Discussion

The weakening of the $C1_q$ binding may be explained by the increase in ionic strength (which is known to decrease $C1_q$ binding,[2] caused by the addition of edetic acid and subsequent neutralisation of the buffer. The relatively high concentration of C3 in serum and the cascade nature of the classical complement activation pathway may explain the C3-binding observed in this experiment. In any event, the binding of complement components to aggregated γ-globulin did not seem to be due to non-specific adherance, but rather the biological integrity of the classical complement activation pathway.

The ability of the assay to measure different rates of complement consumption was measured by preincubating the complement source (37°C for 30 minutes) in the presence of aggregated γ-globulin at concentrations of 1, 2·5, and 10 μg/ml. In these experiments a clear dose dependent decrease in complement activity was observed (fig 2). Much higher concentrations of aggregated γ-globulin were needed to cause a decrease in complement binding in the immunofluorescence assay (100–500 μg/ml for $C1_q$ and 100 μg/ml for C4 and C3.[4] The different inhibition profiles may reflect differences in the complement binding structures used in the assays.

The ELISA method described here only responded to functionally intact complement and at much lower serum concentrations than the immunofluorescence assay (fig 1). With minor modifications—that is, by changing the anticomplement serum—the same assay records three different components of the classical complement activation pathway ($C1_q$, C4, and C3). The assay also recorded different amounts of complement activity (fig 2), indicating that it could become a simple and useful method for the measurement of complement activity in serum samples.

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Bacterial identification using nitrocellulose blotting technique that incorporates ELISA to bacterial surface antigen

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A major and recurring problem in microbiological diagnosis is the detection of a particular microorganism amongst a vast excess of irrelevant organisms. This is usually overcome by the use of selective media or judicious choice of culture conditions, or both. Several immunological techniques have also been used, particularly in the field of recombinant DNA technology.[1–4] In this context it is necessary to identify a small number of colonies making the required gene product from a library of several hundred thousand bacterial colonies. In one widely used technique a nitrocellulose sheet is applied to the surface of the bacterial culture plate and then peeled off, leaving most of the bacteria behind but transferring a small proportion of every bacterial colony on to the nitrocellulose replica. The bacteria on this replica are then lysed to release the recombinant protein, which then binds to the nitrocellulose sheet. By using antibody to the recombinant protein followed by enzyme labelled or radiolabelled second antibody, colonies making the desired protein can be identified. Viable bacteria can then be retrieved from the original agar plate.

Although recombinant proteins are usually intracellular, the above methodology should be applicable to the immunodetection of bacterial surface antigens. When using the technique for this purpose, however,

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we initially met with repeated failure. This prompted us to develop a model system to resolve the problems, and this paper describes a suitably modified method for the immunodetection of bacterial surface antigens.

Material and methods

Escherichia coli was obtained from the National Collection of Type Cultures (catalogue number 10418). *Staphylococcus epidermidis* was isolated in this laboratory. Nitrocellulose paper was purchased from Schleicher and Schull (BA 85, 0·45 μm).

Hyperimmune rabbit antibody to *E coli* was kindly provided by Dr HC Ryley of this department. Peroxidase conjugated, goat antirabbit IgG was obtained from Sigma.

Diaminobenzidine (DAB) (Sigma) was dissolved at 5 mg/ml in phosphate buffered saline (Dulbecco "A"-Oxoid), filtered through a 0·22 μm filter, and stored at −20°C. Immediately before use 1 ml stock solution was diluted with 9 ml phosphate buffered saline, and 15 μl hydrogen peroxide (30%) was added.

When 4-chloro-1-naphthol (Sigma or Biorad) was used as substrate, 3 mg was dissolved in 5 ml methanol; 20 ml Tris buffered saline (50 mM Tris, 200 mM sodium chloride, pH 7·4) was added followed by 30 μl hydrogen peroxide (30%).

Washing was performed in phosphate buffered saline and Tween 20 (PBST) (0·05% v/v Tween 20 in phosphate buffered saline).

FINAL METHOD

Bacteria were plated out on blood agar plates (9 cm in diameter) and grown aerobically until colonies of about 2 mm in diameter were produced. An autoclaved piece of dry nitrocellulose paper (8 cm in diameter) was laid flat on the bacterial colonies on the surface of the blood agar plate. After one minute the nitrocellulose was carefully peeled off with the bacterial colonies attached.

Blotting was immediately performed from the first nitrocellulose sheet (the master) on to a second dry nitrocellulose sheet (the copy) as follows. The second nitrocellulose sheet was carefully placed on top of the first, and for orientation purposes, a non-symmetrical pattern of holes was made around the perimeter by pushing a sterile needle through both sheets. The sheets were then enclosed within two discs of Whatman filter paper (No 1), placed on a flat surface and compressed by a 1 kg weight for five minutes.

The master and copy were then peeled apart, the colonies on the copy being barely visible. The master nitrocellulose sheet was placed on a fresh sterile blood agar plate and stored at 4°C, as the source of viable bacteria, while the copy was used for immunochemical detection.

Immediately after blotting the copy was immersed in 3% aqueous hydrogen peroxide and rotated at 60 revs/minute at room temperature for 30 minutes to inactivate endogenous bacterial peroxidase. The hydrogen peroxide was removed by three × five minute washes with PBST at room temperature with rotation.

The nitrocellulose copy was then placed on a Petri dish and incubated for three hours at room temperature with primary antibody diluted in 10 ml PBST. (In our model system rabbit anti-*E coli* was effective at dilutions of 1/100–1/2500.) Unbound antibody was removed by three × five minute washes with PBST, again with rotation.

After a further two hours of incubation at room temperature with 10 ml peroxidase conjugated goat antirabbit IgG (1/1000) excess conjugate was removed by two × five minute washes with PBST followed by two × five minute washes with phosphate buffered saline alone.

Finally, the copy was exposed to DAB substrate in the dark at room temperature. After colour development (30 minutes) the DAB was removed by washing with water.

Results

In our initial attempts to show the presence of cell surface antigens on blotted material nitrocellulose was placed on to bacterial colonies on a blood agar plate, peeled off, and the nitrocellulose replica was then used directly for immunochemical detection with a peroxidase conjugated second antibody and 4-chloro-1-naphthol as substrate.

Numerous attempts along these lines with a variety of bacterial strains and antisera were uniformly unsuccessful. Paradoxically, with the same reagents, reactivity could readily be seen when assays were performed in microelisa trays (with the bacteria attached to the plastic) with the soluble substrate o-phenylenediamine. Subsequent experiments with microelisa trays showed that of the two insoluble peroxidase substrates tested, DAB was much superior to 4-chloro-1-naphthol. Even with DAB as substrate, however, satisfactory results were not obtained with the nitrocellulose system, largely because of background staining that could not be reduced by various manoeuvres, including increased dilution of antisera, extensive washing, or inclusion of gelatin or bovine serum albumin (BSA) in incubation and washing buffers.

We wondered whether the bacterial surface anti-

gens on the nitrocellulose sheet were being masked by bacterial secretory products or blood agar constituents, or both. Therefore bacteria were transferred from the first nitrocellulose sheet ("master") to a second sheet ("copy") in the hope of leaving the contaminants behind, the nitrocellulose copy then being probed immunochemically. This modified procedure was successful (figure) and proved highly reproducible.

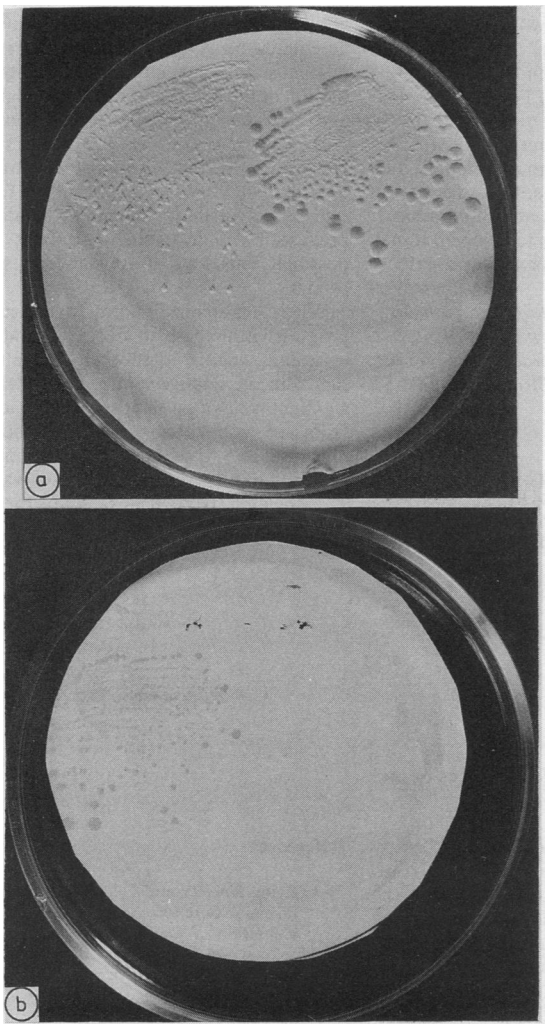

Figure *(a) Nitrocellulose replica (master) of blood agar plate inoculated with E coli (right) and S epidermidis (left). After nitrocellulose copy was made from master this was incubated at 37°C to increase colony size for photographic purposes; (b) nitrocellulose copy of above after probing with rabbit anti-E coli and development with DAB. Reactivity is seen only with E coli. Note that master and copy are mirror images.*

With the modified procedure, DAB was again superior to 4-chloro-1-naphthol. The use of glutaraldehyde to attach covalently the bacteria to the nitrocellulose surface was not of benefit, nor was the inclusion of BSA or gelatin in washing and incubation buffers.

Discussion

When detecting intracellular proteins, the nitrocellulose replica is exposed to chloroform to release the recombinant protein, washed with sodium dodecyl sulphate solution and finally incubated with DNAse to degrade released DNA.[4] Presumably these procedures also remove substances interfering with the immunodetection system. This treatment cannot be used for surface antigens. The second blotting step, as described in this paper, however, circumvents this problem and permits distinction between different microbial species on the basis of their surface antigens. This has obvious applications in microbial diagnosis, the only limitation being the specificity of the primary antibody.

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