Crypt Cell Development in Newborn Rat Small Intestine

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ABSTRACT Three monoclonal antibodies were prepared against luminal membranes from small intestinal cells of 3-d-old rats (YBB 1/27, YBB 3/10) and crypt cell membranes from adult rats (CC 4/80). The antibodies were shown to define specific stages of development of the intestinal crypt cells.

The YBB 1/27 antigen was first detected at the luminal membrane of the epithelial cells in fetal intestine at day 20 of gestation; it was confined to the crypt cells and lower villus cells between 1 and 20–22 d after birth, and could not be detected in any region of the intestine in older animals.

The YBB 3/10 antigen, identified as a set of high M_r proteins, was localized over the entire surface membrane of fetal intestinal cells and of crypt and villus cells after birth; after weaning (20–22 d after birth) it gradually disappeared from the villus cells and became confined to the region of the crypts.

The CC 4/80 antigen, identified as a protein (or a set of related proteins) of molecular mass 28–34 kD, was shown to appear in the crypt cells 10–14 d after birth. Its distribution changed after weaning, when it disappeared from the crypts, and was localized in the absorptive lower villus cells. This change in pattern could, in part, be prematurely elicited by cortisone injection in younger animals.

These results have demonstrated the presence of specific surface membrane components on the intestinal crypt cells, and suggested that fetal antigens may be retained in these cells after birth.

The small intestinal epithelium represents a system of continuous cell renewal: mitotically active cells, present in the region of the crypts, give rise to differentiated nonproliferative cells which migrate along the length of the villi and are eventually lost into the intestinal lumen (6, 24, 41). When the newly differentiated cells reach the top of the crypts or the base of the villi, they acquire distinctive ultrastructural features and surface membrane enzymes which have been studied extensively at the cellular and molecular level. Most of them are typical components of the luminal or brush border membrane of the absorptive villus cells, like alkaline phosphatase, sucrase, maltase, lactase, and various peptidases (20, 34, 37). All the known cellular components that can be considered specific markers for the intestinal epithelial cells are associated with the absorptive or goblet cells present on the villi, or are peptide hormones produced by enteroendocrine cells.

Much less is known regarding many aspects of the functions and biological properties of the crypt cells. Their ultrastructural characteristics and their proliferation kinetics have been studied in great detail. Cell proliferation in the crypts is known to be a steady-state process, with a constant frequency distribution of cells throughout the mitotic cycle, and is precisely balanced by cell loss at the tips of the villi (4, 5, 36). The crypts are populated by a heterogeneous cell population: basally situated crypt cells cycle relatively slowly, and are assumed to represent a pool of stem cells (1, 4, 5). Cells at various stages of differentiation are also known to be present in different regions of the crypts (6). However, no distinctive cellular markers have been conclusively identified for the intestinal crypt cells. A number of reports have suggested that active or inactive forms or precursors to typical villus cell enzymes may be present in the crypt cells (3, 9, 11, 38, 42). However, extensive immunofluorescence and biochemical studies using a large panel of independently derived monoclonal antibodies to sucrase, maltase, lactase, alkaline phosphatase, and aminopeptidase N have produced no evidence for the presence of any of these antigens in the crypts of adult rats, neither during normal postnatal development nor after cortisone-evoked precocious induction (of sucrase) in newborn rats (14, 33, 34, and Quaroni, A., submitted for publication).

Identification and characterization of specific crypt cell markers is likely to be of great importance for the study of the structure and function of the intestinal mucosa, and may

provide insight into basic aspects of cellular differentiation in the intestinal epithelium. The region of the crypts is where the initial events of this process take place, and key inductive and modulating interactions are likely to occur. In this paper, the properties of three monoclonal antibodies that define specific stages of development of the crypt cells in fetal, newborn, and adult rat intestine are described.

MATERIALS AND METHODS

Materials: Sprague-Dawley rats (CD strain, of either sex, weighing 100–170 g), timed pregnant Sprague-Dawley rats, and BALB/C mice (15–17 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Dulbecco's modified Eagle's medium, irradiated fetal bovine (calf) serum, penicillin-streptomycin mixture, and trypsin (2.5% in Hanks' balanced salt solution without calcium and magnesium) were obtained from M.A. Bioproducts (Walkersville, MD). Mitomycin C, thymidine, hypoxanthine, aminopterin, 8-azaguanine, Tris (hydroxymethyl)aminomethane (Tris), HEPES, phenylmethylsulfonyl fluoride (PMSF),[1] aprotinin, leupeptin, and antipain were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide and bis-acrylamide were from Bio-Rad Laboratories (Richmond, CA); sodium cyanoborohydride from Aldrich Chemical Co. (Milwaukee, WI); sequanal grade SDS, from Pierce Chemical Co. (Rockford, IL); polyethylene-glycol 1540 from J.T. Baker Chemical Co. (Phillipsburg, NJ); and Bacto Freund's adjuvant (complete and incomplete) from Difco Laboratories Inc., (Detroit, MI). Protein A-CL Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Affinity-purified goat anti-mouse IgG (H+L), $F(ab')_2$ fragment, and Mouse Immunoglobulin Subtype Identification Kit were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Triton X-100, formaldehyde ([^{14}C], 1% aqueous solution, 40–60 mCi/mmol), methylated ^{14}C-labeled molecular weight markers (carbonic anhydrase, bovine serum albumin [BSA], phosphorylase B, globulins, and myosin), and lysine (L-[4,5-^{3}H(*N*)], 80–110 Ci/mmol in 0.01 N HCl) were obtained from New England Nuclear (Boston, MA). Small volume filtration devices were from Porex Medical (Fairburn, GA).

Membrane Purification: The luminal (brush border) membrane of intestinal epithelial cells was purified by the method of Kessler et al. (21), using different cell/tissue samples for homogenization, as follows: (*a*) mucosal scrapings from adult rat small intestine were used for preparation of brush border membranes from the entire adult intestinal epithelium; (*b*) crypt cell fractions isolated from adult small intestine as described by Weiser (43) were used for purification of crypt cell luminal membrane: fractions 7–9 (43) were pooled as representing crypt cell–enriched fractions; (*c*) the entire intestines obtained from fetal and newborn (until 30 d after birth) rats were used for purification of the corresponding intestinal epithelial luminal membrane: the small size and fragility of the small intestine from fetal and newborn rats made it impractical to obtain mucosal scrapings. A mixture of protease inhibitors (1 mM PMSF, 50 μg/ml leupeptin, 50 μg/ml antipain, 0.1 mg/ml aprotinin) was added to all buffers and solutions used for homogenization and membrane purification. Protein was determined by the method of Lowry et al. (26) and all membrane preparations were monitored for purification (32) by measuring the increase in specific activity of sucrase (adult intestines) or lactase (fetal and newborn intestines), which was 12–20 times higher in the final microvillus-membrane fraction than in the homogenate. Due to the lack of known markers for crypt cells, the extent of purification of their luminal membrane could not be assessed; however, sucrase- and alkaline phosphatase–specific activities were determined in all crypt cell fractions to monitor for villus cell contamination. Specific activities for these two enzymes were, in all cases, found to be at least 20 times lower in crypt cell homogenates and purified membrane fractions than in the corresponding villus cell fractions.

Immunofluorescence Staining: Portions of small intestine from rats of all ages were rinsed with 0.155 M NaCl, cut into small segments (0.5–1 cm long) with a razor blade, immersed in O.C.T. embedding compound (Tissue Tek, Lab Tek Div., Miles Laboratories Inc., Naperville, IL), and quickly frozen in liquid nitrogen. Sections 4–6-μm thick were cut using a Histostat Cryostat (AO Scientific Instruments, Buffalo, NY), spread on glass slides, and allowed to dry at room temperature for at least 1 h. The intestinal sections were then incubated for 1 h at 4°C with 1% formaldehyde in 100 mM sodium phosphate buffer (pH 7.4), washed three times with phosphate-buffered saline (PBS), incubated for 1 h at 4°C with 100 mM glycine in PBS, washed two times with PBS + 0.2% (wt/vol) BSA. All subsequent incubations were performed at room temperature, and all incubations with sera or antibodies were in humidity chambers. The sections were sequentially: (*a*) incubated with goat serum diluted 1:25 in PBS; (*b*) washed three times with PBS; (*c*) incubated for 30 min with mouse serum diluted 1:100 with PBS + 0.2% BSA (controls) or hybridoma antibodies (straight hybridoma-conditioned medium or ascites fluid diluted 1:100 with PBS + 0.2% BSA); (*d*) washed three times with PBS; (*e*) incubated 30 min with fluorescein isothiocyanate–conjugated goat anti-mouse IgG, $F(ab')_2$ fragment, diluted 1:50 in PBS + 0.2% BSA; and (*f*) washed with PBS, incubated 30 s with Evans Blue, 0.1% (wt/vol) in PBS, washed two more times with PBS, mounted in glycerol-PBS 9:1, and viewed in a Nikon Optiphot microscope equipped with epifluorescence attachment.

Binding Assay To Detect Hybridoma Antibodies: Appropriate purified membrane fractions (microvillus membranes from 3–6-d-old rat intestine for YBB antibodies or crypt cell luminal membranes for CC antibodies) were diluted to 100–200 μg/ml in PBS + 1 mM $MgCl_2$ (pH 7.4), and sonicated 20–30 s (Branson Sonifier Model 200, equipped with a microtip, at power setting 3). 50 μl of membrane suspension (5–10 μg protein/well) were added to each well of Immunolon II microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) and incubated overnight at 4°C. After the fluid was aspirated, remaining protein binding sites were blocked by incubation for 60 min at 4°C with 1% (wt/vol) BSA in PBS (350 μl/well). Then the wells were washed three times with washing buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 0.05% [wt/vol] BSA, 0.05% [wt/vol] Nonidet P-40 [Particle Data, Inc., Elmhurst, IL]) and 100 μl of mouse serum diluted $1:10^3$ to $1:10^4$ in washing buffer (controls) or hybridoma-conditioned media were added to each well. After a 90-min incubation at 4°C, the solutions were aspirated and the wells were washed four times with washing buffer; subsequently, 100 μl of affinity-purified, peroxidase-conjugated goat IgG anti-mouse IgG (heavy and light chain specific), $F(ab')_2$ fragment diluted 1:500 in washing buffer were added to each well. After a 90-min incubation at 4°C, the solution was aspirated, the wells were washed four times with washing buffer, and 100 μl substrate solution (48.6 mM citric acid, 102.8 mM Na_2HPO_4, 0.8 mg/ml ortho-phenylendiamine, and 0.024% hydrogen peroxide) were added to each well. The plates were incubated for 15–60 min at room temperature in the dark, then 30 μl of 2.5 M sulforic acid were added to each well and optical densities were determined with a Minireader II (Dynatech Laboratories, Inc.) at 490 nm.

Characterization of Monoclonal Antibodies: Hybridoma-conditioned media were used for determination of immunoglobulin subtype, which was performed with a Mouse Immunoglobulin Subtype Identification Kit following the protocol suggested by the manufacturer. Monoclonal antibodies of IgG class were purified from culture medium and ascites fluid by affinity chromatography on a Protein A-CL Sepharose 4B column (12) as previously described (14). Monoclonal antibodies of IgM class were similarly purified by affinity chromatography, but using a column of affinity-purified rabbit IgG anti-mouse IgM bound to Sepharose 4B. Monoclonal antibodies were metabolically labeled with [^{3}H]lysine by growth of the corresponding double-cloned hybridomas in lysine-free Dulbecco's modified Eagle's medium containing 5 μCi [^{3}H]lysine/ml for 24 h. Labeled immunoglobulins were purified from the conditioned medium by affinity chromatography as described above, and analyzed by two-dimensional slab gel electrophoresis (31) followed by fluorography of the dried gels.

Antigen Identification and Characterization: Monoclonal antibodies were purified by affinity chromatography, bound to cyanogen bromide–activated Sepharose 4B, and tested for their ability to bind Triton X-100–solubilized intestinal brush border membrane enzymes (maltase, sucrase, lactase, trehalase, glucoamylase, alkaline phosphatase, aminopeptidase, and γ-glutamyltransferase) as described previously (14, 33).

Antigens were labeled with [^{14}C]formaldehyde, purified by absorption on monoclonal antibodies bound to Sepharose 4B, and identified by SDS slab gel electrophoresis and fluorography as follows. Purified membrane fractions were suspended in 40 mM potassium phosphate buffer (pH 7.0) at a concentration of 200–500 μg membrane protein/ml; [^{14}C]formaldehyde (40–60 mCi/mmol) was added from a 1% solution in water to a final concentration of 250–500 μCi/ml, immediately followed by 200 μl (per milliliter membrane suspension) of a freshly prepared solution of sodium cyanoborohydride (12 mg/ml in 40 mM potassium phosphate buffer [pH 7.0]) as described for soluble proteins by Dottavio-Martin and Ravel (10). After 90 min at room temperature, the membrane suspensions were diluted with 11 ml of 40 mM potassium phosphate buffer (pH 7.0) and spun in an SW 41 rotor (with a Beckman L8 70 M ultracentrifuge) (Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm for 90 min at 4°C. The supernatants were aspirated and the pellets were either analyzed immediately by SDS slab gel electrophoresis or suspended in 1 ml of solubilization buffer (10 mM sodium phosphate [pH 8.0], 1 % [wt/vol] Triton X-100, 0.1 mg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml antipain, 1 mM PMSF), sonicated (Branson Sonifier Model 200 equipped with a microtip at power setting 3) with 3 × 10 s bursts, and incubated at 4°C for 90 min. After the addition of solid NaCl to a 100-mM final concentration, solutions were

[1] *Abbreviation used in this paper:* PMSF, phenylmethylsulfonyl fluoride.

spun in an Eppendorf centrifuge at 15,000 rpm (15,600 *g*) for 30 min at 4°C. Only very small pellets were observed and discarded. Supernatants (1 ml) containing Triton X-100–solubilized, [^{14}C]-labeled membrane proteins were incubated overnight in 1.5 ml Eppendorf microcentrifuge tubes with 200 μl of mouse IgG-Sepharose 4B beads. After centrifugation in an Eppendorf centrifuge for 15 min at 4°C, the beads were discarded and the supernatants were incubated for 4–6 h at 4°C in 1.5-ml microcentrifuge tubes with 50 μl of monoclonal antibodies bound to cyanogen bromide–activated Sepharose 4B beads. After incubation and spinning in an Eppendorf centrifuge for 3 min at 4°C, the supernatants were discarded and the beads washed sequentially six times with 1 ml washing buffer (10 mM sodium phosphate [pH 8.0] 1% [wt/vol] Triton X-100, 100 mM NaCl, 0.1 mg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml antipain, 1 mM PMSF). The beads were then suspended in 1 ml of H_2O and transferred to small volume filtration devices; most of the liquid was removed by centrifugation in an IEC CRU 5000 centrifuge at 2,000 rpm for 3 min at 4°C. The beads, while still in the filtration devices, were incubated with 30 μl SDS sample solution (62.5 mM Tris-HCl [pH 6.8] 2% [wt/vol] SDS, 10% [wt/vol] glycerol, 2 mM EDTA disodium salt, 50 mM dithiothreitol and 0.01% [wt/vol] bromphenol blue) at 100°C for 2 min. The filtration devices were then spun in the IEC CRU 5000 centrifuge (at 2,000 rpm for 5 min at 4°C) and the eluates collected in 500-μl Eppendorf microcentrifuge tubes; 30 μl of SDS sample solution were again added to the beads, the filtration devices were spun as above, and the eluates combined with the previous ones. Aliquots of the eluates were counted in a Beckman LS 3800 liquid scintillation counter (Beckman Instruments, Inc.) with 5 ml Acquasol II (New England Nuclear, Boston, MA), and appropriate amounts of eluates analyzed by SDS slab gel electrophoresis.

Labeling of Fetal Intestinal Organ Cultures with [^{3}H]Lysine: Fetal intestinal organ cultures, prepared as described in the following paper (35), were incubated with lysine-free Dulbecco's modified Eagle's medium containing 100 μCi/ml of L-[4,5-^{3}H(*N*)]lysine (80–110 Ci/mmol) for 24 h at 37°C. The conditioned medium was removed and spun at 50,000 rpm for 2 h at 4°C in a SW 41 rotor with a Beckman L8-70M ultracentrifuge (Beckman Instruments, Inc). The supernatant, following addition of PMSF (to 1 mM), aprotinin (to 0.1 mg/ml), leupeptin (to 50 μg/ml), and antipain (to 50 μg/ml), was dialyzed against several changes of 10 mM sodium phosphate (pH 8.0), 100 mM NaCl, concentrated 10-fold in an Amicon Ultrafiltration Cell (Amicon Corp., Danvers, MA) equipped with PM10 filter, and made 1% (wt/vol) in Triton X-100. After centrifugation in an Eppendorf centrifuge for 30 min at 4°C, the supernatant was incubated with mouse IgG-Sepharose 4B, and then monoclonal antibodies bound to Sepharose 4B as described above.

SDS Slab Gel Electrophoresis: Electrophoresis and detection of labeled proteins by fluorography were performed as previously described (32).

Injection of Newborn Rats with Cortisone: 9-d-old suckling rats were administered a single subcutaneous injection of cortisone acetate (50 μg/g of body weight) in the back. Control animals were injected the same volume of 0.154 M NaCl.

Preparation of the YBB 1/27, YBB 3/10, and CC 4/80 Monoclonal Antibodies: Hybridoma cells were produced by fusing 10^7 NSI myeloma cells with 10^8 spleen cells from BALB/c mice immunized with suspensions of luminal membranes purified from the intestine of 3-d-old rats (YBB 1/27 and YBB 3/10), or surface membrane fractions prepared from adult intestinal crypt cells (CC 4/80). Primary immunizations consisted of 200 μg of membrane protein in 100 μl PBS mixed with 100 μl of complete Freund's adjuvant, and were followed at monthly intervals by two boosts with the same amount of protein, but mixed with incomplete Freund's adjuvant. Spleen cells were obtained from the immunized mice 3 d after the last booster injection and fused to NSI cells using 50% polyethylene-glycol as described by Galfre et al. (13). After fusion, cell suspensions were divided among 96 wells (in 24-well Costar plates) in the presence of mitomycin-treated (28) 3T3 cells (1.5–2.0 × 10^4 cells/cm^2) and hybrids were selected with HAT medium (25). Hybridoma cultures producing antibodies of interest for our studies were identified using three successive types of tests: (*a*) cultures in all wells were tested for production of antibodies to membrane antigens by an enzyme-linked immunosorbent assay; (*b*) media from cultures positive in that assay were used for immunofluorescence staining of frozen sections from 3-d-old rat intestine (YBB antibodies) or adult rat intestine (CC antibodies); and (*c*) media from cultures positive in the second test were used for immunofluorescence staining of frozen sections from fetal, newborn (6- and 14-d-old), and 30-d-old rat intestine. These time periods were chosen as representative of different known stages of development of the intestinal mucosa (22, 29, 39). Based on the results of the last set of assays, six hybridoma cultures were selected and three of them cloned successfully. Cloning was by limiting dilution plating (half a cell per well) in 96-well Costar plates containing mitomycin C–treated 3T3 cells. Cultures with the highest titer in the enzyme-linked immunosorbent assay were selected and recloned using the same procedure. Double-cloned hybridomas were used for antibody characterization and large scale antibody production in ascites form (14, 33). The monoclonal nature of the hybridoma cell lines was established by three criteria. (*a*) Each cell line was cloned twice by dilution plating, and each time the clonal origin of the cells in each well was assessed by microscopic observation. (*b*) Two-dimensional gel electrophoresis of [^{3}H]lysine-labeled proteins secreted by each cell line demonstrated a single immunoglobulin heavy chain. (*c*) Antibodies produced by sister clones yielded identical results when tested by immunofluorescence staining of intestinal frozen sections. The monoclonal antibodies produced by the three double-cloned hybridoma lines were typed, and found to be of IgG_1 subclass (YBB 1/27 and CC 4/80) and IgM class (YBB 3/10), respectively.

RESULTS

Developmental Patterns of Antigen Distribution in Rat Small Intestine

YBB 1/27 ANTIGEN (FIG. 1, A AND B): This antigen was detected in the fetal intestinal mucosa starting from day 20 of gestation; samples from days 18 and 19 were consistently negative. The antigen was localized at the luminal membrane of the entire population of epithelial cells covering the villi (crypt cells are not present at this time of development of the intestine, first appearing around the time of birth [39]). The intensity of fluorescence reached a maximum at day 22 of gestation in the fetus and at 1 d after birth (Fig. 1 *a*). By 4–6 d after birth, only a weak fluorescence (or no fluorescence at all) was present in the region of the villi; in contrast, the developing crypts were clearly positive (Fig. 1 *b*). The base of the villi was also stained in most samples, and this pattern of staining of the intestinal mucosa remained unchanged until 17–20 d after birth. At the time of weaning (20–22 d after birth), or starting a few days earlier, fluorescence staining became confined to the lower portion of the villi. By 30 d after birth or later, no fluorescence was detected in any region of the intestinal mucosa (data not shown).

YBB 3/10 ANTIGEN (FIG. 1, C–F): Staining of the fetal intestinal mucosa, from day 16 of gestation (the earliest fetal age examined) until birth, was very intense and localized in the epithelial cells. The brightest area of fluorescence corresponded to the luminal aspect of the surface membrane of the intestinal cells. At day 18 of gestation (Fig. 1 *c*), fluorescence was present both at the main lumen and at the intraepithelial secondary lumina typical of this stage of development (39). These secondary lumina appear in the stratified intestinal epithelium between 16 and 17 d of gestation, and seem to play a key role in the remodelling of the mucosa which results in formation of the villi; they do not appear to communicate with the main intestinal lumen of fetal rats (39). Although usually much weaker and variable in intensity, fluorescence was also present at the lateral and basal sides of the intestinal epithelial cells. This was found to be the case in samples from all ages examined, from fetal to adult, and could easily be observed by microscopic observation, but often did not reproduce well photographically, particularly in pictures taken at smaller magnifications. After birth, the entire intestinal epithelial population, comprising both crypt and villus cells, was about equally intensely stained (Fig. 1 *d*), but in samples from rats older than 12–14 d, the upper villus cells showed only faint irregular staining, or no staining at all. Starting with the time of weaning (22–24 d after birth), the fluorescence associated with the intestinal villi became patchy and irregular. In most samples from rats older than 45–50 d, fluorescence was confined to the crypts (Fig. 1 *e*) where, again, both the luminal and lateral basal aspects of the epithelial cells were stained (Fig. 1 *f*). The villus cells were, in all cases,

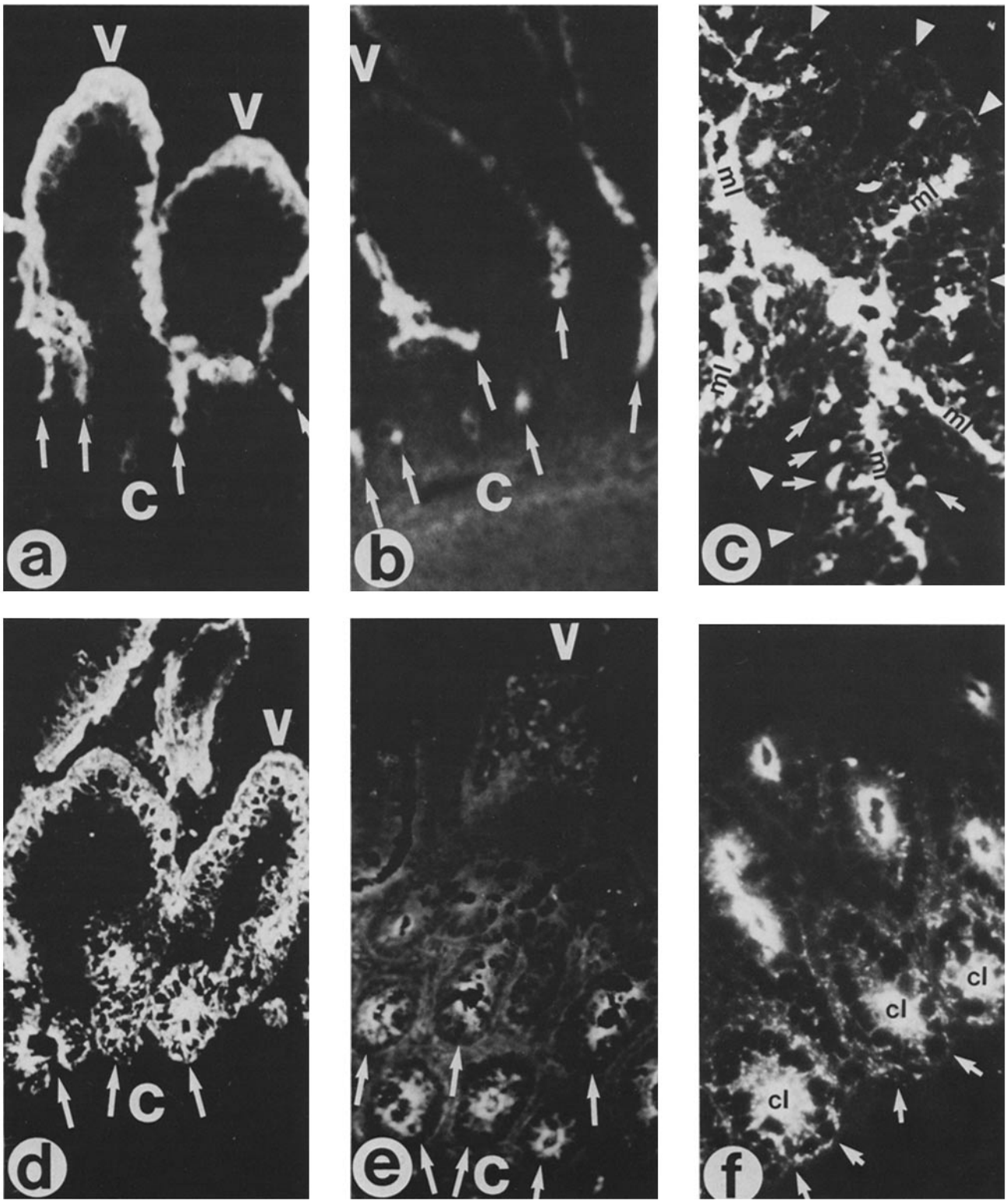


FIGURE 1 Distribution of the YBB 1/27 (*a* and *b*) and YBB 3/10 (*c–f*) antigens in rat small intestine at different stages of development. Frozen tissue sections were fixed with 1% formaldehyde and stained by indirect immunofluorescence. (*a*) In 1-d-old intestine, the YBB 1/27 antigen is present at the luminal surface membrane of the epithelial cells covering the villi, and in the developing crypts (arrows). (*b*) In 13-d-old (suckling) intestine, expression of the YBB 1/27 antigen is limited to the crypts (arrows) and to the lower villus cells; cells present in the upper portions of the villi (top of the figure) are unstained. (*c*) (YBB 3/10 antigen) In fetal intestine, intense fluorescence is present at the luminal aspect of the epithelial cells lining the narrow main lumen (*ml*) and in the intraepithelial lumina (arrows). Weak staining of the lateral and basal (arrowheads) sides of the epithelial cells is also present. (*d*) (YBB 3/10 antigen) In 5-d-old intestine, the entire surface membrane of both crypt (arrows) and villus cells is stained. Cytoplasmic staining also appears to be present. (*e* and *f*) In adult intestine, expression of the YBB 3/10 antigen is limited to the crypt cells (arrows in *e*); the luminal aspect of the cells (*cl* in *f*) is intensely stained, but weak fluorescence is also present at the lateral and basal (see arrows in *f*) sides of the cells. Occasionally, in samples of adult intestine, the YBB 3/10 antibody also stained material present in the intestinal lumen. In figures *a–c* and *e*, white capital letters indicate the regions of the crypts (C) and of the villi (V). (*a* and *b*) × 200; (*c–e*) × 150; (*f*) × 400.

completely negative, but fluorescence was occasionally found associated with material present in the intestinal lumen (data not shown), in part depending on the extent of rinsing of the intestine with saline before preparation and fixation of the samples. This observation has suggested that the YBB 3/10 antigen may be, at least in part, secreted or in some way shed into the intestinal lumen by the epithelial cells. All the staining patterns described above and represented in Fig. 1 correspond to jejunal or duodenal samples (no difference was found between these two regions of the small intestine). In the terminal ileum of adult rats, fluorescence was limited to a few cells present in the bottom of the crypts, and no fluorescence was ever found associated with luminal material (data not shown).

CC 4/80 ANTIGEN (FIG. 2): No staining of the intestinal mucosa was observed with the CC 4/80 antibody until 10–12 d after birth. At this time, fluorescence was first observed associated with the luminal membrane of the epithelial cells in the crypts (Fig. 2 *b*). The intensity of fluorescence increased in this region of the intestinal mucosa with age of the rats (Fig. 2 *c*), but all other parts of the intestine remained consistently negative. After weaning, and in adult animals (Fig. 2 *d*), the pattern of fluorescence staining of the intestine was strikingly different. Only cells at the top of the crypts, where newly differentiated epithelial cells are known to be present (4–6, 14, 18, 24, 29, 33, 41), and absorptive intestinal cells located in the lower half to two-thirds of the villi were intensely stained. The cellular location of the antigen could not be determined with certainty: fluorescence appeared localized over the entire epithelial cells, including the cytoplasm, in contrast with the apparent luminal location of the antigen in the crypt cells of suckling animals (Fig. 2, *b* and *c*).

Effects of Cortisone Injection on Antigen Distribution in Suckling Rats

During the third week of postnatal development in the rat, activities of a number of intestinal enzymes show dramatic changes (9, 14, 18, 22, 29, 38). There is a large amount of evidence to suggest that these changes are mediated, at least in part, by adrenal corticosteroids, because they are precociously induced by administration of glucocorticoids during the second postnatal week, and delayed by adrenalectomy at that time (8, 9, 14, 22, 29). To determine if the changes in the patterns of distribution of the YBB 1/27, YBB 3/10, and CC 4/80 antigens at the time of weaning were also due to an increase in the concentration of plasma glucocorticoids (15), 9-d-old rats were administered a single subcutaneous injection of cortisone acetate, and examined at daily intervals thereafter. Control animals received an equal volume of saline. The precocious appearance of sucrase-isomaltase at the brush borders of the villus cells, detected using the sucrase-specific monoclonal antibodies BB 3/34 and BB 5/8 (14), represented a positive control for the effectiveness of the cortisone injection. As in our previous studies (14), sucrase was detected by immunofluorescence at the base of the villi 48 h after cortisone injection (data not shown). The patterns of distribution of the YBB 1/27 and YBB 3/10 antigens were identical in cortisone-injected and control animals at 1–5 d after injection (data not shown). In contrast, a significant effect of cortisone was observed for the CC 4/80 antigen at 2 d and, more evident, at 4 d after injection. The intensity of fluorescence staining in the crypts was weak and irregular, and staining was observed in the region of the villi (Fig. 2 *f*), in contrast with the exclusive location of the antigen in the crypts of control animals injected with saline (Fig. 2 *e*).

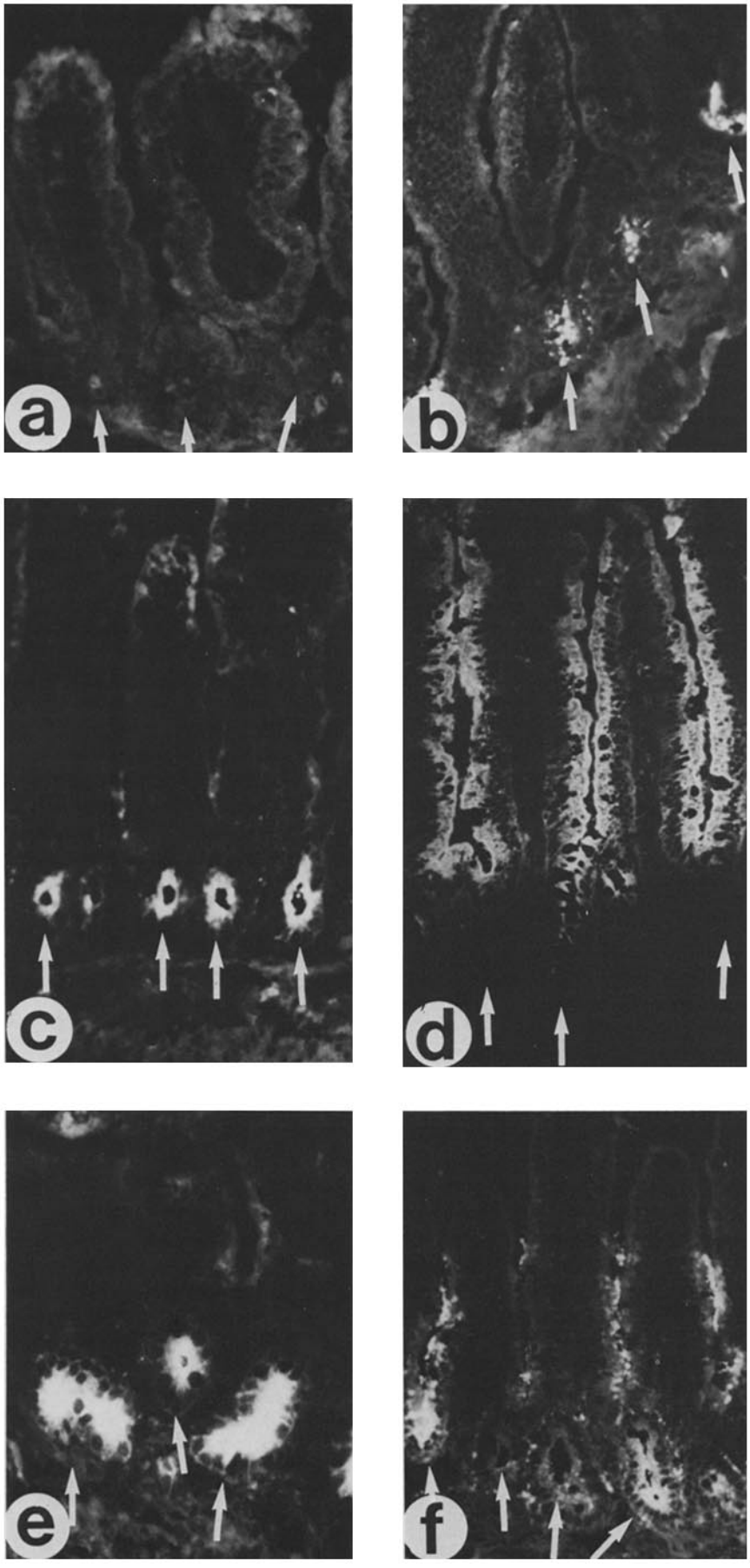
Identification of the YBB 3/10 and CC 4/80 Antigens by Gel Electrophoresis

Proteins of purified luminal membrane fractions from fetal and newborn intestine, adult villus, and crypt cells, were labeled by reductive alkylation with [^{14}C]formaldehyde and sodium cyanoborohydride (10). After solubilization with nonionic detergents, labeled membrane proteins were incubated with monoclonal antibodies covalently bound to Sepharose 4B. Nonbound proteins were washed away, and the specifically bound antigens were analyzed by SDS slab gel electrophoresis under reducing conditions. Controls for nonspecific binding of labeled proteins to the beads consisted of aliquots of labeled membrane proteins incubated with nonimmune mouse IgG bound to Sepharose 4B. In selected cases, unlabeled solubilized membrane protein mixtures were incubated with the insolubilized monoclonal antibodies, and the bound antigens were identified by staining of the gels with Coomassie Blue. Identical results were obtained with labeled and unlabeled samples. Antigens bound to insolubilized monoclonal antibodies were also tested for various enzyme activities which are known to be present in the brush border membrane of the enterocytes (sucrase, maltase, lactase, alkaline phosphatase, aminopeptidase): all assays were negative.

By gel electrophoresis, protein band(s) corresponding to the YBB 1/27 antigen could not be detected for any of the samples examined, which included luminal membranes from fetal (20 and 22 d of gestation), suckling (6, 9, 14, and 18-d-old), and adult rat intestines. There are many possible explanations for the failure to identify this antigen, including the inability of the YBB 1/27 antibody to bind to detergent-solubilized antigen, changes in antigen conformation after solubilization, and low avidity of the antibody. Use of detergents other than Triton X-100 (e.g., Nonidet P-40, deoxycholate, Tween 20) for membrane solubilization produced no positive effects.

The YBB 3/10 antigen was identified as a group of high molecular weight protein bands in samples from fetal (day 20 of gestation) and suckling (3-d-old [Fig. 3 *a*, lane *4*]) intestinal luminal membranes, and from crypt cells luminal membranes of adult rats (Fig. 3 *a*, lane *5*). In all cases, most of the radioactivity was found associated with material barely entering the separation gel, even using 5% acrylamide gels. Its molecular weight could not be therefore determined, but was estimated as greater than 400,000. Two other major protein bands of molecular mass 140 kD and 123 kD, respectively (see arrows to the right of lane *5* in Fig. 3 *a*), were identified in all membrane fractions. When soluble proteins present in the conditioned medium of fetal intestinal organ cultures (35) metabolically labeled with [^{3}H]lysine were incubated with insolubilized YBB 3/10 antibody and the specifically bound radioactivity was analyzed by SDS slab gel electrophoresis, a similar pattern of labeled protein bands was observed for the YBB 3/10 antigen (data not shown).

The CC 4/80 antigen was identified in luminal membrane fractions from 14-d-old suckling rats, and from adult rats, as a broad radioactive band corresponding to a molecular mass of 28 to 34 kD (Fig. 3 *b*, lanes *2* and *3*). No specifically bound radioactivity was found in samples from fetal (days 20–22 of gestation) and newborn (3, 6, and 9-d-old) rat intestine.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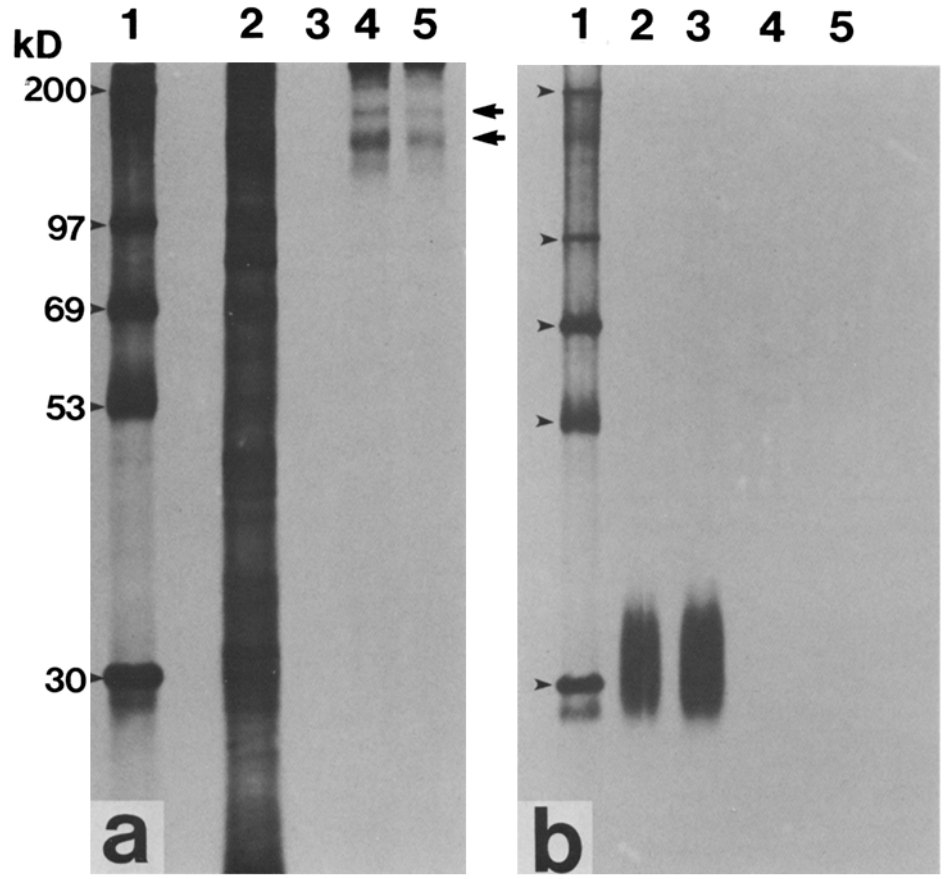

FIGURE 3 Identification of the YBB 3/10 (*a*) and CC 4/80 (*b*) antigens by SDS slab gel electrophoresis. [^{14}C]-labeled, Triton X-100–solubilized membrane proteins were incubated with YBB 3/10 or CC 4/80 antibodies, or nonimmune mouse IgG (controls), bound to Sepharose 4B. Specifically bound proteins were denatured under reducing conditions and analyzed by SDS slab gel electrophoresis on 10% acrylamide gels. (*a*) Lane *2*, total [^{14}C]-labeled luminal membrane proteins from 3-d-old rat intestine; lane *3*, [^{14}C]-labeled membrane proteins from 3-d-old intestine incubated with mouse IgG-Sepharose 4B; lane *4*, YBB 3/10 antigen purified from 3-d-old intestine; lane *5*, YBB 3/10 antigen purified from adult intestine. (*b*) Lane *2*, CC 4/80 antigen purified from 14-d-old intestine; lane *3*, CC 4/80 antigen purified from adult intestine; lanes *4* and *5*, [^{14}C]-labeled membrane proteins from 14-d-old and adult intestine, respectively, incubated with mouse IgG-Sepharose 4B. Lane *1* in both *a* and *b*, [^{14}C]-labeled molecular mass markers (from the top [see arrowheads]: myosin, 200 kD; phosphorylase b, 97 kD; bovine serum albumin, 69 kD; γ-globulin heavy chain, 53 kD; carbonic anhydrase, 30 kD).

DISCUSSION

The immunohistochemical studies described in this paper have demonstrated complex patterns of changes in the cellular distribution of three antigens, defined by the monoclonal antibodies YBB 1/27, YBB 3/10, and CC 4/80, during development of the small intestinal mucosa in late fetal and suckling rats. These results are schematically summarized in Fig. 4. The common feature of these three antigens is their preferential or exclusive localization in the region of the small intestinal crypts at specific stages of intestinal development: the YBB 1/27 antigen during the second and third week of postnatal life, the CC 4/80 antigen during the third week after birth, and the YBB 3/10 antigen in adult (postweaning) intestine (Fig. 4). These antigens appear to be, at least in part, associated with the surface membrane of the epithelial cells, since they were found to co-purify with luminal membrane fractions, and detergents were required for their solubilization.

FIGURE 2 Distribution of the CC 4/80 antigen in rat small intestine at different stages of development. (*a*) 3-d-old intestine. No specific staining is present either in the villi or in the crypts (arrows). (*b*) 11-d-old intestine. Irregular staining of the crypt cells is observed (arrows); this represents the earliest time point this antigen was found in the small intestine. Most of the fluorescence corresponds to the luminal membrane of the crypt cells, but cytoplasmic staining also appears to be present. (*c*) 14-d-old intestine. Intense fluorescence is observed in correspondence with the luminal membrane of the crypt cells (arrows); villus cells are totally negative. (*d*) Adult intestine. No fluorescence is present in the region of the crypts (arrows). Lower villus cells are intensely stained, and the intensity of the fluorescence decreases markedly towards the tips of the villi (top of the figure); the entire surface membrane and the cytoplasm of the epithelial cells are stained. (*e*) Intestine from a 14-d-old rat injected with saline on day 9. Fluorescence is confined to the luminal aspect of the crypt cells. (*f*) Intestine from a 14-d-old rat injected cortisone on day 9. Staining of the crypt cells (arrows) is irregular, and fluorescence is also found in correspondence with the epithelial cells covering the lower portions of the villi. × 150.

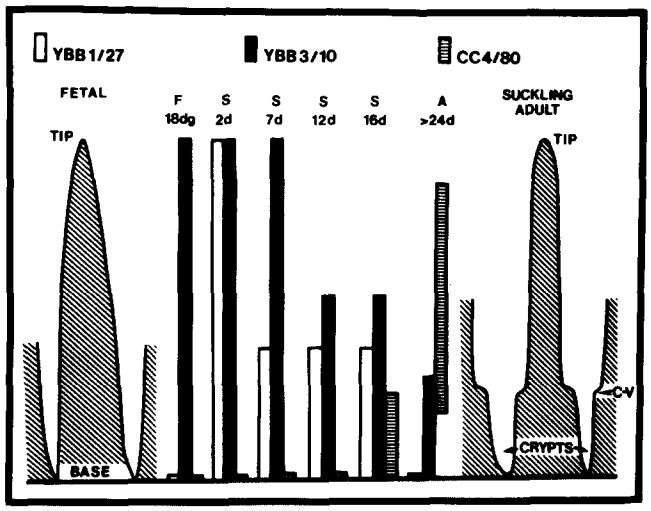

FIGURE 4 Distribution of the YBB 1/27, YBB 3/10, and CC 4/80 antigens in fetal, suckling, and adult rat small intestine. This figure summarizes the results obtained by immunofluorescence staining of intestinal frozen sections, in part illustrated in Figs. 1 and 2, and described in the text. To the left is a diagram of the fetal intestinal mucosa; the base and tip of the villi are indicated. Note the absence of morphologically distinct crypts (which develop in rat small intestine during the first week after birth). To the right is a diagram of suckling and adult intestinal mucosa, the crypts, the crypt-villus junction (*CV*) and the tip of the villi are indicated. No attempt was made to compare, in this diagram, the relative lengths of the villi at different stages of intestinal development. *F*, fetal intestine (18 d of gestation); *S*, suckling intestine (age indicated); *A*, adult intestine (older than 24 d after birth).

A number of factors should be considered in the interpretation of the various patterns of antigen distribution in fetal, suckling, and adult intestine presented in Figs. 1, 2, and 4. Morphologically distinct crypts are not evident in fetal intestine (1), and DNA synthesis and cell proliferation are common over the entire length of the villi until birth (17). The rate of cell migration from the bottom of the crypts to the tips of the villi is much slower in infant rats than in weaned and adult animals (23). For antigens identified as cellular proteins, the stability of the corresponding mRNAs in the cytoplasm and the rate of degradation of the proteins are key factors in determining the observed patterns of antigen expression in a system like the intestinal epithelium, characterized by continuous cell migration and differentiation. For example, even if transcription of a gene corresponding to a crypt cell–specific antigen is completely suppressed at the time cell differentiation takes place in the upper region of the crypts (4, 5, 40), it should be expected that this antigen be detectable up to a certain level in the lower portion of the villi, depending on its mRNA stability, translational activity, and the rate of antigen degradation. Turnover of total cytoplasmic poly (A) containing RNA in the crypt cells occurs with a half life not exceeding 24 h (30). In adult rats, disaccharidases and other brush border membrane proteins of villus cells were found to turn over relatively rapidly, and their half lives have been estimated as 11–18 h (19), but most luminal membrane proteins of crypt cells appear to be much more stable and not to turn over appreciably (2). On the other hand, epithelial cell migration in intestinal crypts and villi is a rapid process, occurring at a rate of 1.2 to 1.5 cell positions per hour (4): it can be estimated that the intestinal cells migrate from the top of the crypts to the middle of the villi in ~24–30 h (36). Thus, the preferential localization of the YBB 1/27, YBB 3/10, and CC 4/80 antigens in the crypts at specific stages of intestinal development suggests that the rates of turnover of these antigens, and of the corresponding mRNAs, are much more rapid than those of typical brush border membrane proteins. This conclusion is also consistent with the marked decline in CC 4/80 antigen expression in the epithelial cells present in the upper half of the villi of adult animals (Fig. 2*d*), which is in contrast with the patterns of expression of most villus cell brush border enzymes (14, 33). Uddin et al. (40) have demonstrated a marked change in the rate of tRNA and mRNA synthesis, and in their biosynthetic pathways, as the intestinal cells pass through the crypt top and villus base. Presumably the mRNA for the CC 4/80 antigen is elaborated in the lower villus region, and, due to its short half life, antigen expression declines rapidly as the cells reach the mid-villus region. Similar considerations also apply to the location of the YBB 3/10 antigen in the crypts of adult rat intestine (Fig. 1*e*).

Structural and enzymatic development of the rat small intestinal epithelium is a continuous process from fetal life to adulthood (17, 18, 22, 23, 27, 29, 39), but two critical periods have been identified. One is just before birth, when many typical brush border enzymes are first expressed or increase dramatically in activity, the crypts start to develop, and DNA synthesis on the villi ceases abruptly. The second one is the time of weaning, when marked structural and enzymatic changes take place. Direct contact between epithelial and mesenchymal cells is maximum during the period delimited by these two critical stages (27), which is also the only period when expression of the YBB 1/27 antigen was observed. The YBB 3/10 antigen was present in the intestinal mucosa at all times considered in this study, but its cellular location varied markedly at different stages of intestinal development. This antigen may be regarded as a fetal product, since it is expressed in apparently large amounts even at day 16 of gestation, when neither the other two antigens considered in this study, nor typical intestinal cell markers like alkaline phosphatase or lactase, are detectable. Its presence in the intraepithelial lumina at day 18 of gestation (Fig. 1*c*) and its synthesis in the fetal intestinal organ cultures (35) demonstrate its origin from the intestinal epithelial cells, but many aspects of its localization and structure cannot, at present, be interpreted with certainty. The presence of this antigen at both the luminal and lateral-basal sides of the intestinal epithelial cells, which are known to be highly polarized (14, 18, 21, 34), is in contrast with the localization of most other intestinal cell surface components studied to date (14, 33, 34). In some samples of adult intestine, immunoreactive material was also observed in the lumen, suggesting that the YBB 3/10 antigen may be, at least in part, secreted or otherwise shed by the cells that synthesize it. Identification of the YBB 3/10 antigen by immunoprecipitation and gel electrophoresis (Fig. 3*a*) demonstrated the presence of a large amount of protein barely entering the separation gel; this protein, presumably, is either of very high molecular weight or corresponds to aggregate(s) not dissociated by SDS even in the presence of dithiothreitol. Two other bands, of much lower intensity and corresponding to proteins with M_r of 140,000 and 123,000, respectively, were also observed in all samples examined. In the absence of more detailed biochemical data, one can only speculate about the relationship of these two lower M_r bands with the material barely entering the separation gels: they may represent

monomeric proteins, not aggregated as the material present at the top of the gels, or degradation products possibly generated by the action of luminal proteases on the high M_r material.

The CC 4/80 antigen is an intestinal cell product which is first expressed at the end of the suckling period, like sucrase-isomaltase (8, 9, 14, 22, 29). Unlike sucrase, however, the time of its appearance does not coincide with the surge in free and bound corticosterone concentration in the blood occurring during the third week of postnatal life in the rat (15). In contrast, its appearance in the intestinal crypts at 10–14 d after birth correlates well with an increase in plasma thyroxine titre in rats at the end of the first postnatal week (7), suggesting that this hormone rather than glucocorticoids may be responsible for its induction.

The pituitary-adrenal system has been known since a long time to control the transition from the suckling to the mature state of the intestine (8, 9, 14, 29). That maturation of the intestinal mucosa at weaning entails more than a glucocorticoid-regulated shift in gene expression is, however, demonstrated by the observation that other changes in intestinal enzyme activities, like the decline in lactase normally occurring after weaning, are not susceptible to precocious induction by cortisone injection (8), and hormone responsiveness of other enzymes (like sucrase) is lost after weaning (16). The results obtained in this study also suggest that, in addition to glucocorticoids, other factors are involved in the development of the intestinal epithelium at weaning, since administration of cortisone to 9-d-old rats did not affect the tissue and cellular distribution of the YBB 1/27 and YBB 3/10 antigens. The presence of three different markers in the crypts at different stages of development indicates that maturation of the intestinal epithelium starts, as it might be expected, with the crypt cells, and the well-known changes in the structure and function of the villus cells with age are not solely the result of the modulation of gene expression in the differentiated cells by hormones, enzyme substrates, and other factors.

The process of cell differentiation in the adult intestine has been the subject of extensive studies, which have well documented the growth kinetics of the epithelial cells, and the morphological and functional characteristics of the villus cells (4–6, 22, 24). However, the cellular and molecular interactions which are responsible for the orderly arrangement of the proliferative, maturing, and functional epithelial cells, and which trigger terminal cell differentiation in the upper part of the crypts are still largely unknown. The results obtained in this study may provide information regarding basic characteristics of the process of intestinal cell differentiation. The expression of specific cell markers in the crypts indicates that the "undifferentiated" crypt cells may be able to perform specific intestinal functions like the villus cells. In immature intestine, functions typical of both crypt and villus cells may be expressed in a single cell type: for example, during the last 2–3 d of gestation, a single epithelial cell may synthesize DNA and proliferate (17), and express typical villus cell markers like lactase, aminopeptidase, and alkaline phosphatase (35), as well as the YBB 1/27 and YBB 3/10 antigens which, at later stages of development, become specific markers for crypt cells. A major challenge for future studies will be to determine how and why expression of these various functions becomes prerogative of different cell types in the intestine shortly after birth.

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