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Julian E. Beesley
Wellcome Research Laboratories

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BIOAPPLICATION OF COLLOIDAL GOLD IN MICROBIOLOGICAL IMMUNOCYTOCHEMISTRY.

Julian E. Beesley

Wellcome Research Laboratories,
Beckenham, Kent. BR3 3BS. UK

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Abstract

Microbiological organisms are an ubiquitous group of animals encompassing bacteria, viruses, protozoa, algae and fungi. They are adapted for survival in many diverse habitats and exert a profound effect on man and his environment. Colloidal gold electron immunocytochemistry is a useful technique for studying these organisms and may be applied in several ways. The post-embedding technique is used to detect internal antigens, whilst the pre-embedding technique is employed for the detection of external antigens. In contrast the immuno-negative stain technique is applied to detect antigens on structures such as viruses or bacterial pili which may be dried down onto an electron microscope grid and immunolabelled in situ. In addition the immuno-replica technique allows the examination of cell surfaces for the appearance of antigens. Together these techniques have yielded valuable information concerning microbiological organisms.

Key Words: Colloidal gold, microbiology, immunocytochemistry, post-embedding, pre-embedding, immuno-negative stain, immuno-replica.

Address for correspondence:
Julian E. Beesley
Wellcome Research Laboratories
Beckenham, Kent BR3 3BS UK
Phone No. 01-658-2211 x5236

Introduction

Microbiological organisms are an ubiquitous group of animals encompassing bacteria, viruses, protozoa, algae and fungi. They are adapted for survival in such diverse habitats as soil, oceans, rivers and streams, ice, water pipes, concrete, hot springs, intestines, plant roots and oil wells. Many of these organisms, such as those responsible for cholera, typhoid fever and acquired immune deficiency syndrome, ruthlessly invade the host body and cause suffering and eventual death. Other organisms dwell in the rumen of cattle and break down ingested cellulose into usable food for the host animal. Some microorganisms spoil food and make it dangerous to eat, while others change one food type into another equally acceptable product such as milk into cheese. There is therefore a complex association between man and microorganisms.

The profound effect of microorganisms on man and his environment can be appreciated by listing the various divisions of microbiology. These study areas are medical microbiology, food and dairy microbiology, water and wastewater microbiology, soil and agricultural microbiology and industrial microbiology (Ross 1986). Genetic engineering, the technique of manipulating bacteria or yeast to produce mammalian protein in virtually unlimited amounts, is a recent development in industrial microbiology.

Immunocytochemistry is an important technique for the study of the relationships between microorganisms, man, and the environment. Immunocytochemistry is the use of antibodies as specific reagents for the localisation of specific proteins. Colloidal gold techniques are the latest, most highly acclaimed techniques for electron immunocytochemistry. Colloidal gold techniques were introduced by Faulk and Taylor (1971) who adsorbed anti-Salmonella rabbit gamma globulins to colloidal gold and used these markers as one step or direct probes for the localisation of antigens. Romano et al (1974) reported the indirect imunolabelling technique with gold probes and also the protein A-gold technique (Romano and Romano 1977) which was later applied to ultrathin sections for the localisation of internal antigens (Roth et al 1978, 1980). Zsigmondy and Theissen (1925) and Geoghegan and Ackerman (1977) have published in

detail the method, theory and application of adsorption of proteins to colloidal gold. The experimental details of these reports remain the cornerstone of today's techniques for complexing proteins with colloidal gold. Early applications and techniques of colloidal gold for scanning and transmission electron microscopy as well as for light microscopy were summarised and reviewed in depth by Horisberger (1981).

Traditionally, gold spheres have been produced by the reduction of tetrachlorauric acid. Frens (1973) reported the production of various sizes of gold by varying the quantity of reducing agent, in this instance sodium citrate in the reaction mixture. Reduction of gold salts with sodium citrate produces the larger 15-150nm gold particles. If the reduction rate is increased by the use of white phosphorus the small 2-12nm particles may be formed (Roth 1982a). Recently Slot and Geuze (1985) have combined a fast reducing agent, tannic acid, with a slow reducing agent, sodium citrate, in various quantities to achieve the precise reduction rate necessary to obtain gold spheres of any chosen diameter between 3 and 17nm.

Adsorption of cytochemically active proteins to colloidal gold is relatively straightforward and may be accomplished in a routine electron microscope laboratory (Roth 1983 and De Mey 1986 for details of techniques). These procedures produce extremely versatile immunocytochemical probes which have been used in many areas of biological research.

To date, there have been relatively few published articles using colloidal gold probes in microbiology. This paper will review the colloidal gold techniques applicable to microbiology and will also review the current usage of colloidal gold techniques in microbiology. It is hoped that it will also demonstrate the future potential of the technique in microbiology.

Materials and Methods

Antibodies

The use of a good antibody is essential because the colloidal gold techniques are so efficient that any unwanted background labelling in the preparation is immediately apparent. A good antibody should be highly specific for its antigen with no unwanted cross reactions. It should possess a high affinity for its antigen and be of high titre. If there are unwanted antibodies in the preparation, these should be of such low affinity and concentration that their contribution to the labelling can be removed by diluting the antiserum.

The choice of whether to use monoclonal or polyclonal antibodies is usually governed by the availability of a suitable antibody rather than a conscious effort to acquire either a monoclonal or a polyclonal preparation. Both monoclonal and polyclonal antibodies are useful in microbiological studies. The gold technique is equally efficient with either.

The specificity of a monoclonal antibody is extremely high. These antibodies usually react with only a single antigenic determinant of a few amino acids on each molecule. They may therefore be designated as monospecific with regard to the antigen. In some cases however this extremely high specificity for a given small epitope may be valueless. If identical, extremely small sequences of amino acids occur on different antigens, then in terms of recognising a given antigen, the advantage of high specificity of the antibody is totally lost.

Although the characteristic of high specificity of these antibodies is desirable for immunolabelling it does cause problems in preparing samples for electron immunocytochemistry. Fixation for electron microscopy involves strong cross-linking of amino acids which destroys the tertiary structure of the protein. If the antigenic determinant is cross-linked then reaction with the antibody will be prevented. Sufficient tissue antigenicity can be preserved by light fixation with freshly prepared 4% formaldehyde together with 0.05% glutaraldehyde, although this fixative is not optimal for preservation of tissue morphology.

Polyclonal antibodies are usually directed against a number of different epitopes on the antigen. In comparison with monoclonal antibodies these can be either monospecific or polyspecific. These antigens are relatively insensitive to aldehyde fixation since not all of the epitopes are destroyed and reactivity of the tertiary structure is assured. Fixation, in this case is achieved using 1% glutaraldehyde which also preserves the tissue ultrastructure. Osmium tetroxide and uranyl acetate, orthodox morphological fixatives, are not usually recommended for immunocytochemical studies. There are however, reports showing that immunolabelling can be successfully carried out in certain cases after osmium treatment. For instance, Bendayan and Zollinger (1982) have described the use of this fixative prior to immunolabelling. Restoration of immunolabelling could be achieved by treating the sections of aldehyde-fixed, post osmicated tissues with a saturated solution of sodium metaperiodate for 30 minutes before immunolabelling.

Studies in this laboratory have shown that uranyl acetate on its own is an excellent fixative for capsular antigens of the bacterium Pasteurella haemolytica although the fine structure of the bacteria was unfortunately very poor. The ultrastructure of the bacteria could be improved with a pre-fixation with 4% formaldehyde but this lowered the antigenic preservation. It therefore appears that uranyl acetate will not be used as the sole fixative in electron immunocytochemistry.

The mechanisms of fixation, with regard to retention of reactivity of a given epitope, are still not fully understood. Mild fixation does not necessarily assure retention of reactivity and the reverse, that strong fixation implies loss of reactivity, are not always correct. It is therefore often advisable in cases of loss of antigenicity to test a range of fixatives on the given antigen.

Whichever antibody is chosen it should be characterised and tested for suitability before use. Apart from Western blot analysis, which is used widely to characterise antibodies, Polak and Van Noorden (1984) recommend that antisera should be tested by either Enzyme Linked Immuno Sorbent Assay (ELISA) against the pure antigen, or by radioimmunoassay, or by being used for immunocytochemical labelling on known positive

tissue. The latter is the preferred test since it most closely corresponds to the experimental conditions.

Colloidal gold probes for microbiological immunocytochemistry

Colloidal gold probes possess many distinct advantages for immunocytochemistry and these have been adequately covered in the series of reviews edited by Polak and Varndell (1984). Appreciation of these characteristics shows that gold probes are applicable to microbiological studies and may be used for immunolabelling even the smallest virus particle.

Gold probes are commonly protein A-gold complexes, antibody-gold complexes or streptavidin-gold complexes. Lectin-gold and enzyme-gold probes are also gaining wide acceptance. Any of these may be used routinely in microbiological studies, the choice being governed by the particular requirements of the study. The production of these different gold probes has been reviewed extensively by Roth (1983) and De Mey (1986) and will not be discussed further in this paper.

Buffer systems

Colloidal gold immunolabelling techniques may be combined with any one of several different specimen preparation techniques in order to obtain specific antigenic information from the sample. The basis of each technique is similar; the specimen is incubated with specific antibody, then washed, and thereafter incubated with a suspension of gold probe before contrasting and examination. The correct choice of antiserum and gold probe concentration is necessary if there is to be sufficient labelling for detection with minimum background labelling. Optimal concentrations are found in each case by testing serial dilutions of the reagents. The buffer system used is important and must be compatible with the immunological reagents used in the experiment. The buffer system found suitable for diluting both antibodies and gold probes and for washing purposes in this laboratory is phosphate buffered saline, pH 7.2, containing 1% bovine serum albumin (Slot and Geuze 1984).

Tissues may possess miscellaneous receptors which are "sticky" for all proteins. These need to be blocked before application of the immunological reagents to prevent non-specific labelling. The presence of bovine serum albumin in the buffer reduces non-specific attachment of antibodies by competing with antibody for these non-specific "sticky" sites. A short pre-incubation of the tissue with 1% gelatin in phosphate buffer also reduces non-immunological sticking of the antibody since the gelatin attached to the "sticky" sites. Gelatin can also reduce non-specific labelling if it is included in the gold probe suspension. The method of action here is that it attaches to free, active sites on the gold spheres, thereby preventing the gold from attaching to non specific "sticky" sites on the tissue (Behnke et al 1986).

If an aldehyde has been used to fix the tissue, free aldehyde groups may remain on the tissue. These may encourage non-specific "fixation" of antibody. This can be overcome by pretreating the sample with 0.02M glycine in phosphate buffered saline.

Immunolabelling techniques

The post-embedding technique. Post-embedding immunolabelling necessitates preparing sections of tissue and incubating these with antibody and probe (Appendix 1) in order to immunolabel antigens exposed by the sectioning process. The overwhelming advantage of this technique is that any antigen, whether internal or external on the sectioned tissue may be immunolabelled. This technique has therefore been widely exploited.

The tissue must first be fixed and embedded. Fixation conditions will be governed by the type of antibody employed (see above) but for embedding the investigator is presented with three choices; a) embedding in resin at room temperature and polymerising the resin at relatively high ($60^{o}C$) temperatures; b) embedding in resin and polymerising at low ($-40^{o}C$) temperatures or c) embedding in a frozen cryoprotectant at liquid nitrogen temperature.

Resin sections have, in the past, been considered easier to prepare than frozen sections although with the introduction of a new generation of cryoultramicrotomes, this is no longer entirely correct. Many resin embedding schedules are routinely employed and produce useful results. The tissue is dehydrated in a graded series of alcohol and embedded in either Epon (Roth et al 1978) or Araldite (Van Noorden and Polak 1985). Some investigators dehydrate their samples in a graded series of alcohol then embed in the acrylic resin LR White (Yoshimura et al 1986) while other investigators dehydrate samples in a graded series of methacrylate and embed in a butyl/methyl methacrylate mixture (Cramer et al 1986). Tissue processing is carried out at room temperature and polymerisation is achieved by either heat at $60^{o}C$, or at $4^{o}C$ using ultraviolet irradiation (Figure 1). One interesting paper (Bayer et al 1985) shows that structural collapse of the capsule in Escherichia coli K29 can be effectively prevented by using dimethyl formamide instead of ethanol for dehydration.

Tissues processed at temperatures below $0^{o}C$ are believed to retain more antigenic activity than those processed at room temperature and above (Armbruster et al 1983). At a low temperature proteins are insoluble in alcohol and molecular movement is hindered thereby preventing protein antigens leaching from the tissue. The acrylic resin Lowicryl K4M is used for this purpose. The tissue is dehydrated in ethanol but on increasing the alcohol concentration the temperature is progressively lowered to $-40^{o}C$. The tissue is embedded in resin which is polymerised, still at $-40^{o}C$, by ultraviolet irradiation. Doubt has been cast on the verity of the resin temperatures specified in these examples (Ashford et al 1986) The conditions used in this paper differ dramatically from those used in embedding protocols and therefore this needs to be investigated thoroughly before firm conclusions can be drawn. Despite this claim, the low temperature embedding technique has, to date, proved valuable.

The technique of cryoultramicrotomy circumvents the necessity of embedding the tissue in resin. The tissue is aldehyde fixed then cryoprotected in 2.3M sucrose for 1h. This procedure prevents ice crystal growth and tissue damage when the sample is plunged rapidly into liquid nitrogen or

nitrogen slush. The sample is warmed to between -100°C and -80°C in the cryoultramicrotome. At these temperatures the sample is sufficiently brittle to allow the preparation of ultrathin frozen sections which are collected on a sucrose droplet and brought to room temperature for immunolabelling and staining (Tokuyasu 1984, 1986). Ultrathin frozen sections possess no intrinsic rigidity and finally the sections must be embedded in methyl cellulose before drying to maintain their three dimensional integrity (Figure 2a). Griffiths et al 1984, consider the cryotechnique to be the method which gives the best preservation of antigenic determinants, since the only potential denaturing step before immunolabelling is the initial aldehyde fixation.

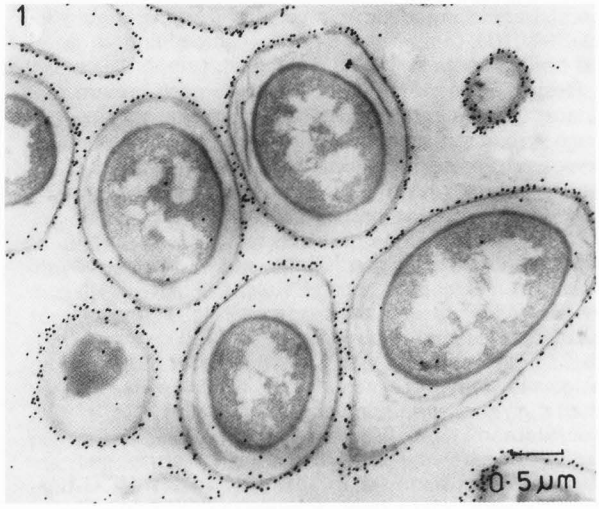


Figure 1. Spores of Bacillus cereus M8 were fixed with 3% glutaraldehyde, dehydrated and embedded in methacrylate resin. Sections were incubated with rabbit antibody against exosporial proteins followed by the protein A-gold probe. There is strong labelling of the exosporium.

The pre-embedding technique. The pre-embedding technique is a high resolution technique for the localisation of external antigenic sites on isolated cells and microorganisms. The technical details of the technique are shown in Appendix 2. A light aldehyde fixation may be used if the cells are to be stored before immunolabelling. Fixation may be omitted if very sensitive antigens are to be examined. Pre-fixation is usually necessary if cultured cells are being immunolabelled since this prevents internalisation of the probe. Alternatively, this may be avoided by incorporating sodium azide into the immunological reagents.

Good fine structural preservation and contrast are achieved by this technique since the tissue is subjected to the orthodox fixation regime of glutaraldehyde, osmium tetroxide and uranyl acetate before embedding (Figures 2b and 2c). This is a distinct advantage for virological studies in which virus particles may be difficult to visualise. This technique is not entirely suitable for the localisation of internal antigens since the specimen must be disrupted before immunolabelling to allow the reagents to penetrate into the cells. This degrades the fine structure, but more seriously false negative results may be obtained because of limited penetration of imunological reagents into the cells.

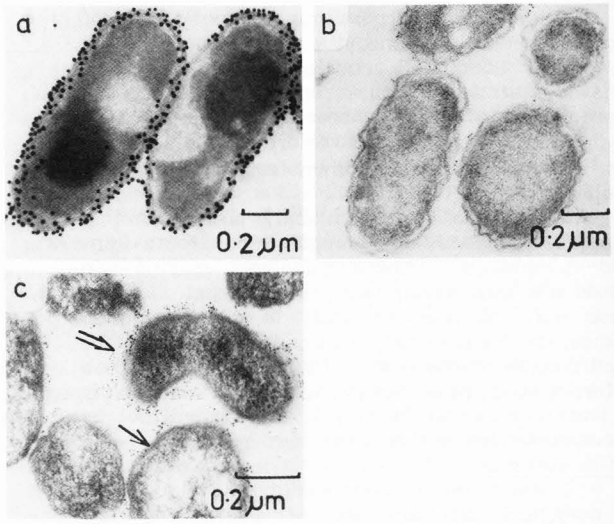


Figure 2a. The bacterium Bordetella bronchiseptica was fixed with 1% glutaraldehyde then cryoprotected with 2.3M sucrose for 1 h. After freezing in liquid nitrogen slush, thin sections of the bacteria were prepared and immunolabelled with antibody against adenylate cyclase related protein and the gold probes. The preparations were contrasted with uranyl acetate before embedding in 1.5% methyl cellulose. Labelling is restricted to the outer membrane.

Figure 2b. The bacterium Bordetella bronchiseptica was incubated with adenylate cyclase related protein antibody and the gold probe before fixation with glutaraldehyde, osmium tetroxide and uranyl acetate, dehydration and embedding in Araldite. Thin sections revealed the presence of very few antigenic sites on the outer surface of the outer membrane.

Figure 2c. Cells of Bordetella bronchiseptica were partially disintegrated then prepared as in Figure 3. There is more labelling in areas where the outer cell wall is disrupted (⇒) compared with where the cell wall is intact (→), showing that many antigens are on the inner surface of the outer membrane.

The immuno-negative stain technique. The immunonegative stain technique is the technique of choice for immunolabelling any structures which may be dried down onto a grid, immunolabeled in situ and then visualised by the negative stain method (Appendix 3). Microbiological organisms, such as viruses and bacteria are therefore ideal candidates for this technique (Figure 3).

The negative stain produces preparations of high resolution, and since the technique is carried out on the grid the technique uses a bare minimum of sample. The schedule is very quick to initiate and carry out, and furthermore the gold probe produces very distinct labelling which can be detected even in samples containing considerable debris. The choice of antisera is critical for this technique since only

external antigens of the sample are exposed. If cross reactions between antisera occur, then the particulate nature of the gold probes allows quantitation of immunolabelling to resolve this difficulty (Beesley and Betts 1985).

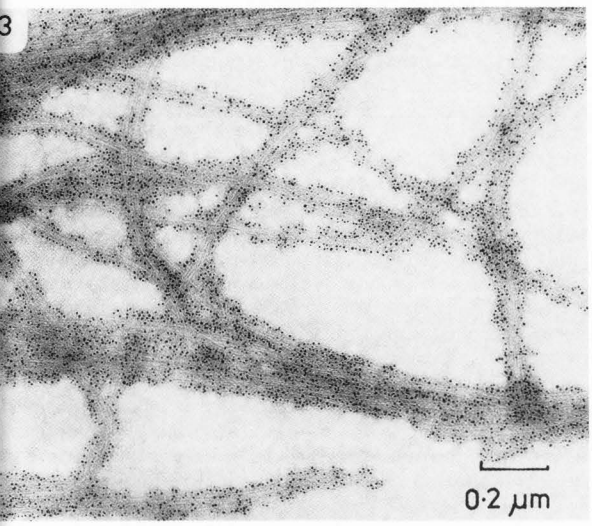


Figure 3. Bacteroides nodosus organisms were dried down onto a Butvar/carbon coated 400 mesh gold grid and immunolabelled in situ with anti-pilus antibody and the gold probe. The labelling is ample, specific and easily detected.

It may be recognised therefore that this is a high resolution technique which possesses considerable potential for both diagnostic and research studies.

The immuno-replica technique The immuno-replica technique was developed specifically for detecting viral antigens on the surface of cultured cells (Mannweiler et al 1982a,b) but surprisingly it has not been used extensively for immunocytochemical studies.

Tissue culture cells, after infection with measles virus, were lightly fixed with aldehyde, then immunolabelled with antiserum and colloidal gold. After washing and post-fixation with osmium tetroxide the preparations were dehydrated in a graded series of alcohol, critical point dried and then replicated with carbon and platinum (Appendix 4). Colloidal gold probes are so dense that they may be distinguished anywhere on the replica. This, of course, is an essential requirement for quantitative studies. The technique is extremely valuable for scanning relatively large areas of cell membrane for isolated patches of antigen.

Double labelling studies. Colloidal gold probes may be produced in different sizes which can be distinguished in the electron microscope. These probes therefore have enormous potential for double labelling experiments. Experiments to date have been restricted to localising two antigenic sites in a tissue but there is no reason why more than two sites should not be identified. Labelling by one probe should not hinder the potential labelling of other probes by steric hindrance or cross-reactions and further, the gold probes used for immunolabelling different antigens should be of clearly distinguishable sizes. Localisation of the different antigens is carried out by the routine incubations described for each of the different techniques. Localisation of the second antigen may be simultaneous with the first by simply mixing the antisera and probes, if the reagents do not cross-react or if they do the different antigens must be localised sequentially. Several principles of multiple labelling have been developed.

If the primary antibodies do not cross-react, then the simplest technique for multiple labelling is to coat each sized gold sol with a different primary antibody then use these probes sequentially. This however is a very extravagant use of primary antibody. A useful extension of this technique is to incubate a protein-A gold probe with primary antibody and then use this in a one-step labelling reaction. This is then repeated for a second antibody using a further size gold probe (Robinson et al 1984).

Pure antigen may be complexed with colloidal gold and used to label tissue which has been incubated with an excess of primary antibody (Larsson 1979). The use of excess antibody ensures that one Fab site on the antibody is free to bind with the antigen gold complex. This is repeated for a second antigen/gold complex. These experiments ensure that there are no cross reactions between probes, but the availability of pure antigen limits the application of the technique.

Tapia et al (1983) have described a very practical double labelling technique. In order to label different antigens these authors employ antibodies raised in different hosts. The tissue may be incubated simultaneously with both antibodies followed by the simultaneous incubation with two different sized probes, one coated with antibody against one host species, the other probe coated with antibody raised against the host species which was used to raise the other primary antibody.

Protein A may be used as a blocking agent, when carrying out double labelling with protein A-gold probes (Roth 1982b, Slot and Geuze 1984). The tissue is immunolabelled with the first antibody then the smallest protein A-gold probe used in the experiment. The tissue is subsequently incubated with free protein A to saturate any remaining Fc sites on the first antibody. This inhibits any further antibody protein A-gold attachment to these sites when the second immunolabelling schedule is followed.

If resin sections are used for immunolabelling they can be mounted on an uncoated grid and labelled on both sides. If care is taken to prevent the grid sinking in the immunological reagents one side of the grid can be labelled with one antibody and one sized probe, and the other labelled with a second antibody and different sized probe (Bendayan and Stevens 1984, Beesley et al 1984a).

Quantitation. Another valuable characteristic of colloidal gold probes is their dense particulate nature. This enables them to be easily quantified, even on the most densely stained tissue or dark replica. Although the first quantitative data with protein A-gold probes were published several years ago (Bendayan et al 1980) quantitation of gold labelling is still in its infancy because the effects of specimen preparation and immunolabelling artefacts

are poorly understood. Quantitation does however yield useful information (Bendayan 1984, Beesley and Betts 1985, Griffiths and Hoppeler 1986) if the experimental variables in the studies are carefully controlled. Van Bergen en Henegouwen and Leunissen (1986) and Voorhout et al (1986) present interesting data relating to probe particle diameter, probe concentration, preparation methods and also problems of steric hindrance which should be of value in quantitative studies.

Results and Discussion

Virology

Electron microscopy continues to be the method of choice for the diagnosis of viral particles. The importance of the technique is shown by the fact that despite elegant tissue culture methods the Norwalk agent, astroviruses and caliciviruses, were first diagnosed using electron microscopy. Indeed, electron microscopy is at least partially responsible for diagnosing 50% of the hitherto undiagnosed non bacterial gastroenteritis in hospitalised children (Riepenhoff-Talty et al 1983).

Identification of viruses using the negative stain technique is relatively straightforward if the viruses are presented as a concentrated suspension and the viruses possess characteristic easily recognised features. Identification of small viruses is more difficult but if they are present in high numbers they can be recognised by their constant size and shape. This technique is unsuitable if the viruses are badly disrupted or if it is necessary to distinguish morphologically similar virus types. An immunological test is then necessary. Although immunoelectron microscope techniques (Almeida and Waterson 1969) have been employed for many years we have found colloidal gold probes in conjunction with the immuno-negative stain technique to be far superior because the gold probes are small and therefore they do not obliterate the virus particles; gold probes are dense, therefore they can be used on samples which contain debris, or may not be optimally stained; and gold probes possess a high immunolabelling efficiency therefore the virus particles are sufficiently labelled for easy identification. The major advantage though, is that the immunonegative stain uses so little material. This means that after an initial examination of the sample in the electron microscope, immunological testing may be carried out, if necessary, on some of the original material prepared for negative stain examination.

This technique has been gaining acceptance recently for identifying viral antigens and typing morphologically similar viruses (Beesley and Betts 1985, Kjeldsberg 1985, Hopley and Doane 1985, Murti and Webster 1986). Alternatively a known antigen or a mixture of several distinct antigens may be dried down onto an electron microscope grid and the technique might be used for identification of unknown antibodies in the serum (Muller and Baigent 1980).

The pre-embedding technique, because it incorporates the orthodox triple fixation regime, is ideally suited for the localisation of viral antigens budding from the cell surface. Yeger and Kalnins (1978) provide the first report of this technique for localising viral proteins. They employed antibody-gold complexes for studying the distribution of major viral glycoprotein, gp70, on cells infected with Rauscher marine leukemia virus. More recently Evans and Webb (1986) combined this technique with a rapid processing schedule for the effective localisation of Semliki Forest viral antigens.

The sensitivity and resolution that can be attained using the post-embedding technique has favoured its application in many virological studies. It has been used to study the movement of viral proteins across the Golgi cisternae of infected host cells (Bergmann et al 1981, Green et al 1981, Griffiths et al 1982, Griffiths et al 1983, Griffiths et al 1985). These studies have contributed significantly to the understanding of the biogenesis and assembly of various membrane components.

In order to target drugs effectively against microorganisms within host cells it is important to understand the replication cycle of the invading organisms and to localise the site of production in their host cells. Post-embedding techniques are most frequently used for these studies since the majority of the antigens are intracellular. For example, antibodies raised against influenza viral haemagglutinin, matrix protein and nuclear protein were used for the localisation of these proteins in the host cell (Beesley and Campbell 1984).

The immuno-replica technique was developed by Mannweiler et al (1982a,b) for the detection of measles viral antigens on the surface of infected cultured cells. This group has continued to apply the technique to the study of viral antigens. They have examined hamster fibroblasts after transfection of Maloney murine leukemia virus mutants (Mannweiler et al 1984) and in studies on virus -induced alterations to regions on the different plasma membrane faces of Hela cells (Hohenberg et al 1985). The technique has also been used by the group to examine the distribution of measles virus antigens on the outer surface of membranes (Rutter et al 1986) and to study actin filament involvement in the budding of measles virus from cultured Hela cells (Bohn et al 1986). They have further developed the technique in combination with the freeze fracture technique to investigate plasma membrane antigens of measles virus (Hohenberg et al 1986).

Bacteriology

The immuno-negative stain technique can be applied with equal facility to the study of bacterial antigens. Beesley et al (1984b) have used the technique for demonstrating multiple antigenicity of Bacteroides nodosus pili, and Levine et al (1984) employed the technique for localising surface antigens 1 and 3 of a colonization factor of Escherichia coli. In the same year the technique was used by Robinson et al (1984) for the ultrastructural localisation of specific gonococcal macromolecules. Hiemstra et al (1986) on the other hand employed the technique to study the induction kinetics and cell surface distribution of E. coli lipoprotein under lac promoter control. A further study using the technique was described by Worobec et al (1986) who have studied the location of the antigenic determinants of conjunctive F-like pili. In addition immuno-negative stain technique has demonstrated five different patterns of spatial arrangements of antigens on the cell surface of Listeria monocytogenes (McLaughlin et al 1986).

The immuno-negative stain technique has been used extensively in the development of a vaccine against foot rot of sheep. There are at least seventeen serotypes of the bacterium Bacteroides nodosus which are responsible for the disease. The inclusion of more than ten serotypes in a vaccine would be difficult to manufacture and the antigenic competition would lead to a reduced response to the individual antigens (Day et al 1986). The selection of strains for use in the vaccine is therefore important. The immunonegative stain technique has helped to elucidate serological specificity of the various serotypes (Figure 3). We have shown that three antisera raised against three distinct pili serotypes exhibit considerable cross-reactions and it now appears likely that each of these pilus types may contain at least four different antigenic sites (Beesley et al 1984b, Day et al 1986). The selection of bacterial strains with appropriate cross reacting antigens would be advantageous and would result in a vaccine giving wider cover in the field.

Diagnostic kits are valuable aids for the rapid identification of bacteria. For instance the serological identification of D and G group β-haemolytic streptococci has recently been facilitated by the introduction of latex agglutination tests. Conventional reference procedures (Lancefield 1933) have confirmed the accuracy of the latex tests, with one exception. The presence of biochemically D organisms possessing additional group G antigenic determinants has been revealed using latex tests. The morphology and antigenic composition of these exceptional bacteria, classified as DG organisms, were investigated using routine morphological techniques and post-embedding immunolabelling of frozen sections.

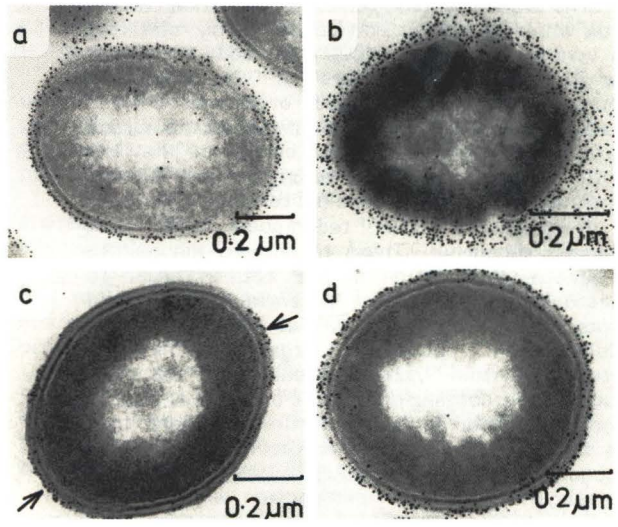
Morphological studies were carried out on representative D, G and DG cultures. These investigations showed that group D organisms were smooth, whereas group G organisms possessed a fluffy outer matrix. DG organisms were intermediate being mostly smooth with only a few wispy projections.

Immunocytochemistry was performed on glutaraldehyde fixed, gelatin embedded ultrathin frozen sections of bacteria. Antisera raised against D antigens labelled antigens tightly bound to the cell wall of D organisms (Figure 4a). In contrast antibody raised against G antigens localised the G antigen on the outer surface of the cell wall (Figure 4b). These antisera showed no cross reactions in heterologous reactions.

Antiserum raised against D antigen showed only minimal labelling on the DG organisms (Figure 4c). Antiserum raised against G antigen however, localised antigen on the DG cells (Figure 4d) in a similar area to the D antigen on D cells.

These studies show that the D and G type organisms possess distinct and separable morphological and immunological characteristics. DG organisms however, possess fine structural characteristics more similar to group D organisms than group G organisms. Immunologically however, the DG organisms possess more G antigenic sites than D sites.

Studies such as these are invaluable in the development and understanding of the limitations of diagnostic kits.



Figures 4a-c. Post-embedding immunolabelling of frozen sections of β-haemolytic streptococci.
Figure 4a. D type organism immunolabelled with D antiserum. The antigens are closely associated with the cell wall.
Figure 4b. G type organism immunolabelled with G antiserum. The antigens are loosely associated with the cell wall.
Figure 4c. DG organism immunolabelled with D antiserum. There are very few antigenic sites on the organism (→), they are mainly polar.
Figure 4d. DG organism immunolabeled with G antiserum. There is heavy immunolabelling and it appears to be D type (Figure 4a) rather than G type (Figure 4b).

The bacterium Pasteurella haemolytica causes pneumonia and septicaemia of sheep and cattle. Vaccines prepared from whole bacteria cause undesirable side effects and attention is now centred on the use of capsular polysaccharide (Adlam et al 1984, 1985, 1986). In each of these studies immunocytochemical labelling of the capsule has been a useful technique for examining the relationships of the capsular polysaccharides from the different serotypes.

Both Mouton and Lamonde (1984) and Orefici et al (1986) have used the pre-embedding technique to study surface antigens of bacteria although the pre-embedding technique is often used to complement results obtained using the post-embedding technique. These techniques have been used to localise the 68,000 mol wt. adenylate cyclase - related protein which is found in abundance in the bacterium Bordetella bronchiseptica (Beesley and Novotny 1984). This enzyme is an important constituent of any vaccine formulated against organisms within the genus Bordetella. It was localised using the post-embedding technique on frozen sections (Figure 2). The outer membrane was heavily immunolabelled but the resolution of the technique was insufficient to detect the exact location of the antigen on the membrane.

The pre-embedding technique was then carried out on whole organisms and despite using relatively high concentrations of primary antibody there was little immunolabelling (Figure 3). Pre-embedding immunolabelling of disrupted organisms showed abundant labelling on areas of disrupted membrane (Figure 4). These experiments confirmed that the majority of antigens were sited on the inner surface of the outer membrane of the bacterium.

Recombinant DNA techniques have been developed recently. These techniques manipulate the genetic information of the cell in order to increase its synthetic potential of useful polypeptides (Paul et al 1983). For instance, foreign proteins from pathogenic organisms may be "cultured" within non-pathogenic or naturally occurring microorganisms. These organisms, altered to contain important and harmless antigens may then be used as an efficient vaccine against the pathogenic organism (Dougan et al 1986). Immunocytochemistry in conjunction with suitable antibodies is being used to monitor the appearance of these proteins within the cell. In this laboratory epidermal growth factor protein (Allen et al 1987), malarial proteins and adhesion proteins have been localised in this manner. Paul et al (1983) have also carried out similar localisation techniques to monitor the appearance of human proinsulin chimeric polypeptide within cytoplasmic inclusion bodies of Escherichia coli. Immunocytochemistry will therefore assume an important role in the development of the techniques of genetic engineering.

Phycology

There appears to be very few reports of colloidal gold cytochemistry applied to phycology. Berger and Schweiger (1986) have employed RNase and DNase linked to gold granules to establish that RNA and DNA occur within the cytoplasmic structures of the Acetabularia cell that appear close to the nucleus during the vegative phase of the life cycle.

Salisbury et al (1984) employed the immunogold system to demonstrate immunospecific labelling of striated flagellar roots and their extensions from the Prasinophycean green algae Tetraselmis striata and conclude that the striated flagella roots are simple ion-sensitive contractile organelles composed predominantly of a 20,000 mol. wt. calcium-binding phosphoprotein which is largely responsible for the motile behaviour of the organelles.

Mycology

Colloidal gold techniques have been used extensively in mycology. As early as 1978, Horisberger et al (1978) were using the technique for the ultrastructural localisation of beta -D - galactan in the nuclei of the myxomycete Physarum polycephalum. Horisberger and Vonlanthen (1979) further developed the gold technique for fluorescent and electron microscopy by coating 12nm gold granules with rhodamine conjugates of Concavalin A and avidin. These probes were used to label cell wall mannan on the yeast Saccharomyces cerevisiae either by the one step or two step method in a biotinyl derivative of Concavalin A. The preparations could then be viewed by either fluorescence microscopy or transmission electron microscopy. The use of these techniques for light and fluorescent microscopy and for transmission and scanning microscopy were later reviewed (Horisberger 1981).

Molano et al (1980) used gold probes coated with either wheat germ agglutinin or chitinases to study the distribution of chitin in Saccharomyces cerevisiae primary septa and cell walls. They concluded that 90% of the total chitin occurs in the septa although the lectin binding receptors as well as chitin is uniformly distributed over the whole cell.

Cabib et al (1983) used colloidal gold markers in conjunction with ferritin markers for double labelling experiments to examine the directionability of chitin synthesis by yeast plasma membranes. Pre-embedding labelling was carried out with a ferritin-Concavalin A probe. The membranes were embedded in Epon and sections were labelled with wheat germ agglutinin - colloidal gold probes to detect chitin. The chitin marker was found close to the ferritin-labelled external face of the membrane.

The highly structured surface of yeast has been used as a test specimen to study the efficacy of detection of 15nm protein A-gold probes by back scattered electrons (Walther et al 1984). These authors extend the usefulness of the scanning electron microscope technique in immunocytochemistry by use of the back scattered image produced by colloidal gold. They found that the combination of the high resolution surface image and the material dependent back scattered image provided an accurate localisation of surface antigens. In addition a secretory enzyme of Candida albicans, Candida protease, has been used as a model system in further comparisons of the efficacy of scanning electron microscope methodology (Borg 1985).

Brawner and Cutler (1986a, 1986b) also studied Candida albicans and carried out a detailed study of the expression of cell surface antigens in order to show this approach would be useful in establishing receptors which mediate adherence.

Chamberland et al (1985) used a chitinase gold complex to localise chitin in tomato root cells infected by Fusarium oxysporum f.sp. radicis - lycopersici, and compared this with the results obtained using a chitin-specific gold-conjugated lectin. It was found that the chitinase-gold conjugates yielded a more uniform distribution of gold particles over the fungus wall compared to that obtained using the lectin-gold technique.

Benhamou and Oullette (1986a, 1986b) carried out two useful studies on the fungus Ascocalyx abietina. One of these studies (1986a) involved pectinase complexed to colloidal gold for the ultrastructural localisation of polygalacturonic acid and the other (1986b) localised glycoconjugates with lectin-gold complexes. These are the first reports of polygalacturonic acid and sialic acids in fungi.

Monensin, a monovalent cation ionophore, was employed to investigate steps of the wall synthesis and morphogenesis in Candida albicans blastospores (Poli et al 1986). Monensin stimulated the development of enormous wall and septum thickenings that reacted intensely and specifically with wheat germ agglutinin and chitinase complexed with colloidal gold and fluorescein isothiocyanate. These aberrant developments can therefore be interpreted as sites of chitin accumulation.

Vesicular stomatitis virus and Semliki Forest virus fuse with the plasma membrane of Saccharomyces cerevisiae (Makarow et al 1987). This reaction was studied with specific antiserum and the protein A-gold probes. It was found that fusion, which was strictly dependent on the prior removal of the yeast cell wall, was fast, calcium-independent and non-leaky, leaving the spheroblasts viable for at least 4h, and furthermore less than 100 vesicular stomatitis virus particles were fused to each spheroblast.

Wheat germ agglutinin complexed to colloidal gold was used as a specific label to visualise chitin in walls of control and polyoxin-treated Candida albicans (Hilenski et al 1986). These results show that the morphological alterations caused by polyoxin D were due to the absence of chitin, a wall component important for formation of primary septa and for maintenance of structural integrity during morphogenesis.

Protozoology

There appears to be approximately one hundred and fifty publications since 1978 which report immunocytochemical applications to protozoology. Approximately 15% of these describe the involvement of colloidal gold techniques.

There have been several reports investigating protective antigens on the surface of Plasmodium spp (Fine et al 1984, Oka et al 1984, Brown et al 1985, and Aikawa et al 1986). One report (Sherman and Greenan 1986) describes the use of lectin-fetuin-gold complexes for detecting regional differences in lectin binding to the surface of the malaria-infected human erythrocytes.

The kinetics of human eosinophil activation upon interaction with Trypanosoma cruzi have been studied using the protein A-gold technique (Kierszenbaum et al 1986). In addition Grab et al (1984) have investigated the intracellular pathway and assembly of newly formed variable surface glycoprotein of T. brucei with the protein A-gold probe. The onset of expression of the variant surface glycoproteins of T. brucei in the tsetse fly have also been investigated using colloidal gold techniques (Tetley et al 1987).

The interaction of Leishmania particles with host cells has been studied using colloidal gold probes. Blackwell (1985) described the role of complement and lectin-like receptors in binding Leishmania particles to host macrophages and later (Wozencraft et al 1986) reported the detection of macrophage-derived complement on the parasite surface by electron immunocytochemistry. Pupkis et al (1986) however, used gold labelled antibodies to show that both cysteine proteinase and arylsulfatase are present in large organelles termed megasomes. These studies were compared with L. mexicana amazonensis, L. donovani donovani, and L. major.

There have been two studies on Theileria parva (Webster et al 1985, Dobbelaere et al 1985). These describe immunoelectron microscopic localisation of sporozoite antigens which are associated with sporozoite entry into lymphocytes.

The earliest colloidal gold studies in protozoology were carried out by Arroyo-Begovich et al (1980) and Arroyo-Begovich and Carabez-Trejo (1982). These two reports describe the localisation of chitin in the cyst wall of Entamoeba spp using colloidal gold-linked wheat germ agglutinin. The latter paper is a post-embedding procedure.

Barbosa and Pinto da Silva (1983) showed, by use of colloidal gold complexed with horseradish peroxidase in order to localise Concanavalin A, that glycolipids were restricted to the outer half of the plasma membrane in Acanthamoeba castellanii.

Vigues et al (1984) described the immunological characterisation of the microfibrillar ecto-endoplasmic boundary in the ciliate Isotricha prostoma. They believe that their 22-23K antiserum will prove to be a useful tool for the comparative study of other non-actin filament systems in protozoa.

There have been many ultrastructural studies on Eimeria spp but to date only one paper (Speer et al 1985) has reported the use of colloidal gold techniques. This describes capping of immune complexes in the sporozoites of E. tenella.

Colloidal gold has been used as a label for secondary lysosomes (Weidner and Sibley 1985). This study showed that phagocytosed intracellular microsporidia block phagosome acidification and phagosome-lysosome fusion in macrophages.

A polyclonal anti-luciferase antibody labelled organelles in the bioluminescent dioflagellate Gonyaulax polyedra (Nicholas et al 1985). This antiserum labelled both dense vesicles and trichocyst sheaths. These were postulated to correspond to the autofluorescent particles observed with the fluorescent microscope.

There has been one report of the use of a DNA-ase gold complex. This was used for monitoring membrane associated DNA in Tetrahymena (Csaba et al 1986).

Paramecium spp has recently been studied in detail. Luthe et al (1986) have examined the lectin binding sites in P. tetraurelia cells, predominantly of secretory components and this was followed (Luthe and Plattner 1986) by analysis of predominantly non-secretory components. Momayezi et al (1986) however use a polyclonal antibody and protein A-gold probes to examine the distribution of calmodulin within the P. tetraurelia cells.

Conclusions

It can be appreciated that microbiological organisms are important to man and his environment. An understanding of these organisms is therefore important if man is to co-exist with these organisms. Immunocytochemistry is a key technique in these endeavours and colloidal gold techniques have shown themselves to be far superior to the immuno-enzyme techniques. Colloidal gold techniques are very simple and produce high resolution labelling of specific receptor sites. These factors have led to the widespread use of colloidal gold immunocytochemistry in the biological sciences. This review has considered in detail the techniques and applications of colloidal gold in microbiology.

Colloidal gold electron imunocytochemistry is now an established technique but it is not static and many new developments are regularly described, such as in situ hybridisation at the electron microscope level (Binder et al 1986). Techniques of in situ hybridisation are already important at the

light microscope level of resolution and it should not be long before the technique achieves wide acclaim for electron microscopy. Furthermore immunoscanning electron microscope techniques in conjunction with back scattered electron detection systems possess high potential for microbiology and should soon become popular.

Finally, colloidal gold has many exceptional attributes as a modern immunocytochemical marker. The new revolution in immunocytochemistry (Beesley 1985) has gained tremendous momentum and these techniques are now ready for widespread application in microbiology.

Appendices

Appendix 1. The post-embedding technique for electron immunocytochemistry.

1. Lightly fix sample with either 4% formaldehyde and 0.05% glutaraldehyde (for monoclonal antibodies) or 1% glutaraldehyde (for polyclonal atibodies).
2. Either a) Embed tissue in resin
   or b) Cryoprotect with 2.3M sucrose for 1h then freeze in liquid nitrogen or nitrogen slush.
3. Cut sections of tissue and mount on Butvar/carbon coated 200 mesh grids.
4. Float grids, section side down sequentially on
   a) 1% gelatin in phosphate buffered saline, pH 7.2 (PBS) for 10 min
   b) 0.02M glycine in PBS for 3 min
   c) 1% bovine serum albumin in PBS (BSA/PBS) for 2 min
   d) Antiserum diluted with BSA/PBS for 1 h
   e) Wash on BSA/PBS droplets, 4 x 1 min
   f) Colloidal gold probe diluted with BSA/PBS 1 hr
   g) Repeat e.
   h) Contrast by routine methods
5. Examine

Appendix 2. The pre-embedding technique for electron immunocytochemistry.

1. Fix cells as in Appendix 1 or use unfixed.
2. Incubate cell suspensions with reagents described in Appendix 1, 4a-g.
3. Fix cells with 1% glutaraldehyde in PBS at room temperature for 15 min.
4. Rinse briefly in water.
5. Post-fix with 1% aqueous osmium tetroxide for 1 h.
6. Tertiary fix with 2% aqueous uranyl acetate for 1 h.
7. Dehydrate in a graded series of ethanol.
8. Embed in Araldite or equivalent resin.
9. Cut thin sections and stain with uranyl acetate and lead citrate before examination.

Appendix 3. The immuno-negative stain technique.

1. Prepare a suspension of sample, then dry down onto Butvar/carbon coated 400 mesh gold grid.
2. Float grid, specimen side down sequentially on,
   a) Antiserum diluted with BSA/PBS for 15 min
   b) Wash with BSA/PBS droplets, 4 x 1 min
   c) Colloidal gold probe diluted with BSA/PBS for 15 min
   d) Wash by floating on droplets of water 4 x 1 min
   e) Negative stain, e.g., ammonium molybdate 1%, pH 6.8
3. Examine

Appendix 4. The immuno-replica technique.

1. Culture cells on coverslips and fix as in Appendix 1, 1.
2. Imunolabel cells on coverslips as in Appendix 1, 4a-g.
3. Wash with 0.05M sodium cacodylate buffer pH 7.2.
4. Post-fix with 2% aqueous osmium tetroxide.
5. Dehydrate with a graded series of ethanol.
6. Critical point dry.
7. Replicate with platinum and carbon.
8. Remove cells and replica from coverslip by immersion in concentrated hydrofluoric acid.
9. Rinse with distilled water.
10. Mount replicas on uncoated grids and examine.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.