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M Cells and Granular Mononuclear Cells in Peyer's Patch Domes of Mice Depleted of Their Lymphocytes by Total Lymphoid Irradiation

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The cytoarchitecture of Peyer's patches that were depleted of their lymphocytes by total lymphoid irradiation (TLI) was examined with particular attention to the effects on M cells in the follicle epithelium and on mononuclear cells in follicle domes underlying the epithelium. Five-month-old, specific pathogen-free Balb/c mice were irradiated with 200–250 rad/day, five times a week to a total dose of 3400–4250, and their Peyer's patches were either fixed for electron microscopy or frozen for immunohistochemistry 1–4 days after completion of irradiation. Control mice were examined at the same time intervals. Follicle domes of TLI mice had approximately one fourth the epithelial surface area of domes of control mice. Within the epithelium, lymphoid cells were virtually depleted after TLI, and yet the epithelium contained M cells. In control mice, most M cells were accompanied by lymphoid cells in invaginations of the apical-lateral cell membrane. In TLI mice, most M cells did not have such apical-lateral invaginations and were columnar shaped. Other than lacking lymphocytes, these cells appeared to be mature M cells. Some M cells did have lymphoid cells or granular mononuclear cells below their basal membranes, adjacent to the basal lamina. Below the epithelium, the proportion of granular mononuclear cells was greatly increased following TLI. The retention of M cells and the increase in proportion of granular mononuclear cells in follicle domes are consistent with selective depletion of lymphocytes following TLI. Persistence of M cells without lymphocytic invaginations after TLI suggests that M cells can differentiate in the absence of, or at least in the presence of very few, lymphocytes, and that invagination by lymphocytes is not necessary to maintain mature M cell morphology. (Am J Pathol 1989, 134:529–537)

Peyer's patches are collections of lymphoid follicles in the intestinal wall specialized for sampling antigens from the lumen. The epithelium covering each follicle contains specialized M cells characterized by short microvilli, an intracellular vesicular transport system, and interepithelial lymphocytes lodged within invaginations of the M cell lateral membrane.[1–5] M cells have also been characterized by the absence of alkaline phosphatase in the brush border,[6,7] absence of secretory component,[8] reduced or absent dense bodies that are found in apical cytoplasm of adjacent enterocytes,[9] and increased cholesterol but reduced concentration of intramembrane particles on the P-face of M cell microvilli.[10] These features appear to be related to the ability of M cells to transfer tracer proteins, viruses, bacteria, and protozoa from the intestinal lumen to the lymphoid compartment of the Peyer's patch.[2,11–14] Beneath the follicle epithelium, the juxtaposition of lymphocytes, macrophages, connective tissue cells, and capillaries provides a locus for cellular interactions[15–18] that may be involved in initiation of immune responses.

Peyer's patches can be depleted of their lymphocytes by total lymphoid irradiation (TLI),[19] a therapeutic procedure whereby high-dose, fractionated irradiation is administered to the major lymphoid tissues of the body to suppress immune responses.[20–22] TLI eliminates both B and T lymphocyte populations from lymphoid organs.[19,23] In

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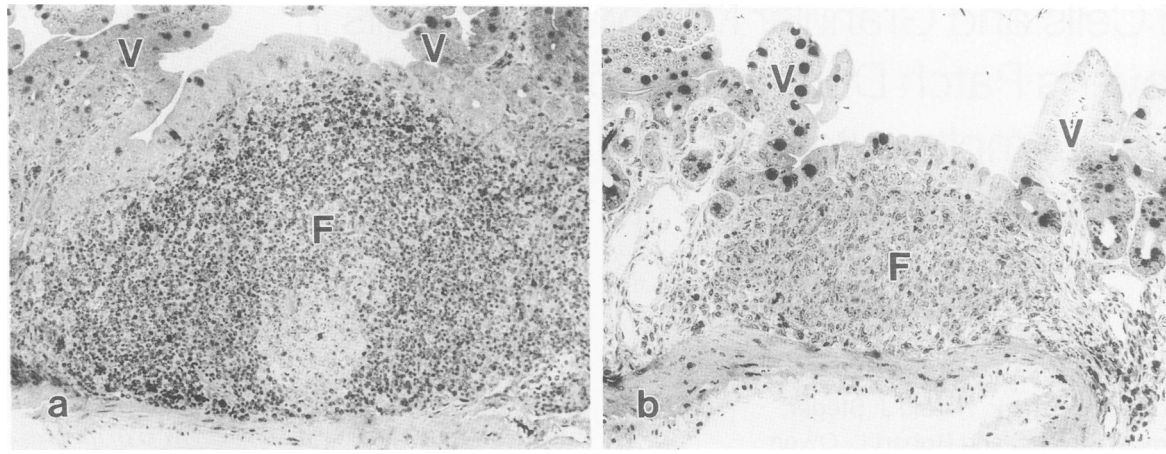

Figure 1. *Effects of TLI on Peyer's patch cytoarchitecture in barrier reared mice. Light micrograph of resin embedded, glutaraldehyde fixed tissue section stained with toluidine blue. Control mouse (***a***) has larger follicle (F) whose dome has a correspondingly larger diameter than TLI mouse. In TLI mouse (***b***), follicle is depleted of lymphocytes and contains an increased proportion of nonlymphoid cells (×140). V, villi.*

Peyer's patches depleted of their lymphocytes by TLI,[19] or by other radiation protocols,[24] the specific cellular domains that define the patch (ie, follicle domes covered by an intact epithelium, follicle B cell areas, and interfollicular areas) become smaller in size but still retain the same spatial relationships as in nonirradiated mice. Because of its selective depletion of lymphocytes, TLI can be used to examine lymphocyte effects on nonlymphoid cellular components of lymphoid organs. In the present study, the cytoarchitecture of Peyer's patches extremely depleted of lymphocytes was characterized by light and electron microscopy. Particular attention was paid to the effects that TLI and the concomitant absence of lymphocytes had on M cells in the follicle epithelium and on the distribution of granular mononuclear cells in follicle domes underlying the epithelium.

Materials and Methods

Animals and Radiation Treatment

Five-month-old, specific pathogen-free Balb/c mice were anesthetized daily with pentobarbital and positioned in an apparatus that allowed radiation of the major lymphoid organs, including all major lymph nodes, the spleen, and the thymus but shielded the skull, lungs, tail, and hind legs with lead.[25] Irradiation was delivered from a single 250 kV (15 A) source (Phillips Medical System Inc., Shelton, CT). The dose rate was 93 rad/min with a 0.35-mm Cu filter and a 52-cm source-axis distance. Polymyxin-neomycin was added to the drinking water during TLI and until the end of the study. Control unirradiated mice were also maintained in the pathogen-free facility. Two TLI mice were given 200 rad/day, five times a week to a total dose of 3400 rad and killed 4 days after treatment. A single control mouse was examined at the same time interval. In a second group of mice, the radiation dose was increased to maximize the effect of TLI on lymphocyte depletion. Five TLI mice were given 250 rad/day for a total of 4250 rad and killed 1 day after the end of treatment. Four control mice were examined at the 1-day time interval. Peyer's patches were identified by dissecting microscopy, removed from the intestine, and either fixed for electron microscopy or frozen for immunohistochemistry.

Table 1. *Effect of TLI on Peyer's Patch Size*

	Area of follicle (sq $\mu \times 10^3$)	Diameter of dome (μ)
Control mice	150 ± 10	540 ± 60
TLI mice	40 ± 2	285 ± 10

Data are mean values ± SEM for N = 4 (control) or 5 (TLI) mice.

Electron Microscopy

Two Peyer's patches from each animal were fixed overnight in 0.8% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed in 2% osmium tetroxide in the same buffer, and embedded in Epon. Sections were mounted on slot grids and examined in a Zeiss EM 10C or a Philips 201 electron microscope. M cells throughout the entire follicle epithelium were photographed and examined in a nonblinded manner because Peyer's patches from TLI and control mice could always be distinguished even if the form of treatment were not known.

For light microscopic morphometry, measurements on two Peyer's patches from the animals given 250 rad/day were made using a Zeiss Videoplan III image analyzer. Follicle size was determined by tracing the perimeter of follicles in equatorial sections. The diameter of each dome was made by tracing the basal surface of the epithelium on equatorial sections through follicle domes.

Immunohistochemistry

The remaining Peyer's patches were mounted in OCT embedding compound (Miles Scientific, Naperville, IL) on cork stubs, and quick-frozen in Freon 22 cooled to its freezing point by liquid nitrogen in a surrounding chamber.[15,19] Acetone-fixed tissue sections 5–7 μ thick were incubated with monoclonal antibody directed against T200 (clone 30-F11) found on all leukocytes and mononuclear cells[26] as described previously.[19] Sections were incubated sequentially with biotinylated monoclonal antibody diluted 1:200 in PBS from an initial concentration of 1 mg/ml for 1 hour, avidin conjugated to biotinylated horseradish peroxidase (ABC, Vector Labs., Burlingame, CA) for 1 hour, and diaminobenzidine tetrahydrochloride (DAB, Organon Teknika, Durham, NC) in 0.1 M TRIS-HCl buffer, pH 7.3, containing H_2O_2 and $CoCl_2$ for 5 minutes.

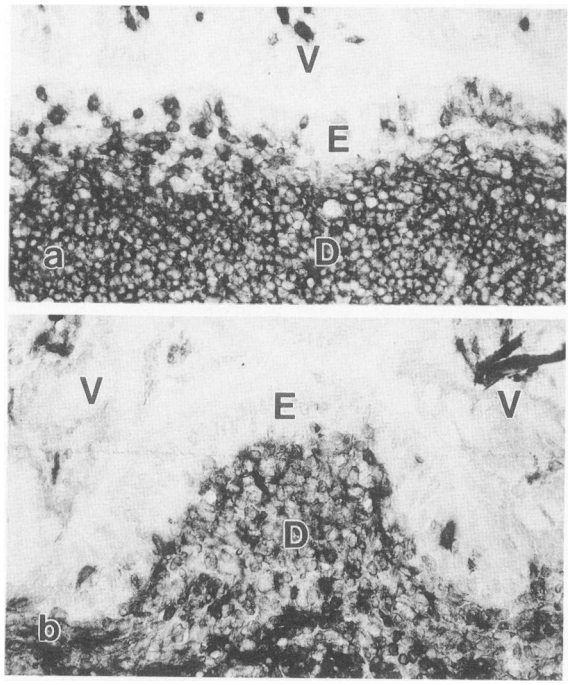

Figure 2. *Depletion of leukocytes from Peyer's patch follicle epithelium (E) by TLI. Immunohistochemical sections of frozen tissue labeled with anti-T200.* **a:** *In a control mouse, T200$^+$ cells are located above and below the level of enterocyte nuclei.* **b:** *In a TLI mouse, T200$^+$ cells are only occasionally observed (×240). D, dome; V, villus.*

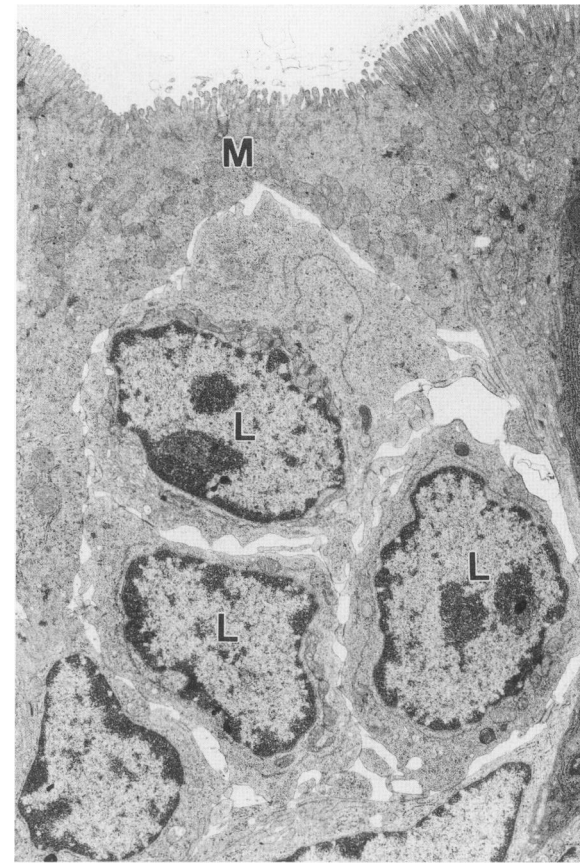

Figure 3. *Electron micrograph of M cell-lymphoid cell association in follicle epithelium of control mouse. The supranuclear portion of the M cell (M) is indented from below by several lymphoid cells (L) (×4400).*

Sections were counterstained in aqueous methyl green and photographed with a Zeiss Photomicroscope III.

Results

Peyer's patch follicles were reduced in size after TLI as shown by equatorial sections through follicles (Figure 1, Table 1). After TLI, the cross-sectional area of follicles was approximately 25% that of control mice, and the diameter of follicle domes was approximately 50% that of controls (Figure 1, Table 1). The 2-to-1 difference in diameter corresponded to a 4-to-1 difference in the calculated surface area of follicle domes.

The follicle epithelium was depleted of lymphocytes following TLI. This loss of lymphocytes from the follicle epithelium was demonstrated immunohistochemically by labeling frozen sections of Peyer's patches from TLI and control mice with pan-leukocyte monoclonal antibody anti-T200 (Figure 2). In control mice, T200$^+$ leukocytes were observed above and below the level of follicle epithelial cell nuclei as single cells or as groups of several

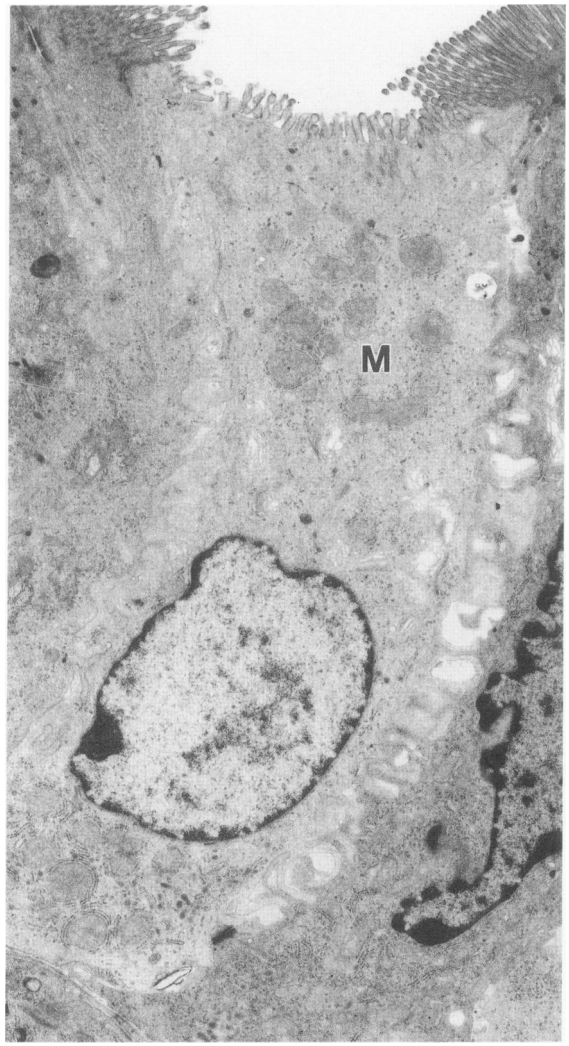

Figure 4. *Electron micrograph of columnar-shaped M cell (M) in follicle epithelium of TLI mouse. M cell lacks invagination by lymphoid cells (×7200).*

cells. In TLI mice, $T200^+$ cells were few in number in the follicle epithelium and occurred mostly as single cells (Figure 2).

TLI mice showed several alterations in the morphology of the follicle epithelium, including a decrease in the number of nuclei per unit length of epithelium, vesiculation of epithelial cells, and occasional cytoplasmic disruption characteristic of radiation damage to intestinal epithelial cells.[27]

M cells were identified at the electron microscopic level by their short microvilli and absence of dense bodies, and were found throughout the follicle epithelium of both control and TLI mice (Figures 3–5). In control mice, typical M cells were seen with one or more lymphoid cells in invaginations of the lateral cell membrane above the level of epithelial cell nuclei (Figure 3). Some M cells had lymphoid cells at the base of the cell or lacked lymphoid cells altogether. In TLI mice, most M cells (80%, N = 20 cells, *vs.* 10% in controls, N = 31 cells) were not associated with lymphoid cells indenting the supranuclear portion of the cell and therefore were columnar-shaped (Figures 4, 5). When typical M cells were occasionally seen, only a single lymphoid or granular mononuclear cell was present in the invagination. Other than their shape and the lack of lymphocytes, columnar-shaped M cells were similar to other typical M cells in their location on the dome, the amount of rough endoplasmic reticulum, and their lack of numerous free ribosomes. Some columnar-shaped M cells did have lymphoid cells or granular mononuclear cells between the basal surface of the M cell and the basal lamina (Figure 5).

Beneath the epithelium, follicles domes were depleted of lymphoid cells after TLI (Figures 6–8). In control mice, Peyer's patch domes contained a mixed population of lymphoid and granular mononuclear cells (Figures 6a, 7). Follicle domes in both groups of mice also contained cap-

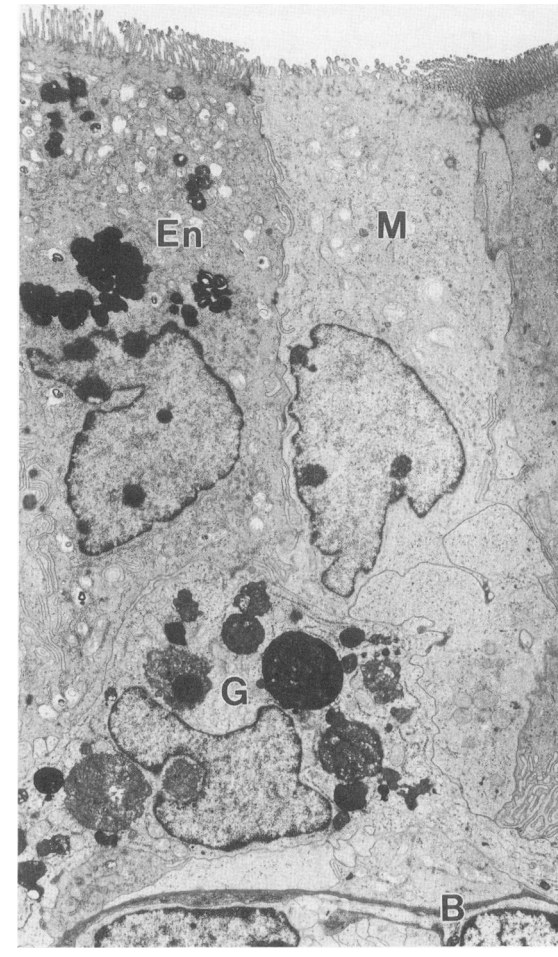

Figure 5. *Electron micrograph of columnar-shaped M cell (M) in follicle epithelium of TLI mouse with granular mononuclear cell (G) at its basolateral surface (×3200). B, basal lamina; En, enterocyte.*

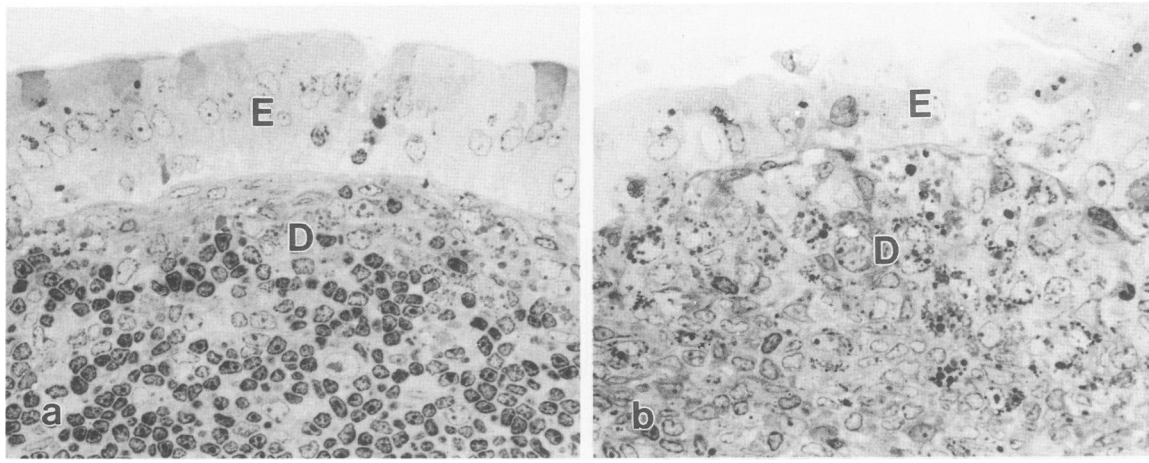

Figure 6. *Effect of TLI on Peyer's patch follicle domes. Light micrograph of resin embedded, glutaraldehyde fixed tissue section stained with toluidine blue.* **a:** *Follicle dome (D) of control mouse contains lymphocytes and granular mononuclear cells.* **b:** *Follicle dome of TLI mouse contains primarily granular mononuclear cells (×470). E, epithelium.*

illaries and reticular cells (Figure 8). After TLI, although lymphocytes were encountered occasionally, granular mononuclear cells predominated (Figures 6b, 8). Most of the granular cells had characteristics of macrophages and contained small, dense granules and large, membrane-bound phagocytic vacuoles. Some macrophages in the dome contained vesicles containing degenerated cellular material.

Discussion

In a previous study we reported that high-dose, fractionated irradiation depleted Peyer's patches of their lymphocytes, although the cellular domains that define the patch retained their spatial arrangement at a reduced size.[19] The lymphocyte depletion produced by TLI was virtually complete in comparison to other experimental systems in which lymphocytes were only partially depleted.[28,29] Because of this extreme depletion after TLI, we examined the follicle epithelium and dome of Peyer's patches in part to determine the effect of TLI on nonlymphoid components and in part to determine whether lymphocytes were necessary for M cell differentiation. The fractionated TLI dosage reduced the surface area of the follicle epithelium but had only a few radiation effects on M cells and follicle epithelium, despite the susceptibility of crypt stem cells to radiation damage.[27] M cells were still present in the follicle epithelium and granular mononuclear cell numbers were enhanced in the dome.

Most of the granular mononuclear cells in Peyer's patch domes had characteristics of macrophages.[30-32] The preponderance of these granular cells after TLI may reflect the relative scarcity of lymphoid cells rather than an actual increase in the number of granular cells per dome. That is, the reduction in the size of follicle domes due to loss of lymphocytes may bring more macrophages into the plane of section. In T cell-deficient nude mice, Peyer's patch follicles are also smaller in size and have a greater proportion of granular macrophages in follicle domes than in normal mice.[28,33] Analysis of dispersed spleen and lymph node cell populations by flow cytometry showed that macrophages increase in proportion after TLI.[23,34] Macrophages are also more numerous in lymphoid organs at birth before lymphoid cells migrate in and populate them.[23] Other granular cells have been associated with either the digestive tract (large granular lymphocytes[35]) or lymphoid organs following TLI (natural suppressor cells[23,34] [Ermak TH, unpublished results]) and could account for some of these granular cells.

The presence of M cells in lymphocyte-depleted Peyer's patches is relevant to current hypotheses regarding the differentiation and origin of M cells. Kinetic autoradiographic studies demonstrated that the follicle epithelium in mice is renewed over a 3 to 4-day time interval; however, the life span and cell renewal rate of individual M cells has not yet been clearly established.[3,4,36] It has been proposed that M cells differentiate from stem cells in the crypts that surround each Peyer's patch follicle.[4,37] This is in agreement with the generally accepted hypothesis for all intestinal epithelial cells, ie, that the main types of intestinal columnar epithelial cells originate from crypt stem cells.[38]

It also has been hypothesized that differentiation of M cells is induced solely by interepithelial lymphocytes through cell-to-cell contact.[39-41] In the present study, columnar shaped M cells were still identified in mice after lymphocytes were depleted from the follicle epithelium. Because the radiation treatment occurred over a 3–4 week period of time and the turnover time of the follicle epithelium is about 4 days in normal mice, we assume

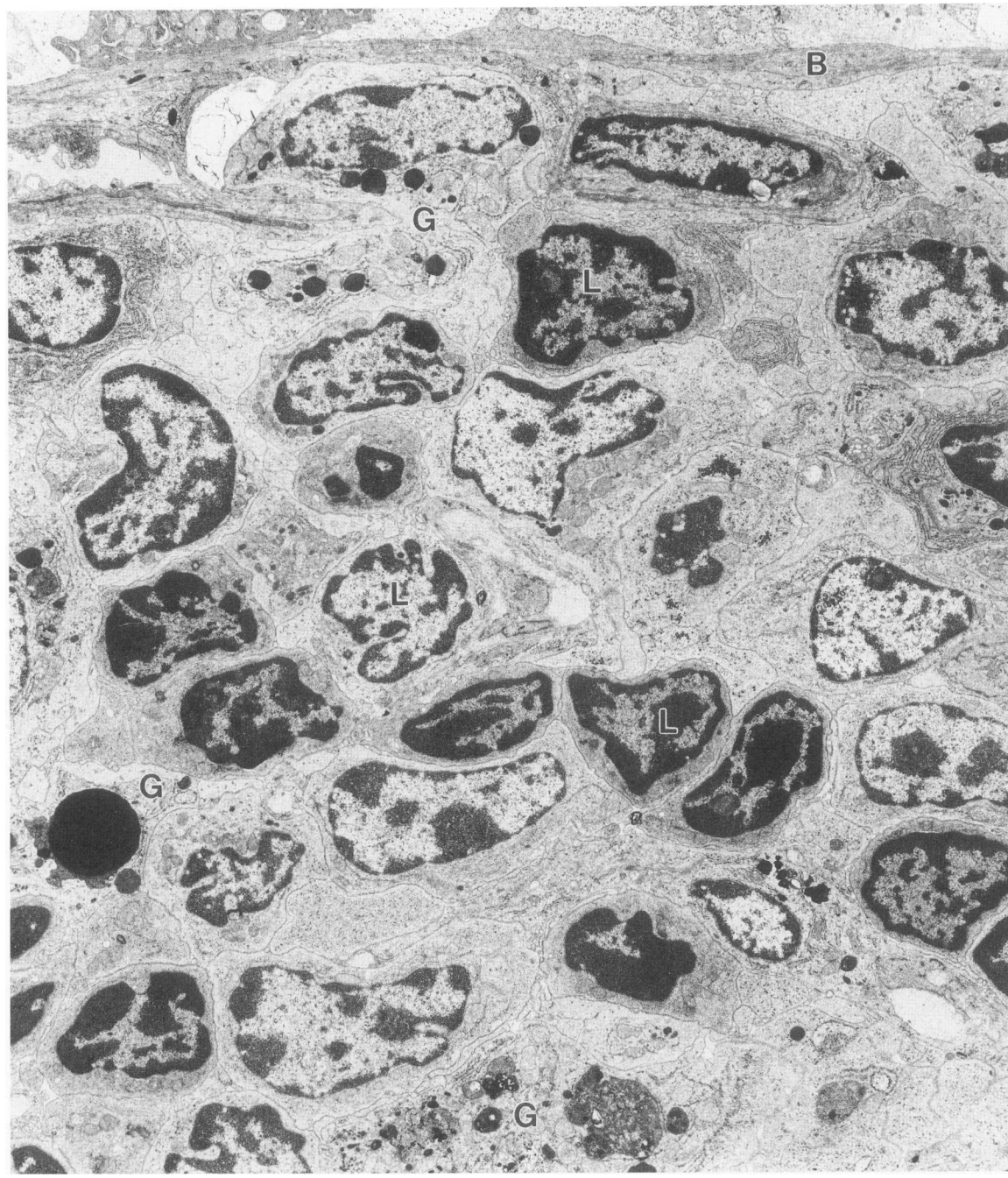

Figure 7. *Electron micrograph of follicle dome of control mouse showing lymphoid cells (L) and granular mononuclear cells (G) below the follicle epithelium (×4,300). B, basal lamina of follicle epithelium.*

that the M cells seen in the TLI-treated mice must have differentiated in the absence of, or at least in the presence of very few, lymphocytes. These results, together with a number of other observations,[4,37] indicate that M cell differentiation may occur independent of induction by lymphocytes. This is not to say that lymphocytes have no effect on M cells, because it has been reported that M cell numbers in mice maintained under specific pathogen-free conditions (as were the mice in this study) increase when exposed to normal flora in conventional facilities.[41] A potential subject for further study would be to examine whether expansion of the M cell population can take place in the absence of lymphocytes in TLI mice.

Based on current discussions of M cell differentiation,[4,37] the columnar-shaped M cells in TLI mice could be described as immature M cells. However, other than lack-

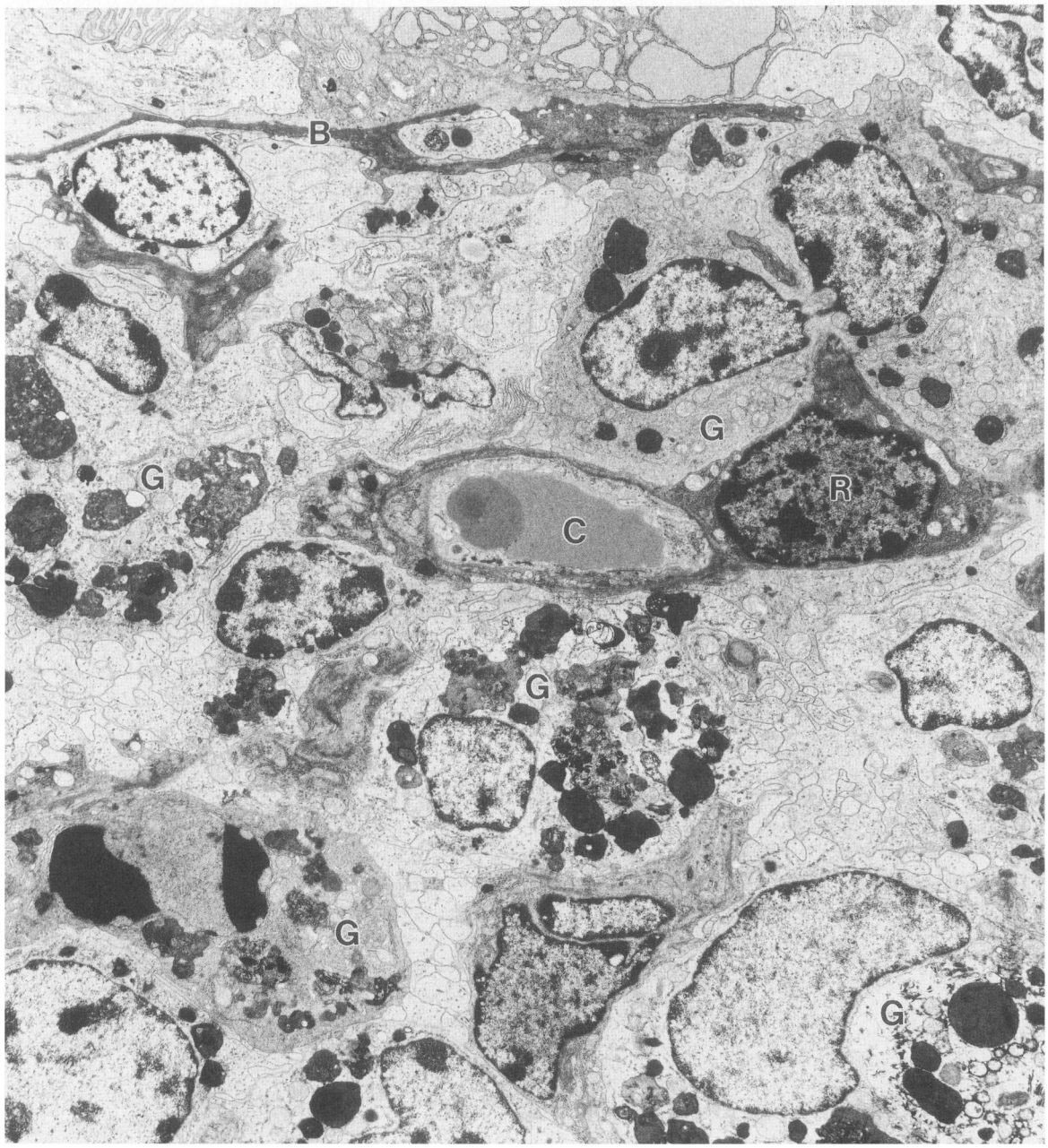

Figure 8. *Electron micrograph of follicle dome of TLI mouse showing depletion of lymphocytes and amplification of granular mononuclear cells (G) (×4300). B, basal lamina of follicle epithelium; C, capillary; R, reticular cell.*

ing lymphocytes, the M cells in TLI mice possessed characteristics of mature M cells, ie, they were found on the upper surface of the dome, they contained rough endoplasmic reticulum throughout the cytoplasm, and they lacked abundant free ribosomes. These findings bring into question the morphologic criteria for defining the degree of differentiation of M cells (eg, invagination by lymphoid cells, characteristics of microvilli). M cells without lymphocytic invaginations have been described as immature M cells at the mouth of follicle crypts.[4,37] Cells in this transitional zone possess cytoplasmic free ribosomes, which are associated with undifferentiated cells. Although these cells may lack maturity as well as lymphocytes, it need not follow that the absence of lymphocytes associated with any M cell necessarily defines it as immature. Indeed, M cells lacking lymphoid cells are also found on the upper half of mouse Peyer's patch domes in normal mice.[4] Lymphocytes migrate into and out of the follicle epithelium,[42] and the presence of lymphocytes may follow M cell maturity, rather than be a prerequisite for it. Another explanation for variation in

M cell morphology may be that the different phenotypic manifestations of M cells relate to the physiologic states of M cells and/or lymphoid cells. In line with this perspective, we note that variations in the apical surface of M cells such as pseudopod or pedestal formation, have been associated with binding and uptake of microorganisms.[11,43] Although there is evidence to indicate an association between the morphology and function of M cells,[4] it may be premature to conclude whether the differences described thus far are due to difference in degree of differentiation, physiologic state, or other factors.

The reduction in lymphocyte populations associated with TLI is accompanied by changes in systemic immune function, most notably suppression of a variety of immune responses.[20-23,25,34] Whether TLI has an effect on M cell function, particularly antigen transport, or whether TLI influences antigen processing or mucosal immune responses in follicle domes, remains to be determined.

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