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**Research Article**

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# Specific Removal of In Vivo Antibody by Extracorporeal Circulation over an Immunoabsorbent in Gel

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**ABSTRACT** A method is described for preparation of a solid immunoabsorbent suspended in an agar gel. The immunoabsorbent in gel retains its specificity. Rabbits were injected with antibody or antigen, and an extracorporeal circulation was established through columns or funnels coated with such immunoabsorbents. It was shown that either antibody or antigen can be specifically and rapidly removed, and that no measurable amounts of antigen from the immunoabsorbent were released into the animals.

## INTRODUCTION

Immunoabsorbents have been used to isolate and to purify antibody (see references 1, 2) and to remove specifically reactive cells from heterogeneous populations of lymphocytes (3, 4). We have recently described the use of a solid-phase immunoabsorbent for the removal of antibody from the sera of immunized animals (5, 6). Portions of plasma were exposed to batches of immunoabsorbent, and the adsorbed plasma was returned together with red cells to the animals. By sequential repetition of this procedure, 50–80% of the intravascular antibody could be removed specifically from actively immunized animals. This maneuver is too complicated for general use, however, and also has the disadvantage that trace amounts of immunoabsorbent are released into the animals.

The present study describes the characterization of a solid-phase immunoabsorbent in agar gel and its use in an extracorporeal circulation to remove antibody from immunized animals. With this technique, approximately 80% of <sup>125</sup>I-labeled antibody to bovine serum albumin (BSA)<sup>1</sup> was specifically removed within 30–60 min from

passively immunized rabbits. No detectable antigen was released into the animals from the immunoabsorbent.

## METHODS

**Antigens.** Bovine serum albumin 5X crystallized (BSA) was purchased from Pentex Biochemical (Kankakee, Ill.), and Keyhole limpet hemocyanin (KLH) was obtained from Pacific Bio-Marine Supply (Venice, Calif.) and further purified by ultracentrifugation at 100,000 *g* for 1 hr. Human IgM was obtained from Immunology Inc. (Glenn Ellyn, Ill.).

Quantities of proteins in solutions used were estimated spectrophotometrically from the optical densities at 280 and 260 *mμ*, respectively (7).

**Antibodies.** IgG fractions were purified from sera of rabbits hyperimmunized against BSA, mouse, or chicken IgG, and also from normal rabbits. IgG was salted out with ammonium sulfate, and the dialyzed precipitate was chromatographed on DEAE-cellulose or DEAE-Sephadex (A-25).

**Iodination of proteins.** Proteins were iodinated with <sup>125</sup>I or <sup>131</sup>I (New England Nuclear Corp., Boston, Mass.) by the method described by McConahey and Dixon (8). The radioiodine was NaI which was free of carrier and thio-sulfate.

**Preparation of solid immunoabsorbent.** Bromoacetyl cellulose (BAC) was obtained from Miles-Yeda, Ltd. (Rehovoth, Israel). BAC-protein immunoabsorbents were prepared by the method of Robbins, Haimovich, and Sela (1). The last step of the procedure, i.e. exposure of the adsorbent to 8 M urea was eliminated, since it proved possible to remove all noncovalently bound protein from the cellulose derivative by extensive washing with saline.

**Preparation of solid immunoabsorbent agar gel.** BAC-BSA or BAC-rabbit IgG was suspended in an equal volume of saline and diluted (1:1 or 1:3) with thorough mixing in melted noble agar (3% in saline) at 50–55°C. The mixture was then poured onto the surface of a glass funnel to form a film or into a mold to form a column. The funnel had been previously coated by a thin film of 1% agar, which was allowed to dry completely. The mold to form the column consisted of a glass tube, 25 cm in length and 0.9 cm in internal diameter into which a glass rod of lesser diameter (0.5 cm) was centered by the use of rubber stoppers. In this manner, an annulus of space was created into which the liquid immunoabsorbent-agar mixture was poured. After hardening of the agar, the glass rod was removed, creating

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<sup>1</sup>Abbreviations used in this paper: BAC, bromoacetyl cellulose; BSA, bovine serum albumin; KLH, Keyhole limpet hemocyanin; PBS, phosphate-buffered saline.

a column lined with immunoadsorbent through which solutions or an animal's blood could be passed.

**Passive immunization.** Albino rabbits weighing 2.5–3 kg were used. About 150  $\mu\text{g}$  of rabbit IgG anti-BSA- $^{125}\text{I}$ <sup>2</sup> or 100–200  $\mu\text{g}$  BSA- $^{125}\text{I}$ <sup>3</sup> was mixed with 4 ml of rabbit antiserum to bacteriophage T<sub>2</sub> or  $\phi\text{X}$  with a titer of neutralizing antibody of 20–50  $k$  ( $k$  = velocity constant of phage neutralization), and injected into the ear vein. In preliminary experiments using radioiodinated purified IgG of similar anti-T<sub>2</sub> activity, it was determined that in antigen excess, T<sub>2</sub> binds 1–3  $\mu\text{g}$  of antibody protein per k. On this basis, the quantity of neutralizing antibody to phage, anti-BSA and BSA administered were approximately similar.

**Extracorporeal circulation.** This procedure was performed 4–7 days after passive immunization. Rabbits were premedicated by intravenous injections of atropine (2 mg) and heparin (1000 U/kg). They were anesthetized 30 min later by a slow intravenous injection of sodium pentobarbital (30 mg/kg). The femoral vein and artery were cannulated with a 16 gauge medium intracath (R. C. Bard, Murray Hill, N. J.). Arterial blood was pumped through narrow gauge Teflon tubing by a peristaltic pump (Imico Co., Stoneham, Mass.) and passed through the column or over the funnel lined with immunoadsorbent. The funnel was rotated slowly. Blood was returned to the femoral vein by gravity via a hemiset (Fenwall Laboratories, Inc., Morton Grove, Ill.) fitted with a fine mesh stainless steel filter and supported 100 cm above the animal. Before extracorporeal circulation, the system was primed with 30 ml of fresh normal rabbit blood. The resultant dilution of antibody was corrected for in all subsequent calculations assuming a blood volume of 70 ml/kg rabbit.

**Antibody assays.** Anti- $\phi\text{X}$  or anti-T<sub>2</sub> was measured by the phage neutralization technique of Adams (9). The proportion of antibody to BSA in the rabbit anti-BSA- $^{125}\text{I}$  IgG was estimated by the following method. 0.1-ml portions of serum were incubated with 1 ml of the BAC-BSA suspension (containing 37 mg of BSA) at 37°C for 10 min followed by the removal of BAC-BSA by centrifugation (10,000  $g$ , 10 min). This absorption was performed three times using 1 ml of fresh BAC-BSA suspension. The third portion of adsorbent remained virtually free of radioactivity. The total amounts of radioactivity removed (corrected for nonspecific adsorption) was used as a measure of antibody specific to BSA.

**Determination of radioactivity.** Radioactive fractions were counted in a Nuclear-Chicago well counter. All counts are net and statistically significant. Spillover of  $^{125}\text{I}$  into  $^{131}\text{I}$  counts was corrected for when necessary.

## RESULTS

**Specificity of binding of BSA-immunoadsorbent in agar.** To investigate this question, the following experiment was performed: 1 ml of the immunoadsorbent agar suspension was added to plastic test tubes and allowed to solidify. To each tube was added 1 ml of equimolar concentrations (100  $\mu\text{g}$ ) of one of the following purified  $^{125}\text{I}$ -labeled IgG's: rabbit anti-BSA, rabbit anti-chicken, rabbit anti-mouse, or normal rabbit IgG. After incubation for 15 min at room temperature, the tubes were decanted. The immunoadsorbent agar gel

<sup>2</sup>  $1.27 \times 10^9$  cpm.

<sup>3</sup>  $6.7 \times 10^6$  –  $1.3 \times 10^7$  cpm.

TABLE I

Specificity of Binding of Anti-BSA to BAC-BSA in Agar Gel

Rabbit IgG- $^{125}\text{I}$ *	Radioactivity	
	Initial counts per minute	Initial counts per minute bound to immunoadsorbent
Anti-BSA	$1.8 \times 10^5$	50
Anti-mouse IgG	$8.1 \times 10^4$	6.5
Anti-chicken IgG	$2.1 \times 10^6$	5.6
Normal	$1.9 \times 10^6$	4.7

\* 100  $\mu\text{g}$  protein in 1 ml buffer.

was washed five times each with 3 ml of phosphate-buffered saline (PBS). The agar was then crushed and washed twice with 3 ml of PBS. The last wash was free of radioactivity. The adsorbent was then counted and the percentage of initial radioactivity remaining bound to the immunoadsorbent was calculated.

As can be seen in Table I, 50% of the radioactivity in the purified IgG fraction of an anti-BSA antiserum remained bound to the immunoadsorbent, whereas only 4.7–6% of similarly prepared IgG fractions of antiserum directed against other antigens was bound.

**Differences in binding between radioactive and non-radioactive antibody.** It was necessary to determine whether radioiodination of anti-BSA influenced its binding by the immunoadsorbent. 1 ml of mixtures of radioactive protein and increasing quantities of nonradioactive protein was added to tubes each containing 1 ml of the agar BAC-BSA. After incubation for 15 min at room temperature, the agar immunoadsorbent was washed and prepared for counting as before.

Fig. 1 shows the relationship between bound radio-

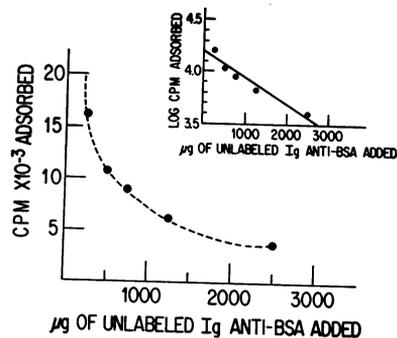


FIGURE 1 Competition for binding sites on BAC-BSA by anti-BSA- $^{125}\text{I}$  and unlabeled anti-BSA. Increasing amounts of unlabeled anti-BSA were added to a fixed amount of anti-BSA- $^{125}\text{I}$  (0.1  $\mu\text{g}$ ), and the binding of radioactivity to immunoadsorbent was measured. The insert shows a plot of the same data as a logarithmic relationship.

TABLE II  
Removal of *In Vivo* Anti-BSA by Extracorporeal Circulation  
over BSA-BAC in Agar Gel

Animal no.	Anti-BSA- <sup>125</sup> I*			Anti-phage		
	Pre-operative	Post-operative†	Removed	Pre-operative	Post-operative‡	Removed
	<i>cpm/0.1 ml</i>		%	<i>k</i>		%
1	519	86	83	0.13	0.12	8
2	347	58	83	0.07	0.06	14
3	306	57	81	0.21	0.19	10
4	752	154	79	0.10	0.09	10

\* Counts per minute, which can be removed from serum after adsorption three times with BAC-BSA, corrected for nonspecific binding of protein to immunoadsorbent.

† Value has been corrected for dilution by "priming" blood volume.

activity and proportion of unlabeled anti-BSA. The insert shows logarithmic plot of these data. The effect of increasing amounts of unlabeled anti-BSA on the binding behavior of radioactive anti-BSA indicates competition of unlabeled and radioactive protein for binding sites on the solid immunoadsorbent.

*In vivo removal of antibody by extracorporeal circulation.* The ability of the immunoadsorbent lined columns or funnels to remove antibody from the circulation of animals was determined. Four rabbits were passively immunized with anti-BSA-<sup>125</sup>I and either anti-φX or anti-Ta. 4-7 days later, the animals were anesthetized, and their blood was circulated through an immunoadsorbent-lined funnel or column. The extracorporeal circulation was maintained for 30-60 min. Levels of antibody to BSA and bacteriophage were determined on portions of serum obtained from bleedings at the beginning and the end of the procedure.

As can be seen in Table II, 79-83% of circulating anti-BSA was removed in the four animals tested. In contrast, the levels of circulating antibody to bacterio-

phage were only decreased by 8-14%. The amount of antibody removed ranged from 17 to 61 μg of protein as calculated from the specific radioactivity of the anti-BSA and the total radioactivity removed. It is not known whether all the antigenic sites on the immunoadsorbent were saturated with antibody.

*Release of antigen from BSA-immunoadsorbent in agar.* In order to determine whether BSA bound to the BAC-agar was released and entered the circulation during the procedure, the BSA in the immunoadsorbent used in the previous experiments had been labeled with <sup>125</sup>I. Three of the above experimental rabbits were sacrificed at the end of the extracorporeal circulation. The entire thyroid, spleen, kidneys, 10% of liver weight, several grams of muscle tissue, 5 ml of blood, and in one case both lungs were collected, and the level of radioactivity due to <sup>125</sup>I was determined.

As can be seen in Table III, no significant <sup>125</sup>I counts could be found in the organs tested though 1 × 10<sup>8</sup> cpm <sup>125</sup>I-labeled BSA was present on the immunoadsorbent used.

TABLE III  
Release of BSA from Immunoadsorbent

Animal no.	Amount of BSA- <sup>125</sup> I in immunoadsorbent	Amount of BSA- <sup>125</sup> I in tissues of treated rabbits							
		Background*	Blood‡	Kidney	Liver§	Lung	Spleen	Thyroid	Muscle
	<i>cpm</i>				<i>cpm in whole organ</i>				
2	1 × 10 <sup>8</sup>	63	63	59	63	—	71	72	—
3	1 × 10 <sup>8</sup>	63	63	58	59	—	72	57	—
4	1 × 10 <sup>8</sup>	66	66	69	68	68	70	67	69

\* Counting background of the well counter.

‡ 5.0 ml.

§ 10% of total liver weight.

|| 5 g.

*Experiments with antibody-containing immunoadsorbent in agar.* Analogous experiments to those described above for the BSA-immunoadsorbent were performed with anti-BSA immunoadsorbent.

(a) Specificity of binding was demonstrated. In an *in vitro* experiment in which equimolar amounts of proteins-<sup>125</sup>I were used ( $1.6-2.9 \times 10^7$  cpm), 52% of BSA was bound in contrast with 4.3% of rabbit gamma globulin and 6.9% of KLH.

(b) No difference in the binding of <sup>125</sup>I and unlabeled BSA was detected. 100-2500  $\mu$ g of BSA was added to a fixed amount of BSA-<sup>125</sup>I ( $1 \times 10^8$  cpm) in an experiment entirely analogous to that described earlier for rabbit anti-BSA. A logarithmic plot of radioactivity bound versus increasing amounts of unlabeled BSA resulted in a straight line.

(c) Specific removal of BSA-<sup>125</sup>I *in vivo* was demonstrated. Two rabbits were injected intravenously with 100  $\mu$ g of unlabeled BSA and  $6.7 \times 10^8$  cpm of BSA-<sup>125</sup>I as well as rabbit anti- $\phi$ X. 5 days later extracorporeal circulation was performed using BAC-anti-BSA in agar gel. 24-27% of the circulating BSA-<sup>125</sup>I was removed, whereas the level of the control protein was decreased by 10-15%. The smaller proportion of specific removal compared with the earlier experiments may be due to an immunoadsorbent agar ratio of 1:3 in contrast with the 1:1 ratio used in the experiments involving removal of anti-BSA. It is also possible that removal using antibody-containing immunoadsorbent will be less efficient since removal depends on the correct orientation of the antibody molecule on the surface of the gel.

## DISCUSSION

Methods for removing antibody *in vivo* have been described in the past, but they have limited potential for general clinical use. Thus, both plasmapheresis (10-12) and exchange transfusion (13) remove antibody non-specifically and, in addition, carry the risk of serum hepatitis.

The present studies indicate that antibody can be removed specifically and rapidly from the circulation of immunized animals. The technique used was to incorporate an immunoadsorbent (e.g. BAC-BSA) into agar, thus forming a solid gel which was used to line the inside of glass columns and funnels. After extracorporeal circulation through such columns or over such funnels, approximately 80% of intravascular antibody was removed in 30-60 min. In contrast, the levels of a control antibody remained virtually unchanged throughout the procedure. No detectable antigen was released from the immunoadsorbent into the animals used in these experiments. The actual amounts injected and removed were small. However, there exists a proportionality between surface area of the immunoadsorbent in gel and

its capacity to remove specific materials. We therefore suggest that with appropriate development in engineering, the capacity for specific removal could be easily and strikingly increased.

The possibility of manipulating humoral antibody levels by this simple and effective method has several experimental and therapeutic implications. Experimentally, it should be possible to probe further into the physiological mechanisms regulating the synthesis of antibody. Therapeutically, this method could be applied to remove autoantibodies involved in the pathogenesis of disease or to remove the "blocking" antibody believed to play a role in the propagation of tumors (14-17). An unresolved problem in therapeutic application is that the removal of antibody in actively immunized animals is followed by a rapid rise of serum antibody back to the original levels (6, 18). A possible solution applicable to autoimmune diseases would be the subsequent administration of specific antibody which cannot bind C' such as Fab' or F(ab')<sub>2</sub> but which can suppress antibody synthesis (19, 20).

We have also shown that antibody conjugated to the solid immunoadsorbent in gel can be used to remove antigen from the circulation. Hence, using the specificity of antibody, it should be possible to remove any circulating molecule, provided an antibody can be prepared to it. Using the appropriate immunoadsorbent, components of complement, hormones, endotoxin, digitalis, or toxic chemicals could be removed. Moreover, different immunoadsorbents could be used in sequence. For example, simultaneous use of antibody and antigen-containing immunoadsorbents would allow removal of immune reactants from patients with immune complex disease without the necessity of establishing whether the patient is in the phase of antigen or antibody excess.

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