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MICROBIOLOGICAL IMMUNOCYTOCHEMISTRY:A REVIEW OF CURRENT TRENDS AND APPLICATIONS

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Abstract

It has been considered worthwhile to update previous reviews of microbiological immunocytochemistry in order to identify areas of current importance in this continually expanding area of research. Publications in virology, bacteriology and protozoology indicate a continued interest in immunocytochemistry. Deployment of colloidal gold techniques is almost universal in these applications. The post-embedding technique was the most widely applied technique although a few studies employed the immunonegative stain, pre-embedding and immunoreplica techniques, thereby reflecting the use of colloidal gold in all other areas of the biological sciences.

Key words: Gold immunocytochemistry, microbiology, virus, bacteria, protozoa, fungi.

Introduction

Viruses, bacteria, protozoa, algae, fungae and helminths compile a wide, varied group of organisms grouped together and defined as the microbiological organisms. Many of these organisms are beneficial to man, his crops and his animals and many are extremely harmful. There is therefore, a complex and tenuous relationship between man and microbiological organisms. The study of microbiological organisms can be grouped in five areas, medical microbiology, soil and agricultural microbiology, food and dairy microbiology, water and wastewater microbiology and industrial microbiology. The fact that a study of these organisms could include human pathogens such as the AIDS virus, soil dwelling organisms which affect man's crops, organisms which ferment fruit juices into wine, organisms which spoil beaches for bathing and organisms genetically engineered by man which produce large quantities of pharmacologically useful proteins underlines the diversity and importance of the group.

Immunocytochemical techniques have recently undergone accelerated development and the emphasis presently resides in their applications. Colloidal gold immunocytochemistry is now at the forefront of these applications. The importance of colloidal gold immunocytochemistry in microbiology is explained in recent reviews (Beesley 1987, 1988, Patterson and Verduin, 1987). The first of these reviews concentrates exclusively on medical microbiology, the second attempts to span the whole of microbiology to the beginning of 1987. It is felt necessary to update these reviews with emphasis on results of the last year, thus enabling the literature review to be brought entirely up-to-date and permitting the identification of areas of importance in microbiological immunocytochemistry.

Materials and Methods

The philosophy of the application of colloidal gold immunocytochemistry to microbiology has been fully explained and detailed in a previous review (Beesley 1988). It would be beneficial to reiterate briefly, several important factors of the technique.

Colloidal gold probes, being small, extremely dense and particulate are almost the universal

choice for microbiological immunocytochemistry and may be used over the whole spectrum of applications from the detection of microbiological proteins in infected host cells down to the identification of small virus particles (Beesley and Betts, 1987). Production of gold probes, which are usually protein A- gold complexes, antibody-gold complexes or streptavidin gold complexes is straightforward and their applications are simple.

The use of an antibody, highly specific for its antigen, with no unwanted cross-reactions and of high titre and affinity for its antigen is recommended. The choice of whether to select a monospecific or polyspecific antibody is usually governed by the availability of an antibody and its immunolabelling characteristics rather than by a predetermined selection of either a monospecific or a polyspecific antibody. Depending on the particular antigenic structure involved, a 1% glutaraldehyde fixative may often be tolerated when using polyspecific antisera and, in some situations, also be acceptable in regimes employing monospecific antisera. Routinely, tissue fixation in preparation for immunolabelling with a monospecific antibody employs 4% buffered formaldehyde, freshly prepared from paraformaldehyde.

Phosphate buffered saline, pH 7.2, containing 1% bovine serum albumin (Slot and Geuze, 1984) is a universal buffer with which to dilute antibody and gold probe. A short preincubation of the tissue with 1% gelatine in phosphate buffer reduces non-immunological attachment of antibody and gold probe, and free aldehyde groups on the tissue may be quenched by pretreatment in 0.02M glycine in phosphate buffered saline.

There are several different immunoelectron microscopical techniques employing colloidal gold probes (Beesley 1988). The pre-embedding technique is a high resolution technique for localising external antigens on tissues, whilst the post-embedding technique is used for localising internal antigens. The immunonegative stain technique however, is used for the localisation of antigens on small particles such as viruses and bacterial pili. The immunoreplica technique is a high resolution technique for the localisation of viral antigens on cultured cells whereas the immunoscanning electron microscope technique is used for surveying large surfaces, such as regions of the gut for external antigens. Finally, the immunofreeze-fracture technique is employed to localise antigens on the fractured faces of membranes. The techniques most widely employed in microbiology are the pre-embedding, post-embedding and immuno-negative stain techniques.

Samples are usually prefixed with an aldehyde before pre-embedding immunolabelling. This prevents internalisation or redistribution of the antigen-antibody complex. The tissue is incubated with antibody and colloidal gold probe before fixation with glutaraldehyde, osmium tetroxide and uranyl acetate and subsequent embedding in an epoxy resin. Ultrathin sections, stained with uranyl acetate and lead citrate reveal high quality ultrastructure with good definition of immunolabelling.

Ultrathin sections of the tissues are prepared in anticipation for immunolabelling using the post-embedding technique. The tissue may be embedded in methacrylate (Cramer et al 1986), Epon (Bendayan and Stevens 1984), Araldite (Van Noorden and Polak 1985), LR White (Yoshimura et al 1986), Lowicryl (Carlemalm et al 1982) or frozen sucrose (Tokuyasu 1986). A recent report (Ingram et al 1988) has detailed the necessity of etching sections of Plasmodium falciparum embedded in LR White with an alcoholic solution of sodium hydroxide followed by sodium metaperiodate in order to achieve optimal immunolabelling of internal antigens. After immunolabelling, the antigen-antibody-probe complex is stabilised and the preparations are contrasted by routine methods. The post-embedding technique now permits the high resolution localisation of antigens thereby helping to realise the importance of the colloidal gold technique in the biological sciences.

The immunonegative stain technique is a simple, rapid, high resolution technique. The antigen is dried onto a carbon and plastic coated electron microscope grid and immunolabelled in situ by floating the grid sequentially on droplets of antiserum and colloidal gold. The antigen is finally contrasted with any one of the routine negative stains. This high resolution technique is very quick, very simple and it may be carried out using minute quantities of antigen. It is useful therefore, for both diagnostic and research studies.

The gold probes are particulate and distinct. By use of two different sized probes, double-labelling of two antigenic sites on a tissue may be accomplished. Furthermore, the immunolabelling may be quantified simply by counting the gold probes attaching to antigens. In most cases, counting of gold probes can be used for quantitative comparison between different samples which are immunolabelled under identical conditions. This, of course, is different from the absolute quantitation of antigens which is now being tentatively explored (Griffiths and Hoppeler 1986). Quantitation is further complicated by variations in gold probe size and the prevailing experimental conditions. Considerable care must therefore be exercised during the interpretation of these experiments. These applications are particularly advantageous in the application of colloidal gold technology.

Results and Discussion

Virology

There is continued interest in virology. Most of the studies reported have used the post-embedding technique; three however, employed the immunonegative stain technique, whilst one employed the immunoreplica technique.

Pekovic et al (1986) report the application of the post-embedding technique to human lymphotropic retrovirus HTLV-III/LAV particles in thin sections of H9 cells. They believe that the technique may be of relevance for simultaneous labelling of viral antigen and detection of infected cell phenotype, using two antisera conjugated with gold particles of different sizes. These authors report the use of the monoclonal antibody P24 which resulted in heavy gold labelling of the

viral particles located on the cell surface, and also in immunolabelling of nascent particles budding from the plasma-membrane. The plasma-membrane itself was weakly immunolabelled. Purified 1gG from an AIDS patient showing specificity against HTLV-III/LAV labelled both viral particles and intracellular antigens in the cytoplasm and chromatin. These authors summarise that the immunogold post-embedding technique appears to be a precise, and sensitive method for both localising viral antigens in infected cells and for the characterisation of antibody specificity. It must however, be remembered that there is always a possibility that either non-specific immunolabelling or incomplete immunolabelling will confuse the sensitivity and precision of the technique.

The role of multivesicular bodies and chloroplast invaginations in tomato bushy stunt virus in Gomphrena globosa cells has been elegantly studied by autoradiography and immunogold labelling of ultrathin sections (Appiano et al 1986). Rabbit antiserum raised against tomato bushy stunt virus immunolabelled the cytoplasmic virus particles and dense granules in infected cells. Multivesicular bodies however, appeared almost unlabelled. By combining this data with autoradiography, the authors conclude that different stages of tomato bushy stunt virus replication occur at different sites. Viral RNA is replicated in multivesiculate bodies, coat protein is made by cytoplasmic ribosomes, and excess protein is deposited in the dense granules. The site of encapsulation of viral RNA is not clear, though it is thought to take place in the cytoplasm.

The silver enhancement technique (Holgate et al 1983) is a colloidal gold amplification technique usually reserved for light microscopy but Marchetti et al (1987) have applied the technique to the intensification of gold directly on ultrathin sections of tissue embedded in Epon. The gold immunolabelling localised Mouse Mammary Tumour Virus antigens in cultured cells derived from mouse mammary tumours. The authors obtained heavy immunolabelling of viral particles, visible even at low microscope magnifications, with negligible background labelling. They feel that the technique could be usefully combined with very small gold particles for high immunolabelling intensity, of high resolution and good definition.

The protein A-gold post-embedding technique has been used by Landini et al (1987) to localise human cytomegalovirus structural proteins p65-69 and p28 both within viral particles and within infected host cells. Protein p65-69 is present in circular structures in the nuclei which are often in contact with the viroplasm. This protein can be detected within the dense body matrix in the cytoplasm. Protein p28 in contrast is present only on the outline of cytoplasmic capsids and on dense bodies which are abundant during the late phases of the viral replication cycle.

Inclusion bodies are ultrastructural markers of hantavirus-infected cells (Tao et al 1987). Three distinctive types of inclusion body occur, granular, granulofilamentous and filamentous. Virus specificity of the inclusion bodies was verified by the authors by use of immunogold labelling for thirteen strains of hantavirus.

Puvion-Dutilleul (1987) localised viral-encoded 21kDa protein in herpes simplex virus type I infected cells by immunoperoxidase and immunogold markers. During early infection the protein was demonstrated in ribosome-rich cytoplasmic areas and in the viral DNA-containing fibrillo-granular material of the virus-specific electron-translucent region of the nucleus. Later, an accumulation of the protein occurred in both fibrillar and granular components of the nucleolus. No immunolabelling occurred in the absence of DNA replication and inhibition of RNA synthesis did not alter the protein distribution. Persistence of cytoplasmic and nuclear protein following inhibition of protein synthesis, late in infection, indicated that the distribution of 21kDa protein represented in part, sites of accumulation and retention of existing molecules.

Hills et al (1987) use low temperature fixation to facilitate gold labelling of intracellular sites of structural and nonstructural tobacco mosaic virus proteins in infected tobacco leaf cells. In cells of TMV-infected tissue, the 126K nonstructural protein (replicase) was limited to the viroplasms and in pockets of virus particles at the viroplasmic periphery, suggesting that these are the sites of virus replication.

Wiley et al (1987) and Narang et al (1987) have reported studies on scrapie infected hamster brains. Wiley et al (1987) report the examination of scrapie infected hamsters for the presence of structures antigenically related to the prion protein (PrP 27-30). Using the colloidal gold technique, they observed that their antibodies labelled 7-17nm diameter filaments. Most of the periventricular and perivascular filaments were extracellular, although some were within processes intimately associated with ependymal cells and degenerating membranes of astrocytes and neurites,

Narang et al (1987), however, employ the immunonegative stain technique to investigate the relationship between large masses of tubulofilamentous structures with irregular fuzzy coats found in hamsters' brains infected with the agent of scrapie and similar tubulofilaments found in mouse brain infected with Creutzfeldt-Jakob disease agent. They could strip the outer coat to reveal thinner fibrillary structures resembling scrapie-associated fibrils which were then identified using colloidal gold immunocytochemistry. As a consequence, they conclude that scrapie-associated fibrils may occur as an internal component of larger structures.

Frankel et al (1987) used the immunonegative stain technique for demonstrating that host cell membranes entrapping Theiler's virus did not incorporate viral capsid antigen.

The use of the immunonegative stain technique for the identification of virus particles in electron microscope diagnosis has been considered and reviewed by Beesley and Betts (1987). They found that the labelling obtained was specific, definitive and very little background labelling occurred. The technique itself is sensitive, very quick, and since a minimum of preparation is needed, appears to possess considerable potential for virus diagnosis.

Bohn et al (1987) report the only recent application of the immunoreplica technique in virology. They examined the dynamic processes of measles virus budding on infected HeLa cells. They conclude that the data suggests that measles virus morphogenesis at the plasmamembrane of cultured cells is dependent upon the function of the cytoskeleton and may be influenced by Ca^{2+} ions.

The use of recombinant DNA techniques employing bacteria was introduced in a previous review (Beesley 1988). These techniques, using viruses (Figure l) as well as bacteria (Figure 2) are assuming increasing importance for the production of pharmaceutically useful proteins. Immunocytochemistry is proving an invaluable technique for monitoring the appearance and location of these products.

Bacteriology

The majority of bacterial antigens studied recently have been localised using the post-embedding technique. There have been several reports detailing studies of Escherichia coli antigens. Kohring and Mayer (1987) reported the distribution of ECorR1 methylase and restriction endonuclease in E. coli cells. Immunolabelling with specific antibodies raised against methylase indicated that two thirds of the labelling occurred in the cell cytoplasm, the remainder occurring at the cell envelope, whereas the use of antibodies raised against restriction enzymes showed only 10-30% of antigen in the cytoplasm, the majority being in the cell envelope. These results coincide with the author's proposed function of the enzymes : cytoplasmic methylase protects the cell DNA from self destruction and the restriction endonuclease cuts foreign DNA whilst entering the cell. Tacket et al (1987) employ the immunonegative stain technique to localise a putative colonisation factor on fimbriae of E. coli. Bayer et al. (1987) however, study the intracellular localisation of thioredoxin in E. coli using post-embedding immunolabelling and correlate the results to previous biochemical data which had suggested that thioredoxin occurs at the inner-outer membrane adhesion sites. Indeed, they report that immunolabel was frequently found at bridges between the inner and outer membranes.

Abraham et al (1987) have used immunogold labelling to substantiate their biochemical findings that about 28% of membrane bound nitrate reductase of Klebsiella aerogenes appears to be tightly associated with the peptidoglycan. This was achieved by positive immunogold labelling of SDS-murein sacculi and thin sections of plasmolysed bacteria.

Surface structures other than frimbriae of bacteria are often important and several studies have detailed antigens occurring on the bacterial surface. Ericson et al (1987) studied the binding sites for beta 2-microglobulin on the non-fibrillar surface structures of mutans streptococci by immunogold as well as beta 2-microglobulin-gold probes. It is interesting to record that for grid-mounted bacteria, the gold was mostly bound in clusters at the periphery of the cells, whereas when the probes were reacted in suspension with bacteria before mounting on the grid, a more even distribution of the probes occurred, but the bacteria, as expected, were aggregated.

Reid et al (1987) studied the immunocytochemical characterisation of a polysaccharide antigen on the surface of Bacteroides fragilis with an IgM monoclonal antibody. Lam et al (1987) employed immunocytochemical techniques to visualise 0 antigens, surface antigens of Pseudomonas aeruginosa, using a protein A- dextran- colloidal gold probe and also with monoclonal antibodies. When the monoclonal antibodies were attached to the cell surface of the bacteria, the foldings and other topographic details could not be visualised by negative staining. Monoclonal antibody pretreatment of the cells resulted in the stabilisation of the lipopolysaccharide structure which prevented the long o-antigen chains from collapsing during the processing necessary for the preparation of ultrathin sections.

Bailey et al (1987) produced monoclonal antibodies to the major axial filament polypeptide of Treponema pallidum. Immunolocalisation of the 37kDa antigen showed it to be on the surface of the axial filament structure. The authors believe that the early appearance of specific antibody to this polypeptide in infected man and rabbit indicates that such monoclonal antibodies are potentially useful for the diagnosis of early syphilis. Post-embedding immunolabelling of these small organisms confirm these observations since immunolabelling often appears to be arranged in longitudinal rows spiralling the organism (Figure 3).

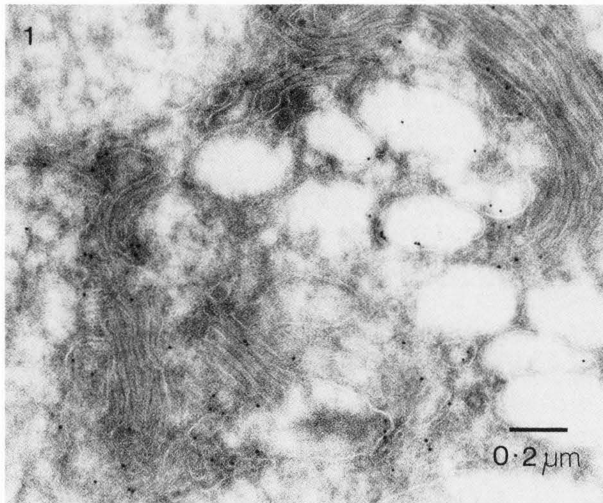
In addition, Baseman et al (1987) identified a 32 kDa protein of Mycoplasma pneumoniae to distinguish the haemadsorption-negative mutant class protein profile from the wild type haemadsorbing strains. This protein clusters at the tip of the organisms.

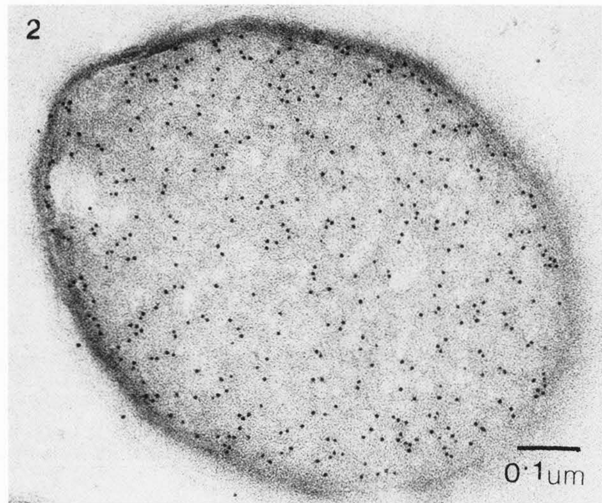
The importance of immunocytochemical studies for characterising surface structures of bacteria has been stressed by Robinson et al (1987b). This group has several recent publications examining antigens of Neisseria gonorrhoeae (Robinson et al 1987a, 1988, Hitchcock et al 1988), as well as a recent publication examining the regulation of pyelonephritis-associated pili expression in E. coli (Low et al 1987).

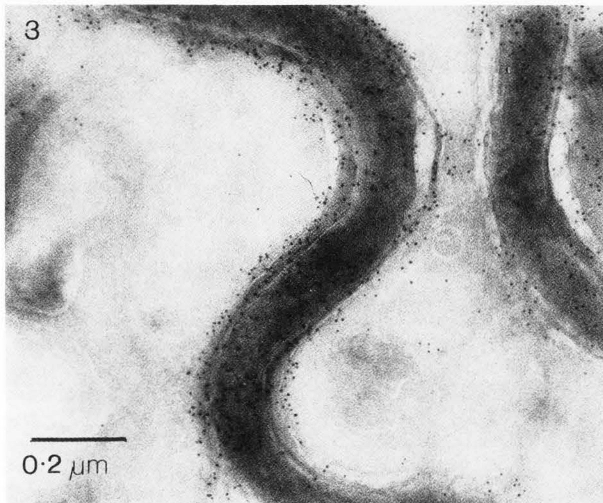
Protozoa

Sinden et al (1987) combined light microscopy, scanning electron microscopy and also transmission electron microscopy in their study of the 21kDa ookinete antigens of Plasmodium berghei. They show the presence of this surface antigen on the macrogamete, zygote and oocyst. It is distributed evenly over the entire surface, but is readily shed from the parasite surface. In addition, the authors detail the potential of the silver enhancement technique in parasitological research. Nagasawa et al (1987) also reported studies of plasmodium antigens, their studies of circumsporozoite proteins in P. ovale oocysts being the first evidence that circumsporozoite protein is present in oocyst sporozoites and sporoblasts of P. ovale.

Nicolas et al (1987a, 1987b) published two papers on the localisation of luciferase in dinoflagellates. One of these papers (Nicolas et







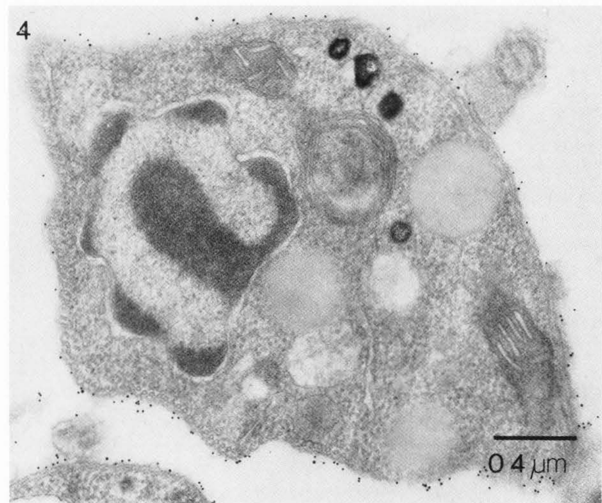


Figure 1. Demonstration of the production of malarial antigens by Baculovirus infecting cultured Spodoptera frugiperba cells. The infected cells were fixed with 4% formaldehyde then cryoprotected with 2.3m sucrose for 1h. After freezing in liquid nitrogen slush, thin sections of the cells were prepared and immunolabelled with specific antibody and the 10nm gold probe. Immunolabelling of specific sites was visualised when the preparations were contrasted with uranyl acetate before embedding in 1.5% methyl cellulose also containing 1% uranyl acetate.

Figure 3. Ultrathin frozen sections of Treponema pallidum prepared as in Figure 1 and immunolabelled with antibody and the 5nm gold probe. The gold probes appear in longitudinal bands spiralling the organisms indicating that the antibody may be specific for the axial filaments.

Figure 2. Escherichia coli cells genetically engineered to contain human interleukin 1β. The cells were prepared as in Figure 1. Immunolabelling shows the protein to be localised, presumably as a soluble protein within the cytoplasm of the cell and not aggregated into dense inclusion bodies.

Figure 4. The pre-embedding technique using Leishmania parasites. The organisms were incubated with biotinylated antibody raised against a membrane surface protein followed by a streptavidin-gold complex. The organisms were fixed with glutaraldehyde, osmium tetroxide and uranyl acetate, then dehydrated in ethanol, embedded in epoxy resin and sectioned. The sections were stained with uranyl acetate and lead citrate and show immunolabelling associated with membrane proteins.

al 1987b) describes the distribution of luciferase in three bioluminescent dinoflagellates as well as two in species of Pyrocystis and Noctiluca. The antibody bound to dense vesicles which correspond in size and position to light emitting bodies detected previously. The possibility is discussed that a protein, different from luciferase but possessing some antigenic similarity, is present in trichocysts and related organelles.

The other paper (Nicolas et al 1987a) is a detailed account of luciferase localisation in the characterisation of the bioluminescent organelles in Gonyaulax polyedra after fast freeze fixation and freeze substitution. As in the previous report, mention is made of the cross reacting antigen occurring in the trichocysts, a non bioluminescent organelle.

Immunogold labelling using a monoclonal antibody, F3 G3, against a protein that induced protection in mice failed to react with the surface of Toxoplasma cells (Sibley and Sharma 1987). After disrupting the cells with Triton X-100 an immunoperoxidase technique localised the protein beneath the surface membrane in the region of the pellicle and within the elaborate vesicles which are released from the cells during entry into host cells. These results show that the epitope recognised by the monoclonal antibody is localised beneath the cell surface membrane and is contained within plasma membrane derived vesicles.

Sudar et al (1986) prepare lactoferrin-gold complexes as a probe for DNA. This probe bound to the ciliary membrane of Tetrahymena, and appeared in coated pits and intracytoplasmic vesicles. After 1h, the gold probes had advanced to a juxtanuclear position. These studies further our knowledge of the cycling of membrane DNA.

A developmentally regulated surface glycoprotein (gp80) has been suggested to mediate the EDTA resistant cell cohesion of Dictyostelium discoideum to form tight aggregates (Choi and Siu 1987). Immunolocalisation of this protein with colloidal gold indicated a non-random distribution of the protein and further, in addition to contact regions, gold particles were found to be localised on filopodia. A distinct advantage of colloidal gold is its particulate, electron dense appearance so useful for quantitation. These authors determined that almost two thirds of the antigen was localised in contact regions between organisms, compared with non-contact areas and the filopodial surface possessed a two-fold higher gold density than the remaining surfaces. This study successfully combines a quantitative approach to immunocytochemistry with transmission and scanning electron microscopy.

Kuo and Chi (1987), compared the microimmunofluorescence test with colloidal gold probes for detecting surface antigens of Chlamydia trachomatis. They found a similar serological specificity between the two techniques in that serovar- and subspecies specific epitopes were the most surface accessible whereas the species- and genus-specific epitopes were the least exposed on the surface of the cells.

There appears to be a scarcity of immunocytochemical studies on Leishmania parasites. Our work continues to show the usefulness of the pre-embedding technique for examining external membrane antigens of these parasites (Figure 4).

Finally, there has been a very recent report (Olenick et al 1988) investigating a flagellar pocket membrane fraction from Trypanosoma brucei rhodesiense. Information on possible common or nonvariant antigens within these protozoa is limited. The authors used immunogold probes for labelling live trypanosome suspensions, which revealed specific staining of the parasite surface at the emergence of the flagellum from the flagellar pocket. This is the first report indicating the combined cellular localisation, nonvariant nature, and protective potential of a membrane fraction from African trypanosomes.

Fungi

Fungal antigens have not been studied widely. During the past year there has been only one immunocytochemical study reported (Wethered et al 1987). These authors studied the differences in fine structure and antigenic determinants of mycetoma fungi in the mycelial phase in vitro and in grains in vivo to facilitate the interpretation of grain formation. Antibodies raised against the fungi in vitro were shown by indirect immunogold labelling to bind to similar sites in fungal material grown in vitro. The grain matrix was not immunolabelled and this suggested to the authors that the structure formed in vivo was composed either of modified antigen or was host derived.

Conclusions

This review has considered the state of microbiological immunocytochemistry throughout 1987 and the early period of 1988. It can be seen that there is continued interest in bacteria, viruses and protozoa. These are the highly infectious organisms which interact, sometimes disastrously with man, his animals and his crops. Colloidal gold probes were the most widely used microscopically dense immunocytochemical marker. The majority of studies employed the post-embedding technique, although some used pre-embedding and immunonegative stain and immunoreplica techniques. Very few studies used the immunoscanning and immunoreplica techniques. This profile reflects the usage of these techniques in the biological sciences.

In conclusion then, it appears that there is still keen interest in microbiological immunocytochemistry and colloidal gold is the method of choice. Colloidal gold techniques are simple and reproducible. The labelling is extremely versatile and is readily apparent, leading to qualitative, quantitative and double-labelling studies, thereby indicating that in microbiology, as in all other areas of biological sciences, colloidal gold is at the forefront of immunocytochemical techniques.

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Fig. 3 is from a collaborative study with Drs. A. Cockayne, D. Strugnall and C. Penn, Department of Microbiology, University of Birmingham, U.K.

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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.