FULL PAPER *Anatomy*

Special Sugar Expression on Apoptotic Epithelial Cells of Peyer's Patches and Intestinal Villi in Rat Small Intestine

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ABSTRACT. Our previous study clarified that the apical regions of both the follicle-associated epithelium (FAE) of Peyer's patches and the intestinal villi are the only adhesion sites of indigenous bacteria in rat jejuno-ileum. To survey the ligands against bacterial lectins, sugar expression patterns on epithelial cells were lectin-histochemically investigated using 21 lectins in the jejuno-ileal Peyer's patches of rats. As a result, $(D\text{-}glcNAc)_{2-4}$, detected by *Solanum tuberosum* (STL) and by *Lycopersicon esculentum* (LEL), and β–D-gal(1–3)-D-galNAc detected by *Peanut agglutinin* (PNA), were strongly expressed on the brush borders of the apical regions of the FAE and the intestinal villi. On the other hand, neither sugar was expressed on the brush borders of the basal regions of both FAE and intestinal villi. The positive intensities for the lectins correlated with the progression of epithelial apoptosis in the FAE and in the intestinal villi. Moreover, the double staining with lectin histochemical method and the *in situ* nick end-labeling method could simultaneously detect the strong expression of both sugars and nuclear DNA fragmentation in epithelial cells at the late apoptotic stage. Other sugar expression patterns in the intestinal villi were similar with those in the FAE. There were no lectins specific for M cells in the FAE. From these findings, the possible sugars of ligands against some indigenous bacterial lectins, expressing specially on the apoptotic epithelial cells, might be narrowed down in rat jejuno-ileum.

KEY WORDS: apoptosis, bacterial colonization, lectin histochemistry, M cell, Peyer's patch.

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In animals, the gastrointestinal tract contains more than 400 species of bacteria, forming the intestinal microflora [33]. The composition patterns of individual bacteria in the intestinal microflora are usually constant but may transiently vary under some pathological situations, such as acute diarrheal illnesses or during antibiotherapy [11]. A crucial and initial step is the adhesion to the mucosal surface in successful colonization or the infection by bacteria [2, 12, 24]. Several bacterial adhesion molecules for recognizing ligand molecules on the mucosal surface have been reported. That is, invasin, an outer membrane protein of *Yersinia*, specifically binds to integrins on the host cell [14]. Intimin, a homologue of the invasin polypeptide, is also related to the binding activity of enteropathogenic *Escherichia coli* to the host cell surface [5]. The most common adhesion molecules of bacteria are adhesin, microbial lectins which selectively recognize glycoconjugates on the surface of intestinal epithelial cells [13, 24]. The mucosal surface of the intestine is always covered with a 400- to 500-nm-thick glycocalyx, where glycoconjugates extend from the apical membranes of epithelial cells [15]. This anatomical advantage also supports the hypothesis that a lectin-sugar binding mechanism plays an important role in bacterial adhesion to the mucosal surface under physiological conditions. However, few studies have been conducted to examine on the ligands on the intestinal epithelium against bacterial lectins *in vivo*.

Our previous study on the colonization sites of microflora clarified that the apical regions of both follicle-associated epithelium (FAE) of Peyer's patch and intestinal villi are the only adhesion sites of indigenous bacteria in rat jejuno-ileum [3]. Therefore, it is speculated that some specific sugars that induce bacterial adhesion might exist on the surfaces of the apical regions of both FAE and intestinal villi. On the other hand, the epithelial cells at the apical regions express several typical morphological characteristics of apoptosis exclusively in murine Peyer's patch and chicken cecal tonsil [21, 32, 34]. However, the relationship between the expression of specific sugars and the epithelial apoptosis has never been clarified. In this study, we first examined whether or not the expression of specific sugars is involved in the progression of apoptosis at the apical regions of FAE and intestinal villi in the normal rat jejuno-ileum.

Peyer's patches are well known as the major sites for the induction of mucosal immunity against luminal antigens in the small intestine. In the FAE, M cells are special epithelial cells that transport the luminal antigens [9]. Lectins are a well-known marker for the histological detection of M cells in various animal species [6, 25, 27]. In rat, however, no lectins that specifically identify M cells have been reported thus far. Our previous study clarified the close relationship of the existence of indigenous bacteria and M cell formation in the FAE of the rat jejunal Peyer's patch [3]. Therefore, in this study we further examined the expression of specific sugars on M cells in the FAE of rat Peyer's patch by lectin histochemistry.

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MATERIALS AND METHODS

Animals: A total of 15 male Wistar rats aged 6–7 weeks (Japan SLC Inc., Japan) were used according to the guidelines for the care and use of experimental animals on the Rokkodai Campus of Kobe University. The rats were permitted free access to water and food (Lab MR Stock; Nosan Corp., Japan). The animal facility was maintained on a 12 hr light/dark cycle, at 21 ± 1°C and 50–60% humidity. Clinical and pathological examinations confirmed that none of the animals had any signs of disorder.

Light microscopy: Animals were killed by cardiac exsanguination under anesthesia by inhalation of diethyl ether. A total of thirty jejuno-ileal pieces containing Peyer's patches were removed rapidly and immersion-fixed in cold periodate-lysine-paraformaldehyde (PLP) fixative for 24 hr at 4°C. The tissue blocks were snap frozen according to the method of Barthel and Raymond [1]. The sections 4 μm thick were cut with a coldtome HM505E (Carl Zeiss, Germany) and placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical, Japan).

Lectin histochemistry: Sections were immersed in absolute methanol for 30 min and 0.5% H_2O_2 for 30 min, followed by treatment with 1.0% normal wild bullfrog serum for 1 hr at r.t. After reaction with 21 biotinylated lectins (Vector Laboratories, U.S.A.) at concentrations of 0.63–2.5 μg/m*l* for 16 hr at 4°C, the sections were incubated with peroxidase-conjugated streptavidin (diluted to 1:500; Dako, Denmark) for 1 hr at r.t. These sections were then reacted with 3, 3'-diaminobenzidine (DAB: Dojindo Lab., Japan) containing 0.03% H_2O_2, and were counterstained with methyl green. The lectins used in this study are shown in Table 1. The specificity of each lectin was confirmed by pretreatment of the lectin solution containing the appropriate sugars to inhibit lectin binding. Sections were also incubated in the streptavidin solution or phosphate buffer alone.

Double staining of lectin histochemistry and in situ nick end-labeling: To investigate the relationship between apoptosis and sugar expressions of epithelial cells in the FAE and intestinal villi, the bromodeoxyuridin triphosphate (BrdUTP) method was applied followed by lectin histochemistry. Briefly, four sections were prepared from each rat (Sections 1, 2, 3 and 4). Sections 1 and 2 were used as the positive controls for Section 3. Section 4 was used as a negative control. These sections were incubated with 1.5 μg/m*l* proteinase K (Sigma-Aldrich, U.S.A.) solution for 10 min at 37°C. Section 1 and 2 were incubated with 1.5 μg/m*l* DNase I solution (Boehringer Mannheim, Germany) for 60 min at 37°C. Sections 1 and 3 were applied with terminal deoxinucleotidyl transferase (TdT) reaction buffer (Roche Diagnostics, Switzerland) containing BrdUTP solution (0.1 nM BrdUTP in 4 μ*l* distilled water; Sigma-Aldrich). Sections 2 and 4 were applied with TdT buffer without BrdUTP solution. All sections were incubated for 2 hr at 37°C. Following incubation with 1% normal wild bullfrog serum for 1 hr at r.t., all sections were reacted with anti-BrdUTP mouse monoclonal antibody (diluted to 1:50; Bioscience Products, Japan) for 16 hr at 4°C. The sections were incubated with anti-mouse IgG rat IgG (diluted to 1:50; Jackson ImmunoRes. Lab., U.S.A.) for 1 hr at r.t., followed by incubation with mouse peroxidase-antiperoxidase complex (PAP) (diluted to 1:50; Seikagaku Corp., Japan). The sections were further incubated with the

Table 1. Lectins and sugar specificities

Lectin	Abbreviation	Major sugar specificity	Consentration (μg/m*l*)
Canavalia ensiforis	ConA	α-D-Man, α-D-Glc	1.25
Pisum sativum	PSA	α-D-Man	1.25
Lens culinaris	LCA	α-D-Man	2.50
Griffonia simplicifolia -I	GSL-I	α-D-Gal, α-GalNAc	1.25
Sophora japonica	SJA	β-D-GalNAc	1.25
Ricinus communis	RCA_{120}	β-D-Gal-4GlcNAc	1.25
Arachis hypogaea	PNA	β-D-Gal(1-3)D-GalNAc	2.50
Trythrica cristagalli	ECL	β-D-Gal(1–4)D-GalNAc	0.63
Glycine max	SBA	α-D-GalNAc-3Gal	2.50
Vicia villosa	VVA	D-GalNAc-3GalNAc	0.63
Dolichos biflorus	DBA	α-D-GalNAc	1.25
Triticum vulgaris	WGA	$(GalNAc)_n$, Neu5Ac	1.25
Griffonia simplicifolia -II	GSL-II	D-GlcNAc	1.25
Solanum tuberosum	STL	$(GlcNAc)_{2-4}$	1.25
Lycopersicon esculentum	LEL	$(GlcNAc)_{2-4}$	1.25
Succinylated WGA	S-WGA	$(GlcNAc)_n$	1.25
Datura stramonium	DSL	$(GlcNAc)_2$	0.63
Ulex europaeus-I	UEA-I	α-L-Fuc	1.25
Phaseolus vulgaris-erythroaggultinin	PHA-E	Oligosaccharide	1.25
Phaseolus vulgaris-leucoagglutinin	PHA-L	Oligosaccharide	1.25
Artocarpus integrifolia	JCA	β-D-galactopyranoside	0.63

Gal, D-Galactose; GalNAc, N-Acetyl-D-galactosamine; Glc, D-Glucose; GlcNAc, N-Acetyl-D-glucosamine; Fuc, L-Fcose; Man, D-Mannose; NeuNAc, N-acetyl neuraminic acid

SG peroxidase substrate kit (Vector Laboratories). These sections were then processed using the same protocol as for the lectin histochemistry mentioned above.

RESULTS

Lectin staining patterns in the FAE and intestinal villi: The 21 lectins used in this study were divided into four groups according to their staining patterns of glycocalyx on epithelial cells in the FAE and intestinal villi: lectins with strong staining in the apical regions of FAE and intestinal villi (Group 1), lectins with gradually increased staining intensity from the basal toward the apical regions (Group 2), lectins with uniformly staining in the basal to apical regions (Group 3), and lectins with faint staining in the basal to apical regions (Group 4) (Fig. 1). Most lectins also reacted with the cytoplasms of epithelial cells in both the FAE and the intestinal villi. There was no difference in the lectin staining patterns between jejunum and ileum.

STL, LEL and PNA were categorized into Group 1. STL and LEL hardly reacted with the surfaces of basal regions of FAE, and never labeled the apical surfaces of typical M cells (Fig. 1a). They strongly reacted with the apical surfaces of one-fourth of the apical regions of FAEs and their staining intensities were strongest at the FAE tips (Fig. 2a, b). PNA strongly reacted only with several apical surfaces of epithelial cells within the FAE tips, whereas it was weakly stained on the other epithelial cells (Fig. 2c). Group 1's lectins also stained the apical epithelial cytoplasms containing variously sized granules, which were stained with various intensities in correlation with those on the apical surfaces of epithelial cells in the FAE or intestinal villi (Fig. 2b). The staining patterns of group 1 lectins in the intestinal villi were the same as those of the FAEs (Fig. 2d-f). In cases where the intestinal lumen was full of contents and the intestinal villi were fallen down, all lectins in group 1 were

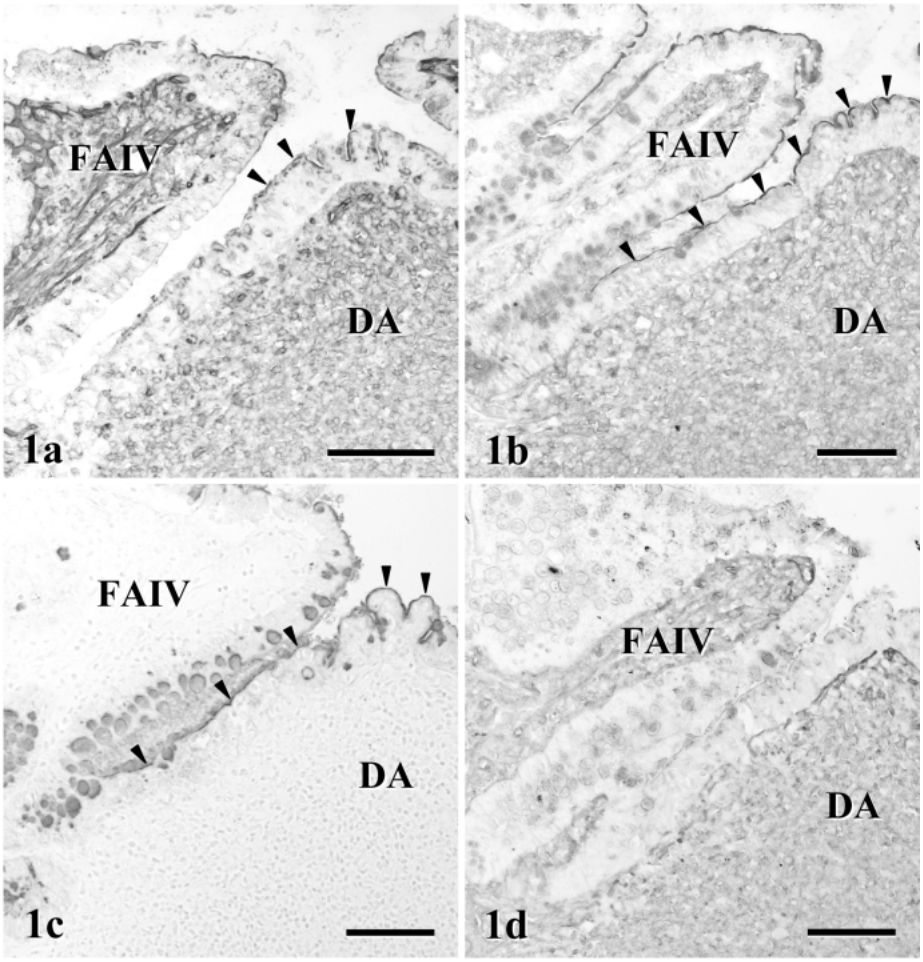

Fig. 1. Four staining patterns of lectins on the apical membrane of epithelial cells in the FAE of Peyer's patches. a) LEL in Group 1 strongly reacts with the striated borders of epithelial cells restricted to the apical region of the FAE (arrowheads). The apical membrane is not stained in the basal region of FAE. b) The staining intensity of PHA-L in Group 2 gradually increases toward the apical region of FAE (arrowheads). c) UEA-I in group 3 shows a uniform staining pattern across the whole surface of FAE (arrowheads). d) PSA in group 4 shows little to no reactions to the whole surface of FAE. DA, dome area; FAIV, follicle-associated intestinal villus. Bar = 100 μm.

strongly reacted with the surfaces on the epithelial cells of the villous side which fronted the luminal contents (Fig. 3).

ConA, LCA, PHA-E and PHA-L were categorized into group 2. These lectins reacted moderately with the surfaces of the basal to middle regions, and strongly with the apical regions of FAE and intestinal villi. PHA-L and PHA-E also

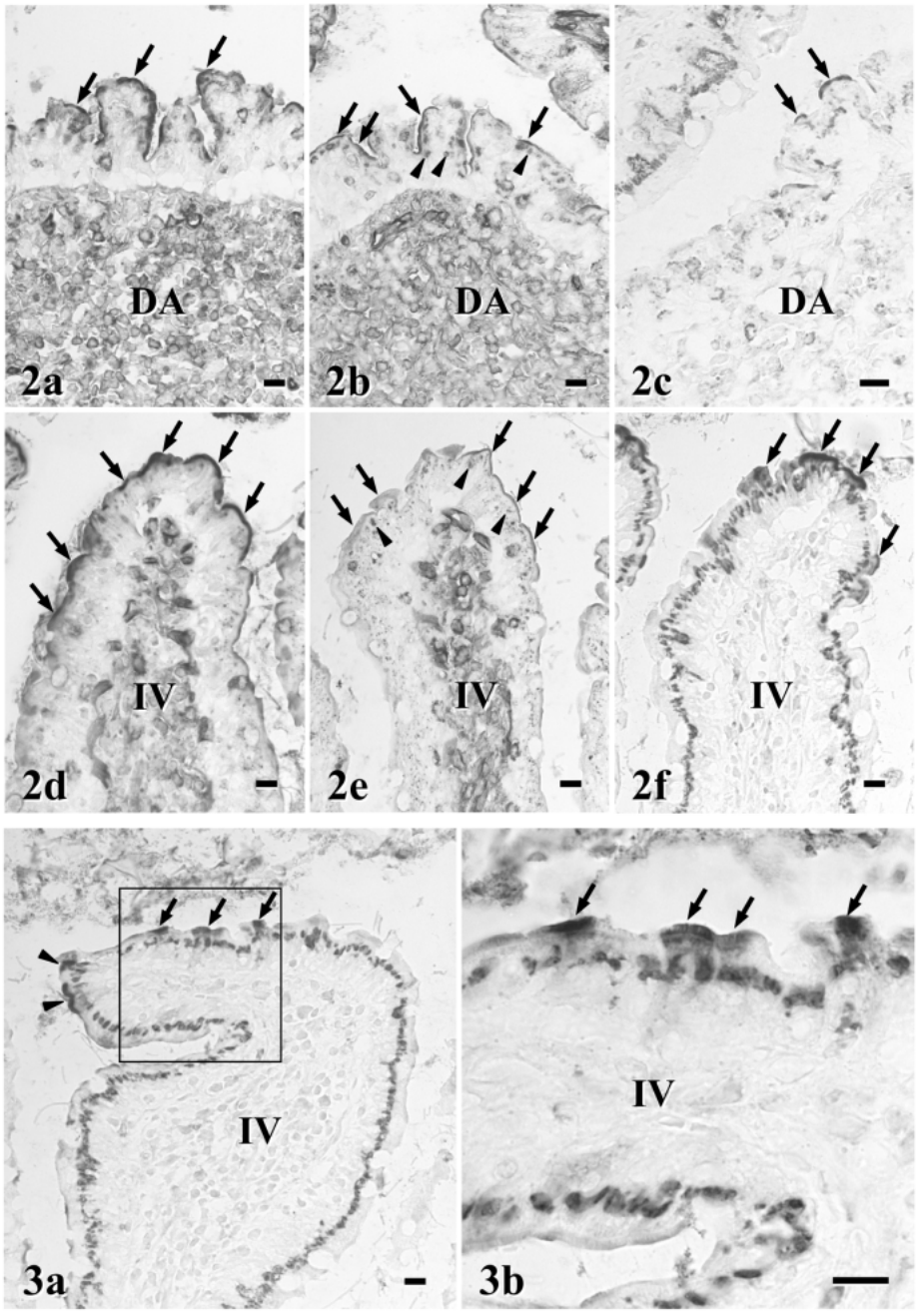

Fig. 2. Special sugar expression detected by group 1's lectins on the surfaces of epithelial cells in the apical regions of FAE and intestinal villi in Peyer's patches. STL and LEL specifically react with the striated borders of epithelial cells in the apical regions of FAEs (a, b) and intestinal villi (d, e), respectively (arrows). The sugars were also stained in the apical epithelial cytoplasms that contained variously sized granules (arrowheads). PNA reacts only with the striated borders of several epithelial cells in the apices of FAE (c) and intestinal villi (f) (arrows). DA, dome area; IV, intestinal villus. Bar = 10 μm.

Fig. 3. a) The lumen is full of contents and the intestinal villus is pushed down. Specific sugar expressions are seen in the striated borders of epithelial cells (arrows) in intestinal villus facing the lumen as well as at the villous tip (arrowheads). b) High magnification microscopic photograph of squared area in a). Epithelial cells detected by PNA are distributed only in the epithelium in the area facing the luminal contents (arrows). IV, intestinal villus. Bar = 10 μm.

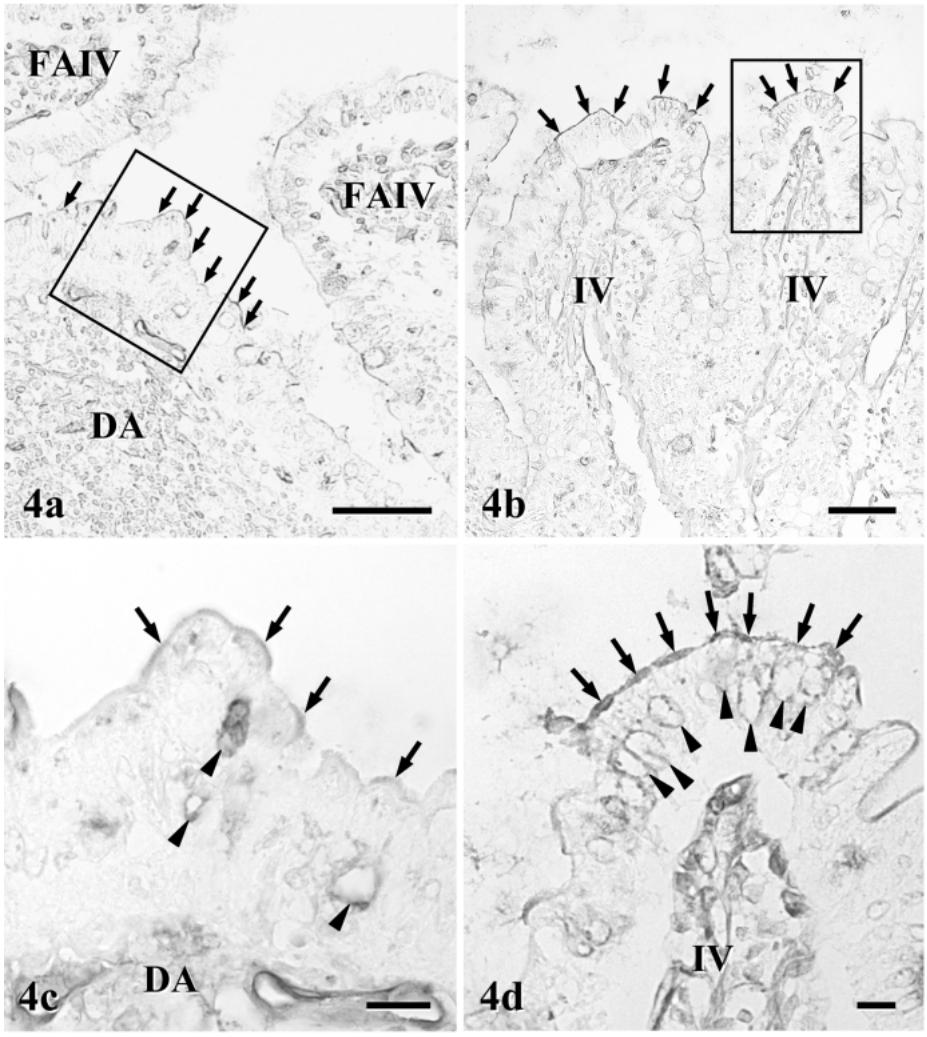

Fig. 4. Double detection of DNA fragmentation and sugar expression. a, b) Low magnification micrographs of the apical regions of FAE and intestinal villi. c, d) Higher magnification micrographs of squared areas in Fig. 4a and b. LEL (arrows in Fig. 4a-d) are expressed on some epithelial cells that coexpressed nuclear DNA fragmentation (arrowheads in Fig. 4c, d). Some DNA fragmentation positive nuclei are shifted toward the luminal portions. DA, dome area; IV, intestinal villus. Bar = 10 μm.

labeled the apical surfaces of most typical M cells, while ConA and LCA did not (Fig. 1b).

ECL, WGA, GSL-II, s-WGA, UEA-I and DSL, and JCA were categorized into group 3. UEA-I reacted very strongly, and both DSL and ECL did moderately with all surfaces of FAEs and intestinal villi, including the apical surfaces of typical M cells (Fig. 1c). WGA and s-WGA also reacted moderately with the surfaces of the basal to apical FAE and intestinal villi, except for faint labeling on M cells. GSL-II and JCA reacted faintly with the surfaces of all FAE and intestinal villi.

PSA, GSL-I, SJA, RCA_{120}, SBA, VVA and DBA were categorized into group 4. None or faint reactions of these lectins were observed with the surfaces of entire FAE and intestinal villi (Fig. 1d).

Double detection of nuclear DNA fragmentation and special sugar expression: Weak signals of nuclear DNA fragmentation were observed in the epithelial cells of the apical regions of FAE and of the middle regions of intestinal villi. Both intensities of nuclear DNA fragmentation increased toward the tips of FAE and intestinal villi. Some positive nuclei were shifted toward the luminal sites (Fig. 4). In the intestinal villi that had fallen down, DNA fragmentation was detected at the villous epithelium on the side fronting the luminal contents. In these villous sides, epithelial cells with morphological apoptotic signs, such as condensation of heterochromatin and shrinkage of both nuclei and cytoplasms, were found.

Only group 1's lectins reacted more strongly with the surfaces of apoptotic epithelial cells, as the nuclear DNA fragmentation was more positively expressed in FAE or intestinal villi (Fig. 4). In lectins of other groups, no

correlation was found between the nuclear DNA expression and the special sugar expression.

DISCUSSION

M cells have been identified by ultrastructural observation of their morphological characteristics in various animals. That is, typical M cells possess thin apical cytoplasms and no or sparse irregularly shaped microvilli: in addition, their epithelial pockets harbor several migrating cells [6, 35]. Several histochemical methods have also been developed to detect M cells more easily. A decrease or absence of alkaline phosphatase activity has been found in the apical membranes of M cells in Peyer's patches of various animal species [18, 27]. Vimentin, an intermediate filament protein, is co-expressed with cytokeratin in M cells in rabbit Peyer's patches and appendix, and is used as a good positive marker for M cell detection in rabbit [17]. Cytokeratin 18 can be also used as an immunohistochemical marker for M cells in porcine Peyer's patches [8]. Villin, an actin-bundling protein in microvilli, shows an unusual localizing pattern in the cytoplasm of M cells [19]. On the other hand, the glycocalyx shows the characteristic expression pattern on M cells in various animal species. UEA-I lectin selectively binds the M cells in mouse Peyer's patches [4], while UEA-I, DBA, PHA, SBA, VVA and WFA do so in the cecal lymphoid patches of rabbit [27]. In the present study, however, no particular lectin specific to M cells was detected in rat Peyer's patch. This result coincides with the report that no M cell-specific lectins exist in chicken cecal tonsils [20]. Moreover, the repertoire of sugars on M cells may be influenced by the characteristics of lectins of resident microflora or newly invaded bacteria [20]. Therefore, lectins might not be an appropriate marker of M cells in some animal species.

The sugar expression patterns on the mucosal surface differ among the intestinal segments, individuals or animal species [10, 30]. The lectin-binding properties are changed along the crypt-villous axis in the intestinal villi and the crypt-dome axis in the gut-associated-lymphatic tissues [7, 26]. In general, epithelial cells of the intestinal villi or the FAEs are generated in the intestinal crypts, followed by migration toward the tips of the intestinal villi or domes in the small intestine. Finally, the epithelial cells expressing of apoptosis are exfoliated from the tips of intestinal villi [16]. Some signs of epithelial apoptosis appear at the midpoint of epithelial migration. In the chicken cecum, weak fragmentation is expressed in the epithelial cells from the upper half to the upper quarter of the intestinal villi, whereas intense DNA fragmentation is restricted in the epithelial cells of the apical villi [34]. In rat small intestine, mRNA-specific DNase is detected from the basal third of rat intestinal villi [36], and nuclear DNA fragmentation gradually increases toward the villous tips [28]. In the human small intestine, epithelial DNA fragmentation is detected not only at the tips of villi but also around the tips, where only a few epithelial cells express the typical morphological characteristics of apoptosis [31]. In the present study, weak DNA fragmentation of epithelial cells was also detected from the middle regions of intestinal villi by the *in situ* nick end-labeling method, and its intensity was higher in the tips. Interestingly, this increasing expression of DNA fragmentation corresponds with the increasing expression of sugars detected by STL, LEL and PNA. Moreover, the double staining of apoptosis and lectin simultaneously detected the strong expression of both the heavy nuclear DNA fragmentation and the specific sugars. These findings suggest that the sugars detected by STL, LEL and PNA are strongly expressed on the surface of intestinal epithelial cells in the late stage of apoptosis. This suggestion is demonstrated by the ectopic expression of both the apoptotic features and the specific sugars detected simultaneously in the villous epithelial cells facing the luminal contents.

The intestinal microflora is usually quite stable in an animal species under normal physiological conditions [11]. In the upper part of the small intestine, lactobacilli are most predominant, but other bacterial species, such as streptococci or coliform bacteria, are rare in rats [23]. In contrast, a large number of microorganisms with long chains of either coccal-shaped or rod-shaped were histologically observed between the intestinal villi of the lower part of small intestine in rats [29]. Segmented filamentous bacteria (SFB), autochthonous, apathogenic bacteria are colonized in the upper one-third of the intestinal villi in the small intestines of a wide range of animal species, including rats [22]. On the other hand, lectins of various bacterial species can bind to specific sugars on the host mucosal surface. Type 1 fimbriated *Salmonella typhimurium* adheres to rat intestinal brush border membrane in a mannose-sensitive manner. *E. coli* K88 fimbriae recognize Gal-Cer and β1-linked galactosyl residues in glycosphingolipids [24]. Moreover, our previous study clarified that the most constant and fundamental colonization site of indigenous bacteria is the apices of FAEs in Peyer's patches and intestinal villi in rat [3]. In the present study, some sugars, such as (D-glcNAc)$_{2-4}$ and β-D-gal(1–3)-D-galNAc, were strongly expressed at the apices of the FAEs and intestinal villi, thus progressing the process of epithelial apoptosis. The result indicates that these sugars might be a ligand against the colonization on the intestinal villi and Peyer's patches of rat small intestine by some lectins of indigenous bacteria. Using histochemistry and scanning electron microscopy, we are further investigating the relationship between specific sugar expression and indigenous bacteria in rat small intestine.

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