Intraepithelial $\gamma\delta$ T Cells are Closely Associated with Apoptotic Enterocytes in the Bovine Intestine

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Received August 20, 1997

Summary. The T cell population in the intestinal epithelium, comparable in size to the T cell pool in the spleen, is characterized by the predominant distribution of T cells bearing $\gamma\delta$ T cell receptors. To determine the functional significance of the intraepithelial lymphocytes, we observed $\gamma\delta$ T cells present in the jejunal epithelium in cattle, in which there is predominance of $\gamma\delta$ T cells. Immunohistochemistry of frozen sections demonstrated that $\gamma\delta$ T lymphocytes were densely distributed in the villous epithelium but there were fewer in the lamina propria and they were not present in the crypt epithelium. Ultrastructurally, intraepithelial $\gamma\delta$ T cells were characterized by possessing electron-dense granules and interdigitating with enterocyte cytoplasm. Enterocytes, which were inserted by processes of intraepithelial lymphocytes or contacted by their cell bodies, showed morphologic changes seen in apoptotic cell death, such as elevated electron density of the cytoplasm and condensation of the chromatin. Apoptotic cells and cell debris were found in macrophages, which gathered in the subepithelial region of villus tips. These findings suggest that in the small intestine of cattle, $\gamma\delta$ T cells are involved in the renewal of epithelial cells by inducing apoptosis of epithelial cells.

T lymphocytes are classified into $\alpha\beta$ T cells and $\gamma\delta$ T cells according to the form of T cell receptors (TCRs). It is well established that $\alpha\beta$ T cells recognize antigens presented on major histocompatibility complex (MHC) products and are engaged in antigen-specific immune responses; however, antigen recognition by $\gamma\delta$ T cells remains to be elucidated. T cells expressing $\gamma\delta$ TCRs have a non-MHC-restricted cytotoxic function, suggesting that $\gamma\delta$ T cells may contribute to the immune system differently than $\alpha\beta$ T cells do (JANEWAY et al., 1988; HAAS et al., 1993). The $\gamma\delta$ T cells are characterized by their rich existence in epithelia such as the epidermis and the intes-

tinal epithelium; they are less numerous in subepithelial connective tissue and rarely distributed in the lymphoid organs (JANEWAY et al., 1988; VINEY et al., 1990). Although intraepithelial lymphocytes (IELs) are thought to provide a first line of defense against microbial pathogens, support for this idea has been obtained for $\alpha\beta$ IELs but not for $\gamma\delta$ IELs. In germ-free mice, the number and functional properties of $\alpha\beta$ IELs in the intestine are strongly diminished, while there is no significant influence on $\gamma\delta$ IELs (BANDEIRA et al., 1990; GUY-GRAND et al., 1991; KAWAGUCHI et al., 1993).

Some ultrastructural studies have revealed the interdigitation of IELs with the basolateral faces of intestinal epithelial cells (OTTO, 1973), suggesting a functional relationship between them. In the guinea pig intestine, IELs are essentially large granular lymphocytes (LGLs) and vigorously interdigitated with the enterocyte cytoplasm by their complicated cytoplasmic processes (IWANAGA et al., 1994). These lymphocytes are densely distributed at the villus tips where aged enterocytes gather. Through studies using guinea pigs, we proposed lymphocyte-mediated killing of aged enterocytes at the villus tips (IWANAGA et al., 1993, 1994; IWANAGA, 1995). On the other hand, ISHIKAWA and coworkers, who analysed the intestinal epithelium of $\alpha\beta$ T cell- or $\gamma\delta$ T cell-deficient mice, demonstrated that the absence of $\gamma\delta$ T cells caused a reduction in epithelial cell turnover, and concluded that $\gamma\delta$ IELs regulate the generation and differentiation of epithelial cells (KOMANO et al., 1995).

The two different ideas concerning the function of intestinal IELs mentioned above suggest a novel role of T cells other than in the immune responses, namely their involvement in the homeostasis of intestinal epithelial cells. However, it remains unclear whether $\gamma\delta$ T cells in the intestine are cytotoxic or trophic to

Fig. 1. Immunostaining of $\gamma\delta$ T cells in the bovine jejunum. Tip regions of three intestinal villi are obliquely cut here. Numerous immunopositive lymphocytes are distributed throughout the epithelium, but fewer are in the lamina propria. $\times 150$

Fig. 2. Histochemical staining for acid phosphatase activity in the bovine jejunum. Macrophages with positive reactions for acid phosphatase are gathered at the subepithelial region of villi. $\times 150$

intestinal epithelial cells. Since there is a possibility that this discrepancy may be attributed to the species difference between the guinea pig and mouse, investigation using other animals is needed. The compositions of $\alpha\beta$ T cells and $\gamma\delta$ T cells are known to vary among species; T cells bearing $\gamma\delta$ TCRs are found in high concentrations in epithelia and peripheral blood of ruminants (MACKAY and HEIN, 1989; MCCLURE et al., 1989; CLEVERS et al., 1990; HEIN and MACKAY, 1991). Therefore, ruminant $\gamma\delta$ T cells provide a model system for studying $\gamma\delta$ T cell function. The present study aims to elucidate the distribution and morphological characteristics of $\gamma\delta$ T cells in the bovine small intestine, with special reference to the relationship between $\gamma\delta$ T cells and enterocytes. The morphological findings obtained here indicate that IELs expressing the $\gamma\delta$ form of TCRs and displaying

LGLs are involved in inducing apoptosis of enterocytes.

MATERIALS AND METHODS

Seven adult female Holstein cattle, weighing about 450 kg, were used in this study. Fresh tissues of the jejunum were obtained in the nearest slaughterhouse and rapidly frozen in liquid nitrogen. Frozen sections, about 20 μm in thickness, were prepared in a cryostat and collected on poly-L-lysine-coated glass slides. Immunohistochemistry for $\gamma\delta$ T cells was carried out according to the avidin-biotin complex method. After fixation in ice-cold acetone for 10 min, the frozen sections were treated with a normal goat serum,

followed by incubation with a mouse monoclonal antibody against ruminant $\gamma\delta$ TCR (TCR1-N24) (Cosmo Bio, Tokyo, Japan) (PARSONS et al., 1993). The site of antigen-antibody reaction was visualized by streptavidin and biotin-peroxidase complex (Histfine; Nichirei, Tokyo, Japan). During this step endogenous peroxidase activities were blocked by immersion of sections in 5 mM orthoperiodic acid. After diaminobenzidine reaction, the sections were slightly counterstained with hematoxylin.

For immunostaining at the electron microscopic level, fresh tissues were fixed in periodate-lysine-paraformaldehyde (PLP) for 3 h at 4°C and immersed in 30% sucrose overnight. Cryostat sections, about 20 μ m in thickness, were cut and mounted on glass slides. After immunostaining as mentioned above, they were postfixed in 1% OsO₄ for 30 min. They were dehydrated through a graded series of ethanol and directly embedded in Epon 812 on glass slides. After the embedded sections were detached from glass slides, ultrathin sections were prepared and stained with lead citrate for electron microscopy.

The PLP-fixed cryostat sections were also used for the detection of acid phosphatase activity according to BURNSTONE (1958). Control experiments for acid phosphatase reactions were simultaneously carried out by incubation with medium containing 10 mM NaF, a potent inhibitor of this enzyme.

For conventional electron microscopy, fresh tissues were cut into small pieces and fixed for 4 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The tissue blocks were washed in the cacodylate buffer and postfixed for 1.5 h in 1% OsO₄ dissolved in the cacodylate buffer. They were dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under a JEOL JEM-1210 type transmission electron microscope.

RESULTS

Histochemistry of $\gamma\delta$ T cells and macrophages

Immunostaining using the anti- $\gamma\delta$ TCR antibody demonstrated numerous lymphocytes distributed in the villous epithelium of the jejunum (Fig. 1), while no immunoreactive lymphocytes were found in the crypt epithelium. The intraepithelial $\gamma\delta$ T cells were more numerous at the villus tips than the basal region of villi. Several immunopositive cells were present in the lamina propria, but their distribution density was considerably low compared with that in the epithelium. The intraepithelial $\gamma\delta$ T cells were to some

Fig. 3. Electron micrograph of the bovine jejunum immunostained for $\gamma\delta$ T cells. The immunoreactivity is localized at the cell surface of two intraepithelial lymphocytes (L). One of them contains electron-dense granules (arrow) in the cytoplasm. E enterocyte. Bar = 2 μ m

extent bigger than enterocyte nuclei and were located apart from the nuclei line of enterocytes. They were situated not only close to the basement membrane of the epithelium, but also in the different levels of the epithelium. No $\gamma\delta$ T cells were observed passing through the microvillous portion of epithelium and entering the intestinal lumen.

Histochemistry for acid phosphatase revealed irregular-shaped macrophages gathering in the subepithelial region of villi (Fig. 2). Macrophages at villus tips were larger, frequently rounded, and more intense in acid phosphatase activity than those at the basal region of villi. Only slender macrophages with a weak acid phosphatase activity were dispersed in the lamina propria between the crypts. Intense activity for acid phosphatase was also localized at the striated border of epithelium (Fig. 2). The activity in the striated border was not inhibited by preincubation of the staining medium with NaF, while that in the macrophages perfectly disappeared.

Immunohistochemistry at the electron microscopic level

The intestinal mucosa immunostained using the anti- $\gamma\delta$ TCR antibody were observed under an electron

Figs. 4 and 5. Electron micrographs showing the epithelium of villus tips. Numerous lymphocytes with pale cytoplasm, embedded in the epithelium, show a mosaic pattern. They are located at various heights of the epithelium, and are frequently close to the striated border. In **Fig. 5**, some lymphocytes (*L*) contact each other forming cellular cords within the epithelium. *E* enterocyte. Bars=2 μ m

Figs. 6 and 7. Electron micrographs showing lymphocytes (*L*) deeply invading the epithelium close to the striated border. They contain electron-dense granules in the cytoplasm and project their irregular-shaped processes to enterocytes. Bars=1 μ m

microscope to examine the ultrastructure of the immunoreactive lymphocytes (Fig. 3). Immunoreactivity for $\gamma\delta$ TCR, identifiable as electron-dense substances, was localized predominantly at the cell membrane of lymphocytes, which were numerous in the epithelium. The $\gamma\delta$ T cells intervened between enterocytes (columnar cells) and were easily discriminated from the enterocyte cytoplasm due to intercellular spaces. They usually contained several electron-dense granules in their broad cytoplasm (Fig. 3).

Ultrastructural analysis of IELs

Electron microscopic observation revealed the existence of numerous lymphocytes with pale cytoplasm in the epithelium of jejunal mucosa (Fig. 4). Although IELs were distributed throughout the villous epithelium, they were present more densely at the villus tips and, in some parts, aggregated to form cell cords within the epithelium (Fig. 5). Mosaic images arising from the dark cytoplasm of epithelial cells and the

clear cytoplasm of lymphocytes were seen in the epithelium of villus tips. On the other hand, there were few IELs in the crypt epithelium. IELs in the villi were located at various heights of the epithelium from on the basal lamina close to the striated border (Figs. 4, 5). As observed by a light microscope, no IELs went across the striated border and entered the intestinal lumen.

IELs were characterized by broad and clear cytoplasm containing electron-dense granules and extending irregular-shaped cytoplasmic processes (Figs. 5-7). The cytoplasmic granules were membrane-bound and variable in number and size, ranging from 0.1 to 0.5 μ m in diameter (Figs. 6, 7). Most of the granules gathered near the Golgi apparatus. IELs extended out their sharp and, frequently, branched cytoplasmic processes deeply into the cytoplasm of adjacent enterocytes (Figs. 5-7). IELs appearing at the apical region of the epithelium invaded the microvillous portion with their cytoplasmic processes (Figs. 6, 7). IELs were basically present in the intercellular

Figs. 8-10. Electron micrographs showing an intimate relationship between intraepithelial lymphocytes and apoptotic enterocytes. In **Fig. 8**, two enterocytes (*E*) showing slight condensation of the cytoplasm are juxtaposed with lymphocytes indicated by *arrows*. In **Fig. 9**, one of enterocytes (*E*) with condensed cytoplasm is invaded by the process (*arrow*) of an intraepithelial lymphocyte (*L*). An empty space is seen in the cytoplasm of another enterocyte. A granular lymphocyte (*L*) in **Fig. 10** invades, with the whole cell body, an apoptotic enterocyte (*E*) showing chromatin condensation. Bars = 2 μ m

Fig. 11. Three macrophages (*M*) gathering at the subepithelial region of a villus tip. Cell debris containing nuclei with condensed chromatin (*arrows*) are phagocytosed by these macrophages. *E* epithelium. Bar = 2 μ m

Fig. 12. An apoptotic enterocyte (*E*) is engulfed by a macrophage (*M*) present at the basal part of the epithelium. The enterocyte in the macrophage shows condensation of both the nucleus and cytoplasm, and appears to remain undigested. Bar = 2 μ m

spaces, but frequently were wholly embedded in the enterocyte cytoplasm.

Enterocytes invaded by the processes of IELs or touched by IEL cell bodies showed morphologic changes characterized by elevated electron density of the cytoplasm and formation of vesicles (Figs. 8–10). The nuclei of such enterocytes showed greater or lesser the condensation of chromatin along the nuclear membrane (Fig. 10). The microvilli became longer and thinner and were arranged more irregularly than those in intact enterocytes. These morphological changes are figures presented in apoptotic cell death. Round and large empty spaces were found in the cytoplasm of the apoptotic enterocytes (Fig. 9). Their shape and size frequently corresponded to those of processes projected from IELs, indicating that the spaces were formed by the invasion of IEL

processes.

Electron microscopic observation confirmed the plentiful existence of macrophages at the lamina propria of villus tips (Fig. 11). The macrophages, rounded and 15–30 μ m in size, loosely gathered beneath the epithelium. They were characterized by possessing phagosomes and lysosomes that were highly variable in size and electron density. Large phagosomes in the macrophages contained nuclei and cell organella showing different digestive processes (Fig. 11). Several macrophages entered the epithelium, appearing nearly on the basement membrane. The intraepithelial macrophages sometimes phagocytosed undigested apoptotic enterocytes showing electron-dense cytoplasm and condensation of chromatin along the nuclear envelope (Fig. 12).

DISCUSSION

Epithelial cells of the gut are characterized by rapid, constant cell renewal. The epithelial cells in the small intestine proliferate in the crypts, move toward the villi, and die at the villus tips (EASTWOOD, 1977; LEBLOND, 1981). The fact that the intestinal villi maintain a constant length and shape indicates that the turnover of epithelial cells is well-controlled by some, at present unknown, mechanism. In previous studies using guinea pigs, we demonstrated that apoptotic enterocytes are phagocytosed by lamina propria macrophages at the villus tips, and further suggested the possibility that perforin-containing cytotoxic IELs are involved in the apoptosis of enterocytes (HAN et al., 1993b; IWANAGA et al., 1994). In the rat and mouse, however, we failed to obtain any evidence for lymphocyte-mediated killing of enterocytes and subsequent phagocytosis (HAN et al. 1993a; IWANAGA, 1995). On the other hand, KOMANO et al. (1995) reported a significant decrease of bromodeoxy-uridine (BrdU)-labeled epithelial cells in the intestine in $\gamma\delta$ T cell-deficient mice, suggesting that $\gamma\delta$ T cells stimulate the proliferation and differentiation of crypt stem cells. Their idea is partially supported by identification of a growth factor, keratinocyte-growth factor (KGF), produced in activated $\gamma\delta$ IELs (BOISMENU and HAVRAN, 1994) and by demonstration of receptors for KGF on crypt epithelial cells (HOUSELY et al., 1994). Since IELs are mainly located in the villous portion, it is reasonable to consider that KGF released from villous IELs acts on crypt progenitor cells as a local hormone. However, this idea can not explain the vigorous interdigitation of IELs with enterocytes at the villous portion.

IELs in the intestine are characterized by predominant distribution of $\gamma\delta$ T cells. In the small intestine in mice, about half of IELs express $\gamma\delta$ TCRs and are thymus-independent (VINEY et al., 1990; ROCHA et al., 1991; ISHIKAWA et al., 1993). The population of $\gamma\delta$ T cells among IELs is known to be extremely high in ruminants as well as chickens, in which 84–90% of IELs express $\gamma\delta$ TCRs (BUCY et al., 1988). It is reported that $\gamma\delta$ T cells bearing WC1 molecules form up to 75% of the peripheral blood lymphocytes of young ruminants, although they decrease in number in adults (CLEVERS et al., 1990; HEIN and MACKAY, 1991). The $\gamma\delta$ T cells of ruminants also occur in large numbers in epithelia, while there are fewer in the lymphoid organs (MACKAY and HEIN, 1989; MCCLURE et al., 1989). The present immunostaining confirmed the plentiful existence of $\gamma\delta$ T cells in the intestinal epithelium of the bovine jejunum. Furthermore, im-

munohistochemistry at the electron microscopic level showed that most of $\gamma\delta$ IELs in the bovine jejunum were large granular lymphocytes (LGLs). LGLs correspond to a heterogeneous cell population including natural killer (NK) cells, cytotoxic T lymphocytes (CTL) and lymphokine-activated killer (LAK) cells (KANEDA, 1989). They all possess intense killer activity. The cytoplasmic granules are known to contain perforin (PODACK and KONIGSBERG, 1984), serine proteinase (MUNGER et al., 1988; HAYES et al., 1989; ZUNINO et al., 1990) and fragmentin (SHI et al., 1992), which are involved in inducing apoptotic cell death of target cells. It is worth noting that the $\gamma\delta$ T cells were more numerous at the tips of villi, in which aged enterocytes gather, but absent in the crypt region.

The granular IELs in the jejunum of cattle were characterized by extending sharp and branched cytoplasmic processes into the adjacent enterocytes. In previous studies, we obtained similar findings at the villus tips in the small intestine of the guinea pig, but failed to demonstrate morphological changes of enterocytes juxtaposed with IELs (IWANAGA et al., 1994). The present study is the first to show that enterocytes, touched by IELs or stabbed with their processes, indicates unique morphologic changes such as the elevated electron density of the cytoplasm and chromatin condensation, which are typical signs seen in the apoptotic cell death (WYLLIE et al., 1980). Thus, cytotoxic IELs expressing $\gamma\delta$ TCRs may exert cytotoxicity against enterocytes, possibly effete enterocytes, and induce apoptosis of the cells.

Since the expression of cytotoxicity by killer lymphocytes is mainly attributed to the granular contents (PODACK and KONIGSBERG, 1984; ALLBIFITTON et al., 1988), exocytosis of the granules is expected to be observed in the intraepithelial LGLs. The present study, however, could not show the accumulation of granules in the cytoplasmic processes or the exocytotic release of granular contents into intercellular space. KANEDA et al. (1991), who incubated splenic NK cells with a tumor cell line (Yac-1 cell), observed accumulation of granular and vesicular components in the cytoplasm of NK cells which faced the target cells. However, they could not detect exocytosis of dense granules and vesicles (rod-cored vesicles) at the contact surface, possibly due to the fact that the phenomenon was very short. On the other hand, some researchers have insisted on an important role of the cytoplasmic projections extending from killer cells (SANDERSON and GLAUERT, 1979; KANEDA, 1989). Physical damage by their cytoplasmic projections and cell-to-cell contact may lead to changes in molecular conformation on cell membranes of target cells or in the distribution of cytosolic factors, which are

lethal to the cell. The attack by cell projections is suitable for targeting the cells, since there is a possibility that adjacent normal and intact cells are also involved in killing by lymphocytes, if the lymphocyte-mediated killing is caused only by cytotoxic substances released from killer cells.

Recent morphological studies revealed some mechanisms for the elimination of apoptotic enterocytes and that they are different according to species (for review, IWANAGA, 1995). Cell bodies of effete enterocytes in the small intestine in the guinea pig and monkey are phagocytosed by lamina propria macrophages, which gather at the subepithelial region of villus tips (HAN et al., 1993b). In the rat and mouse, enterocytes showing apoptotic signs are exfoliated as a whole from the villus tips into the gut lumen (MADARA, 1990; HAN et al., 1993a; IWANAGA, 1995). The mechanism of the disposal of apoptotic enterocytes in the bovine intestine remains unknown. The present ultrastructural observations suggest the occurrence of a guinea pig-type disposal mechanism in cattle. The aggregation of macrophages at the villus tip and presence of phagosomes containing digested cellular elements in their cytoplasm are comparable with observations in the guinea pig (HAN et al., 1993b). Furthermore, we found some macrophages entering into the epithelium and phagocytosing whole cell bodies of apoptotic enterocytes. These findings indicated that apoptotic enterocytes were phagocytosed by intraepithelial macrophages or pseudopods extending from lamina propria macrophages.

In the present study, we obtained morphological evidence that $\gamma\delta$ T cells in the bovine jejunum induce apoptosis of enterocytes and play an important role for cell renewal. A similar intimate relationship of $\gamma\delta$ T cells and apoptotic enterocytes is seen in the sheep and chicken, which are known to be rich in $\gamma\delta$ T cells (Y. SUZUKI, unpublished data).

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