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REDUCTION IN THE ANTIGEN-BINDING EFFICIENCY OF ANTIBODIES IMMOBILIZED IN POLYACRYLAMIDE GEL

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Under conditions of antibody excess, antibodies that had been immobilized by physical entrapment in a polyacrylamide gel matrix consistently removed less radiolabeled microprotein antigen from solution than did the same antibodies chemically conjugated to Sepharose beads. This reaction is believed to relate to the difficulty of formation of secondary antibody–antigen lattices when the molecules are held in "cells" in the polymer matrix.

INTRODUCTION

Proteins may be immobilized by entrapment in the cross-linked matrix of a polyacrylamide gel of suitable concentration. This method has been employed to prepare solid-phase enzyme reagents for substrate assays (1,2), antigen columns for the preparation of immunopurified antibodies (3), and solid-phase antibody reagent for the radioimmunoassay of hormones (4).

We wished to use this method to detect microprotein antigens in the presence of larger cross-reactive molecules that would be unable to penetrate the gel, and thus would not react with the included antibody. The binding efficiency for radiolabeled microprotein antigens (β_2 microglobulin, Ig light chains, type κ) of antibody physically included in polyacrylamide gel was compared with that of the same antibody chemically attached to Sepharose beads.

MATERIALS AND METHODS

Sera. Anti-β_2 microglobulin serum was raised in a Fauve de Bourgogne rabbit by the injection of purified antigen with Freund's complete adjuvant (FCA) into both hind footpads. After an interval of 1 month four intradermal boosters of antigen without FCA were given at 1-week intervals, and the

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animal was bled out 1 week after the last injection. Rabbit antihuman κ chain serum was obtained from the Centre de Transfusion Sanguine Départmentale, Bois-Guillaume, Rouen. Normal control serum was obtained from young, unimmunized, Fauve de Bourgogne rabbits.

Radioiodinated Antigens. Human β_2 microglobulin was isolated from the urine of a patient with renal tubular dysfunction as described previously (5) and was labeled with ^{125}I (Amersham) using the minimum effective quantity of chloramine T. Human Ig light chains of type κ, obtained from the urine of a patient with multiple myeloma, were similarly radioiodinated. Unreacted isotope was removed by exhaustive dialysis against phosphate-buffered saline, pH 7.4 (PBS), at 4°C, after which at least 95% of the radioactivity was precipitated in 5% trichloroacetic acid.

Preparation of Polyacrylamide-Antibody (pac-Ab) Gels. Acrylamide monomer (Eastman Kodak) containing the desired proportion of *N,N'*-methylenebisacrylamide (BIS) as a cross-linking agent was dissolved in PBS to a total concentration four times that desired for the final gel. To 1 ml of this solution was added 0.5 ml of a 1 : 10 dilution of serum, 0.5 ml of a 0.92% *v/v* solution of *N,N,N',N'*-tetramethylethylenediamine that had been neutralized with dilute HCl, and 2.0 ml of a 0.28 g% solution of ammonium persulfate. The solutions were thoroughly mixed and allowed to polymerize at room temperature for 30 min. The polymer mass was then rinsed with PBS and minced under PBS using an Ultra Turrax model TP 18-10 blender to produce an even suspension of particles about 0.2 mm in size. The particles were allowed to settle and were washed with PBS by decantation until no further protein could be detected in the supernatant. The washed particles were resuspended in 20 ml of PBS containing sodium azide, and were stored at 4°C.

Preparation of Sepharose-Coupled Antibody. Sepharose 4B activated with cyanogen bromide was obtained from Pharmacia (France) and, after activation in 10^{-4} M HCl, was charged with serum proteins in 0.1 M bicarbonate buffer, pH 8.0, containing 0.5 M NaCl. Unconjugated proteins were removed from the beads by three washing cycles with 0.1 M acetate buffer, pH 3.0, and 0.1 M borate buffer, pH 8.0, both containing 1 M NaCl. The beads were stored at 4°C under PBS containing sodium azide.

Reaction of the Radiolabeled Antigen with the Immobilized Antibodies. Into plastic tubes of 2.5-ml capacity were added 0.2ml of a suspension of the immobilized antibody reagent, 0.8 ml of PBS, and the desired amount of radiolabeled antigen contained in 20 μl PBS. The tubes were attached horizontally to a rotary shaker at room temperature for the duration of the reaction period (0.5–24 h). At the end of this time the total radioactivity in each tube was measured in a Packard gamma-ray spectrophotometer, an additional milliliter of PBS was added to each tube, and the solid phase was

sedimented by gentle centrifugation (1000 rpm for 5 min). The supernatant was discarded, and the solid phase was washed four times with PBS and its radioactivity determined.

RESULTS

Pac-Ab gels of 5, 6, 7, 8, and 10% total monomer concentration containing 2.5, 5, 10, and 25% BIS as a cross-linking agent were prepared. IgG antibody was slowly lost from the 5 and 6% monomer gels, but was satisfactorily retained in those of higher monomer concentration. Microprotein antigens entered the 10% gels very slowly, but penetrated the 7 and 8% gels quite readily. The gels containing 10 or 25% BIS were mechanically fragile, whereas those with 5 or 2.5% BIS were firm and easily handled. For the subsequent experiments 8% monomer gels with 2.5% BIS were used. Retention of the labeled antigens by these gels reached a maximum after some 10 h, and this level was unaltered after 24 h. Reaction times of 16 h were thus used. Sepharose-conjugated antibodies also reached a plateau of maximum uptake of labeled antigen before 16 h, so that the same reaction conditions were used for both forms of immobilized antibody.

When increasing quantities of labeled β_2 microglobulin were incubated with Sepharose-conjugated anti-β_2 microglobulin antibody a maximum of 87% of the added radioactivity was retained when the antibody was in excess. Excess of the same antibody included in pac gel could only fix a maximum of about 35% of the added antigen. Labeled antigen was then reacted with excess pac-Ab and the reactivity of the supernatant was tested with Sepharose-conjugated or with further pac-included antibody. The supernatant had essentially the same antigenic characteristics as the original antigen solution toward both solid-phase antibody reagents, the antibody conjugated to Sepharose retaining 85% of the counts and the pac-Ab retaining 34%. The manipulation was repeated, using the supernatant from this second incubation with pac-Ab. Again, the antigenic behavior of the residual radioactive material remained essentially unchanged (Table 1).

A similar situation was observed when the two forms of immobilized antibody to κ chains were used. Only some 52% of this labeled antigen could be retained by a Sepharose–anti-κ chain conjugate, and some 34% by pac-Ab. An attempt was made to purify the antigen by passing it through a column of anti-κ chain pac-Ab, and eluting the absorbed antigen at acid pH. Labeled antigen was applied to a short column containing 5 ml of packed pac-Ab. The unbound material was washed out with PBS. Then the remaining antigen was eluted, using a glycine/HCl buffer, pH 2.3 (Fig. 1). These fractions were immediately neutralized with dilute NaOH. Material from

TABLE 1. **Percentage of Radiolabeled Antigen Retained by Excess Anti-β_2 Microglobulin Antibody Immobilized by Conjugation to Sepharose (Seph-Ab) or by Inclusion in Polyacrylamide Gel (pac-Ab)**

	Retention ± observed variation (%)	
Labeled antigen	Seph-Ab	pac-Ab
(1) β_2 microglobulin	87.2 ± 2.9	34.6 ± 2.7
(2) Supernatant from incubation of 1 with pac-Ab[a]	85.1 ± 2.4	34.2 ± 1.6
Supernatant from incubation of 2 with pac-Ab[a]	80.1 ± 1.8	33.0 ± 1.1
Ig κ chains	2.9 ± 1.0	3.3 ± 0.3

[a] $N = 3$; for all other measures $N = 5$.

either the excluded or the retarded peaks was reacted with antibody either conjugated to Sepharose or included in pac. Both forms of immobilized antibody demonstrated appreciable amounts of antigen remaining in the excluded peak. The retarded peak proved to be a more efficient antigen than the original material when it was reacted with the Sepharose-conjugated antibody, showing that a degree of immunopurification had indeed occurred. However, the pac-included antibody only retained this material slightly more efficiently than it retained the original material or the excluded peak (see Table 2). The reactions of both the Sepharose-conjugated and the pac-included antibodies could be specifically blocked by the addition of homologous unlabeled antigen to the reaction mixture. The presence of unrelated proteins in the mixture did not modify the reaction. When the antiserum conjugated to Sepharose or included in pac was replaced by normal rabbit serum the fixation of labeled antigen never exceeded 5% added radioactivity.

DISCUSSION

Excess antibody immobilized in pac gel consistently takes up less radioactivity from a labeled antigen solution than does excess of the same antibody chemically attached to Sepharose beads. This effect does not seem to be due to an alteration of the antigen in contact with the pac gel; supernatants from incubations with pac-Ab behave in the same way as the original solution toward both forms of immobilized antibody. This finding

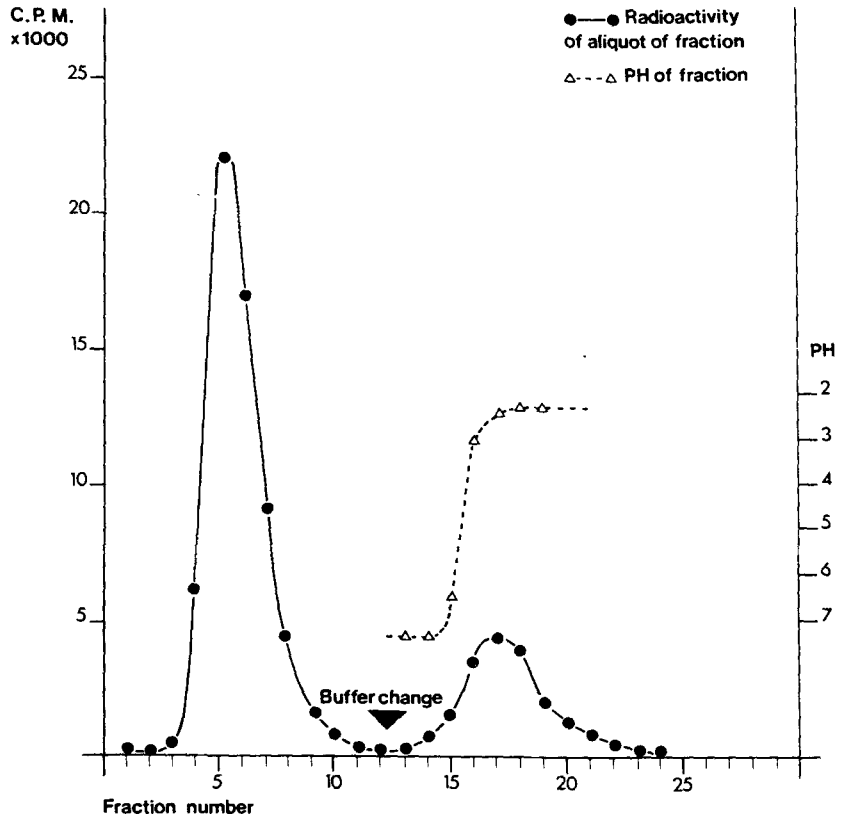

FIG. 1. Elution profile of crude radiolabeled Ig κ chains passed through a column of anti-κ serum included in polyacrylamide gel.

TABLE 2. **Percentage of Radiolabeled Antigen Retained by Excess Anti-κ Chain Antibodies Conjugated to Sepharose (Seph-Ab) or Included in Polyacrylamide Gel (pac-Ab)**

	Retention ± observed variation (%)	
Labeled antigen	Seph-Ab	pac-Ab
Crude κ chains	53.3 ± 2.2	34.4 ± 2.0
Excluded peak[a]	43.4 ± 4.7	36.5 ± 7.2
Retarded peak[a]	73.3 ± 2.4	43.0 ± 4.9
β_2 microglobulin	3.7 ± 0.4	2.4 ± 0.5

[a] From passage of crude labeled κ chains through a pac-anti-κ chain immunoabsorbant column. See Fig. 1. $N = 5$.

also refutes the possibility that antibodies specific for a subpopulation of the antigen molecules are selectively destroyed during the inclusion procedure. Such behavior might be due to the effects of steric hindrance, the antibody molecules attached to the surface of, or within the large porosities in, Sepharose 4B beads being more readily available for reaction than those entrapped within the tight latticework of the pac gel matrix. It is noteworthy, however, that a constant proportion, about one-third, of the offered antigen is retained by the pac-Ab even when the excess of the pac-Ab is very large. This would seem to suggest that the position of equilibrium of the reaction

$$Ab + Ag \rightleftharpoons (AbAg)$$

is displaced to the left when the antibody is immobilized within the pac gel. The displacement may occur because of the considerable reduction in the possibility of lattice formation between the primary antibody–antigen complexes under these conditions. The complexes would be even less able than antibody alone to migrate through the gel matrix; small fragments of lattice could be formed only when a matrix "cell" contained several antibody molecules, or when antibody molecules were present in adjacent "cells" at a distance permitting cross-linking through pores in the gel.

Polyvalent antigen is held in a lattice by several antibody molecules at once. The dissociation of any one of these antibody–antigen interactions will not result in the release of antigen from the complex. Indeed, the antigenic site will probably be retained close to the detached antibody-combining site, favoring recombination. In the absence of lattice formation, dissociation of one or a few antibody–antigen interactions can release the antigen completely, allowing it to diffuse out of combining range.

Sepharose-conjugated antibodies appear to be capable of forming quite stable multiple antibody–antigen interactions between adjacent molecules of the same bead or on different beads (agglutination). However, the dispersal of the same antibodies into the physically delimited "cells" of a cross-linked polymer matrix appears to result in a diminution in the stability of the antibody–antigen complexes formed, with a consequent reduction in the efficiency of antigen retention.

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