JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1985, p. 361–365
0095-1137/85/090361-05$02.00/0
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Vol. 22, No. 3

Immobilization of Microorganisms for Detection by Solid-Phase Immunoassays

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Received 28 February 1985/Accepted 30 May 1985

Several cultures of gram-negative and gram-positive bacteria were successfully immobilized with titanous hydroxide. The immobilization efficiency for the microorganisms investigated in saline and broth media ranged from 80.2 to 99.9%. The immobilization of salmonellae was effective over a wide pH range. The presence of buffers, particularly phosphate buffer, drastically reduced the immobilization rate. However, buffers may be added to immunoassay systems after immobilization of microorganisms. The immobilization process involved only one step, i.e., shaking 100 μl of culture with 50 μl of titanous hydroxide suspension in polystyrene tubes for only 10 min. The immobilized cells were so tenaciously bound that vigorous agitation for 24 h did not result in cell dissociation. The nonspecific binding of ^{125}I-labeled antibody from rabbits and ^{125}I-labeled protein A by titanous hydroxide was inhibited in the presence of 2% gelatin and amounted to only 5.6 and 3.9%, respectively. We conclude that this immobilization procedure is a potentially powerful tool which could be utilized in solid-phase immunoassays concerned with the diagnosis of microorganisms.

Immunoassay methods with radiolabeled or enzyme-labeled reagents have gained extraordinary importance in many fields of research and in routine diagnosis. However, the immobilization of whole bacterial cells for diagnostic purposes by solid-phase immunoassays has received insufficient attention in the literature.

Immobilization of bacterial cells has been achieved by coating microtiter plates (1) or polystyrene balls (6). The disadvantages of this approach are that: (i) it is based upon nonspecific adsorption of antigens, which may be influenced by several factors (3), (ii) after the completion of adsorption, desorption can take place, and (iii) only a small proportion of cells may be adsorbed by this procedure.

Immobilization was also effected indirectly by another concept applied in sandwich-type solid-phase immunoassays by precoating the solid phase with antibody to the cells to be immobilized and then adding the cells (13). This alternative, however, has the same disadvantages as direct immobilization, as both procedures are based upon the nonspecific adsorption of antigen or antibody-antigen to a support. The adsorptive characteristics of several proteins of different molecular weights and ionic charges to polystyrene and their significance in solid-phase immunoassays have been investigated (2). The results (2) showed that each protein had a characteristic adsorptive behavior not explained by simple charge differences, that the proportion of bound protein was independent of the amount added, that the binding increased proportionally with temperature and incubation time, and that above a certain concentration, protein-to-protein rather than protein-to-polystyrene adsorption occurred.

A number of other methods for the immobilization of microbial and animal cells has been reported. Poly-L-lysine precoating of supports has been used for the immobilization of bacterial capsular polysaccharides and diverse cell types (10, 15). The property of glutaraldehyde as a protein cross-linker (7, 16) and its use as a bacterial cell wall cross-linking reagent have been described (8). The immobilization of microorganisms and proteins has been reported with charcoal and insoluble calcium salts (3), plant lectins (17), and activated Sepharose gels (4). Also, carbodiimide has been used in conjunction with appropriate insoluble support systems for the immobilization of whole cells (9) or biochemicals (11). Anion and cation exchange resins have also been used for adsorbing bacteria and other microorganisms (3).

The objective of this investigation was to develop a method for the immobilization of salmonella cells on a nonsoluble support, thereby facilitating the detection of these bacteria by solid-phase immunoassay systems. Ideally, such a method should be simple, rapid, and efficient and should not bind appreciable concentrations of reagents used in immunoassays such as antibodies or protein A, which has been proposed as a general tracer in immunoassays (5, 14).

While the immobilization of salmonella cells was the major concern of this study, the immobilization of other gram-negative and gram-positive bacteria was also investigated.

MATERIALS AND METHODS

Microorganisms. All salmonella serotypes used were obtained from the Salmonella Reference Laboratory, Adelaide, South Australia, Australia. For the sources of other microoganisms, see Table 4.

Immobilization by metal hydroxides. Approximately 2.0 g each of titanous chloride, titanic chloride, and zirconium chloride (BDH, Poole, England) were weighed separately, and 40 ml of distilled water was added to each reagent. The weighing of titanic chloride and the addition of water to this reagent and zirconium chloride were done carefully under a fume cupboard. Ammonia solution (5 M) was added dropwise with continuous agitation until pH 7.0 ± 0.2 was reached. Each metallic hydroxide suspension was then washed three times with 200 ml of saline solution each time by addition, mixing, and aspiration to remove ammonium ions and then stored in screw cap bottles in the dark until required for immobilization of microorganisms.

The metal hydroxide suspensions were dispensed in polystyrene tubes at the rate of 50 μl per tube. Overnight cultures in brain heart infusion (BHI) broth (BBL Microbiology Systems, Cockeysville, Md.) were added (100 μl), and the

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TABLE 1. % Immobilization of salmonellae by metal hydroxides[a]

Salmonella serotype	Cell population ($\times 10^6$)	% Immobilization by:		
		Titanous hydroxide	Titanic hydroxide	Zirconium hydroxide
S. enteritidis	4.3	96.3	93.5	92.1
	43.0	97.0	91.5	93.0
S. lille	4.0	98.5	92.2	91.5
	40.0	94.4	93.2	92.0
S. tennessee	3.5	91.0	94.6	92.8
	35.0	94.9	94.3	94.0
S. waycross	4.0	97.8	96.6	93.1
	40.0	94.2	90.1	90.0

[a] Percentage of immobilization with titanous hydroxide was significantly higher than that obtained with titanic hydroxide and zirconium hydroxide (P = <0.05 and P <0.01, respectively, by paired t test). No significant difference existed between the immobilization by titanic hydroxide and zirconium hydroxide.

tubes were agitated for 10 min at room temperature in a reciprocal agitator at a speed to maintain the metal hydroxides suspended in the tubes except where otherwise specified. Sterilized saline (0.9 ml) was then added to each tube, and after vortexing, the tubes were centrifuged (1,400 $\times$ *g*) for 5 min at 4°C. Portions (100 μl) of the supernatant in the tubes were serially diluted, and the bacterial populations were determined by the standard plate count method. The standard plate count was also conducted on tubes containing no metal hydroxide suspensions (control) which were treated in the same way as those containing the metal hydroxides. The immobilization efficiency was expressed as: % immobilization = (population in control tube − population in supernatant of tube containing metal hydroxide) $\times$ 100/(population in control tube).

Inhibition of binding of radiolabeled antibody and protein A by metal hydroxides. ^{125}I-labeled immunoglobulin G (IgG) to human thyroglobulin raised in rabbits was obtained from Sorin Biomedica, Vercelli, Italy. ^{125}I-labeled protein A was obtained from Radiochemical Centre, Amersham, England. These reagents were used after dilution to produce approximately 15,000 and 20,000 cpm/100 μl, respectively. To each polystyrene tube, 50 μl of metal hydroxide was added, followed by 100 μl of culture suspension in saline. After shaking for 10 min, 100 μl of 0.05 M phosphate buffer containing the appropriate concentration of a quenching material was added. The objective of this step was to saturate (quench) any group(s) on the immobilization matrix which could bind the radiolabeled antibody or protein A. After incubation for 20 min, 100 μl of either of the radiolabeled reagents was added, the mixture was then shaken for 2 h, and then 1.5 ml of saline was added. The tube contents were centrifuged and aspirated, and the residual radioactivity in the tubes was counted with a Packard Selectronic Autogamma Spectrometer. Control tubes containing no metal hydroxides were treated in the same way to deduct the nonspecific binding of the radiolabeled reagents by the polystyrene tubes. Binding of the radiolabeled reagents by metal hydroxides was expressed as: % binding = (residual counts per minute in tube containing metal hydroxide − residual counts per minute in control tube) $\times$ 100/(total counts per minute added per tube).

Quenching materials examined included: sugars, polysaccharides, yeast and beef extracts, sera from normal animals at concentrations of up to 1:50 (sheep, horse, donkey, fetal calf, pig, and goat), amino acids, and several salts.

RESULTS

Immobilization of salmonellae by metal hydroxides. The immobilization data for four salmonella serotypes, each studied at two population levels, are shown in Table 1. All three metal hydroxides were capable of giving a high degree of immobilization, amounting to ≥90%. However, the immobilization percentages obtained with titanous hydroxide were significantly higher than those obtained with titanic hydroxide and zirconium hydroxide (P = <0.05 and P = <0.01, respectively; by the paired t test). The immobilization data were obtained by comparing viable cell counts before and after immobilization. While small numbers of cells were found in the supernatants of suspensions after immobilization, large numbers of cells were recovered by plating of the metallic suspensions. This indicates that the cells were immobilized rather than inactivated by these hydroxides.

Inhibition of binding of radiolabeled antibody and protein A by metal hydroxides. Several materials were examined as possible quenchers to reduce the nonspecific binding of radiolabeled IgG and protein A by the metal hydroxides. However, the results (not shown) were not encouraging, as the binding of ^{125}I-labeled protein A by titanous hydroxide in the presence of these materials ranged from 36.2 to 69.5%. However, the use of 2% gelatin (Difco Laboratories, Detroit, Mich.) gave the highest inhibition to the nonspecific binding of radiolabeled IgG and protein A (Table 2). Such binding amounted to only 5.6 and 3.9%, respectively, with titanous hydroxide. It was further found that quenching by the addition of gelatin solution, followed by agitation for 20 min before the addition of radiolabeled materials, could be simplified by diluting the concentrated radioactive materials in gelatin solution. When this was done, virtually the same low values of nonspecific binding of radiolabeled materials were obtained.

Compared with the other metal hydroxides, titanous hydroxide consistently produced lower binding rates of radiolabeled IgG and protein A. Consequently, it was considered the immobilization matrix of choice. Further testing of the immobilization of other salmonella serotypes (*Salmonella abortus-equi*, *S. kentucky*, *S. lille*, *S. oranienburg*, *S. worthington*, *S. 4,12:d:−*, and *S. tennessee*) by titanous hydroxide revealed that more than 94% of the cells of the serotypes tested were immobilized.

Effect of variables on salmonellae immobilization by titanous hydroxide. The variables investigated were volume and age of titanous hydroxide, shaking time, buffers, pH, and presence of selective and nonselective broths. This was done with *S. enteritidis* and *S. lille* at an average population of 6.0 $\times$ 10^6 CFU/100 μl. As could be expected, some of these variables had a profound effect on immobilization efficiency.

When the volume of titanous hydroxide was increased from 10 to 50 μl, the mean immobilization efficiency increased from 66 to 98%. This increase, however, was not proportional to volume increase. Further increases in volume from 50 to 100 μl resulted in little or no increase in the percentage of immobilization of salmonellae.

The age of titanous hydroxide appeared to have little or no effect on the degree of immobilization. The range of the percentage of immobilization of salmonellae with 100-μl quantities of freshly prepared and 1-year-old titanous hydroxide batches was 92.0 to 99.7%.

Vortexing for a few seconds after the addition of salmo-

TABLE 2. % Binding of radiolabeled IgG and protein A by metal hydroxides[a] in the presence of quenching materials

Quenchers[b]	% Binding of ^{125}I-labeled IgG and ^{125}I-labeled protein A by:					
	Titanous hydroxide		Titanic hydroxide		Zirconium hydroxide	
	IgG	Protein A	IgG	Protein A	IgG	Protein A
Nil (control)	NT[c]	74.8	NT	69.7	NT	71.6
Bovine serum albumin (Calbiochem)	17.5	8.6	24.3	28.4	14.4	15.9
Brain heart infusion (BBL)	21.7	6.0	22.3	17.6	13.3	9.7
Peptone (Oxoid)	25.4	5.6	23.0	11.4	13.5	8.7
Gelatin (Difco)	5.6	3.9	11.9	8.7	9.4	5.8
Gelatin (Sigma)	5.7	5.3	10.8	17.0	9.2	9.3
Normal rabbit serum	20.2	8.2	24.7	43.7	13.4	14.8

[a] Volumes of metal hydroxides used were 50 μl. When 100-μl quantities were used, the binding rates of radiolabeled reagents increased significantly ($P < 0.01$).
[b] Final concentration of quenchers was 2% except for normal rabbit serum (1:50). Lower final concentrations of quenchers resulted in significantly higher percentage of binding of the radiolabeled reagents ($P < 0.01$). Other materials were also used but were less effective as quenchers; see the text. Suppliers: Calbiochem, Calbiochem-Behring, La Jolla, Calif.; Oxoid, Oxoid Ltd., London, England; Sigma, Sigma Chemical Co., St. Louis, Mo.
[c] NT, Not tested.

nella suspensions to titanous hydroxide resulted in only 42.9% immobilization. However, when shaking was done for 5 min, substantially increased immobilization occurred. These increases peaked after a total of 10 min of shaking (97.9%). Further increments of 10-min shaking up to 1 h did not alter the immobilization percentage. Also, prolonged vigorous shaking (200 rpm) over periods of 2, 4, and 24 h had virtually no effect on the immobilization of the salmonella serotypes investigated, indicating a high degree of immobilization stability.

The dilution of salmonella cultures grown in BHI with certain buffers (0.05 M) at pH 7.2 before immobilization had an adverse effect on the immobilization efficiency. The buffers investigated were arranged in order of their increasingly adverse effect on immobilization efficiency: imidazole (81.0%), NaOH-citric acid (71.9%), Tris (71.0%), and phosphate buffer (35.8%).

The effect of pH variation on immobilization efficiency was studied in saline at different pH values. In these experiments, the titanous hydroxide suspensions in tubes were flooded with saline at the desired pH values, left overnight, and then aspirated. Salmonella cultures were then diluted in saline solutions at the desired pH values, and after shaking for 10 min, the final saline volume in each tube was brought up to 1.0 ml with saline solutions at the appropriate pH. The results showed that the percentage of immobilization exceeded 82% over the pH range 3 to 8. However, the highest immobilization rates (>91%) were at pH values in the range 5.00 to 7.25.

Immunoassays for microorganisms are usually carried out on cultures grown in complex medium. The immobilization by titanous hydroxide of three salmonella serotypes in the presence of each of four media is depicted in Table 3. The presence of complex media resulted in some reduction in the immobilization efficiency. The reduction was not consistently restricted to any particular medium or serotype. Moreover, the percentage of immobilization overall ranged from 80.2 to 99.9%.

Immobilization of microorganisms other than salmonellae by titanous hydroxide. Immobilization of other microorganisms was carried out with cultures grown in BHI broth which were diluted with saline. The immobilization efficiency was determined with 100-μl diluted cultures, 50 μl of titanous hydroxide suspensions, and 10-min shaking. The results (Table 4) showed the ability of titanous hydroxide to effectively immobilize all the gram-negative and gram-positive bacteria investigated. The percentage of immobilization ranged from 89.9 to 99.9% and 91.0 to 99.6%, respectively.

DISCUSSION

The successful immobilization of *Escherichia coli*, *Acetobacter* spp., and *Saccharomyces cervisiae* with titanic hydroxide and zirconium hydroxide for the construction of microorganism reactors for commercial purposes has been reported (12). These findings were extended in the current investigation with the same hydroxides and titanous hydroxide for the immobilization of microorganisms to produce a solid phase which could be used for the detection of microorganisms by immunoassays. The percentage of immobilization of salmonellae by titanous hydroxide was significantly higher than those obtained with titanic hydroxide and zirconium hydroxide. Moreover, the nonspecific binding of radio-

TABLE 3. Immobilization of salmonellae present in saline and several broth media by titanous hydroxide

Cell medium[a]	*S. lille*		*S. enteritidis*		*S. waycross*	
	Population ($\times 10^6$)	Immobilization (%)	Population ($\times 10^6$)	Immobilization (%)	Population ($\times 10^6$)	Immobilization (%)
Saline	8.3	99.9	3.1	93.3	3.3	98.2
BHI broth	340.0	99.9	4.0	96.3	3.7	82.0
Nutrient broth	3.4	94.1	3.2	83.1	4.3	93.0
Tetrathionate broth	1.5	80.2	5.3	91.5	5.3	88.7
Mannitol selenite broth	5.0	98.6	2.1	96.4	3.3	80.1

[a] For saline, salmonella cells were first grown in BHI broth and then diluted in saline. For the broths, salmonella cells were first grown in each broth and then diluted in fresh broth.

TABLE 4. Immobilization of some gram-negative and gram-positive bacteria by titanous hydroxide

Microorganism	Source and strain[a]	Cell population ($\times 10^6$)	Immobilization (%)
Citrobacter freundii	UQ 7458	1.21	89.9
Edwardsiella tarda	UQ 1136	0.56	91.6
Enterobacter aerogenes	UQ 1976	4.30	99.3
Erwinia herbicola	UQ 854	4.50	96.3
Escherichia coli	NCTC 8196	4.90	95.6
Hafnia alvei	UQ 1137	23.00	99.3
Klebsiella peumoniae	CIH 248	0.90	98.4
Proteus mirabilis	HC 12201	23.20	99.9
Serratia marcescens	UQ 169	5.90	96.4
Shigella dysenteriae	UQ 321	4.80	98.4
Yersinia pseudotuberculosis	CIH 14	1.78	91.7
Bacillus cereus	ATCC 11778	0.60	97.0
Micrococcus luteus	HAC 85	0.77	98.4
Staphylococcus aureus	ATCC 27664	0.88	91.0
Staphylococcus epidermidis	HAC 82	0.50	93.4
Streptococcus faecalis	HAC 38	17.10	99.6

[a] Abbreviations: UQ, Department of Microbiology, University of Queensland, Queensland, Australia; NCTC, National Collection of Type Culture, London, England; CIH, Commonwealth Institute of Health, University of Sydney, New South Wales, Australia; HC, Health Commission of New South Wales, Lidcombe, New South Wales, Australia; ATCC, American Type Culture Collection, Rockville, Md.; HAC, Hawkesbury Agricultural College, Richmond, New South Wales, Australia.

labeled IgG and protein A was much lower with titanous hydroxide than with the other hydroxides in the presence of gelatin (Difco) as a quencher. A low level of nonspecific binding of these reagents is a prerequisite for the success and sensitivity of immunoassays.

The immobilization of microorganisms by titanous hydroxide is a simple, one-step method involving shaking for only 10 min. The degree of immobilization of microorganisms is usually improved by increasing the duration or the intensity of agitation or both, because of increasing the probability of contact between the cells and the immobilization particles. Nevertheless, a duration of only 10 min was quite sufficient for almost complete immobilization by titanous hydroxide. It is also possible that agitating too violently or for too long a time can cause dissociation of immobilized microorganisms (3). This, however, did not occur with titanous hydroxide in this investigation, indicating a high degree of stability.

Further advantages of using titanous hydroxide as a solid phase in immunoassays are that its particles are insoluble and capable of immobilization of microorganisms over a wide pH range. Large batches may be prepared and stored before immobilization, as the age of the preparation, up to 1 year, had no influence on the immobilization rate. A high degree of salmonella immobilization occurred in the presence of broth media with only 50 μl of titanous hydroxide suspension. The 50-μl volume had an outstanding capacity for the immobilization of large populations of salmonella. An overnight culture of *S. enteritidis* in BHI broth was directly immobilized with 50 μl of titanous hydroxide. While the population was 940 × 10^6/100 μl, the immobilization amounted to 91.9%. Consequently, there is no need to increase the immobilization volume of titanous hydroxide in the immunoassays; otherwise, the nonspecific binding of the reagents used in immunoassays would increase. Titanous hydroxide was also effective in immobilizing gram-negative bacteria other than *Salmonella* spp. and several gram-positive bacteria. Therefore, it may have application in the development of immunoassays for a wide range of bacterial species of pathological significance.

Suspensions of bacteria in buffers (particularly phosphate buffer) should not be used for immobilization, as the presence of buffers could considerably reduce the rate of immobilization. The mechanism of cell immobilization by metal hydroxides is thought to be a result of the replacement of hydroxyl groups on the surface of the metal hydroxide with suitable ligands from cells of microorganisms (12). It appears that this replacement can be competitively inhibited by certain buffer components. However, microorganisms in broth media or saline may be immobilized and buffers may be added subsequently to the system.

Immobilization with metal hydroxides and other immobilization methods were also investigated in the current study with ^{14}C- and ^{125}I-labeled cells of *S. enteritidis*. The results, which are not presented because of space consideration, showed that titanous hydroxide immobilization was superior in terms of efficiency of immobilization and levels of nonspecific binding. Other immobilization methods investigated were microtiter plates in conjunction with either poly-L-lysine or glutaraldehyde, polyacrylamide beads coated with polyethyleneimine, activated charcoal, hydroxylapatite, plant lectins covalently linked to Sepharose, cyanogen bromide-activated Sepharose, ion-exchange gels, and covalent linking with carbodiimide on carboxymethyl cellulose and carboxyl terminal agarose beads.

We conclude that titanous hydroxide has potential as an immobilization matrix for solid-phase immunoassays for microorganisms of pathological significance. This potential derives from its simplicity and rapidity of use, its high efficiency of immobilizing cells, and its low levels of nonspecific binding of rabbit antibody and the general tracer protein A which are frequently used in immunoassays.

ACKNOWLEDGMENT

This research was supported by a grant from the Australian Dairy Research Committee.

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