Immune Reactions in Polysaccharide Media

EXPERIMENTS WITH SPECIFIC ANTIBODIES OF DIFFERENT AFFINITIES FOR SERUM ALBUMIN

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Rabbit antibody fractions of different affinities for human serum albumin were prepared by an immunosorbent technique. The fractions were used in studies on the enhancement of the precipitin reaction by polymers. Dextran increased the immune precipitation to about the same extent regardless of whether antibodies with high and low affinities were used. The effect should considerably facilitate the detection of antibodies with low precipitating ability in immunological assays. The results are discussed in terms of a steric-exclusion mechanism.

It has been demonstrated that polysaccharides enhance immune precipitations, especially in the region of antigen excess (Helsing, 1966, 1969*a,c,d*). An explanation based on the steric exclusion (Laurent, 1968) of immune reactants from the polysaccharide solution was in accord with all experimental observations (Helsing, 1966, 1969*a,b,c,d*).

This phenomenon has important practical implications, since it can be utilized to increase the sensitivity of various immune techniques. To explore this, the present paper deals with the polysaccharide enhancement of the precipitin reaction between albumin and antialbumin of different affinities.

Kitagawa, Grossberg, Yagi & Pressman (1967) have shown that rabbit antibodies with different binding properties for a hapten can be obtained by successive adsorption of the antiserum on small amounts of immunosorbent. The same technique was used in the present investigation to produce nine fractions of antialbumin.

MATERIALS AND METHODS

Unless otherwise stated all experiments were carried out at 4° in 0.05M-phosphate buffer (9.8mM-KH₂PO₄-40.2mM-Na₂HPO₄), pH 7.4, containing 0.1M-NaCl.

Antigen. Human serum albumin was prepared and labelled with ¹²⁵I as described by Helsing (1966). The preparation had a specific radioactivity of 12 µCi/mg. and was used in concentrations of 0.02–1.31 mg./ml.

Antibody fractions. Serum (150 ml.) from two rabbits, which had responded relatively weakly to immunization with serum albumin (Helsing, 1966), was mixed with 5 g. of the immunosorbent, described previously, prepared by coupling albumin to *p*-aminobenzylcellulose (Helsing, 1969*c*). After careful stirring for 30 min. the immunosorbent

was removed by filtration through a fine glass filter and washed with 500 ml. portions of the phosphate buffer until no further material absorbing at 280 nm. was removed. Three washings were usually sufficient. The serum and the combined washing solutions were concentrated by ultrafiltration (Berggård, 1961), by using 23/32 in. Visking dialysis tubing (Union Carbide Corp., Chicago, Ill., U.S.A.), to the original sample volume (150 ml.) and used for repeated adsorption (see below).

Dissociation of the adsorbed antibody was performed by stirring the immunosorbent for 5 min. in 200 ml. of 0.1M-glycine-HCl buffer, pH 2.40, containing 0.1M-NaCl. The immunosorbent was then filtered off and washed with an additional 300 ml. portion of the above buffer. The filtrate and washing solution were combined and the pH of the solution was adjusted to neutrality by addition of 1M-tris-HCl buffer, pH 8.0. This material was then concentrated by ultrafiltration in collodion bags (Mies, 1953) to volumes of 2–8 ml., dialysed against the phosphate buffer and centrifuged at 80000g for 20 min.

The immunosorbent was again transferred to phosphate buffer and the procedure repeated with the concentrated serum recovered after the first treatment. After nine similar cycles anti-albumin activity could no longer be detected in the serum by gel diffusion against a serial dilution of albumin.

The nine antibody fractions all reacted with albumin in gel diffusion and only one band corresponding to IgG* could be seen in immunoelectrophoresis against a sheep anti-rabbit serum (Helsing, 1969*a*). Further, only one line could be detected in gel diffusion experiments when a sheep antiserum against rabbit γ-globulin (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) was used. This antiserum gave three precipitin lines with whole rabbit serum.

The minimum reactivity of the antibodies was tested by agarose-gel electrophoresis in the presence and absence of a

* Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M (macroglobulin).

Table 1. *Fractions of anti-albumin obtained by repeated adsorption on an immunosorbent from a 150 ml. batch of rabbit antiserum*

The amount of protein was determined by a Folin method (Eggstein & Kreutz, 1955) with human γ -globulin as standard. The reactivity of the antibody was estimated by an electrophoretic technique (Helsing, 1969c). The electrophoretic properties of the fractions are expressed as the distance moved towards the anode during agarose-gel electrophoresis for 9 hr. at 2.5 v/cm. in 0.05 M-phosphate buffer, pH 7.4, containing 0.1 M-NaCl.

Fraction no.	Amount recovered (mg.)	Minimum reactivity (%)	Electrophoretic movement (mm.)
1	11.1	85	-1.6
2	7.4	90	-1.0
3	5.9	90	-1.8
4	8.7	95	-1.3
5	3.0	95	-0.9
6	3.8	90	+0.1
7	0.8	85	+0.6
8	2.1	85	+0.8
9	0.6	80	+1.8

large excess of albumin as described before (Helsing, 1969c). For this test samples of the antibody fractions were labelled with ^{125}I essentially as described by Helsing (1966) but on a smaller scale. The incorporation corresponded to 2-4 atoms of iodine/antibody molecule. The minimum reactivity varied between 80 and 95% (Table 1).

The electrophoretic mobility of the antibody fractions varied (Table 1).

Polysaccharide. Dextran 500 (molecular weight 500 000) was kindly supplied by Pharmacia AB, Uppsala, Sweden. It was used in final concentrations of 10, 20 and 40 mg./ml.

Immunological techniques. These were as described by Helsing (1966, 1969a). The antibody solutions used in the precipitin tests had concentrations of 0.5 mg./ml. as measured with u.v. spectrophotometry (Crumpton & Wilkinson, 1963).

Control experiments were performed in which the antibody solutions in the precipitin series were replaced by human γ -globulin (Helsing, 1969d) of the same concentration. No precipitation of albumin could be observed, but a small amount of the radioactive albumin was adsorbed on the glass walls. This amount was independent of the amount of dextran added. As the total concentrations of protein in the experiments were unusually low, a correction for adsorption was introduced in the main precipitin series.

Fig. 1. Precipitin experiments with four of the different antibody fractions: (a) fraction 1; (b) fraction 5; (c) fraction 8; (d) fraction 9. To 100 $\mu\text{l.}$ of antibody solution (0.5 mg./ml.) were added increasing amounts of ^{125}I -labelled albumin (plotted on the abscissa) in a total volume of 400 $\mu\text{l.}$ The experiments were performed in the absence ($\times$) and presence of Dextran 500 (Δ , 20 mg./ml.; $\circ$, 40 mg./ml.). The amount of ^{125}I -labelled albumin found in the precipitate is given on the ordinate.

Table 2. *Maximal precipitation of ^{125}I -labelled albumin in precipitin series performed with different antibody fractions*

The precipitin tests were performed as described in Fig. 1. Maximal precipitation occurred on the addition of the amounts ($\mu\text{g.}$) of albumin given in the rows labelled (a) and the amounts ($\mu\text{g.}$) of albumin precipitated are tabulated in the rows marked (b).

Dextran added (mg./ml.)	Antibody fractions								
	1	2	3	4	5	6	7	8	9
None	(a)	8.3	7.8	7.5	7.6	6.5	7.7	4.8	4.5
	(b)	6.8	6.7	6.3	6.2	4.8	6.1	3.2	3.1
10	(a)	9.5		8.2	8.7		8.2		
	(b)	7.6		6.5	6.4		6.3		
20	(a)	10.1	10.6	9.1		9-10	8.8		
	(b)	7.8	7.6	7.0		5.8	6.5		
40	(a)	11.8	13-17	11.0	11.2	10.5	11-13	8.7	8.0
	(b)	8.3	8.0	7.6	7.6	6.5	7.3	5.2	4.5
								4.5	2.5

The correction was, however, too small to affect the main conclusions of the experiments.

After termination of the precipitin experiments the supernatant solutions were analysed for content of free albumin and antibody-bound albumin by the $(\text{NH}_4)_2\text{SO}_4$ precipitation technique (Helling, 1969a).

Radioactivity. This was determined as described by Helling (1969a).

RESULTS

The antibody fractions that were obtained early in the fractionation procedure are referred to below as high-affinity antibodies and those obtained late are referred to as low-affinity antibodies. The justification for this is given in the Discussion section.

Each of the nine different antibody fractions was used for a series of identical precipitin experiments. Examples are given in Figs. 1(a)–1(d). The high-affinity antibodies precipitated a larger proportion of the added albumin than did the low-affinity ones, and with fraction 9 essentially no albumin was precipitated in the absence of polymer. Table 2 demonstrates this for the whole series of antibody fractions.

The addition of dextran increased the amount of albumin precipitated and moved the point of maximal precipitation towards higher values of added antigen in all experimental series (Fig. 1 and Table 2). The increase in amount of albumin precipitated at the point of maximal precipitation was of the same order in the various experiments (1.2–2.0 $\mu\text{g.}$), although there was a tendency for larger effects with the low-affinity antibodies. The relative increase in the amount of albumin precipitated at the point of maximal precipitation, however, was more pronounced with the low-affinity antibodies, which is most clearly demonstrated by Fig. 1(d). A comparison of the results

obtained in the presence of dextran at different concentrations of antigen showed that at moderate antigen excess (two and three times the amount of antigen added at the point of maximal precipitation) there was a still more pronounced relative increase in the precipitation of albumin with the low-affinity antibody fractions.

The amounts of free and bound albumin were measured in the supernatant solutions from those test tubes where up to 16 $\mu\text{g.}$ of albumin was added. The amounts of soluble bound antigen in the absence and presence of 40 mg. of dextran/ml. are given in Figs. 2(a) and 2(b). There is a considerable scattering of the points in Fig. 2(a) and the amounts of antigen in the soluble complexes cannot with certainty be correlated with any particular sequence of the antibody fractions. The soluble bound antigen in the dextran series showed a different pattern (Fig. 2b). The amount was higher with the low-affinity antibodies than with the high-affinity ones.

The amounts of free albumin in the supernatant solutions are plotted in Figs. 3(a) and 3(b). Both in the absence and presence of dextran there is a trend towards more free albumin in experiments with low-affinity antibodies than with high-affinity ones. On average there is more free albumin in the dextran series than in the control series.

DISCUSSION

The experiments presented here were essentially undertaken to explore the possibility of increasing the sensitivity of immune reactions by polymer enhancement. A fractionation procedure was therefore carried out to produce antibodies having different affinities for human serum albumin.

The fractionation resulted in nine fractions each showing more than 80% reactivity towards albumin. When analysed by immunoelectrophoresis and gel

Fig. 2. Antibody-bound albumin found in the supernatant solutions in the precipitin series. (a) Control experiments; (b) series performed with 40mg. of Dextran 500/ml. Separation of free and antibody-bound albumin was performed by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Experiments with the various antibody fractions are: $\times$, 1; $\circ$, 2; Δ , 3; $\square$, 4; ∇ , 5; $\bullet$, 6; $\blacktriangle$, 7; $\blacksquare$, 8; $\blacktriangledown$, 9.

diffusion, they gave only one precipitation band corresponding to IgG. This is not surprising since the rabbits had been hyperimmunized and could be expected to contain mainly this immunoglobulin. Unfortunately, no specific anti-(rabbit IgM) and anti-(rabbit IgA) sera were available, and trace amounts of these proteins cannot be excluded.

In spite of the similar reactivities, the fractions showed marked differences in precipitability, which is ascribed to a decreasing affinity for the antigen throughout the fractionation series. Another support for this assumption is afforded by the observa-

Fig. 3. Free albumin found in the supernatant solutions in the precipitin series. (a) Control experiments; (b) series performed in 40mg. of Dextran 500/ml. For symbols see Fig. 2.

tion that there was more free albumin in the supernatant solutions of the control series when low-affinity antibodies were used (Fig. 3a).

However, the fractionation also produced a separation of the antibodies according to charge, which is demonstrated by the electrophoretic mobilities (Table 1). One therefore cannot exclude the possibility that an increased solubility of the immune complexes (due to increased negative charge) is partly responsible for the decreased precipitability caused by the low-affinity antibodies.

When dextran was added to the different precipitin series, there was an increased precipitation of albumin, a decrease in the amount of soluble immune complexes and an increase in the concentration of free albumin in the supernatant fluids, in agreement with earlier observations (Helsing, 1966, 1969a,c,d). It is striking that the addition of dextran

to the medium caused about the same absolute increase in the amount of albumin precipitated at the point of maximal precipitation in the series with antibodies of low and of high affinity. This means that, in a reaction with antibodies of high affinity, one obtains a moderate relative gain by the enhancement. Antibodies with low affinity, however, which normally would not be precipitated, can be forced to form a precipitate with the antigen by addition of the polymer (e.g. fraction 9). The polymer enhancement should therefore be of considerable interest for immunological analyses.

There are already reports on the use of polymers for increasing the sensitivity of immune reactions. Reference has been made by Hellsing (1966) to the use of polymers within the field of haematology for demonstrating the presence of antibodies with low affinity ('blocking', 'univalent', 'incomplete' antibodies). Polymer enhancement has recently been reported within the field of immunochemistry. By addition of dextran to the trough after immunoelectrophoresis, sharp and distinct precipitin lines have been obtained even from insulin and anti-insulin (Ceska, 1968). Dextran added to the gel in immunoelectrophoresis experiments sharpens the precipitin lines and reveals the existence of additional lines, which cannot be seen in control slides (Ceska & Grossmüller, 1968). Likewise the sensitivity of the quantitative immunoelectrophoretic method of Laurell (1966) can be increased three- to five-fold in determinations of α -lipoprotein (B. G. Johansson, personal communication).

An analysis of the experimental results indicates agreement with the exclusion hypothesis (Laurent, 1968). Antibodies with low affinity for an antigen should form smaller immune complexes than those with a high affinity when allowed to react under similar conditions. When dextran is added to such systems, large complexes should be removed to a greater extent than small ones (Hellsing, 1969c). From Figs. 2(a) and 2(b) it can be concluded that relatively more of the soluble complexes have been removed from the series containing antibodies with

high affinity than from those containing low-affinity antibodies, which should contain relatively small complexes.

Dextran thus lowers the concentration of immune complexes more in a high-affinity system than in one having low affinity. The main reason that the absolute amount of precipitation caused by dextran is about the same in the different series is the fact that the concentration of immune reactants left in the supernatant fluids before the addition of dextran is higher in series containing low-affinity antibodies than in those containing high-affinity ones. Since the amount precipitated from a saturated system is the product of the excluded solvent volume and the concentration of the compound, the polymer enhancement can be larger in a system with low-affinity antibodies.

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