STUDIES ON TRANSLOCATION OF IMMUNOGLOBULINS ACROSS INTESTINAL EPITHELIUM

I. IMPROVEMENTS IN THE PEROXIDASE-LABELED ANTIBODY METHOD FOR APPLICATION TO STUDY OF HUMAN INTESTINAL MUCOSA

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We adapted the peroxidase-labeled antibody method to localization of immunoglobulins and the secretory component (SC) in human intestinal mucosa at the light and electron microscopic levels. Modifications of the method included (1) the use of digitonin in the first wash step of tissues fixed in periodate-lysine-paraformaldehyde; (2) double coating of glass slides with gelatin and egg albumin before application of cyostat sections, and (3) inactivation of endogenous peroxidase by periodic acid treatment of cyostat sections. With these improvements, tissue morphology was well preserved; antigenicity of immunoglobulins and SC was retained; penetration of labeled antibodies into cellular compartments was complete; and confusion at the light microscopic level between peroxidase-containing cells and cells stained with peroxidase-labeled antibodies was avoided.

In addition to its well known absorptive and secretory functions, the gastrointestinal tract has important immunological functions (24). An appreciation of this has come about largely through the immunohistological demonstration of many immunoglobulin-containing cells and the secretory component (SC) in gut mucosa (3, 4, 7, 20, 23). Nearly all these studies have been conducted at the light microscopic level, usually by immunofluorescence techniques. To further understanding of the immunology of the gut, detailed immunocytochemical examination at the ultrastructural level has been needed. Hence we have applied the peroxidase-labeled antibody method (15, 17) to localize immunoglobulins and

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Dedicated to Professor Hideo Takamatsu in memory of his retirement.

SC in human small intestine. For optimal results, several modifications of the method were required; these were made in accordance with the following principles:

- 1) Morphology of the cells and tissues must be well preserved.
- 2) The antigenicity of the sought antigens *in situ* must be maximally retained.
- 3) Specificity of antibodies against the sought antigens must be well defined.
- 4) The labeled antibodies must gain access to all the sought antigens in the tissue.
- 5) The sought antigens must not be confused with endogenous peroxidase in the tissues.

Modifications of the peroxidase-labeled antibody method that made it suitable for study of intestinal mucosa included 1) addition of digitonin to the first wash step of the periodate-lysine-paraformaldehyde (12) method of fixation, 2) inhibition of endogenous tissue peroxidase by periodic acid, and 3) cementing of cryostat sections to glass slides by a technique that helped preserve tissue ultrastructure.

This report deals mainly with the methodological aspects of the study; the biological aspects are presented elsewhere (6).

MATERIAL AND METHODS

Antibody Preparation. Rabbits were hyperimmunized with purified normal human serum IgG or 19S IgM, myeloma IgA, or free SC from human colostrum. Gamma-globulin fractions of the antisera, precipitated by ammonium sulfate, were rendered antigen-specific by reaction with solid phase immunoadsorbents: anti-IgG was absorbed with Fab fragments of IgG bound to Sepharose 4B (Pharmacia Fine Chemicals, Inc; Piscataway, N.J.); anti-IgM was absorbed with IgA-Sepharose and IgG-Sepharose; anti-IgA was absorbed with IgG-Sepharose; anti-SC was absorbed with IgA-deficient serum Sepharose and a small amount of soluble lactoferrin. Antibody specificity was documented by immunodiffusion in agar gel and immunoelectrophoresis (6). The anti-SC reacted with SC bound to colostrum 11S IgA as well as with free SC; it did not cross-react with anti-lactoferrin.

Fab' fragments of the rabbit gamma-globulins were prepared by digestion with pepsin (Worthington Biochemical Corp., Freehold, N.J.; 1 : 100 w/w) in 0.1 M acetate buffer, pH 4.0, followed by reduction-alkylation with β -mercaptoethanol (0.01 M) and iodoacetamide (0.12 M) (8). The Fab' fragments were isolated by Sephadex G-100 (Pharmacia) gel filtration and labeled with horse radish peroxidase (Type VI; Sigma Chemical Corp., St. Louis, Mo.) as described (16).

Previously prepared (17) rabbit anti-human chorionic gonadotropin (peroxidase-labeled Fab') was used in certain control experiments.

Tissues. Specimens of normal human intestinal mucosa were taken during abdominal operations. Pieces about $2 \times 2 \times 3$ mm were cut and immediately placed in fixative.

Tissue Fixation. Several fixatives were tried: 1) 1% glutaraldehyde for 1 hr, 2) periodate-lysine-2% paraformaldehyde (12) for 4 hr, 3) periodate-lysine-4% paraformaldehyde for 4 hr, 5) periodate-lysine-4% paraformaldehyde for 6 hr with or without digitonin (18, 19) wash, 6) 2% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4, for 4 hr, 7) 2% paraformaldehyde in the same buffer for 6 hr with

or without digitonin wash, 8) 4% paraformaldehyde for 4 hr, 9) 4% paraformaldehyde for 6 hr with or without digitonin wash.

Tissue Mounting. Adherence of cryostat sections of fixed tissues to glass slides throughout lengthy immunohistological procedures is difficult to attain. Therefore, we experimented with several cementing compounds. These included egg albumin as used in conventional histological preparations; egg albumin with ammonium hydroxide (14); commercially available glues, such as rubber cement, Elmer's glue, and Carter's mucilage; 1% or 2% gelatin; watersoluble, pigment-free latex paints. Some slides were doubly coated, first with 6% gelatin then with egg albumin-ammonium hydroxide.

Inhibition of Endogenous Peroxidase. Normal as well as pathological intestinal tissues contain many polymorphonuclear leukocytes and erythrocytes. In enzyme immunohistochemical work at the light microscopic level, pseudoperoxidatic activity of these cells may make them difficult to distinguish from immunoglobulin-containing cells reacting with peroxidase-labeled antibodies (5, 11, 26). The pseudoperoxidatic activity can be abolished by oxidation with periodic acid. A series of experiments, therefore, was performed to determine conditions which would inactivate the endogenous enzyme without reducing the antigenicity of immunoglobulins and SC in intestinal tissues.

Sections mounted on glass slides were incubated with periodic acid in distilled water at concentrations of 0.0005 M to 0.01 M or with 0.005 M periodic acid in citric acid-phosphate buffer adjusted to various H^+ concentrations. To reduce aldehyde groups formed by the periodic acid, the sections were reacted with 0.003 M sodium borohydride in distilled water for 30 min. After incubation of the sections with the peroxidase-labeled antibodies, the intensity of residual endogenous peroxidase activity, antibody staining, and nonspecific background staining were scored.

Some tissue specimens were examined by immunofluorescence techniques as described (4) as well as by the peroxidase-labeled antibody method; the numbers of immunoglobulin-containing cells identified by the 2 methods were compared.

RESULTS

Tissue Fixation. All fixatives except glutaraldehyde satisfactorily retained antigenicity of the immunoglobulins and SC. Cellular ultrastructure was best preserved with 4% paraformaldehyde in 0.0375 M phosphate buffer containing 0.01 M sodium periodate and 0.075 M lysine (final pH 6.2) (12), followed by digitonin wash.

Tissue Mounting. Among the various compounds used for cementing the tissue sections to glass slides, only the egg albumin with ammonium hydroxide provided satisfactory adhesiveness. However, by this method the cells became widely separated during processing for electronmicroscopy. This problem was largely overcome by the use of slides sequentially coated with gelatin and egg albumin-ammonium hydroxide.

Inhibition of Endogenous Peroxidase Activity. Endogenous peroxidase activity of polymorphonuclear cells could be eliminated by periodic acid in water at a concentration of 0.005 M or more with incubation for 5 min or longer (Table I).

TABLE I. *Effect of various concentrations of periodic acid on activity of endogenous peroxidase and antigenicity of IgA in intestinal tissues**

Periodic acid in distilled water	pH	Endogenous peroxidase activity	Intensity of IgA staining	Nonspecific background staining
0.01 M	2.20	—	++	++
0.005 M	2.45	—	++	+
0.001 M	2.90	++	++	+
0.0005 M	3.1	++	++	+

* All specimens were treated with periodic acid for 5 min, sodium borohydride for 30 min, anti-IgA for 2 hr; and diaminobenzidine for 10 min.

Under these conditions, the intensity of immunoglobulin and SC staining was not diminished (Fig. 1). At concentrations of periodic acid of 0.01 M or more, the level of non-specific background staining became unacceptably high.

Increasing the pH of periodic acid to 3 or above reduced its effectiveness against endogenous peroxidase (Table II). That low pH alone was not responsible for the inactivation of peroxidase was shown by ineffectiveness of glycine-HCl buffer (Table II).

The numbers of immunoglobulin-containing cells identified at the light microscopic level in tissues studied by fluorescein-labeled antibodies and by peroxidase-labeled antibodies were similar.

Schedule for Immunocytochemical Study of Intestinal Tissues. Based on the above observations, the following schedule was used for optimal immunocytochemical identification of immunoglobulins and SC in intestinal tissues.

1. Tissue pieces were immediately immersed in periodate-lysine-4% para-formaldehyde (4°C) and gently agitated for 6 hr. The fixative was changed 2 to 3 times.
2. The tissues were washed overnight with PBS (0.01 M phosphate buffer containing 0.15 M sodium chloride, pH 7.2) containing 4×10^{-5} M digitonin and 10% sucrose (2 to 3 changes), then washed with PBS containing 18% sucrose for 2 hr.
3. The tissues were transferred to a solution of PBS containing 5% to 10%

TABLE II. *Effect of periodic acid and variation in pH on endogenous peroxidase activity in intestinal tissues*

	pH	Endogenous peroxidase activity
Periodic acid (0.005 M) in citric acid phosphate*	2.5	—
	3.0	++
	4.0	++
	5.0	++
	6.0	++
Glycine-HCl Buffer (0.2 M)*	2.2	++

* Five min incubation.

a

b

FIG. 1. Immunohistochemical localization of SC in human ileal mucosa by peroxidase-labeled anti-SC. Reaction product indicating the presence of SC is seen on columnar epithelial cells of gland crypts. a). Cryostat section treated with periodic acid to inactivate endogenous peroxidase. b). Untreated control section showing densely-stained, peroxidase-containing polymorphonuclear cells in the lamina propria. Intensity of anti-SC staining of epithelial cells is similar in the 2 sections. $\times 225$

glycerol and 18% to 25% sucrose for 2 hr or longer.

4. The tissues were embedded in OCT (Lab-Tek) in an aluminum foil boat.
5. Frozen sections (6 to 12 μm) were cut in a cryostat at -30°C to -35°C .
6. The frozen sections were mounted on gelatin- and egg albumin-coated slides for electron microscopy and on egg albumin-coated slides for light microscopy.
7. After drying for 30 min in room air, the sections were ready for immunohistochemical staining.
8. For light microscopy:
 - a. The slides were washed with cold PBS for 15 min (3 changes).
 - b. The tissue sections were immersed in periodic acid (0.005 M in water) for 10 min at room temperature, then washed with PBS.
 - c. The slides were immersed in 0.003 M sodium borohydride for 30 min then washed with PBS.
 - d. Ten percent normal rabbit serum in PBS was applied to the sections for 30 min, and the slides were washed.
 - e. Appropriate dilutions of the peroxidase-labeled antibody preparations were applied to the sections and incubated for 2 hr at room temperature.
 - f. The sections were treated with Karnovsky's diaminobenzidine solution (9) for 10 min. The slides were washed in PBS, washed, dehydrated in alcohol, and cleared in xylene.
 - g. The sections were mounted in Permount (Fisher Scientific Co.) and examined under the light microscope.
9. For electron microscopy:
 - a. The slides were washed with chilled PBS containing 12% sucrose.
 - b. Ten percent normal rabbit serum in PBS-12% sucrose was applied to the sections for 30 min; slides were washed in PBS-12% sucrose.
 - c. The peroxidase-labeled antibody preparations were applied for 4 to 5 hr at room temperature; slides were washed in PBS-12% sucrose.
 - d. The sections were treated with 2.5% glutaraldehyde in PBS for 30 min at room temperature, then washed with PBS.
 - e. The slides were immersed in incomplete Karnovsky's solution (0.25 mgm diaminobenzidine/ml of 0.05 M Tris-HCl, pH 7.6) containing 1% dimethyl sulfoxide but no hydrogen peroxide for 30 min at room temperature.
 - f. The slides were transferred to Karnovsky's solution containing 0.005% hydrogen peroxide for 2 to 5 min, then washed with PBS.
 - g. The sections were fixed in 1% osmium tetroxide in PBS for 1 hr at room temperature, then washed with PBS.
 - h. The sections were dehydrated in graded ethanol to 100%.
 - i. While the sections were still wet, a gelatin capsule filled with Epon-Araldite was inverted over the slides. Epon-Araldite was polymerized at 37°C for 24 hr, then at 60°C for 24 to 48 hr.
 - j. The slides were heated on a hot plate (90°C) for 30 to 60 sec, and the polymer-filled capsules were removed.
 - k. Ultrathin sections were cut in an LKB III ultramicrotome, applied to #150-#200 mesh grids without membranes, and examined by an electron microscope.

FIG. 2. Immunocytochemical localization of SC in apical portions of crypt cells of human small intestinal epithelium. Electron-dense reaction product marking the sites of SC is present in the perinuclear spaces (PS); in elements of the Golgi complex (G); in cisternae of rough endoplasmic reticulum (ER); in cytoplasmic vesicles (V); and on lateral interdigitating plasma membranes ($\Rightarrow$) of columnar secretory cells. Reaction product is not present on the apical plasma membranes of these cells or in mucus of a goblet cell (GC). The presence of reaction product in perinuclear spaces, endoplasmic reticulum and Golgi complexes is evidence of complete penetration of the labeled antibodies into cellular spaces. Lumen (L). $\times 13,000$

The quality of ultrastructure preservation, completeness of antibody penetration, and some of the immunocytochemical evidence of antibody specificity in tissues handled by this schedule are demonstrated in Fig. 2 and Fig. 3. Additional evidence of antibody specificity is presented elsewhere (6).

DISCUSSION

This study achieved satisfactory conditions for immunocytochemical localization of immunoglobulins in human gastrointestinal tissues at the light and electron microscopic levels. The peroxidase-labeled antibody method, with certain modifications, provided good preservation of tissue morphology and antigenicity of immunoglobulins, penetration of the labeled antibodies into cellular compartments, and elimination of interfering endogenous peroxidase activity.

Preservation of morphology. The periodate-lysine-paraformaldehyde fixative, which cross-links carbohydrate moieties of tissue glycoproteins, proved to be satisfactory for intestinal tissues, as it has for other tissues (12). Morphologic preservation was improved slightly by increasing the concentration of paraformaldehyde from 2% to 4%, and was improved further by the addition of digitonin to the first wash solution. Digitonin stabilizes cell membranes by fixation of the 3- β -hydroxy position on cholesterol (18, 19). An important innovation was the layer of gelatin between albumin and glass slides. This cushion permitted shrinkage of the tissues without disruption during the steps in preparation for electron microscopy. The use of a relatively high concentration of sucrose during tissue fixation probably also contributed to maintenance of tissue morphology.

Antigenicity of tissue immunoglobulins. The extent to which reactivity of antigens with labeled antibodies is retained during immunocytochemical procedures cannot be assessed objectively. Nevertheless, evidence indicates that the antigenicity of tissue immunoglobulins and SC was not seriously reduced: the staining intensity of labeled antibodies to these antigens was satisfactory, and the numbers of identified immunoglobulin-containing cells corresponded well with cell counts by the immunofluorescence technique (6).

Penetration of labeled antibodies. In previous work (15), penetrability of enzyme-labeled antibodies into tissues has been enhanced by use of Fab' fragments of antibody molecules and by quick-freezing and thawing tissues. The present study demonstrated that these conditions are also satisfactory for intestinal tissues fixed by periodate-lysine-paraformaldehyde.

Inhibition of endogenous peroxidase. In the few published immunohistochemical studies of gut mucosa by peroxidase labeled antibodies (5, 11, 26), difficulty was encountered at the light microscopic level in distinguishing cells reacting with labeled antibodies from endogenous peroxidase-containing cells. Although the endogenous peroxidase can be inhibited by various methods, none had been shown conclusively to be satisfactory in intestinal mucosa. Sodium azide and hydrogen peroxide (2) were not consistently effective (5, 26); methanol and nitroferrocyanide (21) reduced reactivity of antigens in certain tissues, and might do so also in the

FIG. 3. Immunocytochemical localization of SC in basal portions of crypt cells of human small intestinal epithelium. Reaction product is present in perinuclear spaces (PS); in cisternae of rough endoplasmic reticulum (ER); and on lateral interdigitating plasma membranes of columnar secretory cells. Evidence of the antigenic specificity of the anti-SC is the absence of reaction product within or on the basal membrane of an endocrine cell (E). Basement membrane area (BM). $\times 10,800$

gut; the effect of hydrochloric acid (25) or methanol and hydrogen peroxide (22) on antigenicity of human immunoglobulins has not been assessed. Brown and Burns (5) circumvented the endogenous peroxidase problem by staining the peroxidase-containing cells and the antibody-reacting cells with contrasting dyes. In the present work, periodic acid, probably through its oxidative action at low pH on the heme component of peroxidase, abolished histochemically reactive endogenous peroxidase, while leaving antigenicity of the immunoglobulins and SC unimpaired. Hence, this adjunct to the immunoperoxidase method is satisfactory for studying these antigens in gut tissues at the light microscopic level. We did not evaluate its applicability at the electron microscopic level because ultrastructural characteristics of peroxidase-containing cells are distinctive, and the small amount of peroxidase in subcellular organelles, such as peroxisomes, is easily differentiated from immunoglobulins or SC. Whether the periodic acid technique will be satisfactory in immunohistochemical studies of other antigens in gut tissues is conjectural; in unreported studies from this laboratory, periodic acid substantially reduced the antigenicity of hepatitis B antigens in liver.

This study has further documented the advantages of using frozen sections of fixed tissues in enzyme immunocytochemistry (13). Re-use of light microscopic sections for electron microscopy permitted correlation of light and electron microscopic observations and efficient screening of large areas of tissue by light microscopy before electronmicroscopic examination.

Previous immunohistochemical studies of mucous membranes, despite their undisputed significance, were limited by the resolution constraints of light microscopy or failings in application of the peroxidase-labeled antibody method. In the few attempts at ultrastructural localization of SC and immunoglobulins (in rabbit breast and pig intestine), tissue preservation and penetration of peroxidase-labeled antibodies were suboptimal (1, 10). We believe that the improvements in the peroxidase-labeled antibody method made through this study will permit further advances in research on the immunology of the gastrointestinal tract and other externally secreting organs.

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