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Polyamide Resin: A Novel Immunoadsorbent

I. Preparation of Polyamide Resin–Antigen Conjugates for Use in a Solid Phase Assay for Radiolabeled Antibody Synthesized In Vitro

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Abstract

A solid phase assay for radiolabeled antibody synthesized *de novo* in vitro has been described (*1*). The solid phase consists of antigen covalently bound to bromacetyl cellulose, a useful but difficult to prepare immunoadsorbent. Herein, we describe the preparation of polyamide resin immunoadsorbent and the procedure for coupling antigen to the polymer. Data are presented that show that polyamide resin–Ag‡ conjugates can replace bromacetyl cellulose–Ag conjugates. The usefulness of this easily prepared and inexpensive immunoadsorbent is discussed.

Index Entries: Immunosorbent, a novel polyamide resin; polyamide resin–antigen conjugates; method, for assay of radiolabeled *de novo* antibody synthesis; bromoacetyl cellulose, and polyamide resin immunosorbents; solid phase polyamide resin immunosorbent; antibody synthesis, a novel polyamide resin immunosorbent for.

Introduction

A specific, sensitive, and precise assay for antibody synthesized *de novo* in lymphocyte cultures has been perfected by Self et al. (*1*). The assay is based on pulsing

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‡Abbreviations used in this paper: BAC, bromacetyl cellulose; FIB, bovine fibrinogen; HBSS, Hanks' balanced salts solution; HRP, horseradish peroxidase; IA, immunoadsorbent; KLH, keyhole limpet hemocyanin; MEM, Eagle's minimal essential medium supplemented with non-essential amino acids, penicillin, streptomycin, HEPES buffer and normal rabbit serum; PAR, polyamide resin; PBS, phosphate-buffered saline.

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cultured lymph node cell with L-(^{14}C) leucine and incubating the culture supernatants with antigen bound to the surface of bromacetyl cellulose immunoadsorbent. Under appropriate assay conditions, the specific antibody bound can be enumerated.

We have used this assay in our laboratory with excellent results. However, preparation of the immunoadsorbent is a rather time-consuming process requiring specialized equipment and reagents not normally found in an immunology laboratory (*2*). Therefore, we searched for a material that would have physical properties making it useful as an immunoadsorbent and would covalently couple antigens with relative ease. Materials with free amino groups fulfill the latter criterion because of their ability to be activated by glutaraldehyde (*3*); therefore, we decided to test whether polyamide resin (PAR)[5]—a product used in the textile industry—would be used as an effective immunoadsorbent. The following communication presents our results.

Materials and Methods

Animals

Randomly bred New Zealand White rabbits of either sex were used throughout the study. They were maintained on water and laboratory rabbit chow *ad libitum*.

Production of Antisera

Rabbits were injected with 1 mg alum-precipitated antigen into each hind foot pad. One week later, the animals received three intravenous injections of 1 mg of soluble antigen in phosphate-buffer saline, pH 7.2 (PBS), every other day. One week after the last injection, the rabbits' titers were assayed by the ability of their sera to form a visible precipitin ring against a solution of antigen at 1 mg/mL. If this criterion was satisfied, rabbits were bled by cardiac puncture and the sera recovered and stored at −20°C until used. Animals with low titer were reinjected using the same protocol. The sera of several rabbits were pooled and were used in all experiments.

Preparation of Immunoadsorbents (IA)

Bromoacetyl cellulose was prepared according to the method of Robbins et al. (*2*). Polyamide powder (product #5132B) was the generous gift of Bostik Division, USM Corporation (Middletown, Massachusetts). Five grams of powder were mixed with approximately 100 mL 0.6*N* HCl *in* 95% EtOH in order to hydrolyze the polymer and thereby create free amino groups. The mixture was stirred vigorously at room temperature for approximately 1 h at which time any insoluble material was removed by filtration. The filtrate was transferred to a beaker of at least 1 L capacity and distilled water was added slowly to effect precipitation of the polyamide. As the precipitate formed, the rate at which the water was added was increased. Approximately 1 L of water was used in the precipitation. The resultant suspension was allowed to settle at 4°C for at least 1 h and the supernatant fluid

decanted to remove suspended particles. The precipitate was washed twice at 400*g* and then three times at 3000*g*. Each wash was for 10 min with PBS. The immunoadsorbent was suspended in PBS such that the packed particle volume following centrifugation at 3000*g* for 10 min was 6.7%. This degree of suspension was chosen since BAC packed in this manner in our hands. The suspension was stored at 4°C and contained NaN_3 at a final concentration of 0.02%.

Coupling of Antigen to Immunoadsorbents

Antigens were coupled to the BAC as described by Robbins et al. (2). The procedure was slightly modified in that coupling was performed at pH 4.6 for each of the antigens. Antigen coupling to the PAR was as follows: 25 mL of suspended polyamide was washed twice at 3000*g* for 10 min with 0.1*M* phosphate buffer, pH 6.9. Sufficient glutaraldehyde (25% aqueous Sigma, Grade II diluted to 5% with the phosphate buffer) was added to form a stirrable suspension (usually about 10 mL). The mixture was stirred vigorously at room temperature for 1–2 h and the glutaraldehyde was removed with three washes of the buffer. A solution of antigen at 1 mg/mL in the buffer was added to the resin and the mixture stirred slowly in the cold for 12–18 h. The volume of the antigen solution was the same as for the glutaraldehyde. After the incubation period, the antigen solution was washed out and the resin suspended in the same volume of 0.2*M* glycine, pH 6.9, and stirred in the cold for 2 h. Finally, the resin was washed several times with PBS, brought to a volume of 25 mL, and stored at 4°C in the presence of 0.2% NaN_3.

Antigens

Keyhole limpet hemocyanin (KLH) (Schwarz/Mann), horseradish peroxidase grade II (HRP) (Boehringer Mannheim), and bovine fibrinogen fraction I (FIB) and (ICN Pharmaceuticals) were stored at 4°C as filter sterilized solutions in PBS.

Experimental Protocol

Nine rabbits were injected with 1 mg alum-precipitated antigen in each hind foot pad. The animals were subsequently sacrificed and their popliteal lymph nodes removed. Single cell suspensions were prepared in Hanks Balanced Salts Solution (HBSS) with the aid of a stainless steel screen (Cellector, Bellco Glass; Vineland, NJ). Clumps were removed by passing the suspension through gauze and the suspension was washed twice with HBSS at 400*g* for 10 min. The resultant cell pellet was suspended in Eagle's Minimal Essential medium (GIBCO) lacking L-leucine and supplemented with: 10% (v/v) normal rabbit serum (KC Biologicals), 10 mm HEPES buffer (GIBCO), 1% (v/v) MEM nonessential amino acids (GIBCO), penicillin 100 U/mL (Pfizer), and streptomycin 50 μg/mL (Pfizer). This mixture is referred to as MEM hereinafter. The cells were counted with the aid of a hemocytometer and adjusted to 1×10^7 viable cells/mL; viability was assessed by the exclusion of trypan blue.

Cultures were carried out in triplicate in 12 × 75 mm polystyrene tubes (Falcon). Each culture consisted of 1 mL cell suspension and 0.1 mL of either PBS or

antigen at the appropriate concentration. Cultures were incubated at 37°C in a humidified atmosphere at 5% CO_2. Twenty-four hours after the initiation of the cultures, antigens were washed out by two washes with 2 mL HBSS. The cells were resuspended in 1 mL fresh MEM and subsequently incubated for another 48 h, at which time the medium was replaced with fresh medium containing 0.5 μCi L-$I^{14}C$) leucine/m L (specific activity 351 mCi/mmol). After 48 h of culture, the cells and debris were removed by centrifugation, the culture supernatants that contained radiolabeled antibody were either assayed immediately or stored frozen. Frozen samples were thawed and then centrifuged prior to assay to remove insoluble material, which has been found to interfere with the assay.

Assay for Specific Antibody

Antibody was determined according to the method of Self et al (*1*). Briefly, the assay distinguishes between radioactive proteins synthesized by the cultured cells that nonspecifically bind to the IA–Ag complex and radiolabeled specific Ab bound to the IA-Ag complex. Total radioactivity bound is measured (in duplicate) by incubating the IA–Ag complex with cell culture supernatant and determining the counts per minute (CPM) in the precipitate obtained after centrifugation. Nonspecific radioactivity is determined by first incubating the IA–Ag complex with high titer antiserum to the bound Ag; the culture supernatant is then incubated with the IA–Ag–Ab complex. Radioactivity in each precipitate is determined by washing the tube with saline and collecting the precipitate onto a glass fiber filter pad (2.4 cm, Whatman). The filters are dried, added to scintillation fluid, and then counted in a scintillation counter. Results are expressed as stimulation index (SI) = net CPM in cultures with Ag/net CPM in control cultures, where net CPM = mean total CPM − nonspecific CPM.

Statistical Analysis

Stimulation indices were analyzed by the paired *t*-test for related samples. Plots of SI were analyzed by linear regression; equations for the lines and all other numerical data were determined according to standard statistical methodology (*4*).

Results

Physical Properties of Polyamide Resin Immunoadsorbent

When prepared as described, the PAR is virtually identical to the BAC with respect to physical properties. Both are fine white powders that are easily suspended in buffer; the suspensions remain uniform long enough to carry out all the pipeting procedures required in the assay. Polyamide resin–antigen conjugates have shown stability for at least 6 months in our hands. Thus, the PAR appears to possess those properties outlined by Robbins et al. (*2*) for a useful immunoadsorbent.

TABLE 1
Measurement of Anti-KLH Synthesized In Vitro from KLH-Immunized Animals[a]

Immunization interval, d	Antigen, μg			Stimulation index	
	KLH	HRP	FIB	BAC	PAR
3	—[b]	—	—	1.00	1.00
	1			1.13	0.99
	10			0.69	0.61
	100			1.13	0.95
		10		1.02	0.94
7	1			5.06	2.39
	10			5.75	2.39
	100			16.40	5.40
		10		0.58	0.89
			10	0.91	1.22
12	1			18.13	9.47
				13.22	7.78
	100			9.00	6.25
	10			1.33	1.06

[a]Seven rabbits were immunized with 1 mg of alum-precipitated KLH in each hind food pad at 3-, 7-, and 12-d intervals. Post-immunization animals were sacrificed and the popliteal lymph nodes were prepared for tissue culture. 1×10^7 lymphocytes were challenged with the various antigen concentrations and *de novo* antibody synthesis was measured and expressed as the stimulation index.

[b]No antigen was added to these control tissue culture cells. They received 100 mL of PBS instead of antigen.

Measurement of Anti-KLH Ab Synthesis

Nine rabbits primed with KLH 3, 7, and 12d prior to the removal of their lymph nodes were utilized. Table 1 shows that an antigen priming interval of only three days was too short to allow for the expansion of the clone of KLH-reactive B cells; antibody synthesis in these cultures was undetectable with either immunoadsorbent—not a surprising result in view of the findings of Roszman et al. (1971).

Analysis of the stimulation indices by the paired *t*-test, shows no significant difference ($P > 0.05$) between the SI values obtained with BAC-KLH vs the SI obtained using BAC-KLH. A linear regression analysis plot shows that the data fall on a straight line with $r = 0.926$ (Fig. 1).

Table 1 also shows that the 7- and 12-d priming intervals were sufficiently long to allow for clonal expansion of the KLH-reactive B-cells. Upon in vitro antigenic stimulations, *de novo* radiolabeled antibody synthesis was detected. The magnitude of the SI for the 7- and 12-d immunizing intervals is different for the BAC and PAR immunoadsorbents. However, Figs. 2 and 3 show the statistical treatment of the data in the form of a linear regression analysis with an *r* value of 0.988 at both immunization intervals. Both BAC and PAR show linear assay characteristics at

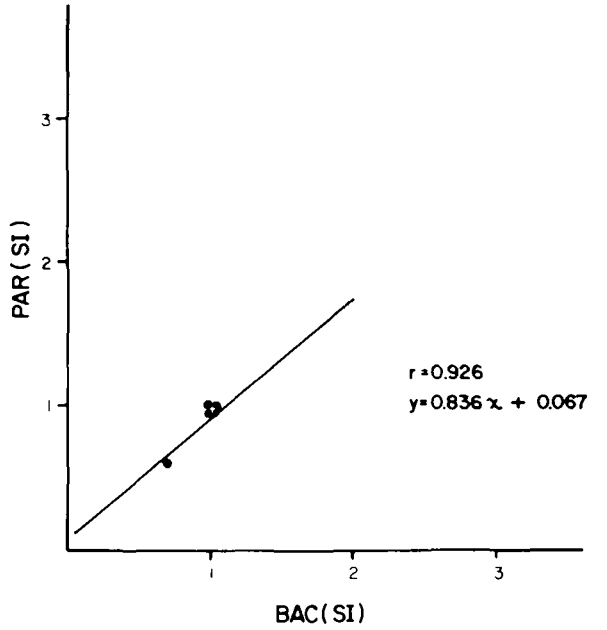

Fig. 1. Linear regression analysis of the measurement of anti-KLH synthesis in vitro. Popliteal lymph nodes were removed and prepared for tissue culture 3 d after immunization. The polyamide resin stimulation index [PAR(SI)] is plotted vs the bromoacetyl cellulose stimulation index [BAC(SI)].

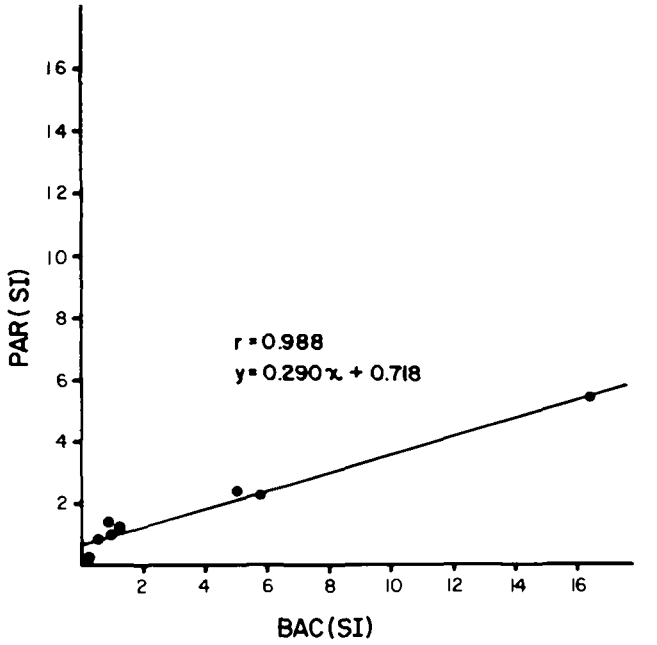

Fig. 2. Linear regression analysis of the measurement of anti-KLH synthesized in vitro. Popliteal lymph nodes were removed and prepared for tissue culture 7 d after immunization. The polyamide resin stimulation index [PAR(SI)] is plotted vs the bromoacetyl cellulose stimulation index [BAC(SI)].

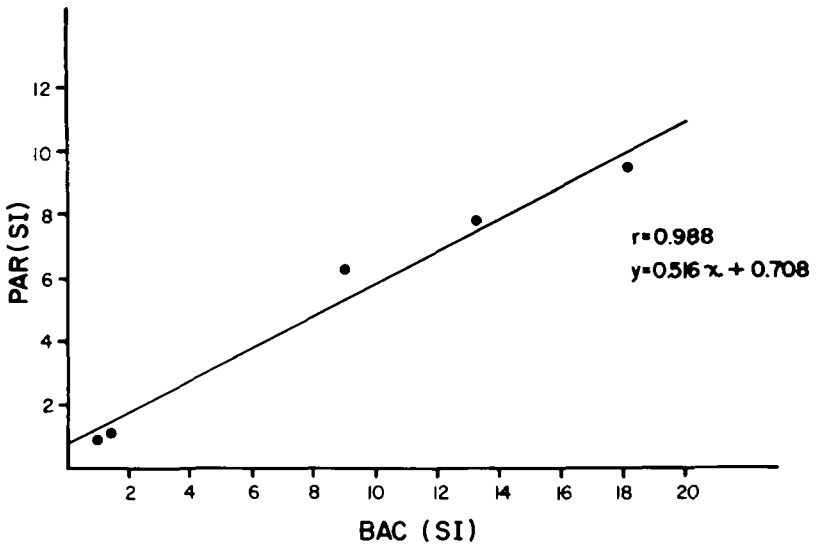

Fig. 3. Linear regression analysis of the measurement of anti-KLH synthesized in vitro. Popliteal lymph nodes were removed and prepared for tissue cultures 12 d after immunization. The polyamide resin stimulation index [PAR(SI)] is plotted vs the bromoacetyl cellulose stimulation index [BAC(SI)].

the various antigenic challenge doses and both show the same trend of *de novo* antibody synthesis.

Antibody Binding to PAR–Ag Complex is Linear over a Wide Range

Antibody binding to BAC–Ag complex is linear over a wide range of antibody dilutions (*1*). To determined if this is the case for the PAR–Ag complex, the supernatant obtained incubating KLH-educated cells with 1 μg/mL KLH was diluted 1 : 10, 1 : 100, and 1 : 1000 and incubated with PAR–KLH as was the undiluted supernatant. These dilutions were assayed for total and net CPM bound; the results are presented in Fig. 4. Analysis of the data in Fig. 4 by linear regression shows that both total and net CPM are linear over the range investigated ($r > 0.9$ for both).

Measurement of Anti-HRP Synthesis

Table 2 shows the data obtained using HRP as an additional antigen with characteristics that differ from KLH as the priming antigen and a priming interval of 6 d. Statistical analysis of these data showed that there was no significant difference between the SI obtained using the BAC and the PAR immunoadsorbents. Both small and large molecular weight antigens can be bound to the PAR and detect low levels of antibody from as few as 1×10^7 lymphocytes.

Discussion

Our studies on antigen-primed rabbit lymph node cells required that we assay cultures of these cells for *de novo* synthesis of specific antibody. Self et al. (*1*) have

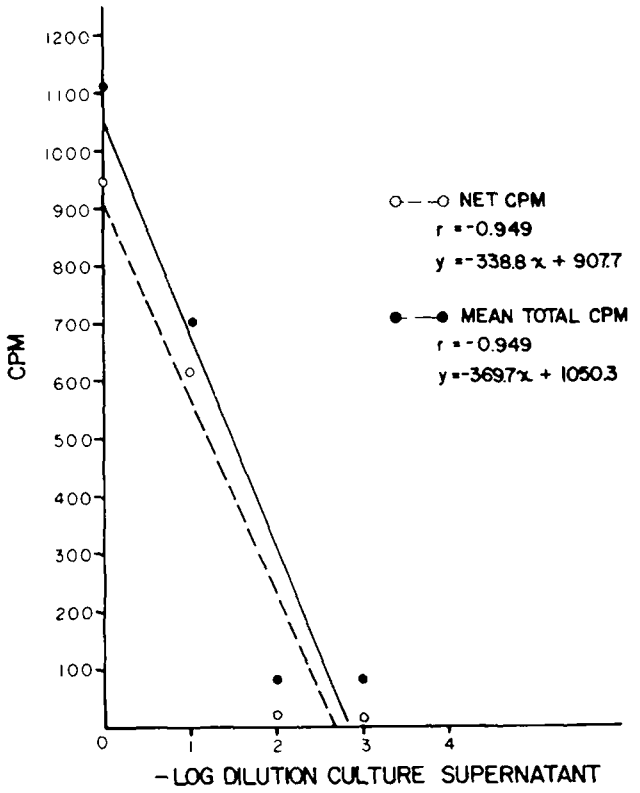

Fig. 4. Antibody binding to PAR–Ag complex is linear over a 3 log dilution range. Linearity is determined by linear regression analysis.

TABLE 2
Measurement of Anti-HRP Synthesized In Vitro from HRP-Immunized Animals[a]

Immunization interval, d	Antigen, μg		Stimulation Index,	
	KLH	HRP	BAC	PAR
6	—[b]	—	1.00	1.00
	10	0.79	1.06	
		1	2.40	2.04

[a]Two rabbits were immunized with 1 mg of alum-precipitated HRP in each hind foot pad at 6-d post-immunization. The animals were sacrificed and the popliteal lymph nodes were prepared for tissue culture. 1×10^7 lymphocytes were challenged with the antigen concentrations and *de novo* antibody synthesis was measured and expressed as the stimulation index.

[b]No antigen was added to these control tissue culture cells. They received 100 μL of PBS instead of the antigen.

devised an assay that is sensitive enough for this purpose. Although this assay works well, the immunoadsorbent used (BAC) is difficult and time-consuming to prepare. Furthermore, the apparatus and reagents needed were not available in our laboratory. Consequently, we searched for an insoluble material that contained

free amino groups; such free amino groups are readily activated with glutaraldehyde using common laboratory equipment and reagents (*3*). Our search for a suitable immunoadsorbent ended with polyamide resin, an inexpensive product more frequently used as a textile adhesive.

The experiments presented here show that the PAR can bind sufficient antigen to detect the antibody synthesized in vitro by as few as 1×10^7 lymph node cells. Furthermore, the binding is linear over a 3 log range (Fig. 4). Examination reveals that in all but one experiment, the BAC–Ag complex tended to bind more radiolabeled antibody than the PAR–Ag complex; this is shown by the generally higher SI obtained with the former as compared with the latter. This increased binding is likely the result of more total antigen being bound by the BAC.

Although the BAC–Ag complex can bind more net CPM than PAR–Ag complex under the same conditions, both sets of data provide the same dose–response profile of the cells to antigen. As pointed out by Self et al. (*1*) this immunoadsorbent assay can only provide information on relative and not absolute amounts of antibody synthesized by the cultured cells. Thus, the fact that the PAR–Ag complex tends to bind less antibody is not crucial.

It might be possible to increase the amount of antigen bound to the PAR by incubating the resin in HCl for a longer period of time (see Materials and Methods). Such a lengthened incubation would make more free amino groups available for antigen coupling. Unfortunately, such a treatment also would reduce the integrity of the polymer. Ideally, it is necessary to determine what degree of hydrolysis provides a reasonable compromise between polymer integrity and available free-amino groups. Such studies are currently under way and the results will be presented in another article in this series.

Most studies carried out in this laboratory have used KLH as the priming antigen. In order to determine whether the PAR was capable of binding proteins other than KLH, experiments were carried out using HRP as the priming antigen. Clearly, the cultured cells produce much more anti-KLH antibody in response to KLH than anti-HRP in response to HRP. This is likely the result of a difference in the immunogenicity of the molecules—not unexpected because of their vastly different molecular weights and the fact the KLH tends to form aggregates that enhance antigenicity.

Fibrinogen was used as an in vitro antigen to challenge the KLH and HRP immunized lymphocytes at the 7-d priming interval (Table 1) to ensure that the immunoadsorbent–Ag complex would not detect a nonspecific lymphocyte product in response to a heterologous antigenic challenge. A 10-μg challenge of fibrinogen showed only background activity with respect to antibody synthesis. To ensure that the immunoadsorbents would only detect specific antibody, the 3-d immunization interval was chosen. This priming interval is insufficient to provide memory lymphocytes for in vitro antigenic challenge. When the complete antigenic dose range was used to challenge these immunized lymphocytes, no antibody synthesis was detected.

Immunoadsorbents are useful for a variety of immunological procedures other than this assay, most notably affinity chromatography. Currently, the most widely used material for such studies is CNBr-activated Sepharose®, a material that

works well but requires great care to prepare because of the harzardous reagents employed. With this in mind, studies are now under way in this laboratory to develop a polyamide resin suitable for affinity chromotography. The results will appear in a forthcoming publication.

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