

## A METHOD FOR STAINING INTRACELLULAR ANTIGENS IN THIN SECTIONS WITH FERRITIN-LABELED ANTIBODY

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### INTRODUCTION

Localization of macromolecules in biological specimens by the use of ferritin-conjugated antibody was first investigated by Singer and Schick (1961), and since that time the technique has been used to stain extracellular antigens by several workers (Baker and Loosli, 1966; Easton et al., 1962; Morgan et al., 1961). The identification of intracellular antigens was hampered by the lack of adequate penetration of the conjugated ferritin into cells. It was therefore necessary to disrupt the cell membranes by various methods such as dissection of tissue with a razor blade (Andres et al., 1962) or by freezing and thawing (Morgan et al., 1963) to allow access of the ferritin-conjugated antibody to the intracellular antigens.

A better method would be the application of the ferritin-conjugated antibody to thin sections of embedded cells so that those antigens on the surface of the sections would be able to combine with the antibody. Two main problems in applying this technique are the destruction of the antigenic determinants by the fixation and embedding and the nonspecific attachment of conjugated antibody to the surface of the embedding plastic (Striker et al., 1966). These problems have been partly overcome by McLean and Singer (1964) by the use of cross-linked polyampholyte and, more recently, by the use of cross-linked bovine serum albumin (McLean and Singer, 1970). A review of studies on the problems of staining of thin sections with the immunoferritin technique has been presented by Sternberger (1967).

We report here the results of experiments dealing with the staining of intracellular antigens in thin sections of cells fixed in formaldehyde and em-

bedded in the water-soluble medium, glycol methacrylate (GMA).<sup>1</sup> In the one case the cells were adenovirus-infected cells (Shahrabadi and Yamamoto, 1970), and in the other the cells were either *Micrococcus sodonensis* or purified cell walls of *M. sodonensis*.

### MATERIALS AND METHODS

Antiserum to canine adenovirus and vaccinia virus was obtained from rabbits hyperimmunized with virus purified by two centrifugations in CsCl. Purified *Micrococcus sodonensis* cell wall preparations (less than 0.01% phosphate) and rabbit antisera to the cell walls were obtained from Dr. J. N. Campbell, Department of Microbiology, University of Alberta. In all cases the antibody was separated in the  $\gamma$ -globulin fraction from the sera by passage through a diethylaminoethyl (DEAE)-sephadex column (Dedmon et al., 1965). Ferritin (6  $\times$  crystallized, Cd-free; Polysciences, Inc., Rydal, Pa.) was conjugated to the  $\gamma$ -globulin by using toluene-2,4 diisocyanate (Singer and Schick, 1961). A canine adenovirus (Ditchfield et al., 1962) was used to infect a canine cell line, MDCK (Madin and Darby Canine Kidney—American Type Culture Collection, Rockville, Md.). Cells were grown in monolayer in Minimal Eagle's Medium (Gibco, Grand Island Biological Co., Grand Island, N. Y.) containing 5% calf serum and infected with virus at a multiplicity of 100 plaque-forming units (pfu) per cell. At 18 hr after infection the cells were harvested, fixed in 4% formaldehyde in 0.1 M phosphate buffer 7.2 at 4°C for 30 min, washed in the same buffer for 16 hr, and embedded in GMA (Leduc and Bernhard, 1967). *M. sodonensis* cells and purified

<sup>1</sup> The following abbreviations were used in this paper: DEAE, diethylaminoethyl; GMA, glycol methacrylate; MDCK, Madin and Darby Canine Kidney; pfu, plaque-forming units.

cell walls of *M. sodonensis* were similarly fixed and embedded. Sections were cut with a diamond knife and floated on distilled water. The ferritin-conjugated antibody was allowed to react with the antigens in the section by transferring the sections with a wire loop to a 0.05 M phosphate buffer solution pH 7.2 containing ferritin-conjugated antibody at a concentration of 0.5–1 mg protein/ml. The reaction was allowed to occur for 30 min at room temperature. In order to eliminate nonspecific reactions the conjugated antibody was absorbed twice with rabbit liver and/or culture cell homogenates before its application. Excess conjugate was removed by floating the sections on three 3-min changes of phosphate buffer in a porcelain spotting dish. The sections were then picked up on grids supported by a carbon-coated formvar film and rinsed in distilled water. After drying, the sections were stained with uranyl acetate. In order to test the specificity of the reaction the controls were prepared as follows: (a) treatment of sections with ferritin conjugated to unimmunized rabbit  $\gamma$ -globulin, and (b) treatment of sections with ferritin conjugated to heterologous (vaccinia) antibody.

## RESULTS

The canine adenovirus-infected cell 18 hr after infection is characterized by the appearance of virus particles, light-staining inclusions, and dark-staining inclusions consisting of protein (Yamamoto, 1969; Yamamoto and Shahrabadi, 1971). The relationship of these inclusions to virus replication is unknown, but the inclusions were suspected to contain virus structural antigens. When a section of infected cell was treated with ferritin conjugated to the antibody of the canine adenovirus, it was possible to show strong attachment of the ferritin conjugate to the virus particles as shown in Fig. 1. The specificity of this attachment is indicated by the lack of attachment of ferritin to the cell area surrounding the virus particles. Fig. 2 is a similarly treated portion of the cell nucleus showing a light-staining protein inclusion. The ferritin conjugate can be observed to stain this inclusion, indicating the presence of virus antigen in these bodies. The specificity of this reaction was tested by using both ferritin conjugated to antibody of vaccinia virus and ferritin conjugated to unimmunized  $\gamma$ -globulin. In both cases there was no attachment of ferritin to the adenovirus-infected cells. Fig. 3 shows such a negative reaction of ferritin conjugated to vaccinia virus antibody with a section of adenovirus-infected cell.

In order to test the application of this technique

to another antigen-antibody reaction, whole cells and purified cell walls of *M. sodonensis* were tested for their antigen reaction in thin sections. Conjugation of ferritin to the cell wall antibody was done as described earlier. Sections of cell walls in the purified preparation were found to specifically stain with the ferritin conjugate. Fig. 4 shows the heavy attachment of ferritin molecules to the purified cell walls. Similarly, the cell walls of whole cells could be shown to stain specifically with the ferritin conjugate as in Fig. 5. In contrast, when the cell wall sections were treated with ferritin conjugated to heterologous (adenovirus) antibody, there was no staining of the cell wall with ferritin (Fig. 6).

## DISCUSSION

This method of directly staining intracellular antigens on thin sections with ferritin-conjugated antibody has proven to be successful for antigens produced in a eucaryotic cell and procaryotic cell. Also, its specificity was demonstrated to be excellent provided the antibody was purified as  $\gamma$ -globulin and absorbed with cell homogenates as described.

The advantages of being able to stain with ferritin on thin sections are, firstly, that any number of antigen-antibody reactions can be carried out on a single block of cells, and secondly, that the ferritin conjugate can attach directly to the antigens exposed at or near the surface of the sections without the destruction of membranes to allow access of the ferritin to the site of the intracellular antigens (Morgan et al., 1963).

Although formaldehyde as fixative leaves a lot to be desired, its use was found to preserve the antigens much better than glutaraldehyde. Initial experiments showed that glutaraldehyde largely inactivated the antibody-combining site of intracellular proteins, contrary to their preservation reported by McLean and Singer (1970).

In an early study McLean and Singer (1964) reported the use of polyampholyte as the embedding medium for ferritin labeling, but attempts in our laboratory showed that this medium was difficult to section. More recently, McLean and Singer (1970) were able to use cross-linked bovine serum albumin as the embedding medium. In this study we have demonstrated that GMA also can be used successfully.

An inherent problem which is present in the staining procedure is the nonspecific attachment

of ferritin to the embedding medium and to the supporting film. This nonspecific attachment was eliminated by first floating the sections on the solution of conjugated antibody, followed by washing with phosphate buffer, and then picking them up on coated grids.

One major difficulty with the formalin-fixed, GMA-embedded sections was the spreading of the sections when floated on water or phosphate buffer during the washing stages, resulting in some loss of preservation. This problem could be partially reduced by the use of a low concentration of bovine serum albumin in the washing solutions.

#### SUMMARY

The staining of both viral and bacterial antigens in thin sections of cells has been demonstrated by using formaldehyde fixation and GMA embedding. The specificity of these reactions indicates the possible general application of this technique to localization of intracellular antigens.

This study was supported by a National Research Council of Canada research grant.

Received for publication 1 December 1970, and in revised form 29 January 1971.

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FIGURE 1 Section of canine cell (MDCK) (18 hr after infection) treated with ferritin conjugated to canine adenovirus antibody, showing the ferritin attached to virus particles in nucleus. Marker, 0.5  $\mu$ .

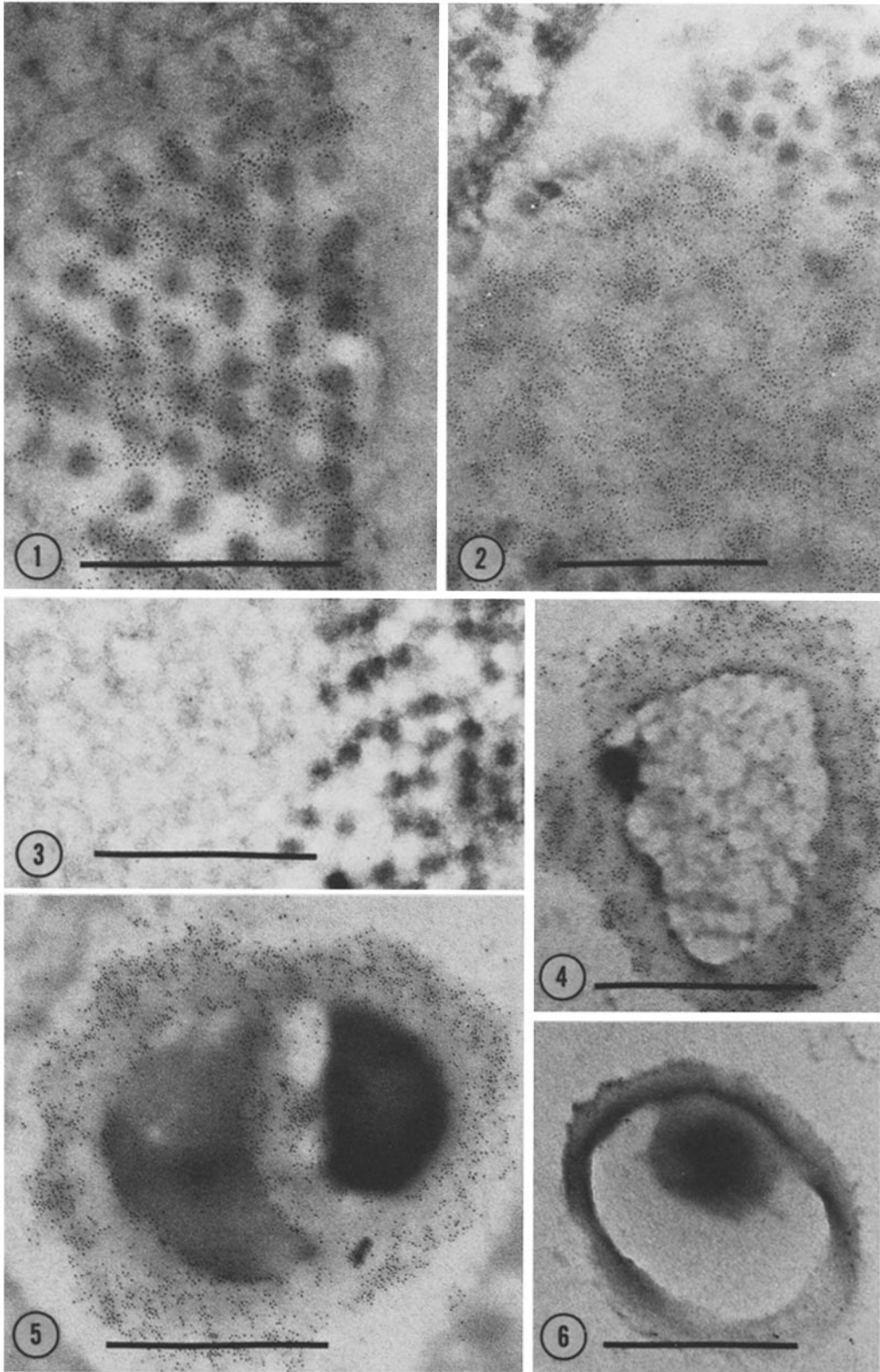
FIGURE 2 Infected cell as in Fig. 1, showing a light-staining inclusion. Ferritin is attached to the inclusion and virus particles.

FIGURE 3 Infected cell as in Fig. 1 but treated with ferritin conjugated to vaccinia virus antibody, showing no attachment of ferritin to either virus particles or inclusion.

FIGURE 4 Section of purified cell wall of *M. sodonensis* treated with ferritin conjugated to *M. sodonensis* cell wall antibody, showing the attachment of ferritin to the cell wall.

FIGURE 5 Section of *M. sodonensis* whole cells treated as in Fig. 4, showing the attachment of ferritin to the cell wall.

FIGURE 6 Cell wall as in Fig. 4 treated with ferritin conjugated to heterologous (adenovirus) antibody, showing a lack of ferritin attachment.



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