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Principles, Problems, and Strategies in the Use of Antigenic Mixtures for the Enzyme-Linked Immunosorbent Assay

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Competition between proteins and other macromolecules for adsorption sites on plastic was studied with the enzyme-linked immunosorbent assay (ELISA) to determine effects of the use of antigenic mixtures or extracts of organisms on assays of antibodies and antigens by ELISA. A comparison of a number of different polystyrene microplates with bovine albumin and human immunoglobulin G (IgG) as antigens showed two major classes of plates: those which adsorbed albumin poorly and those which adsorbed albumin well. IgG adsorbed well on all plates, but plates which adsorbed albumin best also gave significant background levels of nonspecific binding of conjugate. When mixtures of IgG and bovine serum albumin were used as coating antigens, significant competition was observed; the component present at 1% or less in the mixture was essentially undetectable unless excessive amounts of conjugate were used. The important factor was the ratio of competitor to antigen, not the absolute amount. Other proteins (ovalbumin, rabbit albumin, human albumin, and gelatin) were equally effective competitors for adsorption sites on plastic. Nonionic detergents (Tween 20, and Triton X-100) were strong competitors even at 10:1 competitor-to-antigen ratios. In antigen capture assays, normal serum components blocked attachment of antigen-specific IgG, but this competition could be lessened to a degree by the use of strongly binding polystyrene plates. In indirect ELISA for measurement of serum antibody, the use of antigenic mixtures gave significantly lower antibody titers when the desired antigen was less than 1% of the total protein coated. Therefore, the use of mixed or crude antigens in ELISA presents significant problems concerning the sensitivity and specificity of tests.

The enzyme-linked immunosorbent assay (ELISA) (9) is a major tool for detecting antigens and antibodies in a wide variety of diseases (20, 21). Although the method was initially devised for measurement of antibodies to individual proteins, a large number of assays for individual disease agents have been devised using partially purified or even unpurified antigens. Such assays are not uniformly successful, and when they are it is not possible to define the reacting antigen(s). If we were to wait until suitably purified antigens were available, a number of currently useful assays would not be available for serodiagnosis. It has been recognized that components of antigenic mixtures (i.e., an extract of a microorganism) likely compete with each other for the limited sites on plastic surfaces (4, 5, 13, 16). Thus, studies to determine the nature of competition are necessary to define conditions under which ELISAs can best be performed with partially purified or unpurified antigens.

Proteins apparently attach to plastic surfaces by hydrophobic interactions (12, 19), and the protein adsorbed may form a monomolecular film (5, 12). At small concentrations of pure proteins, the amount bound to the plastic is proportional to the concentration of the protein in solution, and at saturation a constant amount of protein is bound regardless of concentration (5). In the linear range, the amount of protein bound may be as great as 80% for bovine immunoglobulin M (IgM) or 10 to 20% for bovine albumin and lactalbumin (5). When adsorption with mixtures of proteins is attempted, the bound proteins are not adsorbed in proportions similar to those of the solution (13). The type of plastic employed also affects adsorption rates (13).

The present study was an investigation of the competition among proteins and other molecules for adsorption sites on plastic surfaces with a variety of commercially available microplates and ELISA procedures commonly used for serodiagnosis (20). The purpose was to determine the ratios of competitor to antigen in mixtures which might cause minor components to be represented in such small quantities on the plas-

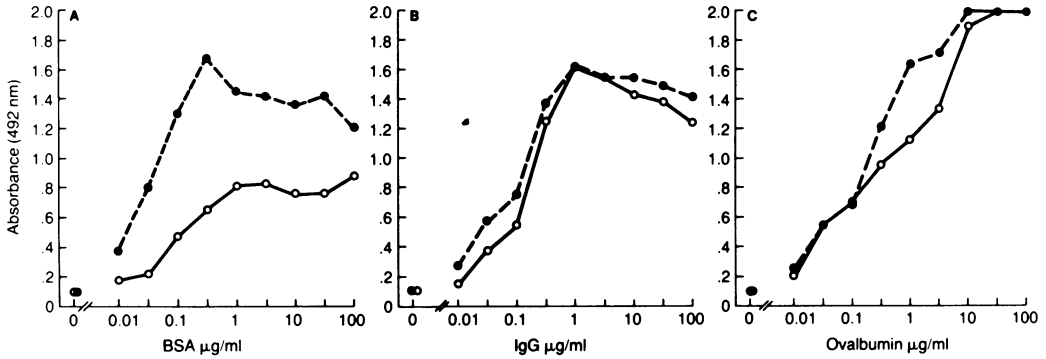

FIG. 1. Comparison of the adsorption of BSA (A), human IgG (B), and ovalbumin (C) to two types of polystyrene plates. Optical densities observed with type A plates (○) and type B plates (●) are shown in each panel. Concentrations of antigens used for coating are shown on the x-axis. Direct ELISA was performed with appropriate monospecific peroxidase-conjugated antisera at the following dilutions: anti-BSA, 1:32,000; anti-IgG, 1:100,000; and anti-ovalbumin, 1:32,000. Plate types are defined in Table 1.

tic surface as to be indetectable by ELISA. An ancillary goal was to determine the conditions under which simultaneous assay of antibodies to different antigens might be possible by using antigenic mixtures.

MATERIALS AND METHODS

Antigens and competitors. The antigens used for most experiments, unless otherwise specified, were from Sigma Chemical Co., St. Louis, Mo., and included bovine serum albumin (BSA), human IgG, and chicken ovalbumin. Antigens were assumed to be anhydrous, and the tables and figures refer to specific weights of each antigen per ml. Protein values as determined by the Lowry method for 1 mg (dry/weight) of each antigen were 1.0 mg for BSA, 1.4 mg for human IgG, and 1.1 mg for ovalbumin when bovine albumin was used to standardize the test. All lots of human IgG gave protein values in excess of 100% of dry weight with this standard. For comparison and reference purposes, BSA was also obtained from Calbiochem, LaJolla, Calif., and two different lots of ovalbumin were obtained from Worthington Diagnostics, Freehold, N.J. The following materials were obtained from Sigma: human albumin, rabbit albumin, rabbit IgG, gum ghatti, Tween 20, and cetrimide (hexadecyltrimethylammonium bromide). Lysozyme, Triton X-100, and Zwittergent (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) were obtained from Calbiochem. Tryptone and gelatin were obtained from Difco Laboratories, Detroit, Mich., sodium dodecyl sulfate from BioRad Laboratories, Richmond, Calif., sodium deoxycholate from Gallard Schlessinger, Carle Place, N.Y., and types 4 and 14 pneumococcal polysaccharides from Eli Lilly & Co., Indianapolis, Ind. Normal goat serum was obtained from GIBCO Laboratories, Grand Island, N.Y.

Antibodies. Peroxidase-conjugated antibodies were obtained from Cappel Laboratories, West Chester, Pa., as the IgG fraction and included goat anti-human albumin, goat anti-rabbit IgG (heavy and light chain specific), goat anti-human IgG (heavy chain specific), rabbit anti-bovine serum albumin, rabbit anti-human IgG (heavy chain specific), and rabbit anti-chicken ovalbumin. As first antibody for indirect ELISA, rabbit anti-human IgG was obtained from Dako (distributed by Accurate Chemical and Scientific Co., Hicksville, N.Y.). Affinity-purified antigen-specific goat anti-rabbit IgG (heavy and light chain specific) was obtained from Zymed Laboratories, South San Francisco, Calif.

ELISA procedure. The suppliers of the ELISA plates used are listed in Table 1. The coating buffer (carbonate-bicarbonate, pH 9.6), phosphate-buffered saline with 0.05% Tween 20 (PBS-Tween), and *o*-phenylenediamine substrate solution were prepared exactly as described by Voller et al. (20). The volume used per well in each microtiter plate for all additions was 200 μl, with a calculated area of 155 mm^2 of plastic surface in the test. Antigens were diluted in coating buffer and allowed to attach to plastic surfaces overnight (16 to 18 h) at 4°C. Plates were washed three times with PBS-Tween. For the direct assay, conjugated antisera were diluted as indicated and added to wells. After 2 h of incubation at 37°C, plates were washed three times with PBS-Tween. Substrate was added, and the plates were incubated in the dark at room temperature (~25°C) for 0.5 h. Sulfuric acid (8 N, 25 μl) was added, and optical densities were determined with a Titertek Multiskan filter photometer (Flow Laboratories, Inglewood, Calif.) at 492 nm. The bank contained substrate and acid. For the indirect test, washed antigen-coated plates were treated with dilutions of rabbit antiserum in PBS-Tween and incubated for 2 h at 37°C. Plates were washed and developed with peroxidase-labeled goat anti-rabbit IgG (heavy and light chain specific). The remainder of the assay was as for the direct test.

Assays for competition during adsorption. To determine competition among various proteins during adsorption, specific concentrations of antigens were mixed with increasing concentrations of a competitor protein in glass test tubes to yield the concentrations stated, using coating buffer as diluent (glass tubes were used because attachment of proteins to glass is minimal [6]). The remaining procedures were as described above for either the direct or indirect ELISA.

Antigen capture (sandwich) assay. Affinity-purified

TABLE 1. Comparative attachment of BSA and human IgG to various microtitration plates

Plate				Absorbance (492 nm)				
Plate type	Name	Vendor[a]	Lot or control no.	BSA[b]	BSA control[c]	Human IgG[d]	Human IgG control[c]	Nonspecific binding[e]
A	Immulon I	Dynatech	CR-124	0.34	0.08	0.80	0.06	0.10
B	Immulon II	Dynatech	CR-123	1.61	0.10	0.83	0.08	0.10
C	MicroElisa Substrate plates	Dynatech	29-11-77	0.58	0.09	0.66	0.09	0.13
D	M 129A Substrate plates	Dynatech	32680	0.22	0.03	0.72	0.04	0.06
E	M 129A Substrate plates	Dynatech	71080	0.52	0.12	0.59	0.10	0.13
F	M 129A Substrate plates	Dynatech	Unknown	0.77	0.09	0.64	0.10	0.11
G	Nunc Immunoplate I	Vangard	Not given	1.69	0.13	0.73	0.12	0.58
H	Nunc Immunoplate II	Vangard	Not given	1.71	0.12	0.75	0.11	0.73
I	Linbro Titertek	Flow	7623001	0.98	0.07	0.82	0.07	0.07
J	Linbro Titertek	Flow	76203001	0.74	0.08	0.74	0.10	0.13
K	Serocluster EIA plate 3590	Costar	1284	1.84	0.19	0.80	0.10	0.53

[a] Addresses: Dynatech Laboratories, Inc., Alexandria, Va.; Vangard International, Neptune, N.J.; Flow Laboratories, Inc., McLean, Va.; Costar, Cambridge, Mass.

[b] BSA at 1 μg/ml for coating; detecting antibody, 1:32,000.

[c] Coating antigen omitted, plate incubated with coating buffer, detecting antibody same as with coating antigen.

[d] Human IgG at 1 μg/ml for coating; detecting antibody, 1:320,000.

[e] No antigen attached; detecting antibody, 1:10,000 goat anti-human IgG (same lot as the controls).

antigen-specific goat anti-human IgG (1 μg/ml) was mixed with various dilutions of normal goat serum in coating buffer and permitted to attach to plates. Plates were washed with PBS-Tween three times. Human IgG (10 μg/ml) was added to plates, and the plates were incubated for 2 h at 37°C. Plates were again washed with PBS-Tween three times, and the captured IgG was detected with peroxidase-conjugated goat anti-human IgG.

Replication. Assays were conducted in triplicate or in duplicate for very large experiments. All experiments were conducted several times, and the results shown in tables and figures are from single experiments but are representative of our general experience.

RESULTS

Differences in adsorption characteristics of various plastic plates. BSA, ovalbumin, and human IgG were chosen as antigens for the studies because they are readily available in purified form, and a number of manufacturers produce directly conjugated antisera. No cross-reactions were found between these antigens and the commercially available monospecific conjugated antisera within the range of dilutions of conjugate used in this study. Furthermore, BSA and IgG differ significantly in isoelectric point, molecular weight, and binding ability to plastics (5). The antigen titration curves for BSA and human IgG were compared on the type A and B plates by using direct ELISA. BSA adsorbed relatively poorly to type A plates in contrast to type B plates (Fig. 1A). Even at a higher antibody concentration (1:3,200), the optical density on type A plates did not exceed 1.0, whereas less antibody (1:10,000) gave an optical density of 2 or greater on type B plates (not shown). As a consequence, the amount of BSA required for detection appeared to be about 10- to 30-fold higher on type A plates than on type B plates. Three different lots of BSA gave similar results (not shown). In contrast, IgG attached well to both type A and B plates (Fig. 1B). The adsorption of ovalbumin differed from that of both IgG and BSA in that the optical density did not peak but continued to increase as ovalbumin was increased (Fig. 1C). Two additional lots of ovalbumin gave similar results. The adsorption characteristics of 11 commercially available plates were compared, using BSA and IgG for comparison (1 μg/ml). Two classes of plates were obvious (Table 1): those which adsorbed BSA well (types B, G, H, and K) and those which adsorbed BSA poorly (types A and D), with some plates giving intermediate adsorption (types C, E, F, I, and J). Adsorption of IgG was similar on all plates. To detect nonspecific attachment of the labeled antibody, a 32-fold excess of antibody was tested (Table 1, last column); plates G, H, and K gave high background levels. Plate type B was the only plate which gave both high BSA attachment and a low background. Nonspecific binding of conjugates on plate types H and K was reduced progressively by increasing the amount of Tween 20 in the diluent from the standard 0.05% to 1.6%. This reduced the optical density from 0.7 to about 0.25 for both plates (Table 2). Increased detergent did not interfere with detection of antigen by antibody (not shown). Since nonspecific binding of type A and B plates was already low, no effects were seen with increased detergent.

TABLE 2. Effect of increases in Tween 20 concentration on nonspecific adsorption of conjugate by microtitration plates[a]

% Tween 20 with PBS	Absorbance (492 nm) with plate type:			
	A	B	H	K
0.05	0.10	0.10	0.75	0.66
0.16	0.10	0.12	0.49	0.33
0.5	0.11	0.11	0.38	0.26
1.6	0.10	0.11	0.29	0.23
5.0	0.11	0.10	0.24	0.21

[a] Plate designations are defined in Table 1. Goat anti-human IgG conjugate was used at 1:10,000 dilution to detect nonspecific attachment on plates pretreated and washed with the indicated concentrations of Tween 20.

Competition between molecules for attachment sites on plastic. To determine the adsorption characteristics of mixtures of proteins, given amounts of a protein were mixed with increasing concentrations of a competitor protein, polysaccharide, or detergent. The mixture was then coated onto plastic, and the protein attached was detected with a directly labeled monospecific antibody (Fig. 2A). Competition between proteins for adsorption sites was readily demonstrated: a concentration of 10 μg of BSA per ml reduced the optical density of 1 μg of IgG per ml by about half (Fig. 2A), and a 100:1 ratio decreased the optical density from 1.3 to 0.2. The important finding was that the amount of inhibition was a function of the ratio of the proteins, not the absolute amount. In each case, a 100:1 ratio of BSA reduced the optical density for IgG by 90% to a value near background. The results were similar on types A, B, and C plates in a number of trials. Furthermore, the same proportionalities between competitor and antigen were found in experiments using less efficient conditions (e.g., a 0.5-h incubation period for conjugate attachment). Tween 20 was an even more effective competitor, requiring only a 10:1 ratio of detergent to IgG to reduce optical density values to low levels. Again, the competition was a function of the ratio of Tween 20 to the protein being attached (Fig. 2B). The same proportionalities held on type H plates for Tween 20. Various proteins, polysaccharides, and detergents were tested for competition with IgG (Table 3). Most tested proteins reduced optical densities to nearly background levels on type A plates when employed at 100:1 competitor-to-antigen ratios. Lesser but parallel inhibition was observed on type B plates. Tryptone was an ineffective competitor. Polysaccharides gave only limited competition, but a 500:1 ratio of pneumococcal polysaccharide type 14 (positive charge at pH 8.6 [11]) was a significant competitor on type A plates. Type 4 pneumococcal polysaccharide (negative charge at pH 8.6 [11]) was an ineffective competitor. Both nonionic detergents (Tween 20 and Triton X-100) were strong competitors, whereas charged detergents were less effective. Cetrimide (positively charged) and sodium dodecyl sulfate (negatively charged) were the least effective competitors, requiring a 1,000:1 ratio to inhibit adsorption of IgG. Sodium deoxycholate (negatively charged) and Zwittergent (both positive and negative charges) were intermediate competitors.

Effect of concentration of conjugate on apparent competition. Since detection of attached protein in ELISA is dependent upon the amount of conjugate used to measure the protein, it was possible that the apparent competition observed was an artifact of insufficient detecting antibody. Mixtures of BSA and IgG (ratios of 10,000:1 through 1:10,000) were adsorbed to plates. The attachment of each species was measured at several concentrations of each monospecific antibody. IgG was detected at BSA:IgG ratios of 10:1 but not at 100:1 on type A plates (Fig. 3). A threefold increase in the amount of detecting antibody increased the optical density at the 10:1 ratio, but IgG was not detectable at a 100:1 ratio even with 30-fold antibody increase. Similar proportionalities were observed when BSA was detected in the presence of increasing amounts of IgG (Fig. 3); BSA was detected marginally when 100-fold IgG was present. Increasing the amount of conjugate gave better detection of IgG on type B plates (Fig. 4), whereas IgG was detectable at 100:1 but not 1,000:1 BSA-to-IgG ratios when the two higher antibody levels were used for detection. Similarly, BSA was detectable at 1:100 ratios of BSA to IgG. Accordingly, the ability to detect a given antigen was dependent upon the ratio of competitor to antigen, the type of plate used, and the amount of conjugate used to detect the antigen.

Effect of competition by normal serum components on coating plates for antigen capture assays. Plates are ordinarily coated for antigen capture assays with high dilutions of powerful antisera or by using the IgG fraction of sera (15). In the first case, normal serum components could be competitors, and in the second case nonspecific IgG could compete with the specific IgG for sites. Affinity-purified antigen-specific goat anti-rabbit IgG (1 μg/ml was used; the minimal amount required for effective capture was 0.1 to 0.3 μg/ml) was mixed with dilutions of normal goat serum and coated to determine competition from normal serum components. The antigen captured was rabbit IgG (10 μg/ml), which was detected by several dilutions of goat anti-rabbit IgG (Fig. 5A). On type A plates, dilutions up to and including 1:10,000 normal goat serum in the coating mixture gave significant inhibition of

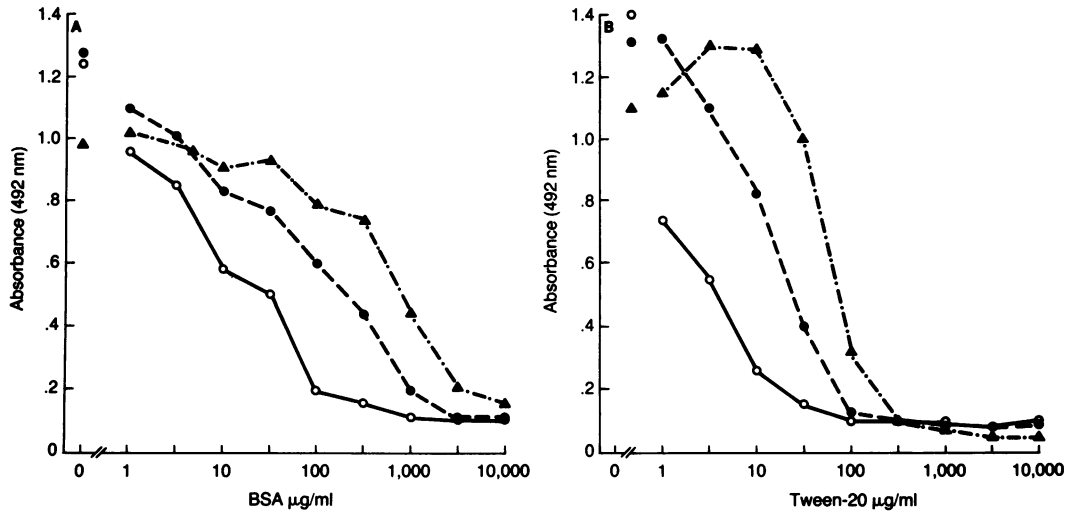

FIG. 2. BSA (A) and Tween 20 (B) as competitors for adsorption sites with human IgG on type B plates. The concentrations of IgG as coating antigen were 1 (○), 10 (●), and 100 μg/ml (▲). The amount of competitor is shown on the x-axis. Direct ELISA was performed with peroxidase-conjugated goat anti-human IgG antiserum at a 1:100,000 dilution.

capture of IgG when 1:32,000 conjugate was used for detection. Even at 1:1,000 conjugate, 1:3,200 normal goat serum prevented effective capture. The results were similar on type C plates (not shown). Capture was more effective on type B plates; at 1:32,000 conjugate, inhibition by normal goat serum was 10-fold less than on type A plates (Fig. 5B). Since the goat serum contained 85 mg of protein per ml of normal goat serum, a 1:3,200 dilution contained 27 μg of competitors per ml for adsorption sites with the 1 μg of antigen-specific IgG used for capture, a 27:1 ratio. Therefore, antigen capture assays were vulnerable to competition effects from normal serum components; competitor-to-capture antibody ratios were similar to those determined for competitor-to-coating antigen ratios (Table 3). At higher conjugate concentration, competition by normal goat serum appeared less, but nonspecific binding on type B plates (but not types A or C) increased greatly. This nonspecific binding was related to the normal goat serum, since omission of the capture antibody did not reduce the optical density at high concentrations of normal goat serum. When plates were coated with 1:100 normal goat serum and tested with conjugated goat antiserum (1:1,000) to human albumin or human IgG (irrelevant antibodies), optical densities were between 0.6 and 1.4, whereas control plates without goat serum but blocked with detergent gave optical densities between 0.05 and 0.15 (Table 4). On type C plates (binding similar to type A plates), pretreatment with normal goat serum gave optical densities ranging from 0.14 to 0.34 compared with untreated plate values of 0.06 to 0.09. Coating with 1:100 normal rabbit serum gave similar results when tested with conjugated rabbit antiserum (1:1,000) to BSA or human IgG; coated type B plates showed significantly higher optical densities than uncoated type B plates or coated type C plates. As expected, reducing the conjugate level progressively lowered nonspecific binding. These results indicate that the nonspecific binding of labeled immunoglobulins to homologous normal serum is likely a protein-protein interaction and not an antigen-antibody reaction, since one would have expected an antigen-antibody reaction to have occurred on type C plates as well.

Effect of antigen competition on the indirect ELISA. In the indirect ELISA, the antigen is attached to the plastic and an unlabeled antibody is applied, and this antibody is detected by a conjugated anti-globulin antibody. Possibly the results of competition between coating antigens in indirect ELISA could be different from direct ELISA or antigen capture assays. Furthermore, the sensitivity of an indirect ELISA for measurement of antibody can be enhanced by increases in conjugate within limits. Type B plates were coated with human IgG (1 μg/ml) in the presence of increasing amounts of BSA as competitor, and IgG antibody was assayed at various dilutions (Fig. 6A). The sensitivity of the test for detection of antibody to human IgG was decreased modestly by BSA competitor at ratios of up to 10:1, but at 100:1 the sensitivity of the test was decreased at least 10-fold, and the slope was also lessened. The IgG antibody titers obtained

TABLE 3. Inhibition of the attachment of human IgG to type A and type B plates by proteins, polysaccharides, and detergents[a]

Competitor	Run no.	Absorbance (492 nm)									
		Plate type A competitor concn (μg/ml)					Plate type B competitor concn (μg/ml)				
		0	10	100	1,000	Control	0	10	100	1,000	Control
Proteins											
Bovine serum albumin	5	1.03	0.29	0.10	0.07	0.09	1.22	0.86	0.42	0.13	0.15
Human albumin	7	1.33	0.46	0.17	0.10	0.10	1.14	0.92	0.49	0.17	0.10
Rabbit albumin	5	0.95	0.35	0.12	0.06	0.10	1.12	0.96	0.68	0.27	0.10
Chick ovalbumin	7	1.37	1.07	0.75	0.25	0.11	1.27	1.11	0.90	0.40	0.10
Rabbit IgG	6	0.68	0.48	0.28	0.13	0.11	0.71	0.60	0.43	0.22	0.14
Gelatin	3	1.08	0.18	0.10	0.06	0.08	1.31	0.30	0.14	0.11	0.11
Lysozyme	5	0.99	0.38	0.24	0.15	0.07	0.99	0.63	0.47	0.28	0.09
Tryptone	7	1.34	1.16	0.95	0.44	0.10	1.40	1.21	1.18	1.09	0.10
Polysaccharides											
Streptococcus pneumoniae type 14	1	0.69	0.43	0.31	0.16[b]	0.09[b]	0.91	0.73	0.56	0.51[b]	0.09[b]
Streptococcus pneumoniae type 4	7	1.20	1.14	1.08	0.83	0.10	1.38	1.33	1.15	1.08	0.10
Gum ghatti	7	1.18	0.87	0.78	0.59	0.10	1.38	1.09	0.92	0.71	0.11
Detergents											
Tween 20	3	1.07	0.12	0.10	0.10	0.10	1.13	0.36	0.12	0.12	0.11
Triton X-100	3	0.84	0.08	0.08	0.08	0.10	1.11	0.52	0.13	0.12	0.11
Sodium deoxycholate	2	1.13	0.42	0.11	0.09	0.07	1.42	1.19	0.86	0.27	0.11
Sodium dodecyl sulfate	2	1.19	1.15	0.63	0.09	0.06	1.39	1.38	1.21	0.12	0.10
Cetrimide	4	0.95	0.62	0.77	0.46	0.10	1.08	0.89	0.43	0.16	0.11
Zwittergent	4	0.93	0.67	0.05	0.05	0.05	1.10	0.95	0.12	0.08	0.07

[a] Human IgG (1 μg/ml) was mixed with various concentrations of competitors and permitted to attach to type A and B plates. Human IgG was detected by 1:32,000 conjugated goat anti-human IgG. Comparisons may be made between plates and concentrations of a given competitor because these sets were in a single run. But, because of run-to-run variation in optical density, caution should be used in comparing runs. The control was competitor by itself tested at maximum concentration as coating antigen against conjugate.

[b] Competitor concentration, 500 μg/ml.

in the presence of competitor closely resembled those seen when IgG antibody was titrated against decreasing amounts of coating IgG (Fig. 6B); for example, the curve for the IgG antibody at the 100:1 BSA-to-IgG coating mixture resembled that obtained on plates coated with 0.01 μg of IgG per ml. When threefold more conjugate was used for detection of IgG antibody, apparent serum titers were increased about sixfold, but as BSA competitor increased in the coating mixture, titers of IgG antibody decreased (data not shown) in a manner similar to that shown in Fig. 6A. Thus, the presence of competitors in coating mixtures had the same effects in indirect ELISA as they have in the direct test, namely, the sensitivity of the indirect test was reduced at 100:1 ratios of competitor to antigen, and antibodies could not be detected at 1,000:1 ratios.

Utilization of deliberate mixtures for simultaneous assay of antibodies to multiple antigens. When extracts of microorganisms are tested as ELISA antigens, the antigen titration is commonly carried out with several human sera or a pool of human sera to determine the optimum antigen concentration. To provide a model for such experiments and to further determine whether deliberate mixtures could be prepared which would permit simultaneous detection of antibodies to several different antigens, we prepared mixtures of BSA, human IgG, and ovalbumin. Dilutions of these mixtures were then permitted to attach to type B plates and titrated with directly labeled monospecific antisera. When a mixture of equal parts of each antigen was prepared, it was found possible to assay all three antibodies simultaneously, provided that the total antigen level was above 1 to 10 μg/ml and assuming that the optical density for each antigen would be similar (Fig. 7A). However, since the BSA assay was the most sensitive (required the least antigen [Fig. 1A]), a test which was mostly specific to BSA would result if the antigen titration were adjusted to optimum with BSA antiserum, whereas titration with antibody to ovalbumin (the least sensitive test [Fig. 1C]) would result in a test specific to all three antigens. If the antigenic mixture were biased to favor ovalbumin by making a mixture of 89% ovalbumin, 10% human IgG, and 1% BSA, all antibodies were detected when the optimum

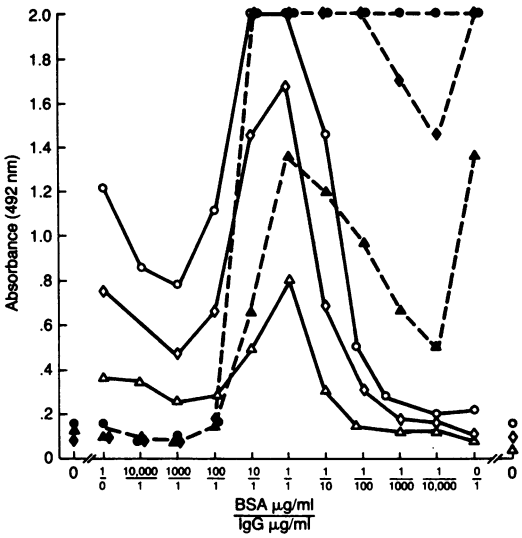

FIG. 3. Effects of various conjugate dilutions on detection of competition between BSA and human IgG on type A plates. Solid lines indicate optical densities obtained with peroxidase-conjugated BSA antibody at three dilutions: 1:1,000 (○), 1:10,000 (◇), and 1:32,000 (△). Dashed lines indicate optical densities obtained with peroxidase-conjugated goat antibody to human IgG at three dilutions: 1:3,200 (●), 1:32,000 (◆), and 1:100,000 (▲). Wells were coated with mixtures of antigens in the amounts indicated on the x-axis (final concentrations). The lines for detection of BSA are superimposed at 1:1,000 and 1:10,000 dilutions of antibody. Direct ELISA.

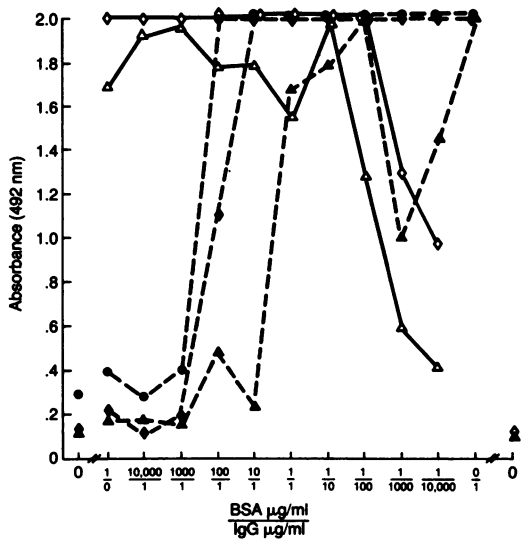

FIG. 4. Effects of various conjugate dilutions on detection of competition between BSA and human IgG on type B plates. Solid lines indicate optical densities obtained with BSA antibody at two dilutions: 1:10,000 (◇) and 1:32,000 (△). Dashed lines indicate optical densities obtained with peroxidase-conjugated antibody to human IgG at three dilutions: 1:3,200 (●), 1:32,000 (◆), and 1:100,000 (▲). Wells were coated with mixtures of antigens in the amounts indicated on the x-axis (final concentrations). Direct ELISA.

antigen level was determined with any of monospecific sera (Fig. 7B). On the other hand, biasing the test in favor of the most sensitive antigen (BSA) by making a mixture of 1% ovalbumin, 10% human IgG, and 89% BSA resulted in a test which at best could measure only BSA and IgG (Fig. 7C). In each case, parallel titrations of the antigens individually gave titration curves similar to those for the individual components in the mixture (not shown). It is important to note that when human sera are used to standardize mixtures of antigens, one would never know how many antibodies to different antigens were present, and thus it would be impossible to know how many different antigenic specificities the test could measure.

DISCUSSION

It is generally known that various brands and lots of polystyrene plates differ in adsorption properties for antigens (3, 4, 10, 15). Indeed, a common procedure when adopting ELISA to measurement of antigens or antibodies in a new system is to test several different types of plates in the hope that one may give better results than another. In our study, we found that adsorption of IgG was quite similar on all types of plates tested and that any of the plates could be used for a satisfactory assay of IgG. However, adsorption of BSA varied to the extent that certain plates were ineffective for measurement of its antibody by direct or indirect ELISA. Our experiments did not distinguish between decreased binding and increased detachment from the plastic during washing steps, but either effect would be deleterious to ELISAs. Although Cantarero et al. (5) found little leakage from the polystyrene they used, Engvall et al. (8) as well as Lehntonen and Viljanen (14) found significant leakage from plastic during washing steps. The differences we found between plastics in this study could explain this discrepancy. Plates which adsorbed BSA well also were more satisfactory for coating with mixed antigens (Fig. 4) and for antigen capture assays (Fig. 5). However, a price was paid for this increased binding power in that conjugate bound to these high-binding plates and yielded a higher background at increased conjugate levels, an effect enhanced by pretreatment with normal homologous serum (Table 4). Binding by conjugate to detergent-satisfied plates could be lessened by increasing the amount of Tween 20 in the diluent (Table 2).

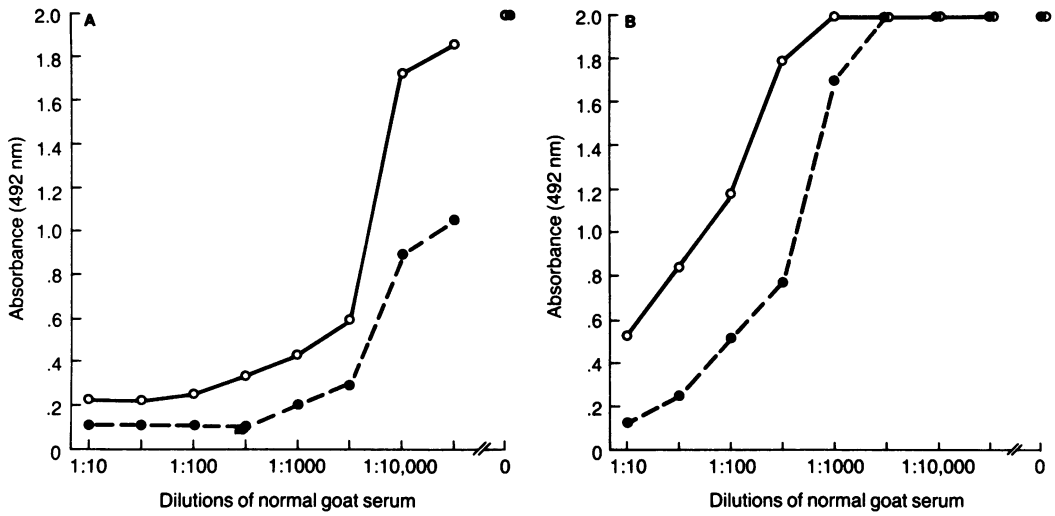

FIG. 5. Competition by normal serum components in antigen capture assay. Capture antibody, 1 μg of affinity-purified antigen-specific goat anti-rabbit IgG per ml. Antigen captured, 10 μg of rabbit IgG per ml. The dilutions of normal goat serum used for competition for adsorption sites are shown on the x-axis. (A) Type A plate and (B) type B plate. Two concentrations of goat anti-rabbit IgG conjugate were used: 1:1,000 (○) and 1:32,000 (●). The normal goat serum contained 85 mg of protein per ml.

Overall, three types of plates were found with gradations in between: (i) those which bound BSA poorly and had low background binding of conjugate; (ii) those which bound BSA well and gave moderate to low backgrounds (plate type B, Table 1); and (iii) plates which bound BSA well and gave high backgrounds with conjugate only. To us, it appeared that we were observing an increase in affinity of the plastic for proteins, which culminated in increased nonspecific binding. However, any of the plates could be used for assay of IgG because the amount of conjugate required for a useful assay was less than the amount which caused significant nonspecific binding. It would appear that for antigens which bind well, plates of the (i) category would work best because large amounts of conjugate could be used if required. For antigens of unknown binding power, we suggest testing representatives of each of the above three classes of plates, since a much better assay will result if binding can be optimized with minimal background. In a comparative manner, it is possible to distinguish between the three classes of plates by using readily available commercial reagents. Plates which bind BSA poorly can be detected by the inability to obtain an optical density much over 1.0 in direct ELISA when plates are coated with BSA, whereas the strongly binding plates will give an optical density of 2.0 at 0.1 to 1.0 μg of coating BSA per ml at reasonable conjugate dilutions (~1:10,000). Plates which show a high

TABLE 4. Differences in nonspecific attachment of conjugates to various types of plates[a]

Conjugate		Coating serum	Absorbance (492 nm)					
			Plate type B			Plate type C		
Source	Specificity		1,000[b]	3,200	10,000	1,000	3,200	10,000
Goat	Human albumin	Goat	1.42	0.64	0.38	0.34	0.20	0.20
Goat	Human albumin	None	0.15	0.16	0.13	0.09	0.08	0.06
Goat	Human IgG	Goat	0.61	0.38	0.24	0.14	0.14	0.12
Goat	Human IgG	None	0.10	0.10	0.09	0.06	0.07	0.08
Rabbit	BSA	Rabbit	0.59	0.29	0.22	0.22	0.14	0.12
Rabbit	BSA	None	0.15	0.13	0.11	0.13	0.11	0.07
Rabbit	Human IgG	Rabbit	0.93	0.49	0.34	0.30	0.18	0.16
Rabbit	Human IgG	None	0.22	0.17	0.13	0.13	0.12	0.10

[a] Normal serum was diluted 1:100 in coating buffer and permitted to attach. After attachment, plates were washed and conjugate was added, and the plates were washed again and developed for color. Since the conjugate was homologous to the coating serum in each case, no antigen-antibody reactions should have occurred.

[b] These values are the reciprocals of the dilutions of the conjugate.

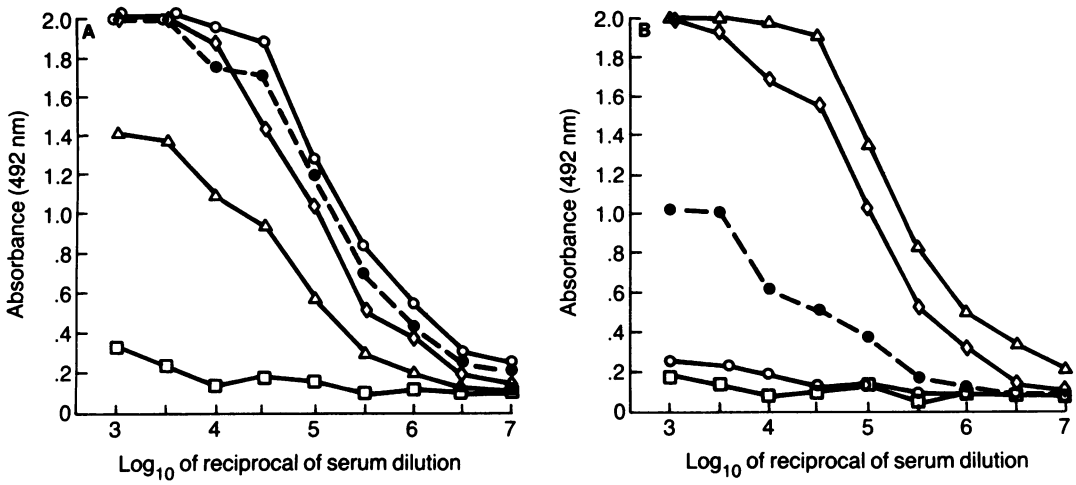

FIG. 6. Effects of competition between BSA and human IgG on antibody detection by indirect ELISA on type B plates. (A) IgG (1 μg/ml) was coated onto plastic in the presence of the following quantities of BSA competitor: none (○), 1 (●), 10 (◇), 100 (△), and 1,000 (□) μg/ml. Dilutions of rabbit anti-human IgG were added as indicated on the x-axis. The rabbit IgG was detected with 1:100,000 goat anti-rabbit conjugate. (B) The control shows the effect of coating with various amounts of IgG as antigen on the apparent antibody titer without competitor present. Five concentrations of coating antigen were used: none (□), 0.001 (○), 0.01 (●), 0.1 (◇), and 1 μg/ml (△). The same conjugate dilution was used as that in (A).

background can be detected by adding an excess of conjugate (~10-fold) to wells satisfied with detergent; optical densities of 0.5 or greater may be expected.

The finding that significant competition for adsorption sites exists in the ELISA is not a new concept (5, 6, 10, 13, 16–18). Cantarero et al. (5), using radioactively labeled proteins, showed that the amount of protein bound was dependent upon the concentration of the protein until saturation of the plastic surface occurred, at which point a constant amount of protein was bound regardless of the amount of protein in the solution. Our findings flow directly from this principle in that it is the ratio of one antigen to another which is critical in determining whether adequate antigenic coating can be obtained in the ELISA. In general, minority components could be detected with difficulty at a 100:1 ratio of competitor to the minority component and could not be detected at all at a 1,000:1 ratio. The effects of competitors were further modified by the type of plastic surface, type of competitor (some were inactive), amount of conjugate, and the sensitivity of the assay for a given antigen. No doubt other variables such as pH and ionic strength (not tested in this study) may have additional effects on competition. Protein-protein interactions may be involved both in solution and as competitors for adsorption sites as shown by Lee et al. (13). In our case, even though BSA bound poorly to type A plates, it was an effective competitor for IgG (Fig. 3), suggesting an interaction between the proteins in solution. Similarly, the strong binding of conjugate to high-binding plates coated with homologous serum suggests a protein-protein interaction. Overall, all proteins were competitors; the least effective was lysozyme, a result which might be expected because binding capacity of proteins to plastic has an apparent inverse relationship to molecular weight (5). The protein hydrolysate, tryptone, also gave little competition. Two pneumococcal polysaccharides of opposite charge were selected for testing; only the positively charged one was a competitor. Gum ghatti, a heterogeneous collection of polysaccharides, also failed to compete. The fact that nonionic detergents are more effective competitors compared with ionic detergents was described in 1966 by Van Oss and Singer, using radiolabeled proteins for attachment to polystyrene (19). They also showed that competition between proteins and nonionic detergents is a function of the ratios of protein to detergent competitor as is shown in our study. A 10:1 nonionic detergent-to-protein ratio effectively prevented binding of IgG or BSA, and this was the lowest competitor-to-antigen ratio found in the study, indicating that the widely practiced use of nonionic detergents to prevent secondary interactions is certainly correct. However, our data suggest that nonionic detergent concentrations greater than the 0.05% commonly used may be necessary to lessen background on polystyrene plates with strong binding capacity (Table 2). The finding that strongly charged detergents are poor competitors has an important

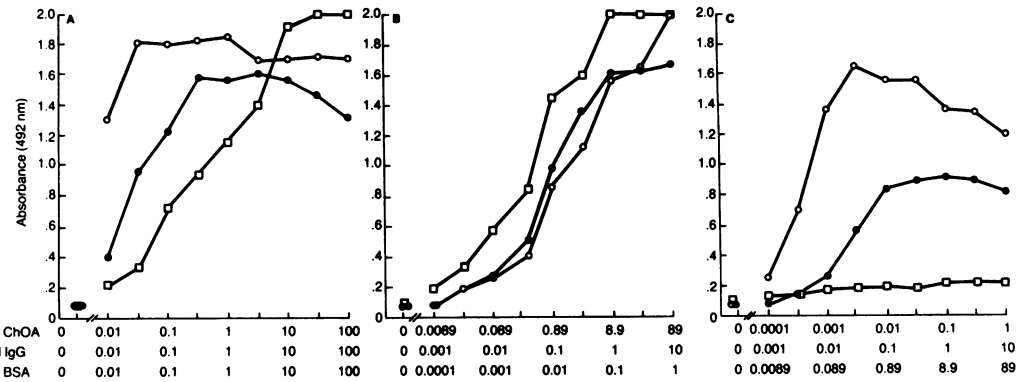

FIG. 7. Antigen titrations of mixtures of ovalbumin (ChOA), human IgG, and BSA by direct ELISA on type B plates. (A) An equal mixture of three antigens. (B) A mixture of 89% ovalbumin, 10% IgG, and 1% BSA. (C) A mixture of 1% ovalbumin, 10% IgG, and 89% BSA. Antigenic mixtures were coated at the indicated concentrations, and individual antigens were detected with monospecific peroxidase-conjugated antisera to ovalbumin at 1:32,000 (□), IgG at 1:100,000 (●), and BSA at 1:32,000 (○).

practical use in that organisms and tissues can be solubilized with such detergents and directly coated onto plastic, provided that the material is diluted sufficiently to limit competition and provided that background levels do not increase (Table 3). Nonionic detergents would be much less suitable for this purpose.

Although ELISA is highly useful in clinical immunology for detection of antibodies by indirect ELISA, few purified antigens are available for coating plates. Instead most assays are performed with extracts of the organism or agent in question. Since such extracts must contain many antigens, only some of these antigens will be represented in large enough proportions to coat the plastic effectively. Furthermore, the particular collection of antibody specificities in sera used to standardize the assay will control whether the test measures one or several antibodies (Fig. 7). This problem has been well recognized (17, 18), but our studies are the first to provide a specific quantitative approach. Strandberg-Pedersen et al. (17) showed that the specificity and sensitivity of ELISA for diagnosis of syphilis was greatly improved when a purified flagellum extract was used rather than sonicated Reiter treponemes. They suggested that the inferior reactivity of the sonicate could be accounted for by competition between antigens for the limited adsorption sites. Although the use of purified antigens is ideal, partial purification of the crude extracts may reduce amounts of competitors and thus permit coating of important antigens. Ballad et al. (1) obtained an improved ELISA for *Alveococcus* (*Echinococcus*) sp. by using a large-molecular-weight fraction obtained by gel filtration for coating plates. In devising tests for viruses by using antigens grown in cell culture (or even using cell culture supernatants), it is important to consider the large amounts of serum competitors present. At 1% concentration of calf serum in the medium, potential competitors would be 700 to 900 μg/ml per ml, an amount which would cause significant competition at lower dilutions. Small-molecular-weight serum components could be readily removed from viral preparations by centrifugation or gel filtration. A similar approach involving partial purification might be useful for many extracts. Purification of IgG from sera is a well-known tactic for antigen capture assays (15). Even so, our data suggest that the specific IgG molecules must represent at least 1% of the total IgG for capture to be effective, suggesting a need for affinity purification of IgG from weak antisera. When an antigenic extract is suspected of containing competing substances (i.e., adequate coating of polystyrene cannot be obtained), competition can be detected by mixing dilutions of the extract with a constant amount of a defined antigen such as IgG (our experiments indicate a concentration of 1 μg/ml for human IgG). After coating plates, the IgG is detected directly by a labeled anti-IgG (as in Fig. 2A). Concentrations of the extract which cause significant inhibition of attachment of IgG likely are not useful in ELISAs.

Comparison of antibody titers in indirect ELISA among sera (i.e., acute and convalescent serum samples from a patient) is difficult when the antibody slope is shallow (i.e., optical density decreases slowly with decreasing antibody concentration [4, 7]). Our results show that shallow slopes may be generated by inadequate coating of plates either because of competition between components in antigenic mixtures (Fig. 6A) or because too little pure antigen has been used (Fig. 6B). Quite likely other factors are

involved as well, such as the affinity of antibody and the heterogeneity of antigenic determinants (4). However, when mixed antigens are employed, we believe that competition between antigens for adsorption sites should be suspected.

Serodiagnosis is complicated by the fact that many agents may cause a given disease; searching for antibody responses one at a time is most expensive. A screening method using multiple antigens in a single test would be useful. For example, pneumococcal infections may be caused by any of the some 80 serotypes. Berntsson et al. (2) used a mixture of polysaccharides of six prevalent serotypes for coating plates for detection of antibody responses. We show that assays can be devised for multiple antigens provided that monospecific antiserum to each antigen is available for standardization of the antigenic mixture (Fig. 7). Each antigen would have to represent at least 1% of the antigenic mixture, limiting the number of antigens detectable to about 10 because not all antigens give equal coating. On the other hand, the use of extracts of organisms and tissues in ELISA or other immunoassays for detection of monoclonal antibodies has distinct limitations, since even under optimum conditions only a few of the many different antigenic specificities could be detected. Hence, antibodies to minor components or components which coat poorly will be missed.

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