against the starting buffer, into a column of 1.5 x 15 cm. The third step of precipitation is also easy and rapid and brings out a product which is so pure that even immunodiffusion tests in wells of 4 mm diameter do not exhibit any lines of contaminants.

The rabbit immunized with this product developed an antiserum with a single line of precipitation against Fab and IgG and a faint trace of an anti-Fc line. It must be admitted that the yield of Fab is not high, but that is no serious disadvantage since immunoglobulin G is easily available as a substrate. In a given run we digested IgG with 1% papain for 4 hr at 37°C and obtained 680 mg = 68 per cent of digest. After two chromatographies and the ammonium sulfate precipitation, the yield was 11.4 per cent of the digested protein. We found that the commercial bovine gammaglobulin fraction II B and A grade (Calbiochem) could replace pure 7 S IgG from Sephadex G 200 or IgG from the first peak of anion-exchange. However, its final Fab product developed a faint unidentified cathodaline against anti-Fc whereas Fc is fast moving anodically, but this contaminant did not weaken the anti-heavy chain antibodies of an anti-IgG serum.

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REFERENCES