ANTIBODIES TO INTESTINAL MICROVILLOUS MEMBRANES

I. Characterization and Morphologic Localization*

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Injection of suitable preparations of intestinal mucosa from another species into rabbits can induce the production of antibodies which combine in vitro with both heterologous and autologus intestine (1, 2). That such antibodies can interfere with absorptive processes, however, has not been reported.

To study intestinal antigens Nairn and coworkers injected rabbits with a microsomal fraction of human colonic mucosa (1). They prepared fluorescent antisera which reacted with acid mucopolysaccharide in superficial glands of stomach, in goblet cells of small intestine, and in cytoplasm of colonic epithelial cells. Complement fixation and precipitin tests in agar revealed minor cross reactions with human kidney. Holborow, Asherson, and Wigley injected rabbits with homogenized mucosa of rat ileum and colon (2). With an indirect immunofluorescent technique they observed that the antisera reacted with epithelial cells of autologous rabbit ileum and colon. The most intense fluorescence occurred in goblet cells, although the cytoplasm of other epithelial cells also fluoresced. Sera of rabbits injected with rat gastric mucosa reacted only with gastric tissue and not with small or large intestine.

On the other hand, Broberger and Perlmann demonstrated that sera of some persons with ulcerative colitis contain antibodies to colon (3). To support the hypothesis that such antibodies might result from cross-reactions with intestinal bacteria, Asherson and Holborow immunized rabbits with bacteria in Freund's complete adjuvant (4). Antibodies to *Escherichia coli* and a few other intestinal

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bacteria caused indirect immunofluorescent reactions with colonic mucosa of some injected animals. In some instances the antisera also reacted with ileum and stomach. They observed several patterns of fluoresence of goblet cells, mucus, and cytoplasm of epithelial cells.

With recently developed methods (5), it is now possible to prepare relatively pure preparations of the apical brush border portion of intestinal absorptive cells. Moreover, microvillous membranes (MVM), which can now be isolated from such preparations of brush borders (6), have been useful in studying the initial phases of intestinal absorption and digestion (5–7).

In this paper we describe the preparation of rabbit antibodies to MVM prepared from the small intestine of hamsters and some immunologic specificities of these antibodies. A companion paper (8) describes experiments in which we used these antibodies to examine intrinsic factor–mediated attachment of vitamin B_{12} to brush borders of hamster distal small intestine.

Methods

Isolation of Brush Borders and Microvillous Membranes.—Brush borders were isolated from the proximal and distal halves of small intestines of golden hamsters by modifications of the method of Miller and Crane (5), as previously described (7). In brief, villous cells were collected by light scraping of the mucosal surface of small intestine. After homogenization of this material in 5 mM EDTA, pH 7.4, and filtration through bolting silk, brush borders were harvested by differential centrifugation of the filtrate. MVM were prepared from these brush borders by disruption with 1 M tris buffer, pH 7.0, and density-gradient sedimentation in a discontinuous glycerol gradient by the method of Eichholz and Crane (6). The preparations of brush borders and of MVM were pure and well preserved when examined with the light and electron microscopes.

Preparation of Antisera.—MVM from either the distal or proximal half of the small intestines of 12 hamsters were suspended in 0.5 ml of Krebs-Ringer bicarbonate buffer at pH 7.4 (KRB). Each preparation contained 2–4 mg of protein. Each suspension was mixed vigorously with an equal volume of Freund's complete adjuvant by repeated passage through a 20 gauge needle. The adjuvant contained 9 parts Drakeol 6-VR (Pennsylvania Refining Co., Butler, Pa.) and 1 part Arlacel A (Atlas Chemical Industries, Wilmington, Del.) with 3 mg per milliliter of *Mycobacterium tuberculosis*, which had been exhaustively extracted with ether, ethanol, and chloroform.[1] 1/10 ml of the 1 ml suspension was injected into a toe-pad of each foot and the remainder subcutaneously on several sites on the back of white, 5 kg New Zealand female rabbits. Two rabbits were injected with MVM from the proximal small intestine and three with MVM from the distal small intestine. At 3 wk the rabbits were reinjected. Blood was collected weekly from a marginal ear vein and assayed for antibodies to MVM. The rabbits were reinjected with MVM after antibody activity had decreased significantly. Potent antisera were subsequently pooled for study.

To prepare antisera to hamster serum, two rabbits were injected subcutaneously with an emulsion of 1 ml of hamster serum and 1 ml of Freund's incomplete adjuvant. After 3 wk the rabbits were injected again and blood was collected weekly. Another rabbit received injections of only Freund's complete adjuvant.

Gel Diffusion Studies.—To prepare soluble antigens, brush borders obtained from proximal

[1] Kindly provided by Doctor Donald W. Smith.

or distal small intestine of hamsters were thoroughly homogenized in 4 ml of 0.5% sodium deoxycholate with Potter-Elvejhem glass homogenizers (9). After incubation at room temperature for 2 hr, the homogenates were centrifuged at 28,000 *g* for 30 min. The supernatant was stored at −20°C. Double diffusion was performed in 0.5% agarose in 0.15 M NaCl on microscope slides. The reactions were observed after 24 hr at room temperature, and the slides stained with Amidoschwartz. Immunoelectrophoresis was performed in 0.5% agarose in veronal buffer, μ 0.1, pH 8.6, on microscope slides.

Gel Filtration.—5 ml of rabbit antisera were fractionated by ascending chromatography through a 2.5 × 100 cm column of Sephadex G-200 in 0.1 M Tris, 0.2 M NaCl, pH 8.0 at 4°C. 10-ml fractions were collected.

Immunofluorescent Studies.—Serum from normal and immune rabbits were conjugated with fluorescein isothiocyanate by modifications of the method of Riggs, Loh, and Eveland (10). Globulins were precipitated by 50% saturation of the sera with ammonium sulfate, dialyzed against phosphate-buffered saline, and incubated overnight at 4°C with 0.010 mg of fluorescein isothiocyanate (Baltimore Biological Laboratories, Baltimore, Md.) per mg of globulins. The fluorescent mixture was passed through Sephadex G-50 in 0.125 M NaCl, 0.175 M phosphate, pH 6.3, and then through diethylaminoethylcellulose in this buffer. The labeled globulins were concentrated by dialysis against 10% polyethylene glycol 20 M (Union Carbide Corp., New York) to a concentration of about 3 g per 100 ml. The molar fluorescein:protein ratio varied from 1.0 to 1.2.

Hamsters and rats were sacrificed by a blow on the head and rabbits by injection of thiopental. Segments of proximal or distal small bowel and of colon were rinsed immediately with cold saline. 1-cm segments were fixed in 10% neutral isotonic formalin for 4 hr and in 30% sucrose in water overnight at 4°C. The segments were embedded in OCT (Ames Laboratories, Elkhart, Ind.) in isopentane at −70°C and sectioned at −20°C. 4–6 μ sections were fan-dried on microscope slides. Normal human jejunal mucosa, obtained by peroral biopsy and normal human colonic mucosa, obtained by biopsy during proctoscopic examination, were similarly processed. Other tissues were frozen immediately after death of animals and sectioned without chemical fixation.

The sections were incubated with fluorescein-labeled globulins for 30 min at room temperature, washed thoroughly in buffered saline for 30 min, covered with buffered glycerol, and sealed beneath a cover slip. Controls included incubation of sections with fluorescein-labeled globulins from normal rabbits and preincubation of sections with unlabeled antiserum before exposure to labeled antiserum.

Specimens were examined with darkfield illumination through a Zeiss fluorescence microscope with an Osram HBO 200 lamp, BG 12 exciter filter, and 500 mμ barrier filter.

To study cross-reactions of the fluorescent antisera with microorganisms of the bowel, hamster ileum and its contents were homogenized in 0.15 M NaCl in a Waring Blendor. Serial dilutions of the homogenate were cultured aerobically and anaerobically on selective media (11). Streptococci, staphylococci, lactobacilli, *Pseudomonas aeruginosa*, rhodotorula, and an unidentified yeast were cultured from the homogenates. Pure cultures of *Candida albicans* and some bacteria were obtained from the clinical microbiological laboratory. The microorganisms were heat-fixed to slides and stained with fluorescent globulins as above.

Ferritin Labeling.—Globulins from normal and immune rabbits were conjugated with ferritin by Dumonde's modification (12) of the method of Singer and Schick (13). Twice crystallized horse-spleen ferritin (General Biochemicals, Chagrin Falls, Ohio) was washed twice in 0.1 M borate buffer, pH 9.45 at 0°C and resuspended in buffer to a concentration of 15 mg per ml. 5 ml were stirred vigorously in an ice bath, and 0.1 ml of toluene 2,4-diisocyanate (TDIC) (Allied Chemical Corp., New York) was slowly added. The mixture was stirred for 45 min and centrifuged at 200 *g* at 0°. The pellet was discarded, and the supernatant, containing ferritin-TDIC, kept at 0°C for 1 hr.

Globulins were precipitated from antisera and from normal rabbit serum by 40% saturation with ammonium sulfate. The precipitates were washed twice and dialyzed against 0.9% saline. After concentration by negative pressure filtration and dialysis against 0.1 M borate buffer, pH 9.45 with 1 M NaCl, the globulins were diluted to 16 mg per ml with this buffer. 2.5 ml (40 mg) of globulins were slowly added to 2.5 ml (37 mg) of ferritin-TDIC at 0°C with constant stirring. After being stirred 2 days at 4°C, the preparations were dialyzed against 0.1 M ammonium carbonate for 24 hr and then against KRB overnight. The globulin suspensions were spun at 105,000 *g* for 4 hr to separate labeled and unlabeled globulins. The pellet of labeled globulins was resuspended in KRB and centrifuged 10,000 rpm for 30 min at 4°C to sediment

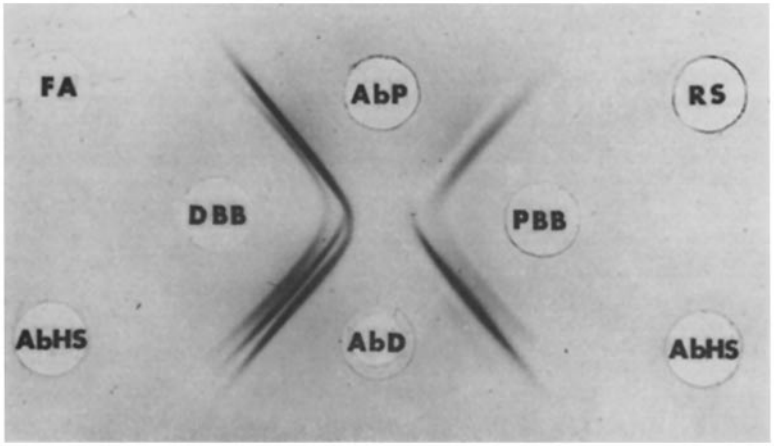

FIG. 1. Immunodiffusion reactions of antibodies to microvillous membranes isolated from proximal (AbP) and from distal small bowel (AbD) with deoxycholate extracts of brush borders of proximal (PBB) and distal (DBB) small bowel. Other sera are from normal rabbits (RS), a rabbit injected with Freund's complete adjuvant (FA), and rabbits injected with hamster serum (AbHS). Stained with Amidoschwartz.

extraneous material. The labeled globulins were passed through 0.45 μ millipore filters into sterile serum vials and were used within 1 wk.

Incubation of Ferroglobulins with Tissues.—3 ml of ferroglobulins from serum of normal rabbits and of rabbits injected with MVM were incubated at 4°C with freshly prepared pellets of brush borders or MVM in 20 ml KRB for 15–45 min in 25-ml Erlenmeyer flasks. As an additional control, brush borders were similarly incubated with washed, unconjugated ferritin. After incubation, the brush borders were recovered by centrifugation at 1500 rpm for 10 min and then washed three times in large volumes of KRB to remove free ferroglobulin. When MVM were studied, the membranes were recovered by centrifugation at 15,000 rpm for 30 min and also washed three times in cold KRB.

In other experiments, 5-mm wide everted segments of hamster jejunum and 5-mm^2 pieces from the body of hamster stomachs were incubated in 25-ml Erlenmeyer flasks with 5 ml KRB and 2 ml of ferroglobulin from serum of control and immune rabbits. Incubation was carried out at 37°C for 30 min in a Dubnoff shaking incubator in an atmosphere of 95% O_2 and 5% CO_2. Immediately after incubation, an excess of cold KRB was added. To remove unbound ferroglobulin, the tissues were rinsed three times for 5 min in cold KRB with continuous agitation.

Tissue samples and pellets of brush borders and MVM were fixed in chilled chrome-osmium (14), postfixed in 10% neutral isotonic formalin, dehydrated in ethyl alcohol solutions, and embedded in epoxy resin (15). Thin sections for electron microscopy were cut with diamond knives and mounted on carbon-coated copper mesh grids. Sections, which were either unstained or very lightly stained with uranyl acetate (16), were examined with either an RCA EMU-3G or a Philips EM300 electron microscope.

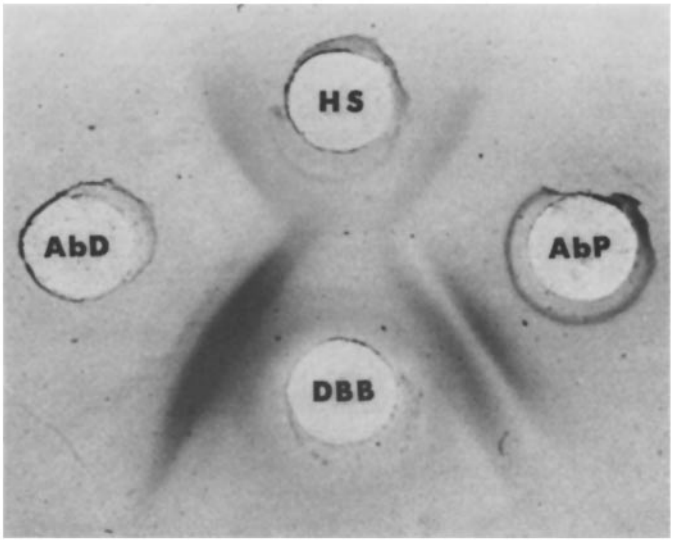

FIG. 2. Immunodiffusion reactions of antibodies of AbP and AbD with hamster serum (HS) and deoxycholate extract of brush borders of distal small bowel (DBB). The well was filled twice with HS to intensify the reaction for this illustration.

RESULTS

Precipitins.—Double diffusion in agarose showed that antisera to MVM both of proximal and of distal small bowel contained precipitating antibodies to deoxycholate extracts of proximal and distal small bowel (Fig. 1). Deoxycholate extracts of proximal and distal small bowel did not react with sera of normal rabbits or with sera of rabbits injected either with hamster serum or Freund's complete adjuvant alone. Antisera to MVM both of distal and of proximal small bowel contained a weak precipitin to hamster serum (Fig. 2). Immunoelectrophoresis of hamster serum against antisera both to proximal and to distal hamster small bowel yielded a barely perceptible line of precipitation in the prealbumin region.

To identify which immune globulins in the rabbit antisera contained precipitins to the deoxycholate extracts, antisera were fractionated through Sephadex G-200. Fractions from each of the three protein peaks were pooled and concen-

trated 20 times by negative pressure dialysis. Only the second peak, which contained 7S globulins, reacted in double diffusion in agarose against the deoxycholate extracts. This result and the reactions of fluorescent antisera, which were IgG by immunoelectrophoresis against goat anti-rabbit serum (Hyland Laboratories, Los Angeles, Calif.), indicated that IgG immune globulins contained the precipitins.

TABLE I

Reaction of Fluorescent AbMVM with Tissues

Species	Tissue	
	Immune fluorescence of brush border	No reaction
Hamster	Small bowel, colon, proximal renal tubules	Gall bladder, stomach, bronchus, liver
Rat	Small bowel, proximal renal tubules	Colon
Rabbit		Small bowel, colon, kidney
Man	Small bowel	Kidney, colon

Immunofluorescence.—Table I summarizes the results of immunofluorescent studies of tissues. Incubation of fluorescent antisera from MVM of proximal or distal small bowel (AbMVM) with proximal or distal small intestine of hamsters caused bright, specific fluorescence of brush borders of villous epithelial cells (Figs. 3 and 4). Mucus and other portions of the cells did not fluoresce. Incubation of small bowel with fluorescent normal rabbit globulins induced no reaction, (Figs. 5 and 6) and preincubation with unlabeled AbMVM specifically inhibited reactions with the fluorescent AbMVM.

In addition, fluorescent AbMVM reacted specifically with the brush borders both of hamster colon (Fig. 7) and of proximal renal tubules of hamster kidney (Fig. 8). Glomeruli and distal renal tubules did not react. These antisera also caused similar, but less intense fluorescence of the brush border of the small bowel and proximal renal tubules of Holtzman rats. Colon from rats, and bowel and kidney from adult New Zealand rabbits, however, showed no fluorescence with these antisera. A biopsy specimen of human jejunal mucosa from a patient without small bowel disease also showed fluorescence confined to the brush border of epithelial cells after incubation with AbMVM (Fig. 9). Renal biopsy specimens from two patients with glomerular disease, but morphologically normal proximal tubules, and rectal mucosa from a normal volunteer showed no

reaction with these antisera. Frozen sections of hamster liver, bronchus, gall bladder, and stomach showed no specific fluorescence.

An oval, saprophytic yeast which was cultured from hamster ileal contents showed intense fluorescence of the cell wall after incubation with fluorescent AbMVM (Fig. 10). Budding portions of these organisms fluoresced less brightly. Incubation with fluorescent normal rabbit globulins caused no visible fluorescence. *Candida albicans*, which was cultured from a patient, showed less intense fluorescence of the cell wall. The following microorganisms which were cultured from hamster ileum showed no fluorescence: staphylococci, streptococci, lactobacilli, *Pseudomonas aeruginosa*, and rhodotorula, another saprophytic yeast. *Esherichia coli* and pneumococcus, Type I, obtained from hospitalized patients also did not fluoresce. In addition, suspensions of *Amoeba proteus I* and of *Amoeba histolytica*, which have a mucopolysaccharide surface coat (17), were incubated with fluorescent AbMVM. After 30 min at room temperature the preparations were centrifuged at 1000 rpm for 10 min and washed twice. The amoebae remained motile, and their brush borders did not fluoresce.

Flourescent AbMVM was also incubated with zymosan (Standard Brands Inc. New York), an insoluble polysaccharide extracted from yeast cell walls, which can inactivate the third component of complement. 1 mg of zymosan in 0.5 ml of saline was agitated with fluorescent AbMVM for 30 min at 37°C. After centrifugation and two washings we observed neither agglutination nor specific fluorescence.

Ferritin-Conjugated Antibody Studies.—Following incubation of ferritin-conjugated AbMVM with brush borders, MVM, and everted jejunal rings, ferritin granules were consistantly identified in close association with the membranes of microvilli (Figs. 11 and 13). The ferritin particles did not appear to be bound to the unit membrane itself; instead, they seemed attached to the fibrillar mucopolysaccharide surface coat on the outer leaflet of the trilaminar membrane. Pellets of MVM incubated with AbMVM demonstrated most clearly this association of ferritin particles with the surface coat (Fig. 13). Ferritin particles in these preparations were observed almost exclusively in the surface coat; very few ferritin particles were seen within the inner leaflet of the unit membrane of the MVM.

After these tissues were incubated with ferritin-conjugated globulins prepared from normal rabbit serum, ferritin particles were rarely associated with the membrane of the microvilli or its surface coat (Figs. 12 and 14). Moreover, unconjugated ferritin did not attach to preparations of brush borders of small intestine.

Ferritin-labeled AbMVM was also incubated with pieces of hamster stomach, whose epithelial cells also contain a mucopolysaccharide surface coat. However, no ferritin particles were observed in association with the apical plasma membrane of the epithelial cells of these gastric segments.

DISCUSSION

These studies have demonstrated that heterologous antibodies can be prepared to MVM of hamster small bowel. Previous investigators of mucosal antigens of intestine have used whole homogenates or microsomal fractions of cells as antigenic material (1, 2). The resulting antibodies reacted variably with the cytoplasm of cells and with mucus. In contrast, the morphologically pure preparations of MVM used in the present studies produced rabbit antibodies which reacted specifically with the absorptive surface of hamster bowel. In frozen sections of hamster small bowel, only the bursh border fluoresced specifically. Cytoplasm, nuclei, and basement membranes of cells as well as mucus of goblet cells, did not react with AbMVM. Although immunologic reactions also occurred in certain other tissues, these were confined to cellular surfaces. In gel diffusion the antisera also reacted slightly with an unidentified component of hamster serum, which migrated like prealbumin in immunoelectrophoresis.

Electron microscopy with ferritin-conjugated AbMVM more precisely localized the antigen-antibody reaction to the surface mucopolysaccharide coat of the MVM of hamster small bowel. We did not find consistent attachment of ferritin-conjugated AbMVM to other components of the MVM. The function of this mucopolysaccharide layer, which is present on the apical surface of all epithelial cells of the small and large intestine, is not known. This surface coat is probably synthesized in the cytoplasm of the cells to which it is applied and is an integral part of the apical plasma membrane (17, 18). The surface coat–apical membrane complex provides the initial contact between nutrients in the gut lumen and absorptive cells. Moreover, digestive enzymes including disaccharidases, alkaline phosphatase, and leucine aminopeptidase have been recovered in high concentration from the surface coat–apical membrane complex (6). Thus, the apical plasma membrane and its surface coat probably participate in initial phases of intestinal absorption and digestion.

In studies of other tissues, fluorescent AbMVM also showed specificity for brush borders of hamster colon and of proximal tubules of hamster kidney. Conversely, Edgington and coworkers recently observed that fluorescent antisera to renal tubules of rats had affinity for both brush borders to proximal renal tubules and of rat jejunum (19). The cross-reactions of renal and jejunal absorptive surfaces are of interest in view of the absorptive defects which simultaneously occur in these tissues in Hartnup disease (20) and in cystinuria (21).

AbMVM did not, however, show specificity for all hamster tissues with a mucopolysaccharide surface coat. Fluorescent AbMVM, for instance, did not react with hamster gall bladder, and both fluorescent and ferritin-labeled AbMVM did not react with hamster stomach.

Studies of tissues of other species indicated that AbMVM was not species specific and that its specificity did not correlate well with the morphologic

characteristics of the brush borders and their mucopolysaccharide surface coat (Table I). After incubation with fluorescent AbMVM, brush borders of jejunum of rats and humans fluoresced brightly. While the enteric surface coat on human intestinal villi is thick, that on intestinal cells of rats is thin (17). The absence of fluorescence in rabbit tissues, however, differed from the observations of Holborow and coworkers who found that rabbit intestinal epithelial cells could bind rabbit antibodies prepared to a homogenate of rat gut mucosa (2).

Since some intestinal bacteria and rat colon have common antigenic determinants (4), we also studied reactions of AbMVM with microorganisms. Fluorescent AbMVM did not react with several species of heat-fixed bacteria. It did, however, cause specific fluorescence of an unidentified saprophytic yeast which was isolated from hamster ileum, and of *Candida albicans* cultured from a patient. Rhodotorula, another saprophytic yeast in hamster intestine, did not fluoresce with AbMVM. Thus, cell walls of some yeasts and microvillous membranes of some tissues have common heterophilic antigens. However, the primary affinity of AbMVM for the superficial mucopolysaccharide coat of the bowel suggested that it might be a useful tool to study the initial phases of absorption and digestion by intestinal cells.

SUMMARY

Antibodies (AbMVM) were produced in rabbits to microvillous membranes isolated from hamster small bowel. Incubation of frozen sections of hamster small bowel with fluorescein-labeled AbMVM showed specific reaction with brush borders, but not with other intestinal cellular components. Electron microscopy with ferritin-conjugated AbMVM localized the antigens more precisely to the surface mucopolysaccharide coat of the brush borders. AbMVM also reacted with the brush border of colon and of proximal renal tubules of hamsters but not with those of hamster stomach or gall bladder. It also reacted with the brush borders of some rat and human tissues, but not with those of rabbits. In addition, fluorescent-labeled AbMVM combined specifically with cell walls of some yeasts, but not of several bacteria. AbMVM also contained a weak precipitin to a component of hamster serum, which migrated like prealbumin in immunoelectrophoresis.

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FIGS. 3–8. Immunofluorescent preparations of hamster tissues. Frozen sections were stained with fluorescein-labeled rabbit globulins.

FIG. 3. Ileum stained with fluorescent AbMVM. Only the brush borders of villi fluoresce. × 100.

FIG. 4. Same preparation as Fig. 3. × 300.

FIG. 5. Ileum stained with fluorescent normal rabbit globulins. There are specks of nonspecific fluorescence in the lamina propria but no fluorescence of the brush border. × 100.

FIG. 6. Same preparation as Fig. 5. × 300.

FIG. 7. Colon stained with fluorescent AbMVM. The thin brush border fluoresces. Mucus (arrow) did not react. × 100.

FIG. 8. Kidney stained with fluorescent AbMVM. The brush border of proximal tubules fluoresces. A glomerulus (*G*) showed no reaction. × 300.

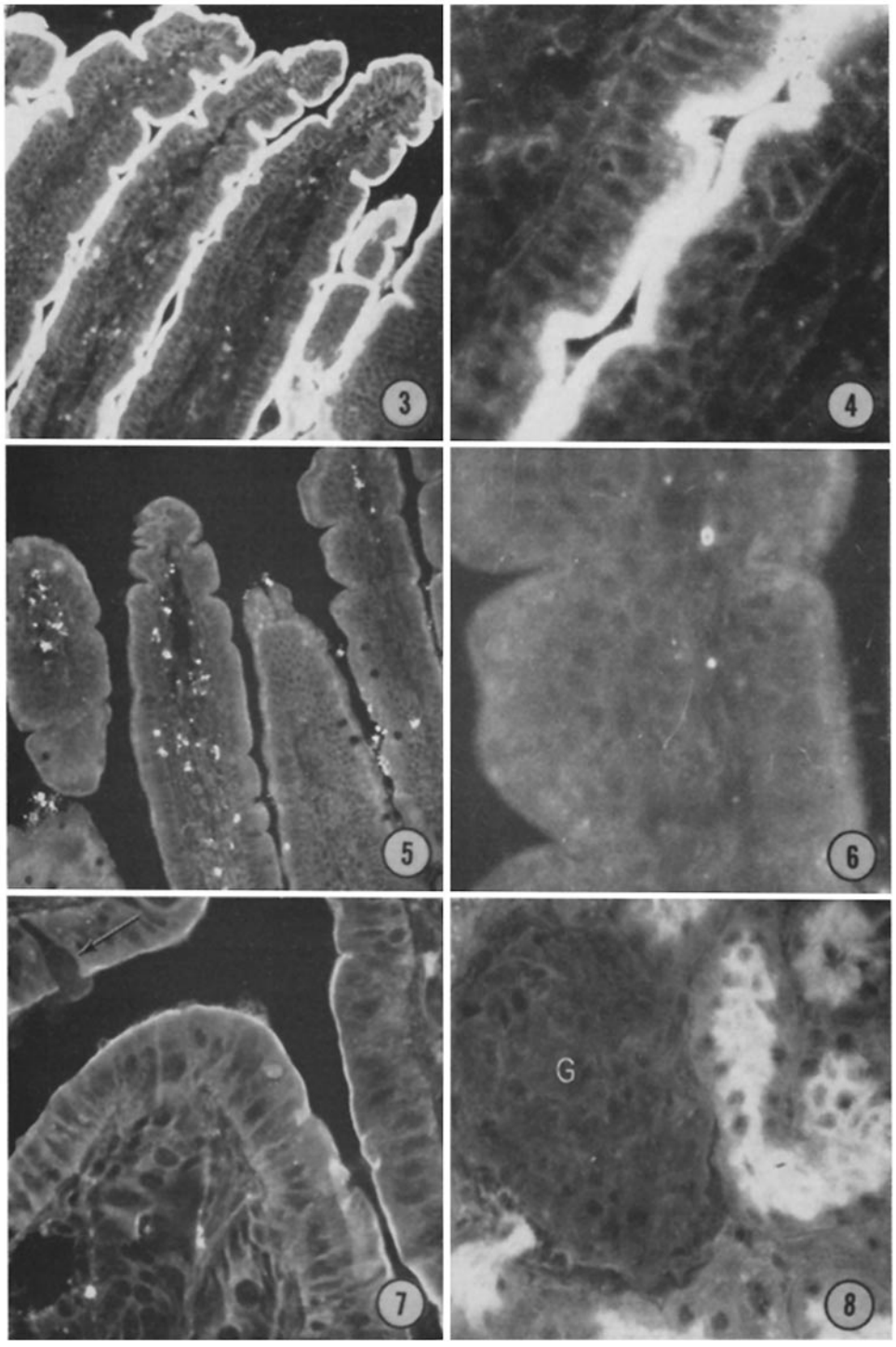
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FIG. 9. Human small intestine incubated with fluorescent AbMVM. Again only the brush border fluoresces specifically. × 300.

FIG. 10. A saprophytic yeast which was grown in culture from hamster bowel and incubated with fluorescent AbMVM. The cell wall fluoresces specifically except where it is budding (arrow). × 1000.

FIG. 11. Section of everted segment of hamster jejunum after incubation with ferritin-conjugated AbMVM. Ferritin particles (arrows) are seen adjacent to the surface coat of the microvilli. Ferritin appears to overlay the surface of the microvilli only where the membrane and surface coat have been sectioned tangentially (right). Lightly stained with uranyl acetate. × 117,000.

FIG. 12. Section of everted segment of hamster jejunum after incubation with ferritin-conjugated normal rabbit globulins. No ferritin particles are seen. × 117,000.

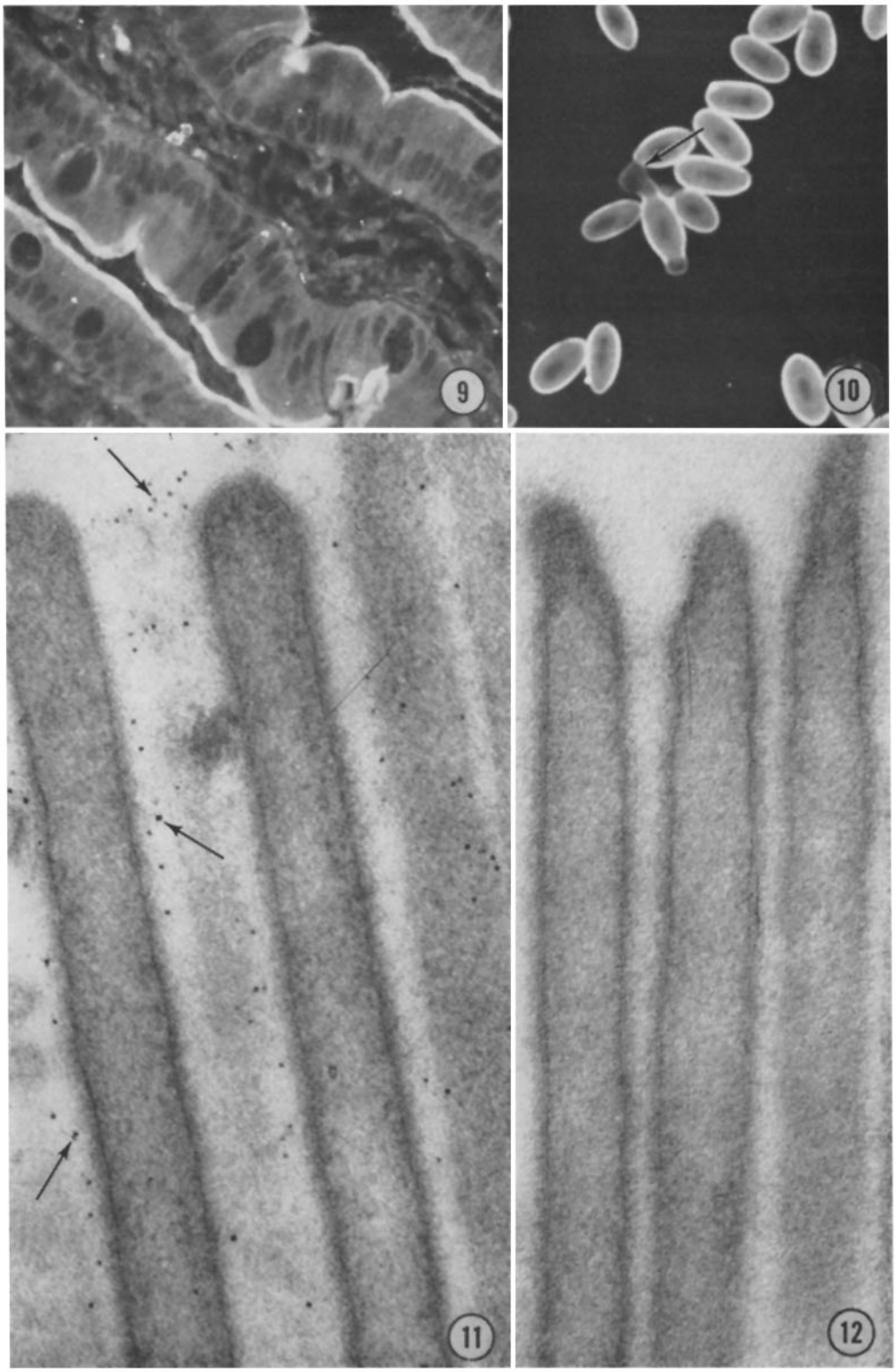
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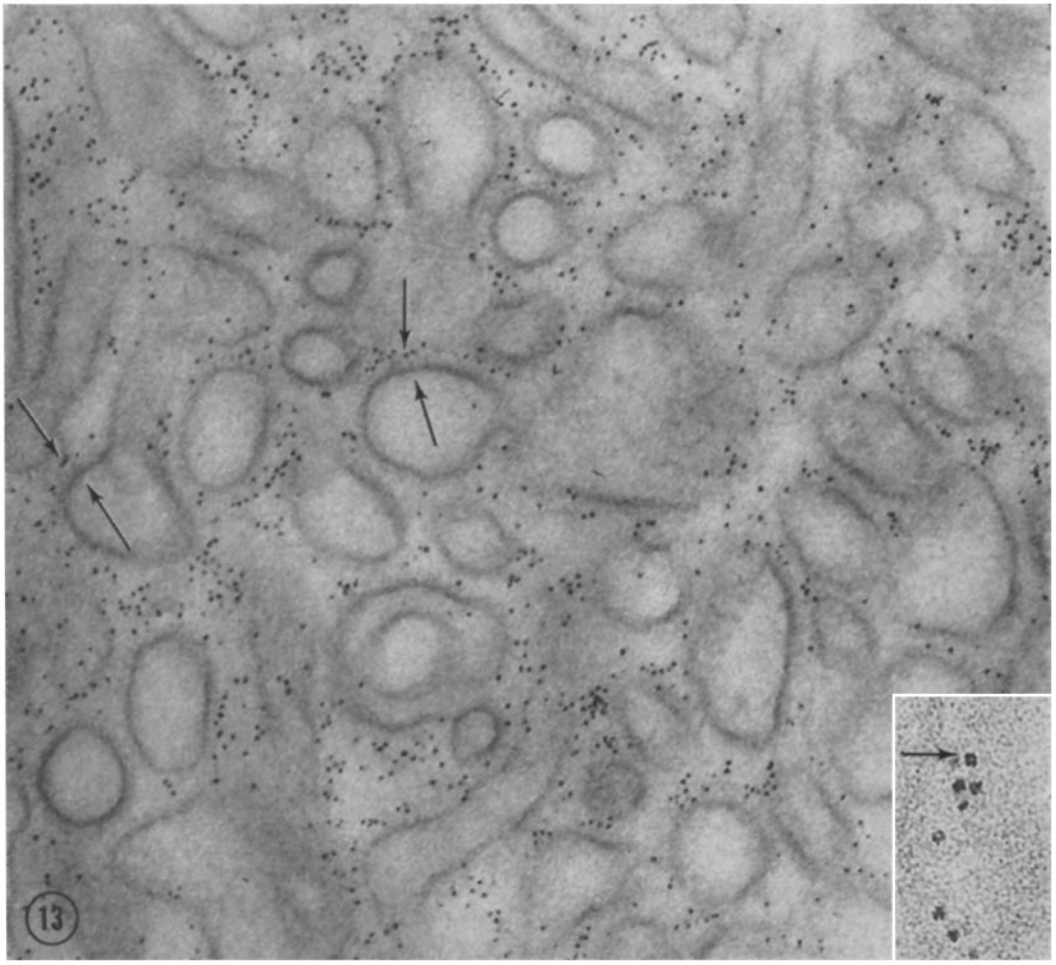
FIG. 13. Isolated microvillous membranes (MVM) of hamster jejunum incubated with ferritin-conjugated AbMVM. Numerous ferritin particles are attached to the surface coat of the MVM (long arrows), rather than to the unit membrane itself. Moreover, little or no ferritin appears bound to the inner aspect of the MVM (short arrows). Lightly stained with uranyl acetate. × 102,000.

Insert: higher magnification of the same preparation, illustrating the typical tetrad substructure (arrow) of the ferritin moiety of the ferritin-AbMVM complex. × 220,000.

FIG. 14. Isolated MVM of hamster jejunum incubated with ferritin-conjugated normal rabbit globulins. Only a few ferritin particles are present in this preparation. × 102,000.



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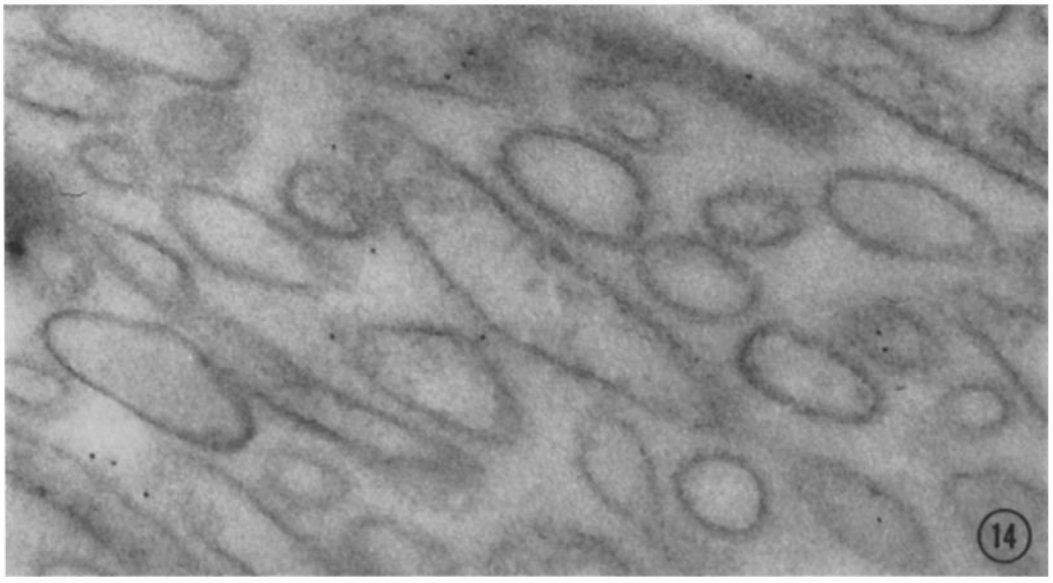

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