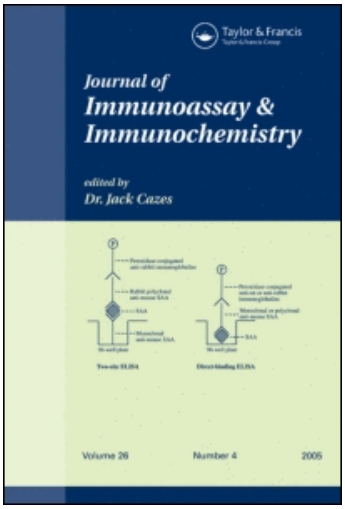

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POLYSTYRENE, POLY-L-LYSINE AND NYLON AS ADSORPTIVE SURFACES FOR THE BINDING OF WHOLE CELLS OF MYCOBACTERIUM TUBERCULOSIS H37 RV TO ELISA PLATES

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ABSTRACT

Several methods of coating whole cells of Mycobacterium tuberculosis H37 RV to ELISA microtitre plates were compared with the aim of developing an ELISA screening assay for murine monoclonal antibodies in culture supernatants and human antibodies in patient sera. Undercoats of nylon or poly-L-lysine were compared to polystyrene as adsorptive surfaces for the bacteria, the effect of increased ionic strength and inclusion of SDS in the coating buffer measured, and methanol (70%) and glutaraldehyde (5%) investigated for their efficiency as fixatives of the bacterial monolayers. The results suggest PBS as a satisfactory coating buffer for the bacterial cells on polystyrene, and 70% methanol the preferred fixative for the dried antigen-coated plates.

(KEY WORDS: Mycobacterium tuberculosis, ELISA, serodiagnosis, nylon, poly-L-lysine)

INTRODUCTION

The evidence that M. tuberculosis infection results in a humoral response which can be measured against mycobacterial antigens is now generally accepted (1). The exact role of the

humoral response in tuberculosis (TB) is still disputed, but it has been shown that, of the total antibody increase, it is the IgG class which is the most specific and discriminatory for TB (2,3,4).

Antibodies to mycobacterial antigens have been identified using a variety of techniques. The controversy surrounding the status of serological reactions in the diagnosis of TB can be attritubed to the fact that few of the antigenic constituents of tubercle bacilli have been successfully applied in serodiagnosis. Moreover, specific antibodies at levels of diagnostic importance are not detected in a large proportion of patients with TB.

ELISA techniques developed for TB almost exclusively measure antibody titers and a few investigators have used highly purified mycobacterial antigens in serological studies (5,6). Both the sensitivity and the specificity of solid phase serodiagnostic techniques to detect anti-tubercular antibodies depend on the type of antigen and how it is presented. Sub-optimal binding of mycobacterial cells to ELISA microtitre plates can influence the outcome of the test.

Many of the biological properties of mycobacteria are due in whole or in part to their unique cell walls (7). The main constituent of the cell wall is a covalent structure of arabinogalactan-mycolate linked to peptidoglycan. This structure has a uniform composition throughout the genus Mycobacterium, with

mycolic acid comprising 20-40%; D-arabinose and D-galactose (5:2) 30-40% and the constituents of peptidoglycan (D-glucosamine, muramic acid, D-glutonic acid, meso-diaminopimelic acid, L- and D-alanine) 18-25%. Some of the interpeptide linkages exist as meso-A_2pmn-meso-A_2pm interpeptide linkages, found only in mycobacteria. The link between the arabinogalactan and the peptidoglycan is through phosphodiester bridges between the C-6-OH of muramic acid residues and arabinose.

Mycolic acids (α-alkyl-β-hydroxy fatty acids) are one the most distinctive features of mycobacteria. Mycobacteria contain saturated C_8 to C_{32} fatty acids, with the prominent long chain fatty acid in human tubercle strains being C_{26}. The esters of C_{16}, C_{18}, C_{24} and C_{26} fatty acids provide the hydrophobic moieties of phospholipids, glycolipids and lipoproteins. These amphipatic molecules probably determine the adsorptive properties of the mycobacterial cell wall.

Proteins in low concentrations adsorb to polystyrene from an aqueous environment by hydrophobic forces (8). Partial denaturation of the protein antigen before or during incubation on a microtitre plate increases its adsorptivity and is usually effected by modulation of temperature and/or acid or alkaline pH (9). Similar procedures have been applied to whole bacterial antigens to create a solid phase antigen. The complexity of mycobacterial cell surfaces however, warrants investigation into other potential adsorptive forces that may be utilized for

coating ELISA microtitre plates with whole bacterial cells. In this study the forces involved in the adsorption of whole M. tuberculosis cells to a solid phase was investigated by evaluating hydrogen bonding, ionic and hydrophobic surfaces for their adsorptive capacity and by measuring the effect of ionic concentration and detergent included in the coating buffer.

The use of poly-L-lysine (PLL) as adsorptive surface for whole M. tuberculosis cells aimed at the utilization of ionic attractive forces between the anionic bacterial cell wall and the cationic PLL surface.

Polyamide 6 (Nylon 6) is a polymer of ε-aminocaproic acid linked by amide-ester bonds. The abundance of amide-esters allows hydrogen bonding between polyamide-coated surfaces and hydrogen bonding components in the bacterial cell wall. The elongated hydrocarbon structure of polyamide allows its adsorption to polystyrene by hydrophobic forces. The principle for coating polystyrene microtitre plates with soluble nylon has been described previously (10).

In previous studies where crude M. tuberculosis antigen was used for coating ELISA microtitre plates, supernatant fractions from sonicated bacteria were employed and the antigen adsorbed to the polystyrene in sodium carbonate buffer at pH 9,6 (11,12, 13). Alternatively, whole bacterial cells were used for coating microtitre plates by glutaraldehyde fixation (11,14). Whole

bacteria may be preferable as antigens for screening hybridomas derived from mice immunized with whole cells, as the mycobacterial cell wall comprises the major antigens responsible for tuberculosis specific humoral immunity (15). In addition, sonicates of mycobacteria contain antigens, including heat shock proteins, which cross-react extensively with intracellular structures of other organisms (16).

MATERIALS AND METHODS

1. Preparation Of Bacterial Antigen

Antigen was prepared as a whole bacterial suspension (17). Mycobacterium tuberculosis H37 RV was grown on Löwenstein-Jensen medium for three weeks and harvested at the mid-log phase. A homogeneous suspension was prepared in phosphate-buffered saline (PBS), pH 7,4 to a concentration of 10^7 cells/ml, corresponding to a McFarland no 1 standard. The bacterial suspension was autoclaved at 121°C for 15 minutes and preserved with 0,05% Thimerosol (Merck, Johannesburg, South Africa). The antigen suspension was stored at 4°C until needed.

2. Production Of Murine Monoclonal Antibodies

Three months' old, C57Bl/6 mice were obtained from the H A Grové Animal Research Centre, Pretoria, South Africa. Primary and booster immunization were administered intra-peritoneally at a concentration of 0,5 x 10^7 M. tuberculosis cells in 0,5 ml PBS, pH 7, on days 0, 14 and 42, the last being administered

three days before cell fusion was performed. Cell fusion between immune spleen cells and SP2/0 myeloma cells (18) at a ratio of 5:1 was performed according to the method of Galfrè and Milstein (19), using 40% (w/v) polyethylene-glycol as fusion agent. Cultures producing antibodies against M. tuberculosis were identified by ELISA (see below), cloned in soft agar and propagated in C57Bl/6 x Balb/c mice primed with incomplete Freunds's adjuvant (0,5 ml, intra-peritoneally) 2-7 days before injection of 10^5-10^6 hybridoma cells (20). Ascitic fluid was obtained by puncture with a 17G short bevelled needle, centrifuged and the supernatant frozen in aliquots at -30°C. Ascitic fluid from mice injected with SP2/0 non-immunoglobulin producing hybridomas was used as a negative control.

3. Human Patient And Control Sera

Three negative control sera were selected from 100 blood samples obtained from persons in whom tuberculosis had been excluded bacteriologically. Selection was done on the basis of low background signal generated in ELISA using whole M. tuberculosis H37 RV cells as antigen rather than previous histories of BCG vaccination or PPD positivity rate. Positive sera were collected from TB patients in whom the disease was confirmed bacteriologically in sputum samples as well as by radiology. Of these, three were selected exhibiting typically high, intermediate and low ELISA signals.

4. ELISA

Whole autoclaved M. tuberculosis H37 RV cells were used as solid phase antigen in the ELISA according to a method previously described (21). Bacterial suspensions at a concentration of 10^7 cells per ml PBS, pH 7,4 were distributed in 96-well microtitre plates (Nunc Immuno II, Roskilde, Denmark) at 100µl/well. The plates were incubated for 1 hour at room temperature, dried under a heating lamp and fan and the bacteria fixed with 70% methanol at 200µl/well for 20 minutes at room temperature. After flicking out the fixative, the plates were dried under the heating lamp and fan and stored at 4°C until needed. Diluent, blocking and washing buffer consisted of 0,5% casein (Merck, Johannesburg, South Africa) in PBS, pH 7,4. After washing, the unbound sites were blocked with casein buffer at 200µl/well for 1 hour at room temperature. Hybridoma culture supernatant (undiluted), or human patient or control sera (1:500) was added at 50µl/well and incubated at room temperature for 45 minutes. Washing was followed by addition of peroxidase conjugates of either goat-anti-mouse IgG (H + L) or goat-anti-human IgG (H + L) sera (1:000) (Cappel, Malvern, USA). After incubation for 30 minutes at room temperature, the plates were washed and the enzyme reaction monitored with substrate solution consisting of 10 mg o-phenylenediamine (Sigma, St Louis, Missouri) and 6-8 mg urea-hydrogen peroxide (BDH Chemicals, Merck, Johannesburg, South Africa) in 10 ml 0,1 M citrate buffer, pH 4,5 at 50µl/well.

Colour development was measured at 450 nm in a Titertek Multiscan MC colourimeter (Flow Laboratories, Eflab Oy, Helsinki, Finland).

5. Nylon-coating of Microtitre Plates

A solution of 1 mg nylon (Polyamide 6 for column chromatography, Woelm, West Germany) in 1 ml m-cresol was diluted in 6 ml of a 67% aqueous ethanol solution and distributed in the wells of a microtitre plate at 100µl/well. After 1 hour incubation at room temperature unadsorbed solution was flicked out and the plate washed with 500 ml tap water using a specially designed shower (22). Plates coated this way were air dried and stored at room temperature or used immediately in the ELISA as described above.

6. Poly-L-lysine-coating Of Microtitre Plates

Poly-L-lysine hydrochloride (PLL) (MW 15 000 - 30 000, Sigma Chemical Company, St. Louis, Missouri) was used to coat microtitre plates as described by Polin and Harris (14). A solution of 0,001% PLL in PBS, pH 7,2 was distributed in a microtitre plate at 50µl/well. After two hours incubation at room temperature, the unadsorbed solution was flicked out and the plate dried under a heating lamp and fan before being used in the ELISA as described above.

7. Modification Of Coating Procedure

The abovementioned procedure for coating of bacteria to the ELISA plates was modified in two ways: In one instance, PBS was

replaced with double-concentrated saline (0,3 M NaCl) and in another, PBS was supplemented with sodium-dodecylsulphate (SDS) detergent to a final concentration of 1%. In addition, 0,5% glutaraldehyde was compared with 70% methanol as fixative.

Glutaraldehyde fixation of bacterial cells was performed by dispensing 100µl of the bacterial suspension per well and centrifugation of the micro-titre plates at 2 000 rpm for five minutes. A solution of 0,5% glutaraldehyde in cold PBS, pH 7,2 was added at 50µl/well. The plates were incubated for 15 minutes at room temperature, washed twice with PBS and vacuum-dried. A solution of 0,1 M glycine in 1% bovine serum albumin, pH 7,6 was added at 100µl/well, followed by incubation for 30 minutes at room temperature. The plates were washed once with PBS, vacuum-dried and stored at 4°C until needed.

RESULTS AND DISCUSSION

Monoclonal antibodies are the preferred probes for optimization of the ELISA as they are homogenous and void of the complexities of competitive affinities and weak reproducibility inherent to polyclonal patient sera. A murine mouse monoclonal IgM against M. tuberculosis H37 RV was selected for use in the optimization of the polystyrene and PLL-coated ELISA. As it is known that IgM binds nonspecifically to nylon (10), the effectivity of nylon as adsorption undercoat could not be evaluated with the murine monoclonal IgM. Instead, Protein A purified IgG from human patient and control sera was used.

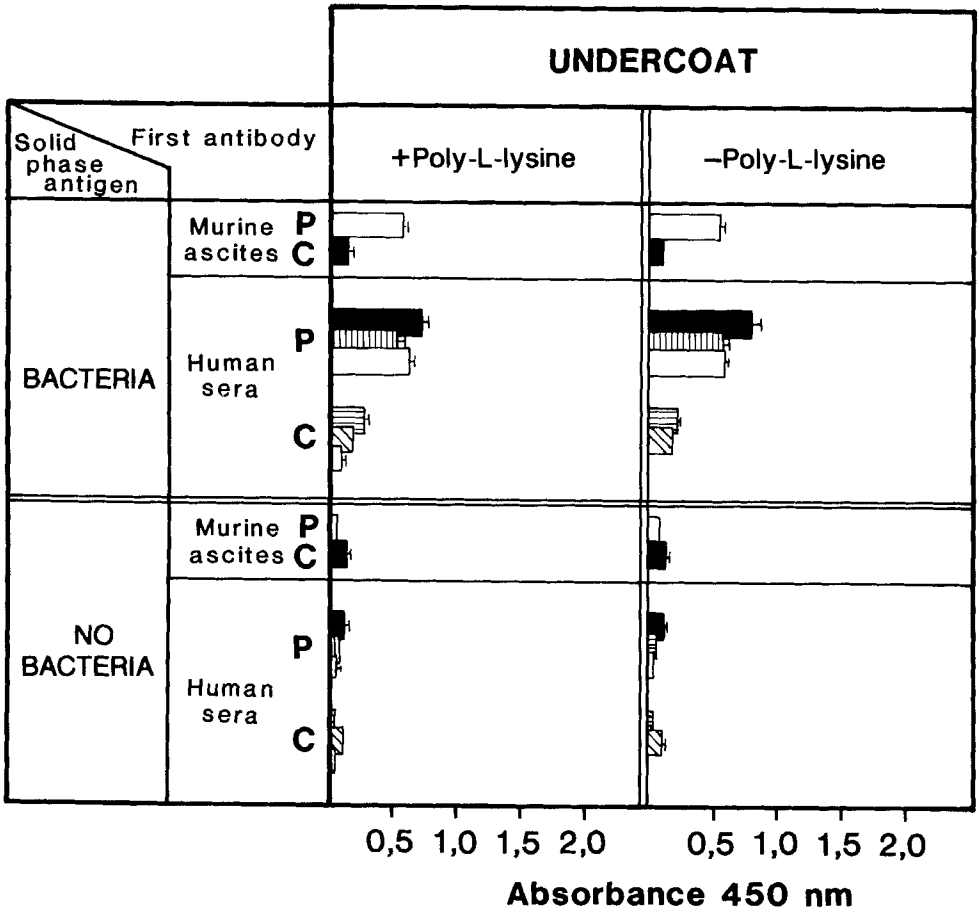

Fig. 1 Effect of poly-L-lysine as undercoat for whole M. tuberculosis H37 RV solid phase antigen in ELISA. "P" and "C" denote positive and control sera/ascites respectively. Three-grouped bars indicate the results of three different sera obtained from TB positive (P) or control (C) individuals. Standard deviations were calculated from quadruplicate assays.

The results obtained when PLL was used as undercoat are illustrated in Fig. 1. Although the PLL undercoat provided a marginally improved signal with the monoclonal IgM, the background signal generated with naive mouse serum was increased by 50% with PLL as compared with the signal produced in wells coated directly with bacteria. This difference in background signal was not as pronounced when human control serum was used as the source of the first antibody. With human patient sera, however, no advantage of PLL as an undercoat for bacterial adsorption could be illustrated. The control experiment, where bacteria were omitted from the ELISA microtitre plate, produced data which proved that PLL by itself did not significantly influence the non-specific adsorption of murine or human immunoglobulins to the solid phase. It is therefore concluded that a polycationic undercoat yields no advantage to direct coating of polystyrene plates with whole cells of M. tuberculosis.

Fig. 2 illustrates the results obtained when bacteria were covalently fixed to a PLL-coated plate with 0,5% glutaraldehyde. A small decrease in ELISA signal was observed with both patient and human control sera, although the background signal was suppressed more effectively in comparison to where bacteria were fixed to the polystyrene directly with 70% methanol. The small improvement brought about by glutaraldehyde fixation does not, however, warrant this more tedious and costly procedure.

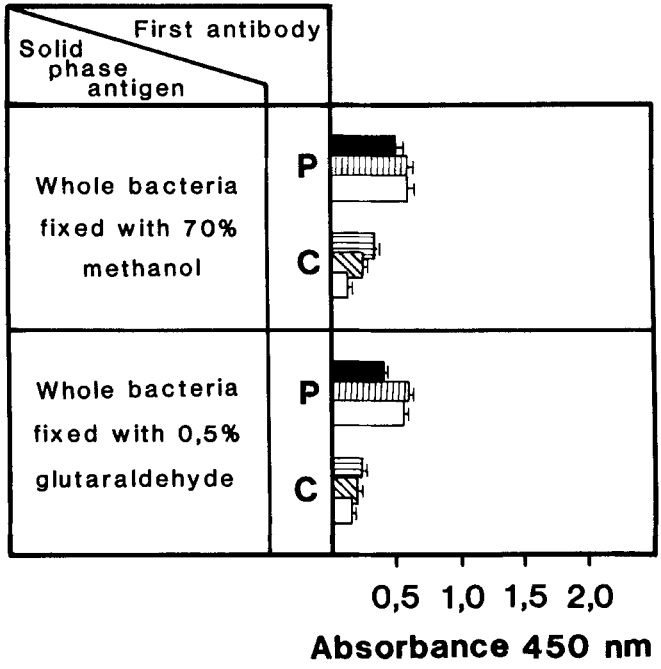

Fig. 2 Comparison of 0,5% glutaraldehyde and 70% methanol fixatives for M. tuberculosis H37 RV on poly-L-lysine coated plates in ELISA. Three-grouped bars indicate the results of three different sera obtained from TB positive (P) or control (C) individuals. Standard deviations were calculated from quadruplicate assays.

The effectivity of nylon as an adsorptive undercoat is shown in Fig. 3 (upperbox). The difference in signal caused by pre-coating with nylon, as shown with human patient serum, was not significant. An increase in the ionic strength of the coating buffer (i.e. 1,8% NaCl) also showed no advantage. However, the inclusion of 1% SDS in the bacterial suspension improved the signal to background ratio on the nylon surface,

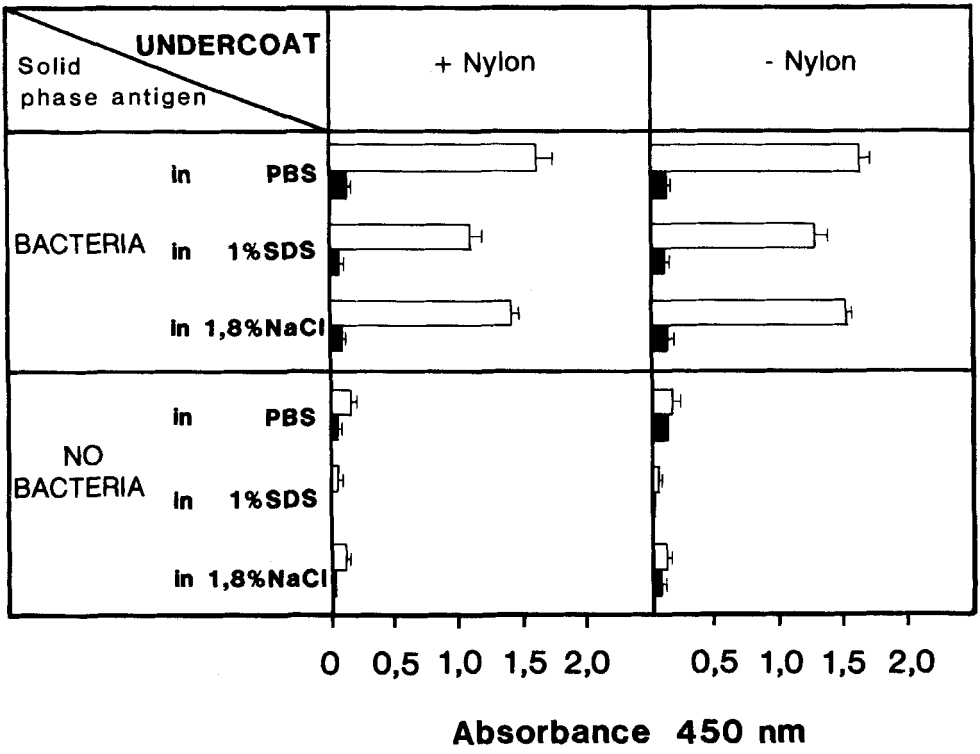

Fig. 3 Effect of nylon as undercoat for M. tuberculosis H37 RV as solid phase antigen in ELISA. Open bars indicate the results for a positive serum; hatched bars for a control serum. Standard deviations were calculated from quadruplicate assays.

even though the signal itself was diminished by about 6% in respect of where nylon pre-treatment was omitted.

When background is defined as the signal generated by a serum on similarly coated wells, but with the bacterial antigen omitted (lower box, Fig. 3), a marked difference was registered with the use of SDS in the coating buffer. Thus, for wells

not pre-coated with nylon, patient serum exhibited a twenty seven-fold signal to background ration on wells coated with bacteria in SDS, compared to a ten-fold signal to background ratio when SDS was omitted, i.e. where bacteria were coated in PBS only. When control serum is used as a background reference, however, this advantage is not manifested. In the clinical situation, where diagnosis depends on ELISA signals of patient sera compared to those produced by control reference sera, the inclusion of SDS during antigen coating is therefore not required.

In conclusion, it can be stated that the utilization of molecular interactions other than hydrophobic forces for the adsorption of whole cells of M. tuberculosis on ELISA microtitre plates does not provide a significantly improved antigen-coated solid phase. The distribution of a bacterial suspension in PBS, pH 7,4 in a microtitre plate, followed simply by drying and fixation with 70% methanol, appears to be the preferred method for coating whole cells to ELISA plates.

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