An Artificial Test Substrate for Evaluating Electron Microscopic Immunocytochemical Labeling Reactions

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We describe an artificial substrate system for optimization of labeling parameters in electron microscope immunocytochemical studies. The system involves use of blocks of glutaraldehyde-polymerized BSA into which a desired antigen is incorporated by a simple soaking procedure. The resulting antigen-impregnated artificial substrate can then be fixed and embedded identically to a piece of tissue. The BSA substrate can also be dried and then sectioned for immunolabeling with or without chemical fixation and without exposing the antigen to dehydrating agents and embedding resins. The effects of various fixation and embedding procedures can thus be evaluated separately. Other parameters affecting immunocytochemical labeling, such as antibody and conjugate concentration, can also be evaluated. We used this system, along with immunogold labeling, to determine quantitatively the optimal fixation and embedding conditions for labeling of hepatitis B surface antigen (HBsAg), human IgG, and horseradish peroxidase. Using unfixed and unembedded HBsAg, we were able to detect antigen concentrations below 20 μg/ml. We have shown that it is not possible to label HBsAg within resin-embedded cells using conventional aldehyde fixation protocols and polyclonal antibodies. (J. Histochem. Cytochem. 35:909–916, 1987)

KEY WORDS: Artificial test substrate; Immunocytochemistry; Immunogold labeling; Fixation; Embedding.

Introduction

One of the more frustrating tasks for the electron microscopist can be the optimization of conditions used for immunolabeling an antigen within cells or cell organelles. The successful outcome of a labeling experiment is dependent on many factors, including choice of fixative and embedding medium, specificity and concentration of the antibody, duration of incubation in reagents, and so forth. The choice of fixative and fixative concentration, for instance, usually results in a compromise between ultrastructural preservation and retention of antigenicity (13,16).

Optimization of all labeling parameters for a particular antigen can be difficult and time-consuming, particularly if the antigen is sparsely distributed within the tissue or is not contained within easily recognized cell compartments. Absence of label in such situations could be due either to the actual absence of the target antigen or to conditions of tissue processing resulting in reduced antigenicity. Since some antigen–antibody systems appear to be especially sensitive to the respective processing treatments, much time and effort might be saved if one could isolate the antigen of interest and create a cell-free system. Labeling conditions could then be experimentally optimized before attempting to label the antigen where it normally resides within cells.

Recently, Valnes et al. (28) described a system that utilized polymerized BSA as an artificial substrate into which selected antigens could be incorporated by simply soaking the BSA blocks in solutions of the antigens. Once impregnated with antigen, the BSA blocks were treated identically to pieces of tissue. These authors used this system for light microscopical evaluation of immunocytochemical labeling in paraffin-embedded sections. BSA has also been used as an embedding medium for EM immunocytochemistry (16,20) to circumvent the problems posed by resin embedding. We reasoned that a combination of these methods could be employed to appraise immunogold labeling at the EM level. The particulate nature of the colloidal gold probes would allow us to quantitatively evaluate the effects of various treatments on labeling of a target antigen (13). Preliminary results derived from use of this test system have been previously reported (11).

We used this system first to determine the optimal fixation conditions for several antigens without subjecting them to resin embedding, and then to determine the best embedding conditions. Other processing and labeling parameters were similarly evaluated. The antigens chosen to evaluate the system were hepatitis B surface antigen (HBsAg), horseradish peroxidase (HRP), and human IgG. HBsAg is a glycoprotein considered to be immunologically identical to the coats of the hepatitis B virus particles. HBsAg has been labeled in tissue at the EM level in several studies using preembedding techniques (2,12,21) but has not, however, been localized using post-embedding methods. Enzymes are frequent targets for...
immunocytochemical labeling, and HRP was chosen as representative of this group of antigens because of its ready availability in purified form. Immunoglobulins such as IgG are important as target antigens in immunocytochemical studies of Ig-secreting cells and are also used as primary antibodies in indirect labeling protocols. Several studies have reported variable success in localizing immunoglobulins in tissue embedded for EM (26, 29), and we felt that the artificial substrate system described here might be of some use in optimization of processing conditions for labeling this antigen.

Materials and Methods

Production of Artificial Substrate. Artificial substrate blocks were produced by polymerization of BSA with glutaraldehyde, as described by Avrameas and Temynck (3). Briefly, 2 ml of a 2.5% aqueous solution of glutaraldehyde were added to 10 ml of a solution containing 60 mg/ml of crystallized BSA (Penter; Miles Scientific, Naperville, IL) in acetate buffer, pH 5.0. A gel was formed in less than 10 min at room temperature. After approximately 1 hr, the gel was cut into small blocks and washed for least 6 days in PBS to remove residual glutaraldehyde. The artificial substrate blocks were then stored in PBS and used as needed.

Antigen-impregnated substrate was produced by cutting the BSA blocks into 1-mm cubes and soaking for 7-10 days in a solution of one of the following antigens: (a) hepatitis B surface antigen (Abbott Laboratories; North Chicago, IL); 550 μg/ml in PBS; (b) homorastalid peroxidase (Sigma; St. Louis, MO); 500 μg/ml in PBS; or (c) human IgG (Cappel Laboratories; Cochrville, PA) 975 μg/ml in PBS. Some of the antigen-impregnated blocks were then placed on a sheet of paraffin and dried in a desiccator without fixing or embedding. Blocks were stored in this manner for up to several months without noticeable loss of antigenicity. Other blocks were processed further as described below.

Table 1. Embedding protocols

<table>
<thead>
<tr>
<th>Epon–Araldite</th>
<th>Lowicryl K4M, low-temperature embedding</th>
<th>Lowicryl K4M, room-temperature embedding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 50% ethanol, 10 min, 4°C</td>
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<tr>
<td>2. 70% ethanol, 10 min, 4°C</td>
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<tr>
<td>3. 95% ethanol, 10 min, 20°C</td>
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<tr>
<td>4. 100% ethanol, 2 × 3 min, 20°C</td>
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<tr>
<td>5. Propylene oxide, 2 × 3 min, 20°C</td>
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<tr>
<td>6. Propylene oxide:Epon–Araldite 1:1, 2 hr</td>
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<tr>
<td>7. 100% Epon–Araldite, overnight</td>
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<tr>
<td>8. Polymerize at 70°C, 24 hr</td>
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<tr>
<td>Lowicryl K4M, low-temperature embedding</td>
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<td></td>
</tr>
<tr>
<td>1. 50% ethanol, 10 min, 4°C</td>
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<tr>
<td>2. 70% ethanol, 10 min, 4°C</td>
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<tr>
<td>3. 90% ethanol, 2 × 30 min, −10°C</td>
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<tr>
<td>4. 90% ethanol:Lowicryl 1:1, 1 hr, −10°C</td>
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<tr>
<td>5. 90% ethanol:Lowicryl 1:2, 1 hr, −10°C</td>
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<tr>
<td>6. 100% Lowicryl, overnight, −10°C</td>
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<tr>
<td>7. UV polymerization, 24 hr, −10°C</td>
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<tr>
<td>8. UV polymerization, 24 hr, 20°C</td>
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<tr>
<td>Lowicryl K4M, room-temperature embedding</td>
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<tr>
<td>1. 50% ethanol, 10 min, 4°C</td>
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<td>2. 70% ethanol, 10 min, 4°C</td>
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<td>3. 90% ethanol, 2 × 3, 20°C</td>
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<td>4. 90% ethanol:Lowicryl 1:1, 30 min, 20°C</td>
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<td>5. 90% ethanol:Lowicryl 1:2, 30 min, 20°C</td>
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<td>6. 100% Lowicryl, 2 × 30 min, 20°C</td>
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<td>7. UV polymerization, 24 hr, 20°C</td>
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</table>

Table 2. Labeling protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
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<tbody>
<tr>
<td>1.</td>
<td>PBS pre-treatment, 5 min</td>
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<tr>
<td>2.</td>
<td>1% BSA in PBS, 5 min (to block nonspecific labeling)</td>
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<tr>
<td>3.</td>
<td>Incubate on primary antibody, 1–2 hr at room temperature</td>
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<tr>
<td>4.</td>
<td>PBS, 3 10-min washes</td>
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<tr>
<td>5.</td>
<td>1% PEG in PBS, 5 min</td>
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<tr>
<td>6.</td>
<td>Incubate on conjugate, 1 hr</td>
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<tr>
<td>7.</td>
<td>PBS, 2 10-min washes</td>
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<tr>
<td>8.</td>
<td>Rinse in triple-distilled water, dry</td>
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Fixation and Embedding Methods. The ability of fixatives to retain antigenicity was first compared using substrate blocks fixed in 1% glutaraldehyde, 2% paraformaldehyde, or periodate–lysine–paraformaldehyde (PLP) containing 2% paraformaldehyde (10). Glutaraldehyde and paraformaldehyde fixatives were made up in Sorenson’s PO4 buffer at pH 7.4, and blocks were fixed by immersion for 1 hr in the respective fixatives. Fixative concentration was studied using blocks fixed in glutaraldehyde concentrations of 0.25%, 0.5%, and 1%, and paraformaldehyde concentrations of 0.5%, 2%, and 4%. After fixation, the blocks were washed for 1 hr in buffer and then dried on paraffin as above.

Once the best conditions for fixation were determined for each antigen, optimally fixed substrate blocks were embedded in either Epon–Araldite or Lowicryl K4M in order to assess the effect of the embedding medium on immunolabeling. In addition, labeling was compared using material embedded in Lowicryl K4M at low temperature and at room temperature. Dehydration and embedding protocols are summarized in Table 1. The Lowicryl resin formulations were identical for both room-temperature and low-temperature polymerization, except that benzoin ethyl ether was used as initiator for room-temperature polymerization.

Production of Protein A-Gold Conjugate. Colloidal gold (10-nm diameter) was produced by a modification of Frens’ (10) method for reduction of HAuCl4 by sodium citrate, as described by DeMey (9). After adjusting the pH of the gold sol to 6.2, 10 ml of the sol were added to 30 μl of protein A (1 mg/ml in distilled water) with gentle stirring. After 1 min, 2 drops of 1% polyethylene glycol 20,000 (PEG) in distilled water were added (14). The conjugate was then centrifuged for 60 min at 15,000 RPM in an SW-41 rotor. After removal of the supernatant, the conjugate was resuspended to a final volume of 1 ml in PBS containing 1% PEG and was then stored at 4°C.

Immunolabeling Procedure. Dried, resinless substrate blocks were glued to blank Epon–Araldite blocks and sectioned with a diamond knife on an LKB Ultratome III ultramicrotome. The blocks are highly hydrophilic, and it was necessary to lower the water level in the trough considerably to prevent wetting of the block face. Section thickness tended to vary considerably but was not critical, since sections with interference colors varying from silver to dark purple were sufficiently transparent to the electron beam. The sections were collected on parlodion- and carbon-coated nickel grids and were stored in a desiccator until needed.

Immunolabeling was performed by floating the grids section side down on 20-μl drops of the appropriate reagents. Washing steps were performed by floating the grids on the surface of PBS contained in the wells of 96-well tissue culture plates (Corning "Cell Wells"). The plates were placed on a shaker and gently agitated throughout the washing period. The standard labeling protocol used for both resinless and resin-embedded substrate sections is given in Table 2. Resinless sections were examined without further processing, whereas resin-embedded sections were stained for 10 min in 2% alcoholic uranyl acetate. Before incubation with the primary antibody, some sections of Epon–Araldite-embedded material were subjected to pretreatment with saturated sodium metaperiodate (60 min) or 10% hydrogen peroxide (10 min) followed by thorough washing with distilled water.

Primary antibodies were used: guinea pig anti-hepatitis B surface anti-
gen (Abbott); rabbit anti-horseradish peroxidase (prepared by M.F. Miller); and affinity-purified goat anti-human IgG (Cooper Biomedical; Malvern, PA). Antisera were diluted in 1% BSA/PBS. Protein A-gold conjugate (10 nm) was used in the labeling step for HBsAg and HRP. Protein A, however, has a very low affinity for the goat immunoglobulins that were used to label human IgG (18). We therefore used rabbit anti-goat IgG conjugated to 10-nm gold particles (Janssen, SPI Supplies; West Chester, PA) for this purpose. Conjugate dilutions were made using 1% PEG/PBS. Controls included use of "blank" substrate blocks lacking antigen, substitution of normal serum for the specific antiserum, and omission of the antibody incubation step.

**Quantitative Evaluation.** Sections on duplicate grids were photographed at × 10,000 and negatives were then projected on the screen of a slide viewer (Creatron; Floral Park, NY) at a total magnification of × 70,000. The slide viewer was fitted with a high-resolution videocamera (Dage–MTI; Michigan City, IN) connected to a LeMont OASYS optical image analysis system (LeMont Scientific; College Station, PA) which digitized the video image and automatically counted the gold particles in the image. At least three fields from each negative were analyzed. Results were recorded as the density of gold particles/µm² of the region analyzed (5).

**Labeling of HBsAg in Tissue-culture Cells.** We attempted to label HBsAg in mouse Ltk⁺ cells (ATCC L929) expressing HBsAg. The cells were fixed in 50% acetone containing 8% sucrose and were incubated in suspension with guinea pig anti-HBsAg (1:10 dilution, 3 hr, room temperature) followed by 10-nm diameter protein A–gold (1:10 dilution, 1 hr, room temperature). After thorough washing the cells were pelleted, fixed in 3% glutaraldehyde followed by 1% OsO₄, and embedded in Epon-Araldite.

**Results**

**Properties of Artificial Substrate**

Artificial substrate blocks were easily produced in large quantity and could be stored in PBS indefinitely until needed. Blocks stored for more than 1 year were successfully impregnated with antigen and immunolabeled. Sections of unembedded dried BSA substrate adhered well to the parlodion-coated grids and were rarely lost during the immunolabeling procedure. Variations in section thickness had no effect on label density, since only antigens exposed at or near the section surface are accessible to the gold probes (4).

Gold particles were easily visualized over the amorphous, moderately electron-dense background of sections (Figure 1). Sections of resin-embedded substrate stained with uranyl acetate revealed the diffuse fibrillar network of cross-linked BSA. Gold particles tended to be localized over these substrate fibers, indicating that the antigens had been cross-linked to the BSA by the fixative (Figure 2). The distribution of antigen throughout substrate blocks was as-

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**Figure 1.** (a) Electron micrograph of BSA substrate which was soaked in human IgG, fixed in 0.5% paraformaldehyde, dried, then sectioned and labeled with goat anti-human IgG followed by rabbit anti-goat IgG coupled to 10-nm gold particles. Label is much heavier over artificial substrate compared to adjacent area of parlodion support film. (b) BSA substrate treated identically to (a) except that substrate was not soaked in IgG. Only scattered gold particles are present. Bar = 0.5 µm.

**Figure 2.** (a) BSA substrate soaked in human IgG, fixed in 0.5% paraformaldehyde, embedded in Lowicryl K4M at room temperature, and labeled as in Figure 1. Many gold particles are located over BSA fibers. (b) BSA substrate lacking IgG and treated identically to (a). Very few gold particles are present. Bar = 0.5 µm.
sessed by evaluating sections taken from various areas within blocks. No significant difference in label density was observed in any area greater than 50–100 μm inward from the substrate block edge (i.e., approximately the width of one grid square of a 300-mesh grid). Label density was slightly lower near the edge of the block, possibly due to some loss of material during processing.

Figure 3 shows antibody titration curves for the three antigens studied. In all cases, background labeling was very low, as measured by the amount of label present on control sections. There was no significant difference between controls using normal serum and controls using “blank” substrate blocks. Specific labeling could be detected at antibody dilutions of up to 1:10,000 for anti-hepatitis B surface antigen, 1:8000 for antihorseradish peroxidase, and 1:25,000 for anti-human IgG.

The relationship between antigen concentration and label density was examined by labeling sections from substrate blocks that had been soaked in serial dilutions of HBsAg. The results (Figure 4) show a linear increase in gold particle density with increasing antigen concentration. Using this system, we were able to detect HBsAg in substrate sections at concentrations of less than 20 μg/ml of the soaking solution.

**Time of Incubation**

The effect of duration of incubation in the primary antibody step was studied for HBsAg. Sections of unfixed, resinless antigen-impregnated substrate were incubated in anti-HBsAg for 1, 2, and 4 hr at room temperature and overnight at 4°C. All other labeling steps were as given in Materials and Methods. The results showed a twofold increase in labeling density between 1 and 2 hr of incubation and with a much smaller increase between 2 and 4 hr (Figure 5). Incubation overnight at 4°C resulted in counts comparable to those achieved with a 4-hr room temperature incubation. Background levels rose only slightly with increasing length of incubation in antibody. Similar results were obtained on HBsAg that had been fixed in 2% paraformaldehyde (Figure 5).

**Effects of Fixation**

The three antigens differed in sensitivity to aldehyde fixation (Figure 6). Hepatitis B surface antigen showed the greatest reduction of labeling in fixed tissue as compared to the unfixed state, with a nearly 90% decrease in label density. The antigens also differed in response to glutaraldehyde- and paraformaldehyde-containing fixatives. The best labeling for HRP was achieved using glutaraldehyde, whereas paraformaldehyde and PLP were superior for labeling IgG. There was no significant difference in labeling of HBsAg with the various fixatives. In all cases, labeling of PLP-fixed material did not differ significantly from that of material fixed in paraformaldehyde alone.

The antigens also differed in their response to various concentrations of glutaraldehyde and paraformaldehyde (Figure 7). Human IgG was most sensitive to fixative concentration, whereas HRP labeling exhibited little variation throughout the concentration ranges tested.

On the basis of the above data, the following fixation conditions were used in subsequent experiments: 0.25% glutaraldehyde for HBsAg; 0.5% glutaraldehyde for HRP; and 0.5% paraformaldehyde for IgG.

**Effects of Embedding Medium**

Experiments comparing labeling of material embedded in Epon–Araldite and Lowicryl K4M demonstrated a clear superiority of...
Lowicryl for two of the three antigens tested (Figure 8). HBsAg proved to be the sole exception, with Epon–Araldite embedding yielding an almost fourfold greater gold particle density as compared to Lowicryl. For both HRP and IgG, Lowicryl embedding yielded higher label densities and lower background levels compared to Epon–Araldite-embedded material. The difference between embedding media was most striking with IgG, which did not label above background levels using Epon–Araldite yet labeled up to 60 times above background when embedded in Lowicryl K4M (Figure 8). In all cases there were only slight differences in labeling efficiency between material embedded in Lowicryl at −10°C as compared to room temperature.

Figure 5. Effect of incubation time in primary antibody on labeling intensity of unfixed HBsAg (left) and HBsAg fixed in 2% paraformaldehyde (right). Shaded bars represent control sections treated with normal serum. Standard errors are indicated by thin vertical bars.

Figure 6. Effect of fixative on labeling of unembedded HBsAg, HRP, and IgG. Shaded bars represent control sections. Standard errors are indicated by thin vertical bars. U, unfixed; G, 1% glutaraldehyde; F, 2% paraformaldehyde; P, periodate–lysine–paraformaldehyde.

Figure 7. Effects of concentration of glutaraldehyde (open symbols) and paraformaldehyde (filled symbols) on labeling of IgG (triangles), HBsAg (circles), and HRP (squares). Vertical bars indicate standard error.

Figure 8. Effect of embedding medium on labeling of HBsAg (0.25% glutaraldehyde), HRP (0.5% glutaraldehyde), and IgG (0.5% paraformaldehyde). Shaded bars represent control sections. Thin vertical bars indicate standard errors. E, Epon–Araldite; L/R, Lowicryl K4M, room-temperature polymerization; L/L, Lowicryl K4M, low-temperature (−10°C) polymerization.
pared to room-temperature embedding. Pre-treatment of Epon–Araldite sections with oxidizing agents, such as sodium metaperiodate or hydrogen peroxide, failed to enhance labeling and, indeed, in some cases even appeared to cause a reduction in labeling (Figure 9).

**Labeling of HBsAg in Tissue Culture**

We attempted to label HBsAg-producing tissue-culture cells using the optimal fixation and embedding conditions as determined by the artificial substrate system (i.e., glutaraldehyde fixation followed by embedding in Epon–Araldite). The results of the substrate experiments suggested that post-embedding labeling of aldehyde-fixed tissue would be difficult or impossible because of the extreme sensitivity of the antigen to fixation and embedding conditions. Indeed, our initial attempts to label HBsAg in the cells were unsuccessful.

We next evaluated the ability of acetone and osmium tetroxide to retain antigenicity using the artificial substrate technique (Table 3). Use of acetone fixation resulted in approximately a 50% decrease in labeling as compared to unfixed antigen, whereas OsO₄ fixation, like glutaraldehyde fixation, resulted in greater than 90% reduction even after pre-treatment with NaIO₄. Despite our knowledge that acetone would be a poor fixative of ultrastructure, we attempted to fix cells in acetone and carry out an en bloc immunogold labeling experiment.

For fixation of mouse cells, 8% sucrose was added to the acetone solution to reduce osmotic damage to the cells. This treatment resulted in acceptable morphological preservation, although far inferior to aldehyde fixation. Cells were labeled in suspension with guinea pig anti-HBsAg followed by 10 nm protein A–gold. Within the cells, gold label was found associated with amorphous material located in the dilated cisternae of the rough endoplasmic reticulum (Figure 10). Controls incubated with normal guinea pig serum were devoid of label in the endoplasmic reticulum cisternae, although a few scattered gold particles could be found in the cytoplasm of the cells. The latter indicated that the reagents apparently had access to potential sites of HBsAg.

**Discussion**

Test substrates used previously for evaluation of immunocytochemical parameters have included filter paper strips, gelatin models, and agarose beads (16,24). The use of polymerized BSA as an artificial substrate has several advantages over these methods including: (a) easy production and long storage life; (b) simple incorporation of antigen into the substrate by a soaking procedure; (c) handling in a manner identical to a piece of tissue; (d) ability to be thin-sectioned and immunolabeled with or without chemical fixation and without exposure of the antigen to dehydrating agents and embedding resins (this feature allows separate evaluation of the effects of fixation and embedding procedures); and (e) potential for quantitative comparisons using a particulate label such as colloidal gold.

Using the artificial substrate system described herein, we were able to determine quantitatively the best fixation conditions for the three antigens studied. We chose to evaluate fixatives containing glutaraldehyde and paraformaldehyde because fixation of tissues for EM almost always involves the use of one or both of these aldehydes. Although aldehyde fixatives produce excellent ultrastructural preservation, their adverse effects on preservation of antigenicity are well known. Stabilization of tissue structure by aldehyde fixation primarily involves cross-linking of the amine groups of proteins (7) which can either mask or destroy nearby antigenic sites. Conformational changes have been observed in proteins subjected to aldehyde fixation (17). Reaction of aldehydes with proteins can cause a significant drop in tissue pH, even when buffering solutions are used (15).

Formaldehyde has long been considered to be a relatively "mild"
fixative for immunocytochemistry in comparison to glutaraldehyde (27), but evidence indicates that this may not always be true (22). Indeed, paraformaldehyde-containing fixatives were clearly superior in antigen preservation for only one of the three antigens (IgG) used in this study. Of the other two, glutaraldehyde was superior for labeling of HRP, whereas all aldehyde fixatives caused a drastic drop in labeling efficiency of HBsAg. This extreme sensitivity of HBsAg to aldehyde fixation may, when combined with the equally detrimental effects of the embedding medium, explain the failure of attempts to label this antigen by post-embedding techniques.

After optimal fixation conditions for each antigen were determined, we examined the effects of embedding media on labeling performance. Embedding resins can have several deleterious effects on preservation of antigenicity and accessibility of antigen to the primary antibody (8). Resins such as Epon, Araldite, and Spurr contain very reactive epoxy groups which could interact with the peptide groups of protein antigens. Epoxy resins also contain many hydrophobic groups which inhibit water absorption by the section and the necessary formation of a gel layer at the section surface to allow antibody access to the antigen. The hydrophobic nature of the resin also increases the likelihood of nonspecific absorption of antibody molecules to the section surface (8). Acrylic resins, such as Lowicryl K4M, have hydrophilic groups built into them, which should result in greater antibody penetration and lower nonspecific absorption. An additional advantage of acrylics is the ability to utilize milder curing conditions than the heat curing used for epoxy resins. Despite the apparent disadvantages of epoxy resins, many successful immunocytochemical studies have been carried out using epoxy-embedded tissue (e.g., 5,6,23,29).

Pre-treatment of epoxy resin-embedded tissue sections with oxidizing agents, such as sodium metaperiodate and H$_2$O$_2$, has become a common practice, particularly for tissue that has been post-fixed in OsO$_4$ (6). Oxidizing agents can increase the hydrophilic properties of a resin by oxidizing the hydrophobic alkane side chains to alcohols, aldehydes, and acids (8). Hydrogen peroxide also may have a slight “etching” effect on the section, removing some of the resin and thus allowing greater access of the antibody to the antigen. Oxidizing agents, however, may have deleterious effects on sensitive antigens.

In the present study, the overall superiority of Lowicryl over Epon–Araldite, in terms of both intensity of labeling and background levels, may be explained by the greater hydrophilicity of the Lowicryl resin. Polymerization temperature may also be a factor, but reducing polymerization temperatures below room temperature does not appear to be necessary. Altman et al. (1) found no essential difference in labeling intensity of tissue embedded in Lowicryl K4M polymerized at 4°C as compared to −30°C. The superiority of Epon–Araldite over Lowicryl, as seen for HBsAg, could be due to some component of the Lowicryl resin interacting with the antigenic determinants of HBsAg. HBsAg is the only glycoprotein among the three antigens studied in these experiments. It would be interesting to experiment with labeling of other glycoproteins embedded in Lowicryl to determine if this could be a factor.

The use of oxidizing agents failed to enhance labeling for any of the antigens tested. In previous studies, Viale et al. (29) successfully labeled human immunoglobulins in glutaraldehyde-fixed, post-osmicated, and Epon-embedded tissue using H$_2$O$_2$ pretreatment. However, Smart and Millard (26) were unsuccessful using H$_2$O$_2$ pre-treatment for labeling of immunoglobulins in Spurr resin-embedded tissue and had to resort to de-resinizing procedures. Apparently the epoxy resin formulation affects both the retention of antigenicity and the ability of oxidizing agents to unmask hidden antigenic groups.

We were able to localize HBsAg in cultured cells only by using acetone fixation and pre-embedding labeling. The distribution of label was similar to that reported by Aoki et al. (2) using a pre-embedding immunoperoxidase technique. The fact that we were not successful in labeling the cells using post-embedding application of label is not surprising, since other immunocytochemical studies of HBsAg have also had to resort to either pre-embedding labeling techniques (12,25,30) or cryosections of gelatin-embedded tissue (21). We should point out that successful immunogold labeling was accomplished by Patzer et al. (21) only when they prepared a special hyperimmune serum to glutaraldehyde-fixed HBsAg. Applying the label en bloc is, therefore, perhaps the only way to visualize antigens such as HBsAg, which are extremely sensitive to fixation and embedding.

The artificial substrate system can be used to evaluate many other immunocytochemical parameters, in addition to fixation and embedding procedures. Examples include evaluation of various agents used to block nonspecific absorption of immunoglobulins onto sections, effect of gold particle size on label intensity and background levels, and optimization of incubation times and washing procedures, and so on. Because the tests can be performed in an identical manner to the way tissue sections would be labeled for EM immunocytochemistry, the results should be directly applicable to tissue samples. The distribution of an antigen within tissue can vary widely from section to section, making direct comparison possible only in a subjective manner. Uniform distribution of the incorporated antigen throughout the BSA substrate permits direct quantitative comparison of various treatments on different sections taken from the same block. The artificial substrate technique should, therefore, find many uses in assessment of the effects of a wide variety of parameters in EM immunocytochemistry.
Literature Cited


