Apoptosis of Villous Epithelial Cells and Follicle-Associated Epithelial Cells in Chicken Cecum

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ABSTRACT. The process of the disappearance of epithelial cells was examined in chicken cecal villi and follicle-associated epithelium (FAE). The apoptotic epithelial cells with intense DNA-fragmentation and their exfoliation were found in the villous tips. The epithelial cells with weak DNA-fragmentation were seen in the upper portion of the villi and their sparse exfoliations were also found there. Numerous epithelial cells in the intestinal lumen expressed the apoptotic features. A row of apoptotic epithelial cells with DNA-fragmentation was also found in the apical FAE, whereas no M cells exhibited any apoptotic signs. In all cecal regions, $CD3^+$, $CD8^+$, and $TCR2^+$ lymphocytes were predominant in the epithelium at the upper portion of the villi and the FAE. $CD4^+$ lymphocytes were mainly seen in the lamina propria. $TCR1^+$ lymphocytes were not abundant in comparison with $TCR2^+$ lymphocytes in the epithelium. $TCR3^+$ T lymphocytes were rarely detected. These results suggest that the chicken cecal epithelial cells exfoliated into the lumen after the induction of the apoptosis, and that the induction may be involved with $CD3^+$, $CD8^+$, and $TCR2^+$ lymphocytes. No death in M cells suggests that M cells may transform into microvillous epithelial cells.—KEY WORDS: apoptosis, chicken, intestine, M cell, T lymphocyte.

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The process of the disappearance of intestinal epithelial cells is different among the mammalian species. In rat and mouse small intestines, most of the villous epithelial cells are induced into apoptosis and exfoliate from villous tips into the lumen. In the guinea pig and the monkey, only the apical pieces of the cytoplasm are shed into the intestinal lumen and the majority of the epithelial cytoplasm is phagocytosed by macrophages in the villous tips [9].

The intestinal epithelium contains numerous intraepithelial lymphocytes (IELs). IELs are classified into $\alpha\beta$ and $\gamma\delta$ T lymphocytes according to the type of T cell receptor (TCR) and $\gamma\delta$ T lymphocytes are preferentially located in the epithelium of the chicken and mammals [6, 15]. Moreover, the majority of IELs express $CD8^+$, the surface T cell marker of killer/suppressor T lymphocytes, and express the morphology of the large granular lymphocytes (LGLs) [15]. Recently, it was proposed that $\gamma\delta$ T lymphocytes may attribute to apoptosis of the intestinal epithelial cells and regulate the proliferation of epithelial cells in mammals [9, 16].

In the adult chicken, the proliferation sites and the kinetics of intestinal epithelial cells have been clarified only in the cecum [23], whereas the process of the disappearance of epithelial cells and the role of IELs in this process are still unknown in all intestines. In this study, apoptotic epithelial cells were light-microscopically investigated in the chicken cecum to detect the sites of the disappearance of the villous epithelial cells and M cells. The subpopulation of the IELs were also investigated in the chicken cecum in order to clarify the relation between the apoptotic epithelial cells and the IELs.

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MATERIALS AND METHODS

Animals: Ten White Leghorn chickens (more than 5 months old) were obtained from our laboratory. They were permitted free access to food and water. Artificial light was utilized between 5:00. a.m. and 9:00 p.m. The chickens were reared with an animal protein-free formula feed to avoid binding between the antibodies used in this study and the immunoglobulins against dietary-animal proteins.

General histology: Five chickens were sacrificed by cervical exsanguination under anesthesia with an intravenous (i.v.) injection of pentobarbital sodium around noon. Tissues obtained from the base including the cecal tonsil, the body, and the apex of the cecum were fixed in Bouin's solution for 24 hr at room temperature (RT). Thereafter, paraffin sections in 4 μm thickness were stained by hematoxylin and eosin (H.E.).

Detection of DNA fragmentation: To detect the apoptotic epithelial cells, BrdUTP (bromodeoxyuridine-conjugated dUTP) was applied as a marker according to a modified method described by Li and Darzynkiewicz [18]. Briefly, the tissues obtained from each cecal region of 5 chickens were fixed in PLP (periodate-lysine-paraformaldehyde) for 24 hr at 4°C, and frozen in liquid nitrogen. Serial sections of 3 μm in thickness were prepared according to the method described by Barthel and Raymond [4]. The sections were cut with a coldtome CM-501 (Sakura, Japan) and were placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shinetsu, Japan). Four sections were prepared per chicken (sections 1, 2, 3 and 4). Sections 1 and 2 were used as positive controls for sections 3. Section 4 was used as a negative control. The sections

were pretreated with 200 μg/m*l* proteinase K solution (Sigma, U.S.A.) for 20 min at 37°C. After treatment with absolute methanol and 0.1% H_2O_2, sections 1 and 2 were incubated with 80 U/m*l* DNase I solution (Boehringer Mannheim, U.S.A.) for 30 min at 37°C. Following immersion in a terminal deoxynucleotidyl transferase (TdT) buffer (Boehringer Mannheim, U.S.A.), sections 1 and 3 were incubated with 20 μl of TdT reaction buffer (Boehringer Mannheim, U.S.A.) containing BrdUTP solution (0.1 nM BrdUTP in 4 μl distilled water; Sigma, U.S.A.) for 90 min at 37°C. After the reaction, all sections were incubated with 1% normal rabbit serum. Thereafter, the sections were treated with anti-BrdU mouse monoclonal antibody (diluted 1:100; Bio-Science Introducts, Japan) for 18 hr at 4°C. Following incubation with anti-mouse IgG rabbit serum (diluted 1:100; Jackson, U.S.A.) for 1 hr at RT, the sections were treated with mouse peroxidase-anti-peroxidase-complex (PAP) (diluted 1:100; Seikagaku, Japan) for 1 hr at RT. Finally, these sections were incubated with 3,3'-diaminobenzidine (DAB) containing 0.03% H_2O_2 and counterstained with methyl green.

Detection of T lymphocyte subpopulations: Serial frozen sections of 3 μm in thickness were prepared from tissues obtained as described above. These sections were treated with absolute methanol followed by 0.3% H_2O_2 and incubated with 1% normal rabbit serum. Thereafter, they were incubated with mouse monoclonal antibodies for chicken TCR1, TCR2, and TCR3, which recognize $\gamma\delta$ TCR, Vβ-1$\alpha\beta$ TCR, and Vβ-2$\alpha\beta$ TCR respectively, and CD3, CD4, and CD8 (diluted 1:50 except for CD3 which was diluted 1:100; Southern Biotechnology Associates, U.S.A.) for 18 hr at 4°C. After incubation with anti-mouse IgG rabbit serum (diluted 1:100; Jackson, U.S.A.) for 1 hr at RT, they were treated with mouse PAP (diluted 1:100; Seikagaku, Japan) for 1 hr at RT. Finally, these sections were incubated with DAB and counterstained with methyl green.

RESULTS

Apoptosis of the epithelial cells: Epithelial cells with typical morphological characteristics of apoptosis were found at the intestinal villous tips in all cecal regions and the apical FAE in the cecal tonsil (Fig. 1A). The typical apoptotic epithelial cells were characterized by a round shape, degradation of the nuclei, chromatin condensation, and cell shrinkage. Moreover, epithelial cells with apoptotic features (chromatin condensation and the cell shrinkage) were also distributed sparsely in the upper half of the villi and the FAE (Fig. 1B, C). These epithelial cells were more slender in shape as compared to the common columnar epithelial cells. The exfoliation of apoptotic epithelial cells into the lumen was frequently found, but phagocytosis by macrophages was extremely rare in the epithelium. Few apoptotic epithelial cells were observed in the intestinal crypts.

Intense DNA-fragmentation of the epithelial cellular nuclei was localized at the villous tips in all cecal regions (Fig. 2A, B). Weak DNA-fragmentation of the epithelial cells was also detected at the upper one-half to one-quarter of the villi, but most of epithelial cells with weak DNA-fragmentation expressed no other morphological features of apoptosis. Few apoptotic epithelial cells with DNA-fragmentation were seen in the intestinal crypts. In the cecal tonsil, DNA-fragmentation of the epithelial cells was localized in the apical FAE, and was especially focused at the transition area from the lateral to the apical FAE (Fig. 2C). No DNA-fragmentation was detected in M cells, which were distributed at the lateral FAE.

T lymphocyte subpopulations in the epithelium: $CD3^+$,

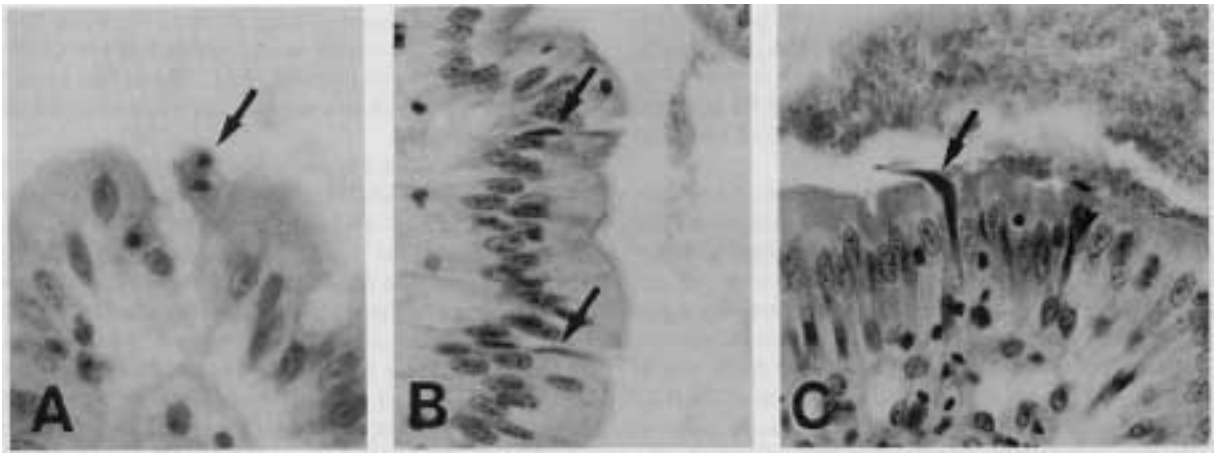

Fig. 1. Apoptotic epithelial cells in the chicken cecum. (A) Top of the intestinal villus. An apoptotic epithelial cell (arrow) with a degradation of the nucleus nearly exfoliates from the villous tip. (B) Upper portion of the intestinal villus. Slender epithelial cells (arrows) with chromatin condensation and cell shrinkage are found on the lateral of side the villus. (C) Apical FAE. Slender epithelial cells (arrow, arrowhead) are found in the epithelium and nearly exfoliate (arrow) into the lumen. H.E. staining. A: × 770, B: × 600, C: × 600.

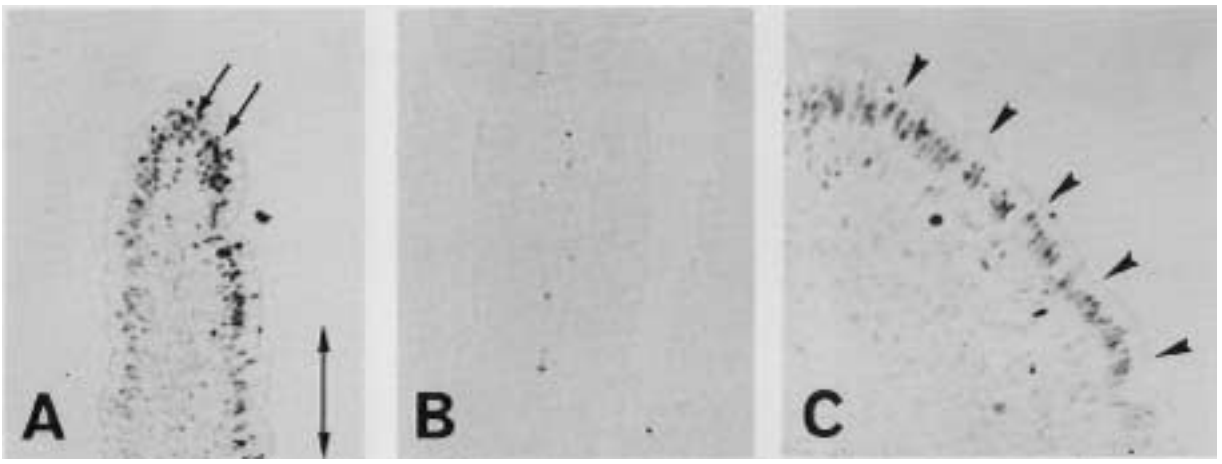

Fig. 2. Epithelial cells that express DNA-fragmentation. (A) Upper portion of the intestinal villus. The epithelial cells with strong DNA-fragmentation (arrows) are mainly localized at the top of the intestinal villus. The epithelial cells with weak DNA-fragmentation (indicated by double-headed arrow) are also found. (B) Lower portion of the intestinal villi. DNA-fragmentation of the epithelial cells is not found. (C) Transition area from lateral to apical FAE. DNA-fragmentation of the epithelial cells (arrowheads) is found in the apical FAE and is especially focused at the transition area. Counterstained by methyl green. A: × 200, B: × 130, C: × 260.

$CD8^+$, and $TCR2^+$ lymphocytes were predominantly distributed in the epithelium of the upper portion of the villi in all cecal regions (Fig. 3). In the cecal tonsil, $CD3^+$, $CD8^+$, and $TCR2^+$ lymphocytes were localized in the apical FAE, and especially numerous at the transition area from the lateral to the apical FAE (Fig. 4). In all cecal regions, $CD4^+$ lymphocytes were seldom seen in the villous epithelium (Fig. 3D). In the cecal tonsil, numerous $CD4^+$ lymphocytes were distributed in the lamina propria (Fig. 4D). In the FAE, $CD4^+$ lymphocytes were distributed, especially in the lateral epithelium, near the orifice of the intestinal crypts. $TCR1^+$ lymphocytes were apparently less than $TCR2^+$ lymphocytes in the epithelium in all cecal regions, and were distributed sparsely throughout the villous epithelium and the FAE (Figs. 3E, 4E). $TCR3^+$ lymphocytes were rarely seen throughout the cecal epithelium (Figs. 3F, 4F). $CD3^+$ lymphocytes seemed to be more numerous than all lymphocytes with $CD8^+$ or $CD4^+$, or all of the TCR^+ lymphocytes.

DISCUSSION

In the present study on chickens, epithelial cells that expressed the typical characteristics of apoptosis were found at the villous tips and the apical FAE. Moreover, the exfoliation of the apoptotic epithelial cells was frequently observed, but phagocytosis by macrophages was extremely rare. These results suggest that most of the epithelial cells in the chicken cecum are exfoliated from the villous tip and the apical FAE into the lumen after being induced into apoptosis.

In the chicken cecum, the migration of epithelial cells become faster as they migrated toward the villous tip [22]. In the upper portion of the villi and the FAE, the exfoliation of slender epithelial cells was found in this study. Therefore, these findings suggest that some of the epithelial cells exfoliate from the epithelium on the way to the top of the villi or the FAE in the chicken cecum.

In human small intestines, numerous epithelial cells express the DNA-fragmentation in the upper portion of the villi, whereas most of them do not express the morphological characteristics of apoptosis, excluding those in the villous tips [21]. Similar findings were observed in the present study on the chicken cecum. The apoptotic epithelial cells express DNA cleavage with 300 and/or 50 kbp fragments prior to the 180–200 bp internucleosomal DNA-fragments [20]. Therefore, weak DNA-fragmentation of the epithelial cellular nuclei in the apical portion of the intestinal villi and the FAE might express large DNA-fragments in the chicken cecum. In mouse small intestines, apoptosis of the epithelial cells is regulated by the *bcl-2* gene [2, 8, 11]. In general, apoptotic cells show various morphological characteristics, i.e., chromatin condensation, degradation of the nuclei, cell shrinkage, and apoptotic body [13]. These apoptotic changes of the epithelial cells might also be inhibited by the *bcl-2* until most of the epithelial cells reach the villous tips.

In the chicken cecum, M cells distribute in the epithelium of the lateral FAE, not in the apical FAE [14]. In the present study, no DNA-fragmentation was found in M cell, whereas the epithelial cells with DNA-fragmentation were found in the apical FAE. In general, the epithelial cells migrate from the lateral FAE to the apical FAE in chicken cecal tonsils [23]. Therefore, these phenomena suggest that M cells may transform to the ordinary microvillous epithelial cells, which are the main constituent of the apical FAE.

In mammals, the intestinal epithelium contains numerous IELs. A majority of IELs express $CD8^+$ and can be further classified into $\alpha\beta$ or $\gamma\delta$ T lymphocytes [15]. More $\gamma\delta$ T

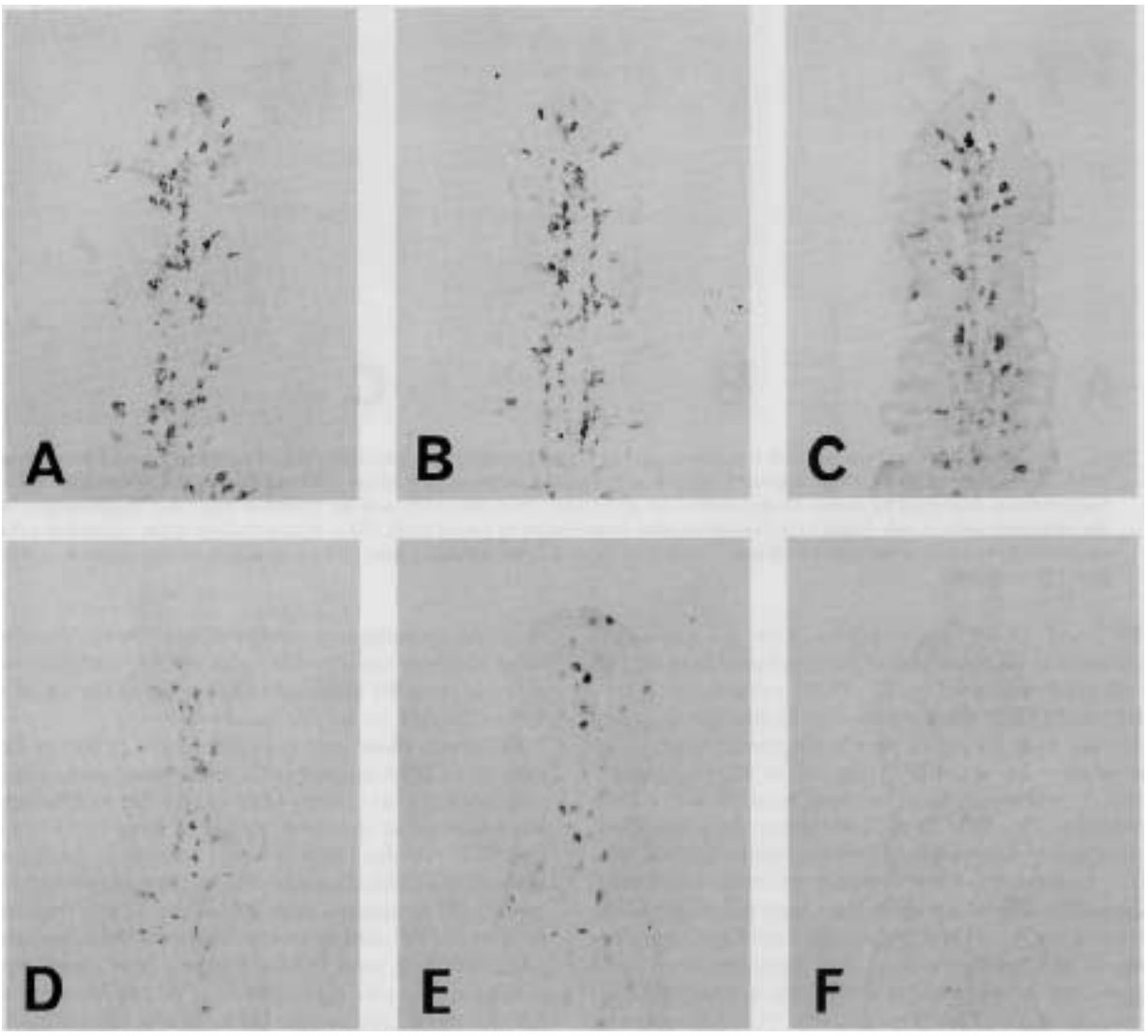

Fig. 3. Phenotypes of the lymphocytes in the upper portions of the same cecal villi. (A) $CD3^+$, (B) $CD8^+$, (C) $TCR2^+$, (D) $CD4^+$, (E) $TCR1^+$ and (F) $TCR3^+$ lymphocytes. Counterstained by methyl green. × 190.

lymphocytes locate in the intestinal epithelium than in the lamina propria, whereas their ratio varies in the epithelium among species [15, 17, 24]. In the bovine jejunum, the intraepithelial $\gamma\delta$ T lymphocytes were more numerous at the villous tips and most of $\gamma\delta$ IELs were LGLs. These findings suggest that $\gamma\delta$ T lymphocytes may contribute to apoptosis of the epithelial cells in the bovine intestine [22]. In this study, however, more $CD3^+$, $CD8^+$, and $TCR2^+$ lymphocytes were distributed in the epithelium than were $TCR1^+$ lymphocytes, and the distribution of $TCR2^+$ lymphocytes was similar to that of the epithelial cells with DNA-fragmentation in all cecal regions. This finding may suggest that $CD3^+$, $CD8^+$ and $TCR2^+$ lymphocytes are involved in the induction of apoptosis of epithelial cells in the chicken cecum.

In the mouse intestine, the existence of microbes increases the number of $\alpha\beta$ IEL, but has little effect on $\gamma\delta$ IELs [12]. In addition, it has been reported that the stimulation of intestinal bacterium accelerates the turnover of the epithelial cells in the intestine of rodents [1, 3]. Numerous bacteria that degradate uric acid into volatile fatty acids and ammonia exist in the chicken cecum [5]. These might suggest that TCR2+ IELs attribute to the apoptosis of epithelial cells in the chicken cecum.

Recently, it has been suggested that the induction of apoptosis may be mediated by perforin [9, 10] or Fas-Fas ligands [7, 19] in the epithelial cells of mammalian intestines. We are now conducting further studies to elucidate how the apoptosis of epithelial cells is induced in chicken intestines.

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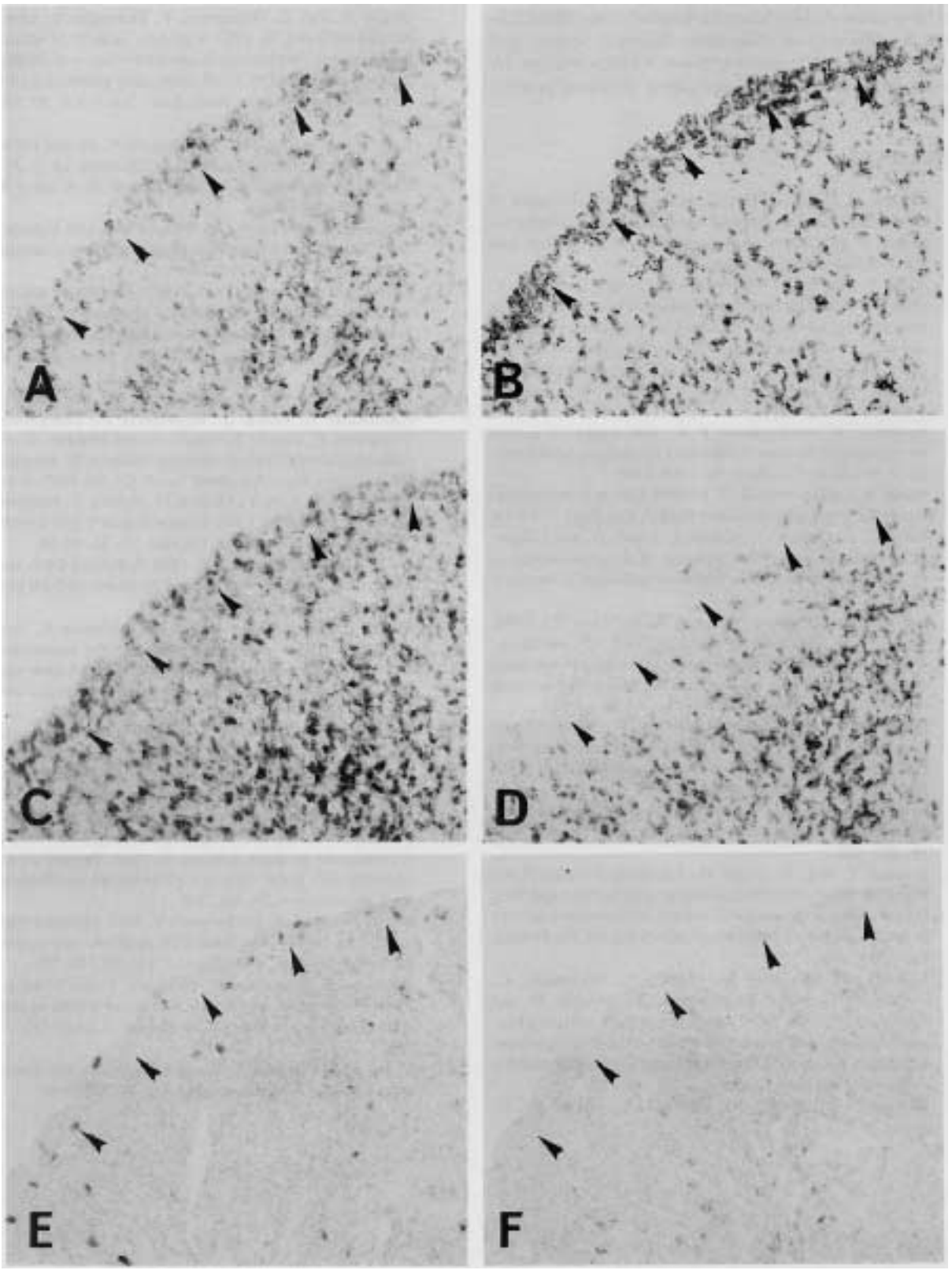

Fig. 4. Phenotypes of the lymphocytes in the same transition area from lateral to apical FAE. (A) $CD3^+$, (B) $CD8^+$, (C) $TCR2^+$, (D) $CD4^+$, (E) $TCR1^+$ and (F) $TCR3^+$ lymphocytes. Arrowheads indicate the basement membrane of FAE. Counterstained by methyl green. × 190.

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