Binding and Transepithelial Transport of Immunoglobulins by Intestinal M Cells: Demonstration Using Monoclonal IgA Antibodies Against Enteric Viral Proteins

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Abstract. M cells of intestinal epithelia overlying lymphoid follicles endocytose luminal macromolecules and microorganisms and deliver them to underlying lymphoid tissue. The effect of luminal secretory IgA antibodies on adherence and transepithelial transport of antigens and microorganisms by M cells is unknown. We have studied the interaction of monoclonal IgA antibodies directed against specific enteric viruses, or the hapten trinitrophenyl (TNP), with M cells. To produce monospecific IgA antibodies against mouse mammary tumor virus (MMTV) and reovirus type 1, Peyer's patch cells from mucosally immunized mice were fused with myeloma cells, generating hybridomas that secreted virus-specific IgA antibodies in monomeric and polymeric forms. One of two anti-MMTV IgA antibodies specifically bound the viral surface glycoprotein gp52, and 3 of 10 antireovirus IgA antibodies immunoprecipitated sigma 3 and mu lc surface proteins. ^{35}S-labeled IgA antibodies injected intravenously into rats were recovered in bile as higher molecular weight species, suggesting that secretory component had been added on passage through the liver. Radiolabeled or colloidal gold-conjugated mouse IgA was injected into mouse, rat, and rabbit intestinal loops containing Peyer's patches. Light microscopic autoradiography and EM showed that all IgA antibodies (antivirus or anti-TNP) bound to M cell luminal membranes and were transported in vesicles across M cells. IgA-gold binding was inhibited by excess unlabeled IgA, indicating that binding was specific. IgG-gold also adhered to M cells and excess unlabeled IgG inhibited IgA-gold binding; thus binding was not isotype-specific. Immune complexes consisting of monoclonal anti-TNP IgA and TNP-ferritin adhered selectively to M cell membranes, while TNP-ferritin alone did not. These results suggest that selective adherence of luminal antibody to M cells may facilitate delivery of virus–antibody complexes to mucosal lymphoid tissue, enhancing subsequent secretory immune responses or facilitating viral invasion.

To enter cells and initiate replication, viruses often adhere to host cell plasma membranes and exploit cellular endocytic activity (16). Certain enteric viruses that enter their hosts at intestinal mucosal surfaces are internalized by epithelial cells, but do not infect the epithelium itself; rather, they enter transepithelial transport pathways to cross the intact epithelium and spread systemically (20, 35). For example, mouse mammary tumor virus (MMTV),[1] which is transmitted in milk from mother to offspring, crosses the intestinal epithelium and subsequently replicates in the mammary gland (21). Reovirus type 1 adheres specifically to M cells, specialized epithelial cells that overlie mucosal lymphoid follicles, and uses M cell transepithelial transport activity to cross the epithelium and spread to neural and lymphoid tissues (35, 48).

M cells are specialized for rapid transepithelial transport of luminal macromolecules and microorganisms to underlying lymphoid tissue (7, 25), a transport process that may lead to development of a specific secretory immune response (37, 42). Thus, despite the fact that M cell transport activity provides an entry point for some viruses, it may also be necessary for subsequent production of specific secretory IgA (sIgA) against these viruses. Considerable evidence indicates that sIgA (but not IgG) protects intestinal epithelial surfaces and prevents enteric infection (1, 2, 4, 5, 24), but there is little information on precisely how such protection is accomplished. It is generally believed that luminal sIgA inhibits absorption of viruses and other microorganisms by blocking the microbial surface molecules responsible for epithelial attachment (44, 46), but this has not been directly demonstrated in the intestine, in part because the relevant

1. *Abbreviations used in this paper*: FAE, follicle-associated epithelium; i.l., intraluminal; i.p., intraperitoneal; MLN, mesenteric lymph node; MMTV, mouse mammary tumor virus; PP, Peyer's patch; sIgA, secretory IgA; TNP, trinitrophenyl.

monospecific IgA antibodies are not available in sufficient quantities. Nor is it known whether IgA can affect binding or transport of any macromolecule or microorganism by M cells.

Our ultimate goal is to elucidate the mechanisms whereby sIgA influences epithelial adherence and uptake of macromolecules and microorganisms in the intestine by testing monospecific secretory antibodies in well-characterized systems of viral infection and antigen transport. We have selected for study two viruses, reovirus type 1 and MMTV, both of which invade the intestinal mucosa by crossing an intact, undamaged intestinal epithelium (21, 35). A first step in these studies is the generation of monospecific antibodies of the IgA isotype, directed against viral surface components that may be important in intestinal attachment and invasion. As a model antigen, we selected the hapten trinitrophenyl (TNP), since it is readily attached to ultrastructural tracer molecules, and monoclonal anti-TNP IgA antibodies are available.

In this report, we describe the production and characterization of monoclonal IgA antibodies directed against MMTV and reovirus type 1. Hybridomas were made by fusing myeloma cells with Peyer's patch (PP) cells from mucosally immunized mice, and the IgA antibodies obtained were characterized according to their protein binding specificity, degree of polymer formation, and ability to bind to liver polymeric immunoglobulin receptors. To test for possible interactions of antivirus and anti-TNP IgA antibodies with specific epithelial cell surfaces, metabolically labeled or gold-conjugated IgA antibodies were injected into intestinal loops and visualized by autoradiography and electron microscopy. Our results indicate that luminal IgA binds selectively to M cell surfaces and thus may enhance delivery of antigens and microorganisms across the follicle-associated epithelium (FAE).

Materials and Methods

Animals

Female BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) between the ages of 6 and 12 wk were used for feeder layer preparation and all fusions. Female CD rats (Charles River Breeding Laboratories, Inc.) between the ages of 8 and 15 wk were used for liver transport experiments. Adult male or female BALB/c mice, female CD rats, and male or female New Zealand albino rabbits were used for injection of labeled antibodies into intestinal loops and for immunofluorescence studies. Neonatal jejunum was obtained from ~12-d-old CD rats.

Cells and Cell Culture

P3X63/Ag 8.653 mouse myeloma cells and hybridoma cells were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% FBS, 2 mM glutamine, 10 mM Hepes, 24 mM $NaHCO_3$, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI). L929 mouse fibroblastic cells were grown in Joklik's S-MEM (Irvine Scientific, Santa Ana, CA) containing 5% FBS, 3 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, GR mouse mammary cells (obtained from Dr. H. Diggleman, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) were grown in DME (Gibco Laboratories) containing 10% FBS, 2 mM glutamine, 10 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Viruses

MMTV particles were purified using described methods from culture medium of GR cells, a mouse mammary cell line persistently infected with MMTV (27). Reovirus type 1 was grown in L929 cells and purified as previously described (29).

Immunization

Immunization protocols are summarized in Table I. For MMTV immunization, BALB/c mice were immunized on day 0 with a suspension of MMTV-producing GR cells, injected either into the intestinal lumen (10^6 cells) or directly into the submucosal tissue of five to six PPs (1–2 × 10^5 cells/per patch). On days 14 and 21, mice were boosted with intraluminal (i.l.) or intraperitoneal (i.p.) injections of MMTV particles at a titer corresponding to 0.5 μg of gp52, the major viral surface glycoprotein.

For reovirus immunization, two protocols were used, both involving administration of virus on days 0, 14, and 21, and fusion of PP cells on day 24 (Table I). A virus suspension of 5 × 10^8 particles was injected i.l. or i.p.; for oral immunization, the same amount was given dropwise.

Cell Isolation and Fusion

For isolation of mouse PP mononuclear cells, the mucosa and submucosa of 8–10 PPs were removed and incubated in complete RMPI medium containing 0.1% collagenase for 1 h at 37°C. Tissues were ground between the ends of two sterile frosted-end microscope slides, and released cells were washed two times in complete RPMI. Approximately 2–3 × 10^7 viable PP mononuclear leukocytes were recovered from one mouse. Spleen and mesenteric lymph node (MLN) cells were isolated using the same technique, except that the collagenase treatment was omitted.

Myeloma cells and mouse mononuclear cells were washed twice with serum-free RPMI and fused in 37% polyethylene glycol 4000 (E. Merck, Darmstadt, FRG) at a leukocyte to myeloma cell ratio of 5:1. After fusion, cells were diluted to 1 × 10^6 cells/ml in complete RPMI and distributed into 96-well tissue culture plates that had been seeded 24 h earlier with 2 × 10^4 irradiated (800 rads) mouse peritoneal exudate cells per well. Hybridomas were selected in hypoxanthine, aminopterin, and thymidine medium using standard methods.

Production and Labeling of Monoclonal Antibodies

Hybridoma wells were screened for specific antibody production by ELISA. Wells were coated overnight at 4°C with 0.1 ml of MMTV at ~0.01 μg gp52 protein/ml, or 1 × 10^{10} reovirus type 1 particles/ml in carbonate-bicarbonate buffer at pH 9.6, and incubated with 100 μl of hybridoma supernatant. Bound antibodies were revealed using peroxidase-coupled rabbit antibody against mouse Ig, or against mouse IgA (Zymed Laboratories, South San Francisco, CA).

Specific IgA-producing hybridomas were expanded and cloned two to three times by limiting dilution until stable antibody production was obtained. To produce antibody for characterization, hybridomas were adapted to grow in complete RPMI containing 1% Nutridoma-SP serum-free media supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) in place of FBS. Antibody was stored at 4°C in PBS containing 0.5% BSA and

Table I. Immunization Procedures

	Immunization			Cells fused
Protocol	day 0	day 14	day 21	day 24
MMTV Immunization*				
Method A	i-PP	i.p.	i.p.	spleen, MLN, PP
Method B	i.l.	i.l.	i.p.	spleen, MLN, PP
Method C	i.l.	i.p.	i.p.	spleen, PP
Reovirus Immunization				
Method A	i.l.	i.p.	i.p.	PP
Method B	oral	oral	oral	PP

* Two mice were immunized by each method shown. For MMTV immunization, MMTV-infected cells were injected on day 0, followed by purified MMTV on days 14 and 21. Purified reovirus type 1 was used both for injections (method *A*) and oral immunization (method *B*). *i-PP*, antigen injected into PP submucosa; *i.p.*, antigen injected intraperitoneally; *i.l.*, antigen injected into the intestinal lumen.

0.01% thimerosal. Hybridomas MB2 and MB3 were injected i.p. in pristane-primed BALB/c mice to produce antibody in ascitic fluid.

Selected antibodies were labeled metabolically by incubating hybridoma cells in cysteine-deficient (2.5 μg/ml) RPMI (Selectamine kit, Gibco Laboratories) with 1% Nutridoma and 250 μCi [^{35}S]cysteine for 18 h.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (14). MMTV-infected GR cells were treated with lysis buffer (10 mM Tris HCl, 1% NP40, 0.4% deoxycholate, 60 mM EDTA, 1 mM PMSF, 5 μg/ml each of pepstatin, leupeptin, and antipain, pH 7.5) and centrifuged. Lysate supernatants and purified MMTV were solubilized in SDS sample buffer containing 2-mercaptoethanol. For endoglycosidase F treatment, 15 μg MMTV protein was solubilized in SDS sample buffer and 5 μl endoglycosidase F (Boehringer Mannheim Biochemicals) in 100 mM phosphate buffer with 50 mM EDTA, 1% NP40, 1% 2-mercaptoethanol, 1 mM PMSF, pH 6.5 was added. Samples were incubated overnight at 37°C, precipitated with TCA, and solubilized in SDS sample buffer. After electrophoresis and transfer to nitrocellulose (43), immunoblots were blocked with 5% nonfat dry milk and incubated with hybridoma culture medium followed by biotinylated rabbit anti-mouse Ig and streptavidin-peroxidase.

Antibodies were denatured in SDS-sample buffer without reducing agent and heated to 100°C for only 1 min to avoid breakdown of IgA polymers. IgA proteins were separated on 3–10% gradient gels and detected after electrophoretic transfer using peroxidase-coupled antibodies specific for mouse IgA (Zymed Laboratories).

Immunoprecipitation

Specificity of antireovirus IgA was determined by immunoprecipitation of ^{35}S-labeled reovirus proteins. L929 cells were grown in monolayers and infected with reovirus type 1 at a multiplicity of infection of 20. After 12 h, cells were incubated for 45 min in methionine-deficient MEM plus 100 μCi/ml [^{35}S]methionine, chased for 30 min in cold methionine-containing medium, harvested into PBS, pelleted, and treated with lysis buffer containing 0.5% deoxycholate and 0.1% NP40. Aliquots of the lysate were mixed with hybridoma supernatant overnight at 4°C and immune complexes were precipitated with rabbit anti-mouse IgG/A/M. Precipitated proteins were separated on 10% polyacrylamide gels and radiolabeled bands detected by fluorography.

Radioimmunoassay

To explore the specificity of IgA antibodies that immunoprecipitated both sigma 3 and mu lc, the major outer capsid proteins common to reovirus types 1 and 3, a solid phase radioimmunoassay was performed using intact reovirus 1, intact reovirus 3, and reovirus type 1 that had been protease-digested to yield intermediate subviral particles that lack sigma 3 but retain the major portion of mu lc. Flexible 96-well plates were coated with 1.25 μg reovirus/well, blocked with 1% BSA in PBS, and exposed to hybridoma supernatants overnight at 4°C. After washing, wells were exposed to ^{125}I-labeled rabbit anti-mouse IgG/A/M, separated, and counted.

Liver Transport of IgA

Bile ducts of adult CD rats were cannulated with PE10 tubing (Clay-Adams, Parsippany, NJ; outside diameter 0.61 mm), and 2.5 to 7 × 10^6 cpm of ^{35}S-labeled IgA or IgG (~2 μg protein) were injected intravenously. Bile was collected continuously from 15 min before to 3 h after antibody injection, and total radioactivity was determined by scintillation counting. For PAGE analysis of labeled protein, samples of stock labeled antibody and bile were diluted in SDS sample buffer and run without reduction on a 3–10% gradient acrylamide gel. Dried gels were autoradiographed with x-ray film without fluorography.

Autoradiography

Monoclonal IgA was metabolically labeled with [^{35}S]cysteine as described above and diluted to 1.2 × 10^7 cpm/ml (~5 μg/ml antibody) in PBS containing 0.5% BSA. Mice were anesthetized with 2,2,2-tribromoethanol in tertiary-amyl alcohol and 50–100 μl labeled antibody was injected into ligated intestinal loops, each of which contained a PP. After 1 h, loops were excised, quickly rinsed with PBS, and fixed in a solution containing 2% freshly depolymerized formaldehyde, 2.5% glutaraldehyde, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4. Tissues were postfixed in 1% OsO_4 in cacodylate buffer, en bloc stained in 0.5% uranyl acetate, dehydrated with graded ethanols, and embedded in Epon-araldite. 1-μm sections were stained with iron hematoxylin, coated with Ilford K5 emulsion diluted 1:1 with distilled water, and exposed at 4°C. After 3 wk, autoradiographs were developed (in D19; Eastman Kodak Co., Rochester, NY) and photographed.

Colloidal Gold Probes and Binding Assays

Colloidal gold particles of 5 nm or 6–8 nm were produced and conjugated using standard methods (36) to mouse polyclonal IgG (Sigma Chemical Co., St. Louis, MO), rat polyclonal secretory IgA that contained secretory component (provided by Dr. A. Plaut, Tufts University Medical School), BSA, or to mouse monoclonal IgA antibodies: either antireovirus antibodies RB2 and RB5, or MOPC 315, a myeloma IgA against TNP (Sigma Chemical Co.). Conjugates were stabilized with 0.5% BSA, washed, and concentrated by centrifugation to a standard volume. Based on our experience with other gold conjugates, we assumed that no more than 25% of the protein used for conjugation remained bound to the gold after washing; the estimated final concentrations of gold-conjugated proteins were monoclonal IgAs RB2 and RB5, 180 μg/ml; MOPC 315 myeloma IgA, 300 μg/ml; rat sIgA, 100 μg/ml; polyclonal IgG, 300 μg/ml; BSA, 2.4 mg/ml. Mice were anesthetized and a standard volume (40–60 μl) of each gold conjugate was injected into a ligated intestinal loop of uniform length containing a PP. After 1 h, loops were excised, quickly rinsed with PBS, and fixed and embedded as described above. Thin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (model 100CX; JEOL USA, Peabody, MA).

For examination of IgA-gold binding in the presence of excess unlabeled protein, a series of ligated loops were injected first with 30 μl of excess competitor protein (either 50 mg/ml MOPC 315 IgA, 10 mg/ml IgG, or an equal concentration of BSA) and returned to the abdominal cavity. After 15 min, 30 μl of either MOPC 315 or RB5 IgA-gold conjugates were added to the loops. Based on estimated concentrations of protein in gold conjugates, this represented a 1,000-fold excess of unlabeled competitor IgA protein and 200-fold excess of unlabeled competitor IgG. After 1 h, intestinal loops were processed for EM as described above. For each condition tested, all clearly visualized M cells in sections from 5 to 6 separate blocks of tissue were photographed and examined for IgA-gold binding. Numbers of M cells with and without surface-bound IgA-gold were tallied and differences compared by chi-square analysis for significance. In addition, we counted the number of IgA-gold particles bound per 10 μm M cell luminal membrane as measured with a digitizing pad. A measuring magnifier was used to identify gold particles that lay within 25 nm of the membrane, a distance consistent with direct membrane binding taking into account the dimensions of the polymeric IgA molecule (6). One factor analysis of variance was used to determine whether differences in surface density were significant.

TNP-Ferritin Binding Assays

Horse spleen ferritin (Polysciences Inc., Warrington, PA) at a concentration of 0.022 mM in PBS was mixed with an equal volume of trinitrobenzenesulfonic acid (Sigma Chemical Co.) at a concentration of 81.8 mM in PBS, and stirred for 1 h in the dark. Unreacted trinitrobenzenesulfonic acid was removed by passing the mixture over a Sephadex G25 column (PD-10; Pharmacia Fine Chemicals, Piscataway NJ). Conjugation of TNP to ferritin was confirmed by ELISA using anti-TNP monoclonal antibodies. To generate immune complexes, TNP-ferritin was mixed with MOPC-315 (anti-TNP) monoclonal IgA (final concentrations: 1 mg/ml TNP-ferritin and 5 mg/ml IgA) with 10 mg/ml BSA. As control, TNP-ferritin was mixed with RB2 (antireovirus) monoclonal IgA under the same conditions. The mixtures were allowed to stand for 1 h before injection into intestinal loops. Visible precipitates did not form over this time, and EM revealed monodisperse ferritin particles with no aggregates. M cell binding assays, quantitation and statistics were done as described for colloidal gold probes.

Immunofluorescence

Tissue was fixed in a solution of 10 mM $NaIO_4$, 7.5 mM lysine, 3.7 mM Na_2HPO_4, and 2% paraformaldehyde for 3 h at room temperature. Tissue was then treated for 45 min in PBS containing 50 mM NH_4Cl, infiltrated with 2.3 M sucrose, mounted on chucks coated with Tissue-Tek II (Lab-Tek Products, Naperville, IL), and frozen in liquid nitrogen-cooled Freon 22. Frozen sections (1 μm) were cut on a cryomicrotome (model FC-4; Reichert

Scientific Instruments, Buffalo, NY). Before immunostaining, free aldehydes were blocked with 50 mM NH_4Cl in PBS, and nonspecific protein binding was blocked with 0.2% gelatin in PBS. Sections were treated with primary antibody diluted in PBS/gelatin and with an appropriate secondary antibody, either rabbit anti-mouse IgG (preabsorbed with rat serum) conjugated to rhodamine (HyClone Laboratories, Logan, Utah) or rabbit anti-rat IgG (preabsorbed with mouse serum) conjugated to fluorescein (HyClone Laboratories). Sections were photographed with a light microscope (model PM3; Carl Zeiss, Inc., Thornwood, NY) using 400 ISO film (TMAX; Eastman Kodak Co., Rochester, NY) for both fluorescence and Nomarski optics.

Results

Production of Anti-MMTV Monoclonal Antibodies

Experiments using MMTV as immunogen were designed to determine whether cells obtained from PPs alone could be used to obtain hybridomas producing specific antivirus IgA. Mice were immunized by three different immunization protocols, one involving injection of antigen directly in PP submucosa and the others involving one or two injections into the intestinal lumen (Table I). Cells from PP mucosa, mesenteric lymph nodes (MLN), and spleen were each fused separately with myeloma cells.

All three immunization methods and all three tissues yielded hybridomas that produced anti-MMTV antibody. Of the eight hybridomas that produced specific IgA antibodies, seven were derived from PP cells (Table II). Two anti-MMTV IgA-producing hybridomas were obtained from mice immunized by method A (intra-PP and i.p. injections), but neither of these proved stable. Six anti-MMTV IgA hybridomas were derived from PP cell fusions after i.l. injections (methods B and C); two (designated MB2 and MB3) remained stable during expansion and were cloned twice before further characterization. Although the number of stable MMTV specific IgA hybridomas obtained in this study was too small to determine which immunization protocol was most effective, the results suggest that injection directly into PP submucosa had no advantage over i.l. immunization, and that IgA-producing hybridomas could be produced by fusing PP cells.

Table II. Production of Anti-MMTV Hybridomas

Immunization	Cell source	Wells screened	Anti-MMTV positive wells‡	
			Non-IgA	IgA§
Method A	PP	152	1	1
(i-PP/i.p./i.p.)*	MLN	120	3	0
	spleen	192	15	1
Method B	PP	124	0	5
(i.l./i.l./i.p.)	MLN	136	1	0
	spleen	192	7	0
Method C	PP	176	1	1
(i.l./i.p./i.p.)	spleen	192	13	0

* *i-PP*, injections into PP submucosa; *i.p.*, intraperitoneal injection; *i.l.*, injection into lumen of intestine. For details, see Table I and Methods.
‡ Number of wells producing anti-MMTV antibody of non-IgA (IgG and IgM) and IgA isotypes. Specific antibody production was determined by ELISA assay.
§ Of the IgA-producing hybridomas identified in early screening, only two continued to produce antibody after expansion.

Table III. Production of Antireovirus Hybridomas

Immunization	Cell source	Wells screened	Anti-MMTV positive wells‡	
			Non-IgA	IgA§
Method A	PP	288	15	8
(i.l./i.l./i.p.)*				
Method B	PP	337	9	4
(oral/oral/oral)				

* *i.l.*, injection into lumen of intestine; *i.p.*, intraperitoneal injection. For details, see Table I and Methods.
‡ Number of wells producing antireovirus antibody of non-IgA (IgG and IgM) and IgA isotypes. Specific antibody production was determined by ELISA assay.
§ Of the IgA-producing hybridomas identified in early screening, only 10 continued to produce antibody after expansion.

Production of Antireovirus Monoclonal Antibodies

To obtain specific antireovirus type 1 IgA hybridomas, PP cells alone were used for fusion after two immunization protocols (Table I). One protocol consisted of two i.l. injections followed by an i.p. boost, and the other consisted of three oral doses of virus with no injections. One-third of the antireovirus monoclonal antibodies generated by both protocols were of the IgA isotype (Table III). From 12 wells initially positive for antireovirus IgA, 10 stable hybridomas were obtained, 6 from mice immunized intraluminally, and 4 from mice immunized orally. These hybridomas were expanded, cloned twice or more until stable, and designated RB1 to RB10.

Specificity of Antivirus Monoclonal IgA Antibodies

The specificity of anti-MMTV IgA antibodies MB2 and MB3 was examined by immunoblot analysis. MB3 did not recognize its antigen on immunoblots, but MB2 bound to a 52-kD band when tested against purified MMTV particles (Fig. 1 *A*). The band co-migrated with gp52, the major envelope glycoprotein of MMTV. MB2 also recognized its antigen after MMTV proteins were deglycosylated with endoglycosidase F. Since the MMTV envelope glycoprotein gp52 does not contain O-linked carbohydrates (3), this indicates that MB2 is not directed against a carbohydrate epitope. To provide further evidence that MB2 binds to gp52, we tested it on immunoblots of lysates from control GR cells, which produce low levels of MMTV, and dexamethasone-

Table IV. Binding of Monoclonal IgA Antibodies to Complete Reovirus Particles (Serotypes 1 and 3) and Intermediate Subviral Particles

Monoclonal IgA antibody	cpm bound per well*		
	Reovirus type 3	Reovirus type 1	type 1 ISVP‡
RB2	30,930	28,995	652
RB3	19,990	16,290	466
RB8	8,374	11,474	10,806

* Binding of antibody was determined by solid-phase radioimmunoassay. Wells were coated with virus particles and treated with the indicated monoclonal antibodies followed by ^{125}I-labeled anti-mouse Ig antibody.
‡ Reovirus type 1 intermediate subviral particles (*ISVP*).

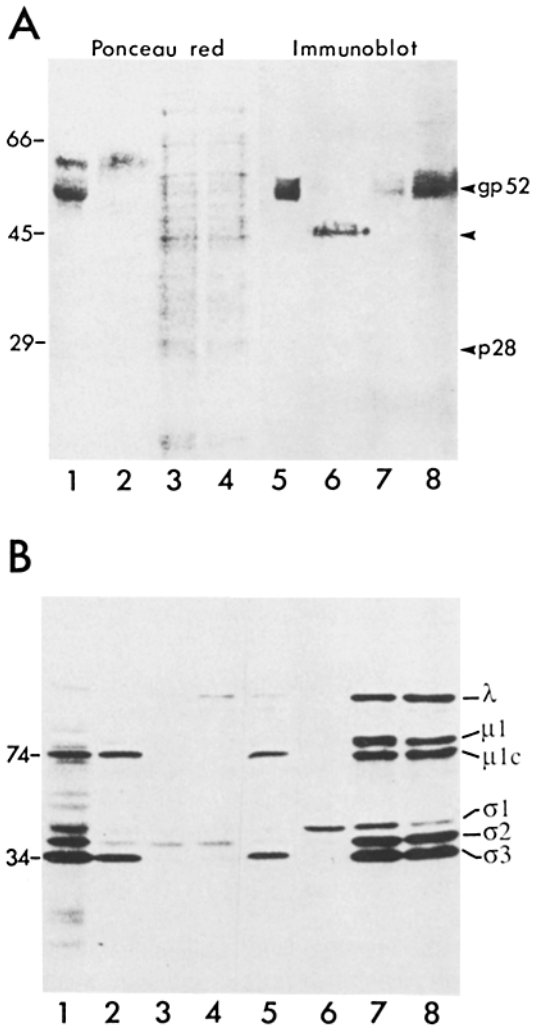

Figure 1. Specificity of antivirus monoclonal IgA antibodies. (*A*) Immunoblot analysis showing that MB2 recognized the major MMTV surface glycoprotein gp52. Proteins of purified MMTV and MMTV-infected GR cells were electrophoresed on a 10–15% SDS polyacrylamide gel and transferred to nitrocellulose. Lanes *1–4* show Ponceau red staining of proteins. Lanes *5–8* show identical lanes immunostained with antibody MB2. Lanes *1* and *5* show proteins of purified MMTV particles. Lanes *2* and *6* show endoglycosidase F-treated MMTV proteins. Lanes *3* and *7* show extracts of control GR cells. Lanes *4* and *8* show extracts of GR cells treated with dexamethasone to stimulate MMTV production. Arrowheads (*right*) indicate major MMTV protein bands: gp52 and gp52 after endoglycosidase F treatment, and p28. Molecular mass standards (*left*) are in kilodaltons. (*B*) Immunoprecipitation of ^{35}S-labeled viral proteins from lysates of reovirus-infected cells. Immunoprecipitates were run on a 10% polyacrylamide gel and radiolabeled bands detected by fluorography. Lanes *1–5* show precipitation by monoclonal IgAs RB2, RB3, RB4, RB5, and RB8. Lane *6* shows precipitation by monoclonal IgG antibody 5C6 against the sigma 1 protein of reovirus type 1. Lanes *7* and *8* show precipitation by antisera against reovirus type 1 (lane *7*) and type 3 (lane *8*). There was decreased precipitation of type 1 sigma 1 by anti-type 3 antibodies. Major reovirus proteins are indicated at right. Molecular masses of viral proteins mu lc and sigma 3 are indicated at left.

treated GR cells, which produce high levels of MMTV. Binding to the 52-kD band was greatly enhanced with dexamethasone-treated cells, suggesting that the band represented an MMTV protein.

All of the antireovirus IgA antibodies that recognized reovirus type 1 in ELISA assays also recognized reovirus type 3; this was confirmed by solid phase radioimmunoassay. Types 1 and 3 are defined by the nature of the serological response to the sigma 1 outer capsid protein, the viral hemagglutinin known to be a potent systemic immunogen (35). These results therefore suggest that none of the mucosally derived IgA antibodies recognized sigma 1. Since none of the antireovirus IgA antibodies detected viral proteins on Western blots, specificity was determined by immunoprecipitation from lysates of ^{35}S-labeled reovirus-infected cells as shown in Fig. 1 *B*. Three antibodies, RB2, RB3, and RB8 (all obtained from intraluminally immunized mice) immunoprecipitated proteins that co-migrated on gels with sigma 3 (34 kD) and mu lc (72 kD), two major components of the reovirus outer capsid that are closely associated in intact virus and in infected cell lysates (35). We therefore wished to determine if these antibodies recognized sigma 3 or mu lc. Sigma 3 can be removed from the virus particle by protease treatment, leaving intermediate subviral particles in which the major portion of mu lc is present as a cleaved product. When intermediate subviral particles were used in radioimmunoassay, binding of two antibodies (RB2 and RB3) was markedly reduced (Table IV), suggesting that these antibodies reacted with sigma 3, or possibly with the portion of mu lc that had been removed.

Polymeric State of IgA Antibodies Produced by PP-derived Hybridomas In Vitro

To determine whether the monoclonal IgA antibodies were produced as polymers, concentrated culture media from IgA-producing hybridomas RB1 through RB10 were run on a nonreducing gel, transferred to nitrocellulose, and reacted with peroxidase-conjugated antibody specific for mouse IgA (Fig. 2). Monomeric and polymeric IgA bands showed lower apparent molecular masses than expected for complete mouse IgA, presumably because of the absence of covalent bonds between IgA heavy and light chains in BALB/c mice (45) that causes dissociation of light chains in SDS sample buffer. The apparent molecular masses of the IgA bands were nevertheless consistent with their being IgA monomers, dimers, and higher polymers. In all cases, the majority of IgA was present in polymeric form.

Transfer of IgA from Blood to Bile

Three monoclonal IgA antibodies (RB2, RB3, and RB4) and one monoclonal IgG (directed against *Vibrio cholerae*) were metabolically labeled with [^{35}S]cysteine and injected intravenously into rats in which the bile duct had been cannulated. Rat liver polymeric immunoglobulin receptors can bind mouse IgA and in rats, IgA is transported from blood to bile much more efficiently than in mice (12). Fig. 3 shows that 34, 45, and 58% of the radiolabeled IgA activity was recovered in the bile over 3 h, suggesting that the monoclonal IgA antibodies bound to liver polymeric immunoglobulin receptors and were transported into the bile. In contrast, only 3% of the injected IgG activity was recovered in the bile. When

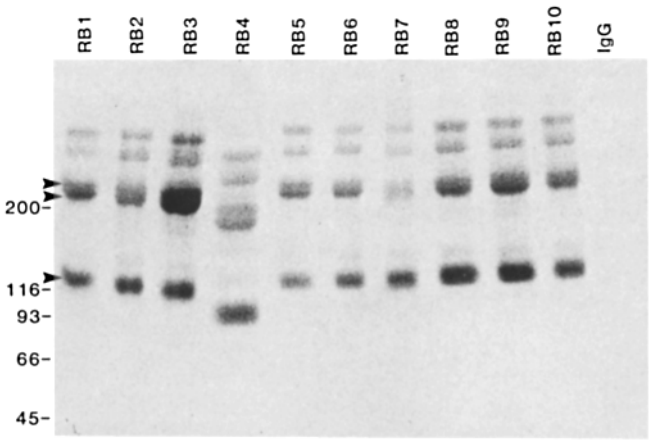

Figure 2. Anti-IgA immunoblot analysis of hybridoma media showing in vitro production of monomeric and polymeric IgA. Concentrated serum-free medium from antireovirus IgA-producing hybridomas RB1-RB10, and one IgG-producing hybridoma, were run on a nonreducing 3–10% polyacrylamide gel, transferred to nitrocellulose, and immunostained with peroxidase-coupled antibody specific for mouse IgA. Each IgA was produced in monomeric (lane *1*, *single arrowhead*), dimeric (lane *1*, *double arrowhead*), and higher polymeric forms. The lack of reaction in the IgG control lane shows that immunostaining was specific for IgA. Positions of molecular mass standards in kilodaltons are shown.

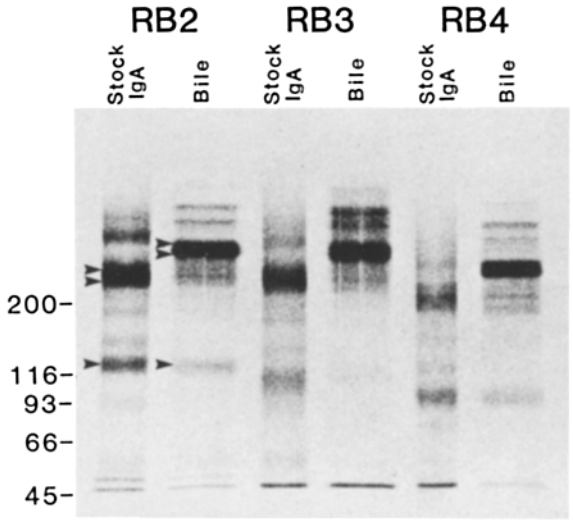

Figure 4. Paired samples of stock ^{35}S-labeled IgA (RB2, RB3, and RB4) that were injected i.v. into rats, and bile from the rat in which it was injected, were run without reduction on a 3–10% gradient polyacrylamide gel and autoradiographed. Monomeric (*single arrowheads*) and dimeric (*double arrowheads*) IgA are identified in the first two lanes. Higher polymer bands are seen above the dimer bands. The shift in mobility of polymeric IgA in the bile samples suggests that it was associated with secretory component. Positions of molecular mass standards in kilodaltons are shown.

labeled IgA antibodies were run on nonreducing acrylamide gels and autoradiographed, radioactive bands were seen at positions corresponding to monomeric and polymeric IgA (Fig. 4). Bile from animals that had been injected with labeled IgA showed radioactive bands at positions corresponding to polymeric IgA, but with apparent molecular masses ~60 kD higher than those of stock labeled IgA, an increase consistent with addition of secretory component.

IgA Binding and Transport

To test whether IgA interacts with the intestinal epithelium, we used light microscope autoradiography and EM of PP tissue treated with radiolabeled IgA or IgA-colloidal gold. M cells were identified in the lymphoid FAE by the presence of intraepithelial lymphocytes. By EM, M cells were recognized by their short, irregular microvilli and the invaginated basal membrane that forms a pocket containing lymphoid cells (Fig. 5). Light microscope autoradiography of PP FAE after 1 h luminal exposure to ^{35}S-labeled antireovirus IgA showed clusters of grains associated with M cells (Fig. 6). No label was detected in epithelial cells of adjacent villi. EM of mouse PP tissue exposed to colloidal gold conjugated to antireovirus IgA antibodies (RB2 or RB5) or to anti-TNP IgA (MOPC 315) revealed selective association of IgA-gold particles with M cell surfaces (Fig. 7). IgA-gold particles adhered to M cell luminal membranes both individually and as clusters. Frequently, IgA-gold was seen in M cell intracellular vesicles (Fig. 8, *A* and *B*) and within the intraepithelial pocket (Fig. 8 *C*). In EM sections from two experiments, 42 and 48% of M cells with surface-bound IgA-gold also showed probe in endocytic vesicles and/or the intraepithelial pocket. Small amounts of IgA-gold bound to non-M columnar cells of the FAE but not to the absorptive epithelium of adjacent villi. In contrast, BSA-gold did not bind to M cells and was rarely internalized. Identical binding and transport patterns were observed in rat and rabbit PPs exposed to mouse IgA-gold and in mouse PPs exposed to rat polyclonal secretory IgA-gold (not shown).

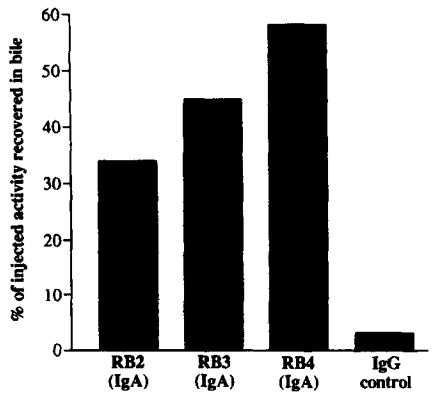

Figure 3. Transport of monoclonal IgA from blood to bile. Three monoclonal IgAs and one monoclonal IgG were metabolically labeled with [^{35}S]cysteine and injected intravenously into adult rats. Over a 3-h period, 34–58% of injected [^{35}S]cysteine was recovered in bile. In contrast, very small amounts of [^{35}S]cysteine were recovered after labeled IgG injection.

Binding of IgA-gold in the presence of 1,000-fold excess unlabeled IgA, or comparable amounts of excess BSA, was examined to test the specificity of binding. Inhibition was measured by scoring the percentage of M cells with IgA-gold bound to their surfaces (Fig. 9 *A*), and by counting the number of IgA-gold particles per 10 μm of membrane (Fig. 9 *B*). By both criteria, unlabeled anti-TNP IgA significantly in-

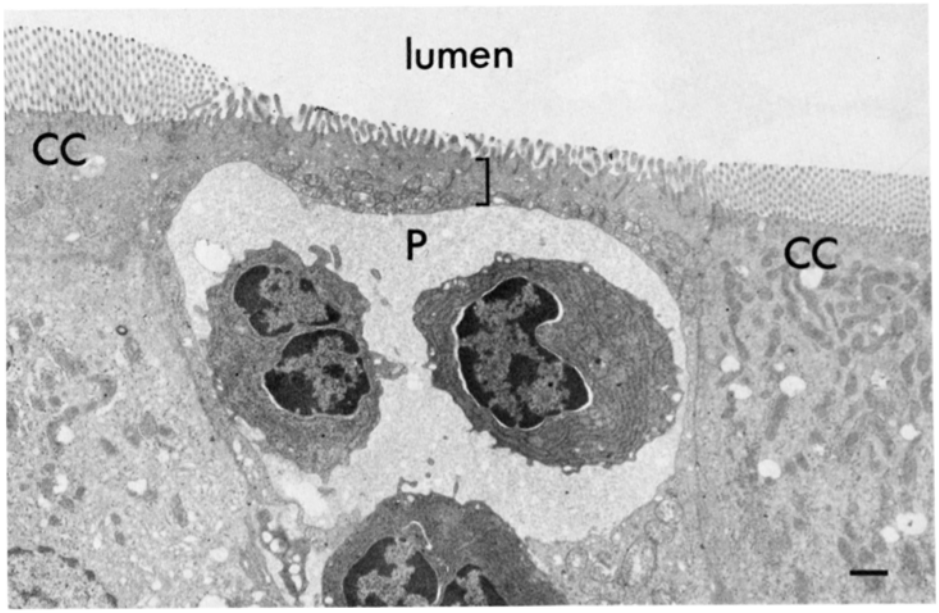

Figure 5. M cell and adjacent columnar cells (*CC*) of mouse lymphoid FAE. The typical M cell has short, irregular microvilli and a basolateral pocket (*P*) into which lymphoid cells (here resembling plasma cells) and macrophages migrate. Luminal antigens are endocytosed, transported across the thin bridge of apical cytoplasm (*bracket*), and delivered to the basolateral pocket. Bar, 1 μm.

hibited binding of both anti-TNP IgA-gold and antireovirus IgA-gold conjugates.

To determine whether binding was confined to antibodies of the IgA class, we examined binding of polymeric mouse IgG conjugated to colloidal gold. IgG-gold, like IgA-gold, bound to M cells but not to villus absorptive cells, and IgG-gold was transported to the M cell pocket. IgA-gold binding was reduced by 52% in the presence of excess IgG (as compared to binding in the presence of an equal amount of excess BSA). Thus, IgA and IgG share M cell binding sites.

Binding of IgA-Antigen Complexes

To determine the effect of specific IgA on binding and transport of antigen by M cells, we used the hapten TNP (rendered visible by conjugation to ferritin) and specific monoclonal anti-TNP IgA. This system was used because TNP-ferritin binding to M cells is readily assessed quantitatively in EM sections; quantitation of virus binding and transport will require extensive studies that are beyond the scope of this report. Mouse PP were exposed to TNP-ferritin mixed with fivefold excess of either anti-TNP IgA or as control, antireovirus IgA. Adherence of TNP ferritin to M cell apical membranes was enhanced about 10-fold by the presence of specific anti-TNP IgA (Fig. 10).

Absence of Previously Described Immunoglobulin Receptors on M Cells

Frozen 1 μm sections of PP FAE were examined by immunofluorescence for the presence of immunoglobulin receptors previously identified on epithelial cells or macrophages. Fig. 11, *A–D*, shows paired fluorescence and Nomarski optical images of rabbit tissue treated with monoclonal antibody 166 against the cytoplasmic tail of the polymeric immunoglobulin receptor (39). The receptor was present on villus absorptive cells, but was not observed on epithelial cells of

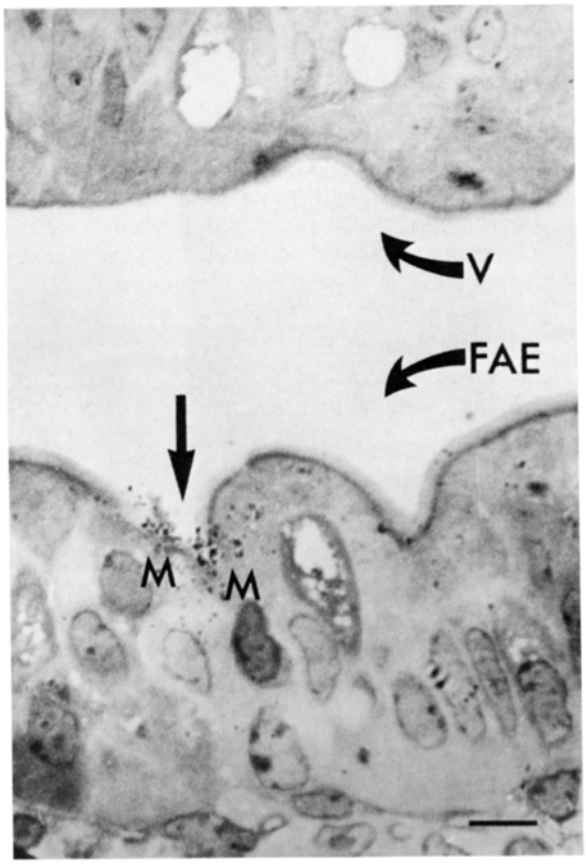

Figure 6. Association of IgA with M cells (*M*) of mouse FAE shown by light microscopic autoradiography. PP mucosa was exposed for 1 h to ^{35}S-labeled monoclonal antireovirus IgA. Autoradiography shows label associated with M cells but not with villus epithelium (*V*). Arrow indicates M cell-associated cluster of silver grains. Bar, 10 μm.

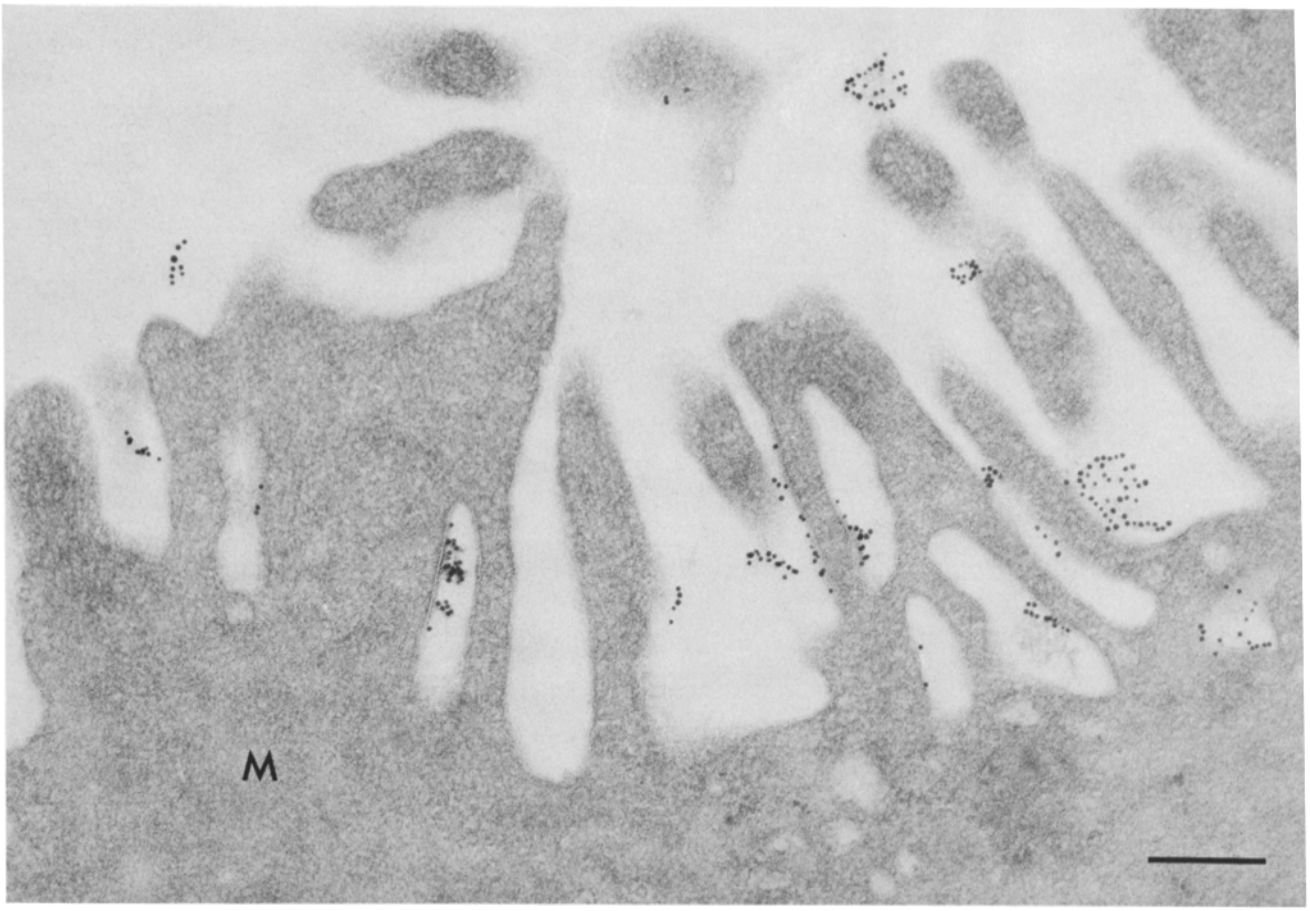

Figure 7. Electron micrograph of the apical surface of an M cell from a mouse PP after 1 h luminal exposure to mouse monoclonal antireovirus IgA conjugated to 6–8 nm colloidal gold. IgA-gold adhered closely to luminal membranes of M cells (*M*) but not to villus absorptive cells. Bar, 0.2 μm.

the FAE, including M cells. Fab fragments of monoclonal antibody 2.4G2 against the high avidity macrophage Fc receptor (19) bound to subepithelial macrophages in sections of mouse PP tissue, but did not bind to cells of the FAE (Fig. 11, *E–H*). Monoclonal antibody MC39 against epithelial Fc receptors of neonatal rat jejunum (32) also failed to bind to M cells or other epithelial cells of the adult rat FAE (Fig. 11, *I–L*).

Discussion

Role of M Cells in Mucosal Immunity

Transepithelial transport by M cells appears to be an important event in generation of secretory immune responses to luminal antigens (4, 25, 37). These specialized cells, confined to lymphoid FAEs of digestive and respiratory mucosal surfaces, are invaginated basally to form an intraepithelial pocket into which lymphocytes and macrophages migrate (4, 7, 25). Rapid transepithelial transport of luminal antigens into this pocket is believed to allow local antigen sampling by cells of the mucosal immune system, but it is not known whether M cells play a more specialized role in modulating the wide variations observed in mucosal immune responses to diverse luminal antigens (17, 37). M cells are devoid of Ia antigen (17), and there is no evidence to date that they are capable of antigen presentation, but they could modulate immune responses by determining the dose, concentration, or form of antigen delivered. For example, we previously demonstrated that luminal lectins that adhere to M cell surfaces were transported across the epithelium at least 50 times more efficiently than BSA, a protein that does not adhere (23). Particulate antigens and microorganisms that can adhere to and colonize epithelial surfaces are generally more effective in eliciting secretory immunity than those that do not adhere (17, 28, 30). If we are to eventually predict or manipulate secretory immune responses, it is important to precisely correlate M cell transport of specific luminal antigens with the mucosal immune response, and also to define the influence of secreted antibodies on subsequent transepithelial transport of antigen by M cells.

Role of M Cells in Transport of Viruses

Reovirus type 1 adheres specifically to the luminal surfaces of M cells of PP (48) and uses the M cell transepithelial transport system to invade and colonize the mucosa before spreading to distant sites (35). The function of several reovirus outer capsid proteins are established; for example, sigma 1 mediates the tissue specificity of systemic spread (11). It is

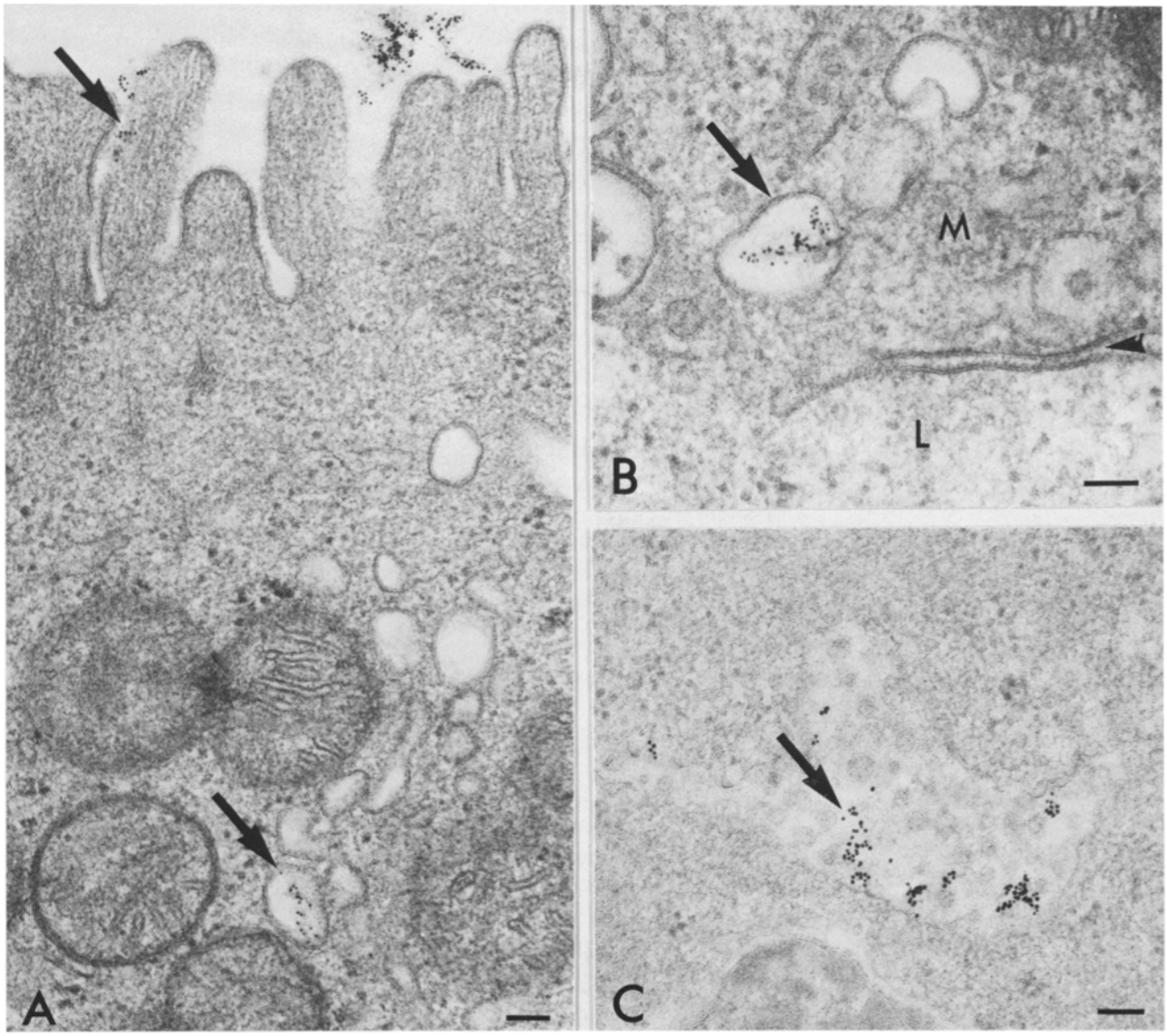

Figure 8. Electron micrographs of M cells from mouse PPs after 1 h luminal exposure to mouse monoclonal IgA conjugated to 5 nm or 6–8 nm colloidal gold. Bars, 0.1 μm. (*A*) Anti-TNP IgA-gold (*arrows*) on the M cell surface and within a vesicle of the type previously shown to transport macromolecules to the basolateral pocket. (*B*) IgA-gold in an M cell (M) vesicle (*arrow*) close to a portion of the basolateral pocket that contains a lymphocyte (*L*). The extracellular space is indicated by an arrowhead. (*C*) IgA-gold (*arrow*) in the extracellular space of the M cell pocket.

not known, however, which viral protein mediates initial attachment to M cell surfaces (47). In any case, adherence of reovirus to M cells enhances manifold the efficiency with which reovirus invades the mucosa via the M cell route, and may also facilitate development of a mucosal immune response. Enteric administration of reovirus has been shown to elicit a mucosal immune response as reflected by intestinal secretion of antivirus IgA antibodies (34), generation of memory B cells committed to IgA production, and long-lived virus-specific cytotoxic T cells in PP (15). MMTV, a retrovirus that is shed into the milk of infected mothers and infects offspring during the suckling period, gains access to host tissues by transepithelial transport across the intestinal epithelial barrier (21). The exact sites of MMTV invasion in the gastrointestinal tract are not established (10). Whether MMTV selectively adheres to M cells to gain access to the host is currently being tested.

Our initial plan was to use monoclonal antibodies of known specificities in attempts to block virus attachment to intestinal epithelial cells and thus identify the viral surface proteins that mediate attachment. IgG antibodies would be unsuitable for these studies because they are degraded in the intestinal lumen whereas secretory IgA, because of the presence of secretory component, is more stable (44). Therefore, our first goal was to generate hybridomas that produce polymeric antivirus IgA antibodies and to test these antibodies for their binding specificities and ability to associate with secretory component.

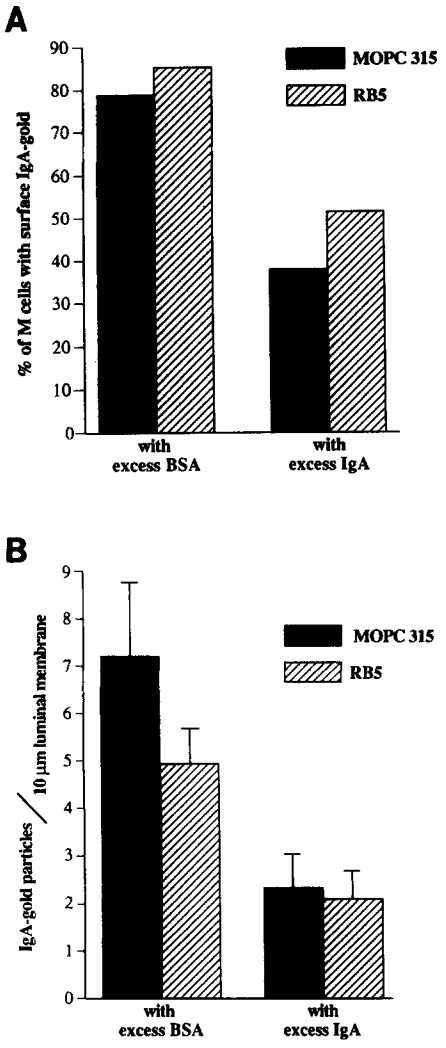

Figure 9. M cell binding of anti-TNP (MOPC 315) and antireovirus (RB5) IgA-colloidal gold conjugates in the presence of approximately 1000-fold excess of unlabeled MOPC 315 IgA, or a comparable excess of BSA. (*A*) The percentage of M cells with surface-bound IgA-gold. Chi square analysis of this data showed that excess unlabeled MOPC 315 IgA caused a significant decrease in binding of both MOPC 315 (p = 0.002) and RB5 (p = 0.004). (*B*) The mean number of surface-bound IgA-gold particles per 10 μm of M cell luminal membrane. Vertical bars indicate the SEM. Analysis of variance confirmed that excess unlabeled IgA caused a significant decrease in binding of both MOPC 315 (p = 0.010) and RB5 (p = 0.007).

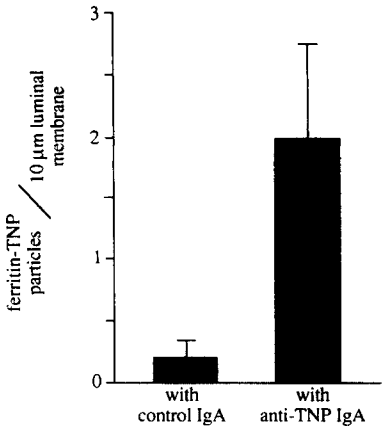

Figure 10. Binding of TNP-ferritin to M cells in the presence of control (antireovirus) IgA (RB2) or anti-TNP IgA (MOPC 315). Bars show the mean number of surface-bound ferritin particles per 10 μm of M cell luminal membrane. Vertical bars indicate the SEM. Analysis of variance showed that binding in the presence of anti-TNP IgA was significantly greater than binding with control IgA (p = 0.004).

PP Cells from Orally Immunized Mice as a Source of Polymeric IgA Antibodies

Our results establish that PP cells are effective in generating IgA-secreting hybridomas when directly fused with myeloma cells. IgA-producing hybridomas have been generated previously using spleen cells from mucosally immunized animals (8, 18), but the technique is not always successful (9). Cells from MLN and thoracic duct lymph (31, 41) or mixed cells from MLN and PPs (13) have been used successfully, and it was suggested that PP lymphocytes alone may be effective in generating IgA-secreting hybridomas (41). Our results support this hypothesis and suggest that PP cells may be better than either spleen or MLN cells for this purpose.

Binding specificities were determined for several of the resulting antivirus monoclonal IgA antibodies, identifying viral proteins that are able to elicit mucosal immune responses. For reovirus, the outer capsid protein sigma 3 proved to be immunogenic after i.l. (but not oral) administration of virus. For MMTV, the major surface glycoprotein gp52 was identified as a mucosal immunogen. Identification of other potential immunogens will require further fusions.

The hybridomas described in this paper produced IgA primarily in the expected polymeric forms, the majority being dimeric. We demonstrated that antireovirus monoclonal IgA antibodies were efficiently transported from blood to bile, confirming their ability to interact with liver polymeric immunoglobulin receptors. SDS-PAGE of bile proteins followed by autoradiography showed that the polymeric antibody molecules were transported intact, and that secretory component was added on passage through the liver. Having confirmed that these monoclonal, polymeric IgA antibodies were analogous to IgA produced in vivo, we proceeded to apply them to mucosal surfaces.

M Cells Express Immunoglobulin Binding Sites

Mouse monoclonal IgA antibodies adhered selectively to M cell luminal membranes in the intestinal epithelia of adult mice, rats, and rabbits, and were transported transepithelially, regardless of their antigen specificities. IgG also bound to M cell surfaces and inhibited IgA binding. Together, these results suggest that a common immunoglobulin domain mediates adherence to M cells and that the cell surface binding site is not species specific. Since sIgA is the principal immunoglobulin present in the adult intestinal lumen (42), Ig binding sites on M cells in vivo would presumably interact primarily with sIgA. Immunocytochemical studies of developing PPs in suckling rabbits showed that endogenous IgA from ingested milk accumulates on M cell surfaces in FAE (33). We have now demonstrated that purified polyclonal sIgA binds

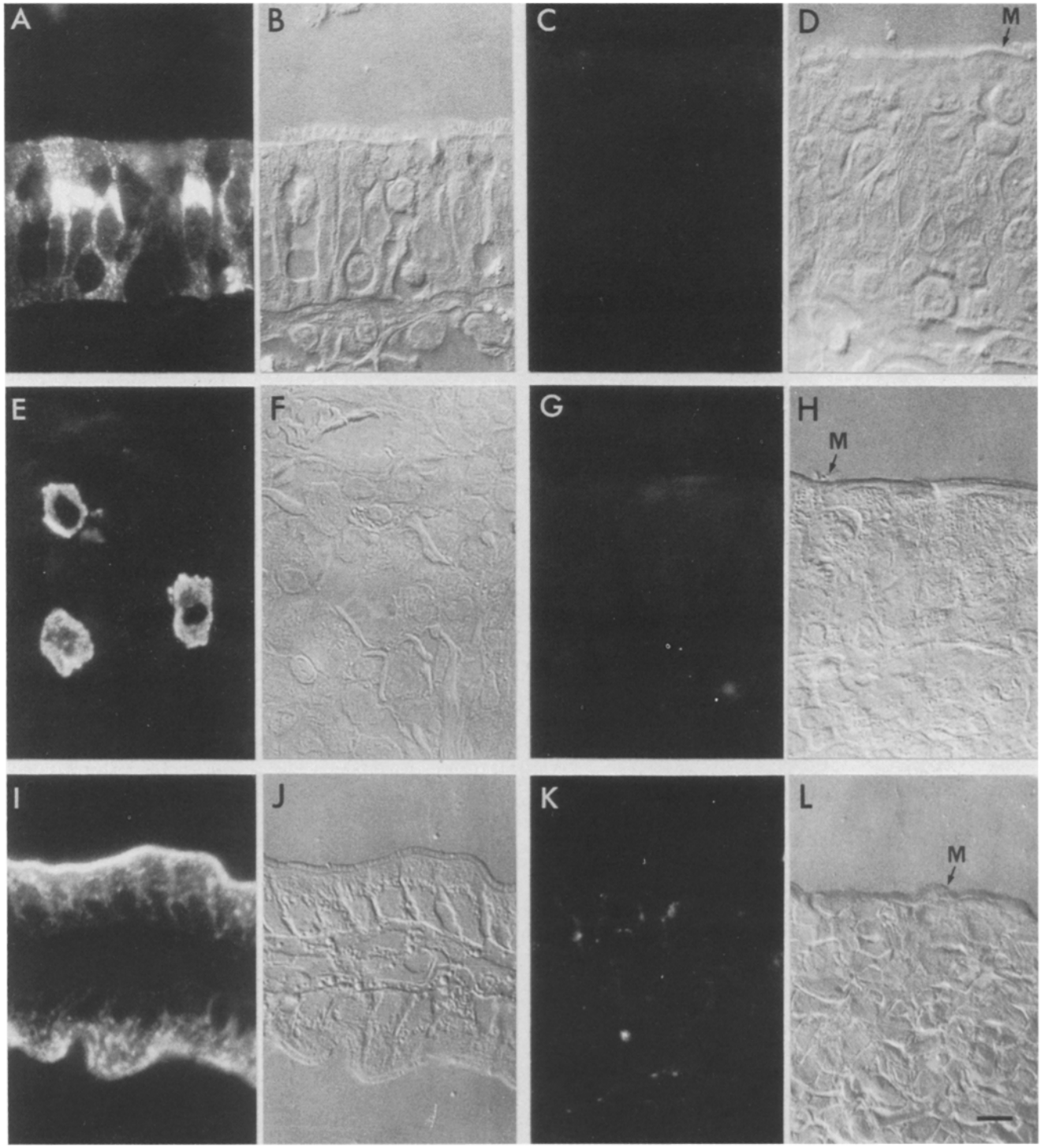

Figure 11. Immunofluorescent staining and matched Nomarski images of 1-μm sections of intestinal mucosa treated with monoclonal antibodies against immunoglobulin receptors. (*A–D*) Rabbit mucosa treated with mouse monoclonal antibody 166 against the polymeric immunoglobulin receptor (39). (*A* and *B*) Villus epithelium showing presence of receptor in absorptive epithelial cells. (*C* and *D*) FAE of PP showing absence of receptor on all FAE epithelial cells, including M cells (*arrow*). (*E–H*) Mouse PP mucosa treated with rat monoclonal antibody 2.4G2 against macrophage Fc receptor (19). (*E* and *F*) Subepithelial area showing receptor-positive macrophages. (*G* and *H*) FAE showing absence of receptor on M cells (*arrow*) or other FAE epithelial cells. (*I–L*) Rat mucosa treated with monoclonal antibody MC39 against the neonatal rat jejunum immunoglobulin receptor (32). (*I* and *J*) Neonatal rat jejunum showing intracellular and brush border staining of absorptive epithelial cells. (*K* and *L*) FAE of adult rat PP showing absence of reactivity with M cells (*arrow*) or other epithelial cells. Bar, 10 μm.

to adult mouse M cells. Since in both these cases the IgA was complexed with secretory component, it is clear that IgA can bind selectively to M cells whether or not secretory component is present. There is indirect evidence that M cells have unique apical surface components: certain microorganisms including reovirus (47, 48) and *Escherichia coli* strain RDEC-1 (37) bind selectively to this cell type. The molecular basis for these interactions is unknown. Proteins previously shown to bind to M cell membranes (certain lectins and cationized ferritin) are nonselective in that they bind to all intestinal cell types (23). Thus, immunoglobulins are the only macromolecules identified to date that bind selectively to M cells in the adult intestinal epithelium. We have further shown that the presence of specific IgA in the intestinal lumen enhances binding of antigen to these cells. Additional studies are required to determine whether IgA also enhances binding and transport of enteric viruses.

Our previous studies showed that adherence to M cells greatly increases the efficiency of transepithelial transport (23). Selective transepithelial transport of IgA-antigen complexes in vivo would serve to deliver these complexes to intraepithelial and subepithelial macrophages and lymphocytes for subsequent antigen processing and presentation. It is thus important to determine whether M cells express immunoglobulin receptors similar or identical to those previously identified on intestinal epithelial cells or macrophages. Using specific antibodies applied to appropriate intestinal tissues, we confirmed that M cells do not express the polymeric immunoglobulin receptor of enterocytes that mediates delivery of sIgA into the intestinal lumen (38, 39). Indeed, this receptor has been shown to be absent from the entire FAE (26). Nor do M cells express macrophage-type high avidity Fc receptors: subepithelial macrophages of PPs were labeled by Fab fragments of specific antimacrophage Fc receptor antibodies (19), but epithelial cells were not. Antibodies specific for the epithelial Fc receptors that mediate transepithelial transport of maternal milk IgG in suckling rat jejunum (32) also failed to recognize any component of M cell membranes. The nature of the M cell membrane components responsible for immunoglobulin adherence is currently under investigation in our laboratories.

Conclusion

Our results suggest that luminal antigens and microorganisms complexed with sIgA could be "targeted" to M cell surfaces, and selectively transported to underlying macrophages and lymphoid cells (Fig. 12). Considerable evidence indicates that sIgA effectively reduces accumulation of antigens and colonization of microorganisms on mucosal surfaces (1, 2, 44, 46). Nevertheless, it is likely that small amounts of antigens or pathogens gain access to the vast epithelial surface of the intestine despite the presence of specific sIgA. Efficient M cell binding and transport of these IgA–antigen complexes could serve to enhance or sustain the mucosal immune response once it has been initiated. This may explain why repeated oral administration of antigens has been shown to result in a persistent secretory immune response (40). It also suggests that the efficacy of oral immunization strategies might in some cases be enhanced by administration of immunogens in the form of IgA–antigen complexes. On the other hand, IgA-mediated M cell transport of viruses could also promote invasion and infection.

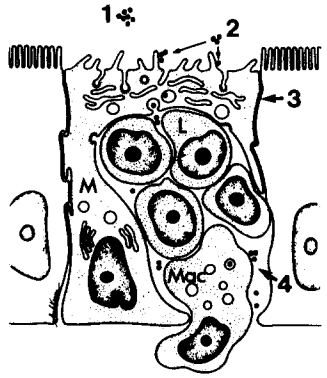

Figure 12. Model of selective binding and transport of antigen-IgA complexes by M cells. (*1*) Most antigens or virus particles that are complexed with sIgA in the lumen would be cross-linked, eliminated by peristalsis, and prevented from binding to absorptive cell surfaces. (*2*) Virus-IgA complexes that contact the mucosal surface would bind to M cell receptors, and (*3*) would be transported transepithelially, into the M cell pocket. (*4*) Virus-IgA complexes could then be phagocytosed by macrophages (*Mac*) and sampled by lymphocytes (*L*), or have access to subepithelial lymphoid tissue. This could lead to presentation of viral antigens, or to infection of macrophages and lymphoid cells.

The monoclonal antivirus IgA antibodies described in this study, with and without secretory component, can now be applied in vivo and in vitro to establish more clearly the role of IgA in adherence and transepithelial transport of enteric viruses and other antigens in FAE. Application of these tools to murine viral systems may provide new insight into the possible interactions of human enteric viruses with the intestinal epithelium and with the mucosal immune system.

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