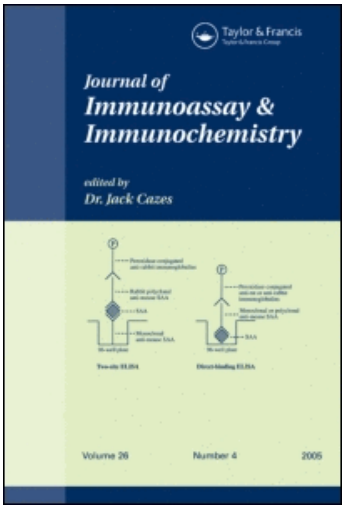

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Enzyme Immunoassay: Binding of *Salmonella* Antigens to Activated Microtiter Plates

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ENZYME IMMUNOASSAY: BINDING OF SALMONELLA ANTIGENS TO ACTIVATED MICROTITER PLATES

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ABSTRACT

A heat extract prepared from radiolabeled Salmonella cells was used to determine if covalent binding to activated surface of polystyrene plates would improve antigen retention thus contributing to increase sensitivity in an enzyme

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immunoassay for Salmonella antigen. The effect of treatment with ethylchloroformate on the retention of antigens passively absorbed to polyvinylchloride and polystyrene plates was also investigated. Chemically modified plates retained more radiolabeled antigens after washing than did untreated plates in which the antigens had been physically adsorbed. However, improvement of assay sensitivity depended on the type of plate used for covalent binding of antigen. N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), was found to be potentially useful for mediation of covalent binding of antigens to activated plates.

Key Words: Enzyme immunoassay - covalent binding - Salmonella

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Abbreviations: EIA=enzyme immunoassay; SPDP=N-succinimidyl 3-(2-pyridyldithio) propionate; PVC=Polyvinylchloride; EDC=1-ethyl-3(3-dimethylaminopropyl) carbodiimide; MES=2(N-morpholino) ethanesulfonic acid; ETCH=ethylchloroformate

INTRODUCTION

Various materials have been proposed for use as solid-phase supports in heterogenous enzyme immunoassays, but plastics such as polyvinylchloride and polystyrene in the form of microtiter plates have gained acceptance in most laboratories. Immobilization of antigens or antibodies on plastic surfaces is usually achieved by simple passive adsorption, a phenomenon attributed to non-specific hydrophobic interactions (Van Oss and Singer, 1966). In our previous studies on immobilization of Salmonella antigens on the surface of microtiter plates, passive adsorption was preferred over centrifugation or antigen capture by antibody or polylysine (Aleixo et al., 1983). However, a considerable degree of antigen desorption occurs during the washing steps in the EIA. In addition, due to the conditions of manufacture of molded plastics, variation in the binding characteristics of different batches or lots of

microtiter plates is expected to occur. These factors adversely affect the sensitivity and precision of the enzyme immunoassay.

Covalent binding of antigens and antibodies to plastic surfaces has been suggested as one approach to minimize the above problems and still retain the functionality of microtiter plates (Clark and Engvall, 1980). Rotmans and Delwel (1983) reported that EIA sensitivity was enhanced when Schistosoma mansoni antigens were immobilized on chemically modified plates through covalent bonds. We report here on the use of different methods for covalent binding of Salmonella antigens to activated microtiter plates and on the effects of such binding on the sensitivity of enzyme immunoassays. The Salmonella model was chosen because our laboratory has been engaged in the development of a sensitive enzyme immunoassay for this organism. Further, the sensitivity of such an assay would depend on the quantity of antigen retained by the solid matrix. Therefore, any improvements achieved in antigen retention would contribute to the achievemnt of our goal.

MATERIAL AND METHODS

Solid phases

Three types of commercially available plastic microtiter plates were used in the experiments. Polyvinylchloride plates were obtained from Costar, Cambridge, MA (Lot No. 1426) and Dynatech Laboratories, Alexandria, VA (Lot No. not available). Polystyrene plates of two types, Immulon I (Lots No. CR 3005 and CR 402), and Immulon II (Lot No. CR 5502), were obtained from Dynatech Laboratories. Polystyrene plates were chemically modified by the following treatments:

(i) Introduction of amino groups: In this procedure, as described by Rotmans and Delwel (1983), the wells of a polystyrene plate were filled with 0.1 ml of a 10% fuming nitric acid solution in glacial acetic acid. Nitration was

allowed to proceed for 3h at 50°C. The wells were washed once with tap water and 0.1 ml of a 1% sodium dithionite ($Na_2S_2O_4$) solution in 0.1M NaOH was added. After an incubation period of 3h at 50°C, the wells were rewashed with tap water and the plates stored at 4°C.

(ii) Introduction of spacer molecule: Suberic acid was coupled through one of its carboxylic groups to the amino group of polyaminostyrene plates obtained as above. One hundred microliter of an aqueous solution containing 0.2% (w/v) suberic acid and 0.1% (w/v) EDC was added to each well; the plates were incubated overnight at room temperature and then stored at 4°C until use.

(iii) Thiolation: Thiol goups were introduced into the polyaminostyrene plate by the use of SPDP (Carlsson et al., 1978). An aliquot of 0.1 ml of a 1mM solution of SPDP in phosphate buffer (0.1M, pH 7.5) containing NaCl (0.1M) was added to each well of a polyaminostyrene plate and left overnight at room temperature. The wells were washed once with tap water, treated for 5 min with 0.1 ml of 25mM dithiotreitol in distilled water and immediately used.

Isotope labeling of antigens

Salmonella milwaukee was used for preparation of radiolabeled antigens. The organism was grown overnight in M9 broth (Maniatis et al., 1982) at 37°C with shaking; the cells were harvested by centrifugation at 1000 g for 15 min, washed once with phosphate buffered saline (PBS, 0.02 M, pH 7.4), resuspended in PBS and starved for 1.5h at room temperature. The cell suspension was then centrifuged, resuspended in M9 broth without NH_4Cl but containing 6 μCi/ml of a ^3H-aminoacid mixture (Amersham, Arlington Heights, IL), and incubated at 37°C for 3h with gentle shaking. The labeled cell suspension was then split in two portions. In order to remove unincorporated ^3H-aminoacids, one portion was washed three times with PBS and the other three times with MES buffer (0.25M, pH 6.0) containing 0.1M (w/v) EDC. Both cell suspensions were steamed for 1h in the appropriate buffer, cooled and stored at 4°C for up to 7 days. These antigens preparations were used in the coupling studies.

Coupling antigens to microtiter plates

To study the efficiency of untreated and activated microtiter plates in binding Salmonella antigens, 0.1 ml of the radioactive antigen preparation (approxymately 3000 cpm) was added to each well and incubated at 37°C for 3h with gentle agitation. The antigen preparation in PBS was used to sensitize untreated plates by passive adsorption, while the preparation in MES buffer was used to sensitize plates containing free amino goups or suberic acid molecules. For plates containing thiol groups, 1 ml of antigen preparation in PBS was treated for 30 min with 10 l of a 20 mM SPDP solution in ethanol before adding to the wells. A schematic representation of the reactions involved in the chemical activation of polystyrene plates and binding of antigens to the activated plates is shown in Figure 1. Fixation of antigens to untreated plates with ETCH was as described by Place and Schroeder (1982). Wells to which antigens had been passively adsorbed for 3h at 37°C were treated with 0.1 ml of a 0.007% solution of ETCH in PBS for 2h at room temperature.

At the end of the reaction time with the antigen preparation, the contents of each well were aspirated and the wells were manually washed 5 times with 0.3 ml aliquots of PBS containing 0.05% Tween 20 (PBST). The wells were manually cut from the plate (polystyrene plates were cut with hot wire and PVC plates were cut with a pair of scissors) and the level of radioactivity retained was measured in a Beckman LS 7000 liquid scintillation counter. The percent radioactivity retained was determined as follows: % radioactivity retained=[mean cpm after washings/mean cpm added x 100]. Mean values were computed from 6 replicates.

Binding treatments and EIA sensitivity

An enzyme immunassay was performed to verify whether the treatments used to improve binding of Salmonella antigens to the surface of microtiter plates affected the sensitivity of an assay using MOPC 467, a monoclonal antibody

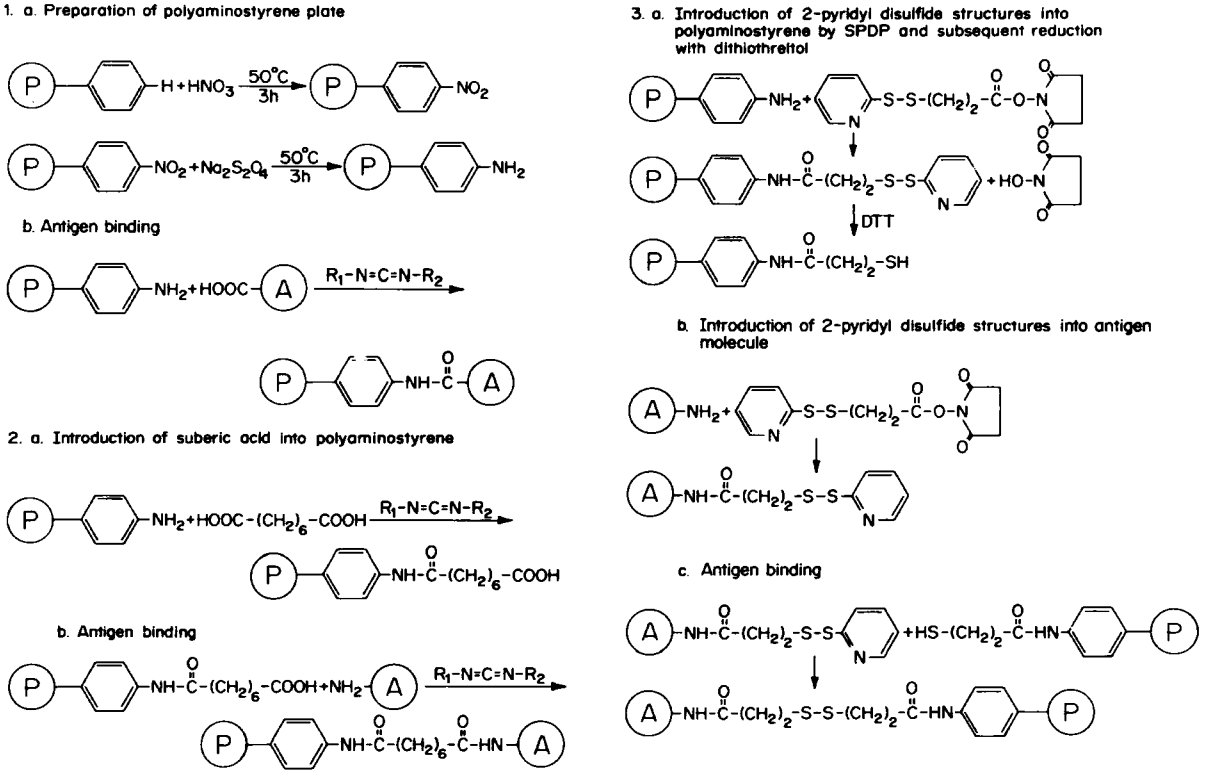

Figure 1. Various chemical reactions for activating polystyrene and for binding antigens to the activated matrix.

A = Antigen P = Polystyrene

specific for flagellar antigenic determinants of many Salmonella serotypes, including S. milwaukee (Smith et al., 1979).

An overnight culture of S. milwaukee in brain heart infusion broth was divided into two portions and the cells were pelleted at 1000 g for 15 min. One portion was washed once with PBS and resuspended in PBS; the other was washed once with MES buffer containing EDC and resuspended in the same buffer. After removing an aliquot for plate counts, the cell suspension was steamed for 1h. Decimal dilutions of the steamed antigen preparation were made in the appropriate buffer and added in triplicate to the wells of untreated and activated plates as described in the previous section.

Unreacted material was removed by washing once with PBST; 0.1 ml of MOPC 467-alkaline phosphatase conjugate (2.5 ug antibody protein/ml; prepared by the one step glutaraldehyde procedure according to Voller et al., 1969) in PBS containing 0.5% Tween 20 was added to each well and the plate was incubated for 1h at 37°C with gentle agitation. After washing the wells 3 times with PBST(using mechanical washer/aspirator from Dynatech Laborartories), 0.2 ml of substrate solution (Sigma 104) in diethanolamine buffer(Voller et al., 1976) was added. The reaction was allowed to proceed for 30 min at 37°C and stopped by adding 0.05 ml of 5 M NaOH solution. The reaction product was quantified at 410 nm in a MicroELISA Mini Reader (Dynatech Laboratories).

RESULTS

Table 1 shows the retention of Salmonella antigens by activated and untreated microtiter plates. Chemical modifications of polystyrene plates significantly ($P<0.05$) increased antigen retention by the solid matrix. Both types of polystyrene plates showed improved antigen retention when their surfaces were modified to contain free amino goups or suberic acid as a spacer molecule. Establishing covalent binding through SPDP resulted in better antigen retention by Immulon I plates. Treatment of antigens adsorbed passively to PVC

Table 1. Percent of radioactivity retained by activated and standard plastic surfaces[1].

ACTIVATION	Plastic surface			
	PVC[2]		Polystyrene	
	A	B	Immulon I	Immulon II
NONE	2.2±0.2[a]	2.0±0.2[a]	1.6±0.2[a]	3.6±0.4[a]
AMINO GROUPS	-	-	2.3±0.2[c]	4.3±0.6[b]
SUBERIC ACID	-	-	2.2±0.1[bc]	4.2±0.6[b]
THIOL GROUPS	-	-	2.8±0.3[d]	3.3±0.2[a]
ETHYLCHLOROFORMATE	2.4±0.3[a]	2.0±0.1[a]	1.9±0.2[b]	3.5±0.3[a]

1. Mean of 6 values(from 6 wells of microtitration plate) ± std dev. Numbers in a column not having common letters are significantly different from each other by the Newman-Keuls procedure ($P<0.05$).

2. Polyvinylchloride

3. PVC A was from Dynatech and PVC B was from Costar.

or polystyrene plates with ethylchloroformate did not significantly improve antigen retention, except with Immulon I plates. Untreated Immulon II plates retained more radioactivity than the other plates used in the study, regardless of whether they were treated or not.

An EIA was performed to determine the effect of the activation treatments on the sensitivity of the assay. A typical example of the results obtained with Immulon II plates is shown in Figure 2. The sensitivity of the assay using untreated plates and passive adsorption of antigen was approximately 10^6 cells/ml. Activation treatments which required carbodiimide for covalent binding of antigen resulted in a 10 fold decrease in the assay sensitivity. Passive adsorption of antigen cross-linked with carbodiimide to untreated plates

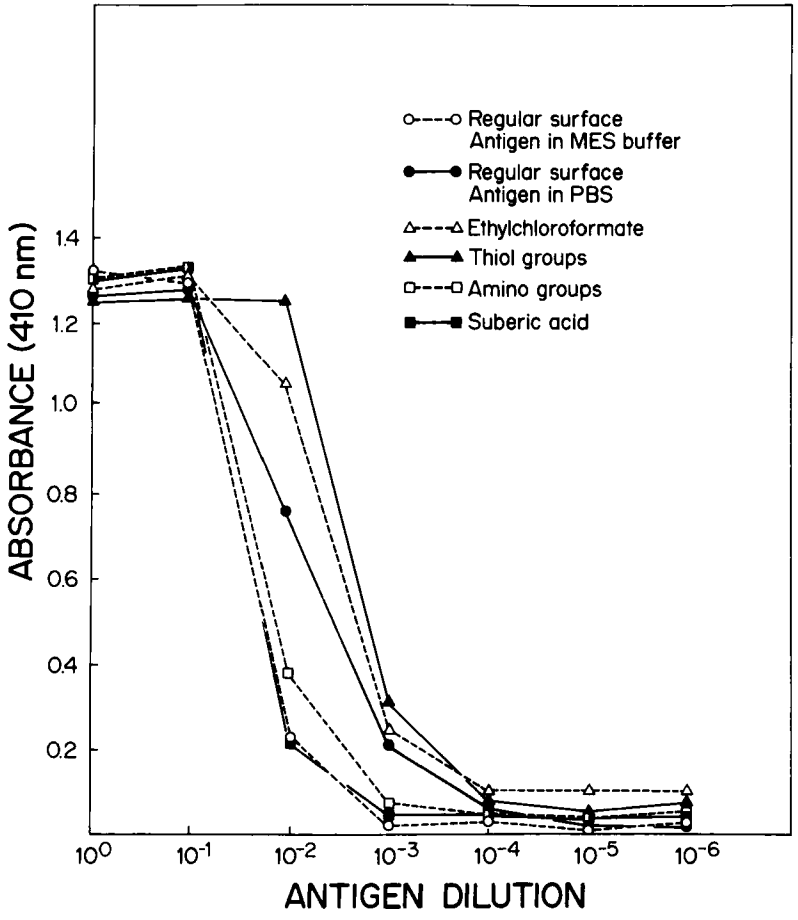

Figure 2. Sensitivity of the enzyme immunoassay (EIA) using activated and regular Immulon II plates. Salmonella milwaukee antigen preparation (1.2 X 10^9 cells/ml) was used at dilutions indicated. For more information on EIA and activation procedures see Material and Methods. "Regular surface" denotes untreated plasic microtitration plate.

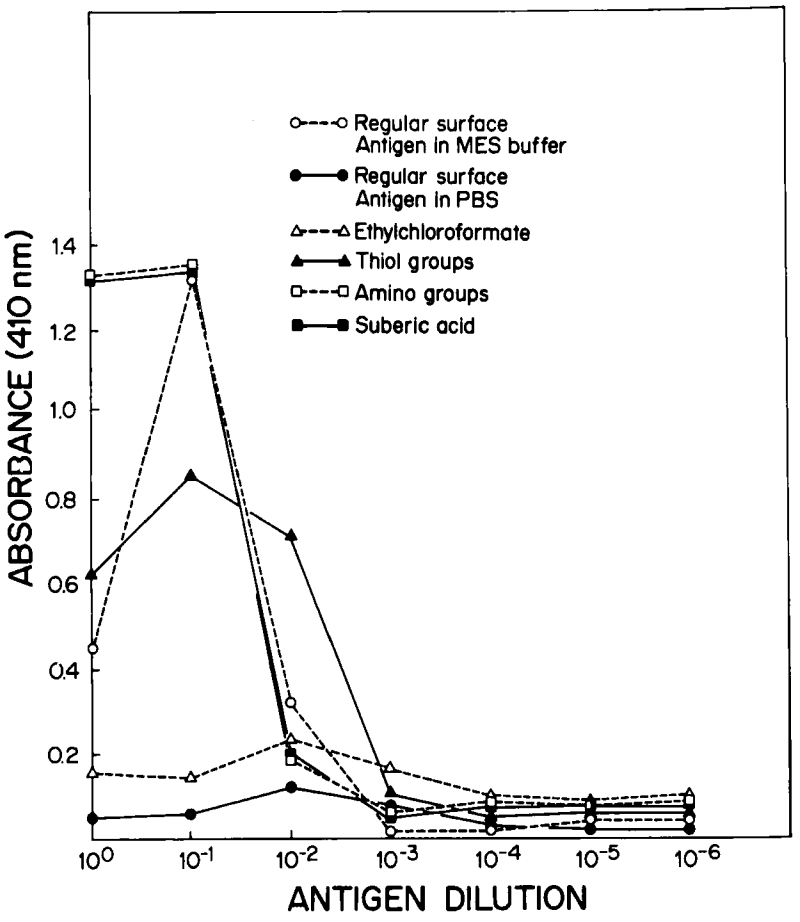

Figure 3. Sensitivity of the enzyme immunoassay (EIA) using activated and regular Immulon I plates. Salmonella milwaukee antigen preparation (1.2×10^9 cells/ml) was used at dilutions indicated. For more information on EIA and activation procedures see Material and Methods.

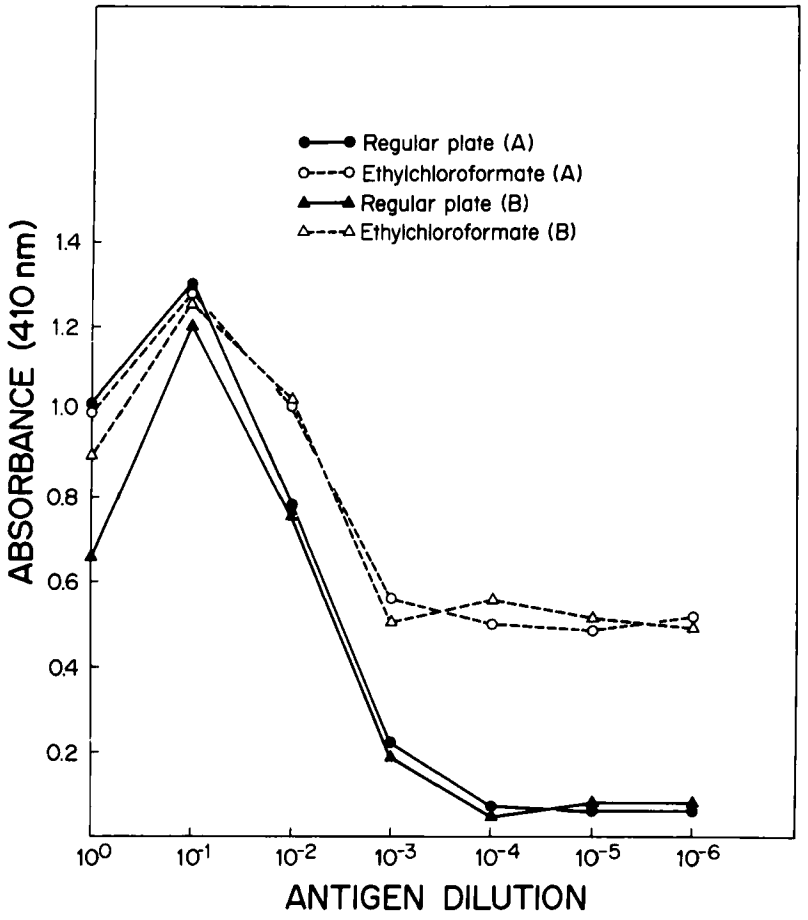

Figure 4. Sensitivity of the enzyme immunoassay (EIA) using two brands (A and B) of polyvinylchloride plates. Salmonella milwaukee antigen preparation (1.2×10^9 cells/ml) was used at dilutions indicated. For more information on EIA and activation procedures see Material and Methods.

also resulted in decreased sensitivity. The use of SPDP or ethylchloroformate did not significantly affect antigen retention or assay sensitivity, though at increasing antigen contrations EIA readings tended to be higher in the treated than in the untreated plates.

In the case of Immulon I plates all activation procedures improved assay sensitivity (Figure 3). Untreated Immulon I plates to which antigens diluted in PBS were passively adsorbed yielded very low EIA readings indicating that a small amount of the enzyme labeled antibody reacted with the immobilized antigen. However, the use of activated plates or untreated plates sensitized with antigen diluted in MES buffer containing carbodiimide led to a dramatic increase in the EIA readings. Such an increase was not observed after ethylchloroformate treatment. Identical results were obtained using two different lots of Immulon I plates. We tested the two lots of plates in a sandwich type of assay, using MOPC 467 as the capture antibody, and in an indirect assay, using a polyclonal Salmonella flagellar antiserum and a protein A-alkaline phosphatase conjugate (Aleixo et al., 1984). Even though a slight improvement in the EIA readings could be observed in both assays, the results were still unsatisfactory (data not shown). It may be argued that monoclonal antibodies, in general, do not function well as capture reagents; however, MOPC 467 antibody has been demonstrated to be an efficient capture antibody in our laboratory.

Use of ethylchloroformate as a fixative in PVC plates caused a loss in assay sensitivity due to an increase in background absorption (Figure 4).

DISCUSSION

An increase in antigen or antibody retained by the solid surface is expected to result in a corresponding increase in the sensitivity of a solid-phase enzyme immunoassay. One approach to achieve the binding of more

ligand to a plastic carrier has been to increase the antigen or antibody concentration in the coating solution. However, it has been observed that this practice can lead to a decrease in EIA readings (Engvall and Perlmann, 1972; Cantarero et al., 1980; Rotmans and Scheven, 1984). This phenomenon is probably due to a greater detachment of ligand from the plastic surface (Engvall et al., 1971; Herman et al., 1979), or due to steric hindrance caused by close or multilayer packing of ligand molecules (Cantarero et al., 1980). Covalent binding of antigen to polystyrene is another approach used to increase the amount of surface ligand available in the assay. Rotmans and Delwel (1983) used a water soluble carbodiimide for coupling schistosomal antigens covalently to polyaminostyrene plates. We used the same procedure to investigate whether covalent binding of Salmonella antigens would improve antigen retention and sensitivity in our assay system. In addition, we investigated the usefulness of SPDP in promoting covalent binding of Salmonella antigens to polyaminostyrene plates. Ethylchloroformate, which was reported by Place and Schroeder (1980) to be efficient in preventing desorption of antibodies physically absorbed to plastic surfaces was also tested.

Experiments with radiolabeled cellular antigens showed that covalent binding generally improved antigen retention by activated polystyrene plates. In the case of activated Immulon I plates, increased antigen retention led to a corresponding increase in assay sensitivity. However, such a phenomenon was not observed for Immulon II plates. For example, a decrease of assay sensitivity was observed for activated Immulon II plates in which carbodiimide was used to establish covalent binding. Two possible explanations for this anomalous behavior are: (i) direct modification of antigenic determinants by carbodiimide or (ii) shielding of antigenic determinants due to inter-or intramolecular cross-linkings among antigen molecules. It is known that carbodiimide can promote extensive cross-linking of protein molecules under mildly acidic conditions (Timkovich, 1977). In contrast to our observation with Salmonella antigen, Rotmans and Scheven (1984) demonstrated that inter-molecular

cross-linking of antigen with carbodiimide led to an increase in immunoassay sensitivity. They attributed this improvement in sensitivity to the increased affinity of polymerized molecules for the plastic carrier. Probably, for the antigens used in the above mentioned study, the negative effects of modification or shielding of antigenic determinants were overcome by the polyclonal nature of the antisera tested. A polyclonal antiserum contains antibodies which react with a variety of antigenic sites on the antigen molecule. Consequently, the fact that some of the antigenic sites were unavailable for antibody binding after carbodiimide treatment was not critical for the performance of the assay. Therefore increased antigen retention resulted in increased sensitivity in their assay system.

Passive adsorption of Salmonella antigen (diluted in PBS) to untreated Immulon I plates was so ineffective that it could not be detected by the direct enzyme immunoassay. An indirect or a sandwich type of assay improved antigen retention only slightly. Identical results were obtained with two lots of Immulon I plates. Low affinity of antigens for certain types of plates has been previously reported (Kenney and Dunsmoor, 1983). However, low surface density of antigen alone does not explain the results obtained in the direct assay since some antigen remains adsorbed to the plate even after several washings (Table 1). It is probable that in addition to incomplete adsorption, the immunoreactivity of the antigen was affected upon binding to the plastic surface. Conformational changes affecting antigen reactivity can occur upon its attachment to plastic surface (Morrisey, 1977; Pesce et al., 1978). Covalent binding of antigens to activated Immulon I plates through carbodiimide or SPDP improved antigen retention as well as assay sensitivity. Cross-linking of antigen (MES buffer containing EDC) before passive adsorption to untreated Immulon I plates also improved antigen retention and assay sensitivity.

SPDP has been used to prepare immunoadsorbents for affinity chromatography (Habeeb, 1981). We generated a solid-phase reagent for covalent binding of antigens by using SPDP to substitute the amino groups of polyaminostyrene plates

with 2-pyridyl disulfide structures. Just before use, free thiol groups were generated in the plates by reducing the disulfide bond with dithiotreitol (Figure 1). Amino groups in the antigen molecule were substituted with 2-pyridyl disulfide groups to establish covalent coupling to the plate through a thiol-disulfide exchange reaction. Our data indicate that this approach can be used to promote covalent binding of antigens to activated surfaces. SPDP mediated binding of antigen to Immulon I plates improved antigen retention and assay sensitivity. For activated Immulon II plates SPDP treatment did not improve antigen retention or assay sensitivity, even though it gave higher EIA readings. In this study, however, we did not attempt to vary the degree of substitution of amino groups in the activated plates or in the Salmonella antigen preparation with 2-pyridyl disulfide groups. Optimizing the degree of substitution with 2-pyridyl disulfide may yield improved EIA sensitivity with Immulon II plates. Advantages of SPDP over other reagents used for covalent binding of antigen to solid matrices are: minimum distortion of the antigen molecule, elimination of intramolecular cross-linkings, and elimination of homopolymers formation (Carlson et al., 1978). Further studies on the application of SPDP for covalent binding of antigens in solid phase EIA are needed.

Fixation of passively adsorbed antigens with ETCH did not improve antigen retention in our assay system. This observation was at variance with the work of Place and Schroeder (1982) who found ETCH treatment effective in improving protein (antibodies) retention by both PVC and polystyrene plates. the action of ethychloroformate is not well understood. It has been suggested that it would prevent desorption by increasing hydrophobicity of the adsorbed protein (Place and Schroeder, 1982).

Untreated Immulon II plates bound more antigen than did PVC plates; however, EIA sensitivity was the same for both types of plates. From the results obtained in this study, it is evident that commercially available Immulon II or PVC plates can be used in our enzyme immunoassay system for

Salmonella antigen without chemical modifications. Since the binding capacity of microtiter plates has been reported to vary depending on the type of antigen used in the assay (Kricka et al., 1980; Kenny and Dunsmoor, 1983; Shekarchi et al, 1984), it has been recommended (Shekarchi et al, 1984) that one should test several types of microtiter plates to find the type which yields optimal results with a particular antigen. Our observations with the monoclomal antibody MOPC 467 highlights the importance of this recommendation. The immunoreactivity of an antigen and antigen-matrix interactions would need to be considered before substituting a monoclonal antibody for a polyclonal antiserum in an existing enzyme immunoassay system.

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