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Original Article

Location and Phenotype of Proliferating T cells in Secondary Lymph Follicles

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Lymph follicles (LF) have been viewed as nests for B cell proliferation and differentiation into memory cells. This report demonstrates that T cells in LF can also divide. We examined lymph follicles of immunized mice by immunohistochemical and autoradiographic techniques. We were able to detect subgroups of T cells in the S phase and localize them. From 0.2% to 0.5% of intrafollicular T cells incorporated ^{3}H-thymidine and thus could have been in the S phase ; such cells were found in every part of the follicle. Not only $CD4^+$ T cells but also $CD8^+$ T cells in LF incorporated ^{3}H-thymidine. This study is the first to demonstrate a proliferation of $CD8^+$ T cells in secondary lymph follicles. However, the ratio of $CD4^+$ to $CD8^+$ T cells in the S phase differed between LF and T-dependent zones ; the ratios were 0.66 in LF and 1.78 in T zones. The difference may stem from differences in their immune response roles. When we counted the dividing T cells in the T-dependent zones, we found more of them in areas adjacent to the follicles than in more-distant areas. This suggests that T cells, engaged in the cell cycle in the T-dependent zones, moved toward the germinal center or actually entered it.
Key words lymph follicle, T cell, proliferation, tritiated-thymidine

INTRODUCTION

T-lymphocytes are distributed mainly in paracortical areas of lymph nodes, but have also been detected in lymph follicles. Most T cells in lymph follicles are $CD4^+$ and have helper/inducer phenotypes. But a few $CD8^+$ cells, suppressor/cytotoxic cells, are also present.[1,2] $CD4^+$ cells are known to help B cells to differentiate into memory B cells,[3,4] whereas $CD8^+$ cells are thought to regulate the proliferative activity of B cells.[5]

Since the work of Fuller, et al., germinal center (GC) T cells have been believed to divide.[6] To show that GC T cells were derived from dividing cells mice were labeled by repeated injection with bromodeoxyuridine before they were sacrificed.

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In the present work, we sought to answer some simple, but fundamental, questions : do follicular T cells divide in situ or do they derive from division cycles occurring elsewhere, namely in the adjacent T-dependent zones ? If T cells can divide in the follicles, in which compartment does this occur and are the dividing cells $CD4^+$, $CD8^+$, or both ?

To resolve these questions, we examined lymph nodes of immunized mice using immunohistochemical and autoradiographic techniques. Double labeling allowed us to detect and localize $CD3^+$, $CD4^+$, and $CD8^+$ cells in the S phase (dividing cells).

MATERIALS AND METHODS

Mice and immunization

Twelve BALB/c mice at 6 weeks of age were intraperitoneally immunized against hapten arsonate coupled to keyhole limpet hemocyanin (Ars-KLH) in Freund's complete adjuvant (FCA). Controls were treated with FCA alone or in combination with phosphate-buffered saline

(PBS, pH 7.6). Six mice (two for each set of conditions) were sacrificed by decapitation 10 or 11 days after primary immunization. One hour before sacrifice, they received ^{3}H-thymidine (37 MBq/ml, 200 μl/mouse) intraperitoneally. Another six mice were boosted on day 21. Six days after this secondary immunization they were injected in the peritoneal cavity with ^{3}H-thymidine one hour before being sacrificed.

Immunohistochemistry

The cryosections of mesenteric lymph nodes taken from each mouse were immunostained by the strept avidin-biotin complex (sABC) method. The monoclonal antibodies used in this study were rat anti-mouse CD3 (1 : 50, SEROTEC, England), anti-mouse CD4 (1 : 50, GIBCO BRL, USA), and anti-mouse CD8a (Ly-2, 1 : 100, Pharmingen, USA) ; all were biotin-conjugated. Some slides were treated with B220 (rat anti-mouse antibody, supernatant) and then with biotinylated rabbit anti-rat Ig (1 : 300, DAKOPATTS, Denmark). After immunostaining, the slides were washed in distilled water and kept in the dark for a week and finally covered with an emulsion for autoradiography.

Autoradiography

Cells that incorporated ^{3}H-thymidine were visualized by in situ autoradiography. The following steps were performed in the dark and at room temperature (RT, 24°C). The immunostained sections were dipped in stabilized emulsion (K-5, ILFORD, England) and then dried. The emulsion curing time was one week. After that, the preparations were developed (Microdol, KODAK, USA), fixed, and observed under a light microscope (Zeiss, Germany).

Statistical analysis

A statistical analysis was performed on comparison among the frequencies of ^{3}H-thymidine labeled T cells between CD4$^+$ T cells and CD8$^+$ T cells, by the test of the median.

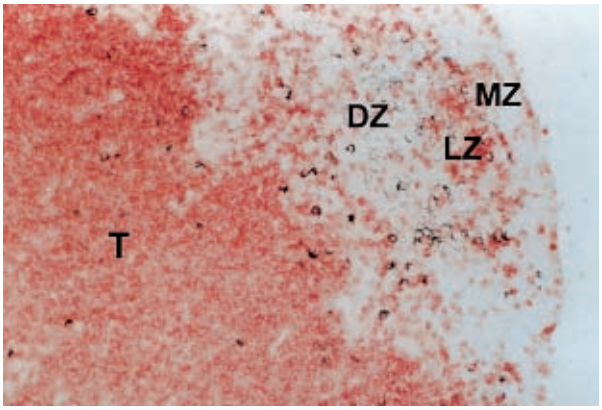

Fig. 1. Morphological aspect of a lymph follicle after anti-CD3 immunolabeling and autoradiography. CD3$^+$ cells (red) are distributed in the light zone of the germinal center as well as in the T-dependent zone. Cells that incorporated ^{3}H-thymidine (dark spots) are found mainly in the dark zone of the germinal center, but are found also in the T-dependent zone, especially in the vicinity of the lymph follicle. MZ, mantle zone ; LZ, light zone ; DZ, dark zone ; T, T-dependent zone (ABC method and autoradiography, bar : 40 μm.)

RESULTS

Incorporation of ^{3}H- thymidine into lymphocytes

As shown in Fig. 1, lymphocytes that incorporated ^{3}H-thymidine were found mainly in the germinal-center dark zones. Less frequently, lymphocytes with ^{3}H-thymidine were located in the T-dependent zones. The former were mostly B cells. The latter were identified as T cells on the basis of their reaction with anti-CD3, anti-CD4, or anti-CD8 antibody. Some ^{3}H-thymidine-labeled T cells were also found in lymph follicles (Fig. 2). B and T cells differed in the amount of ^{3}H-thymidine their nuclei incorporated : generally, the grains of T cells were packed more densely than those of B cells.

The number of ^{3}H-thymidine-labeled T cells per lymph follicle varied from zero to four, and averaged 0.3±0.7. The average number of T cells in LF were 171±83, and thus the percentage of ^{3}H-thymidine-labeled T cells among follicular T cells was about 0.2%.

In lymph follicles, both CD4$^+$ and CD8$^+$ T cells were found to have incorporated ^{3}H-thymidine (Fig. 2), but the former were more frequently double-stained than the latter. The ratio of CD4$^+$ cells to CD8$^+$ cells among the ^{3}

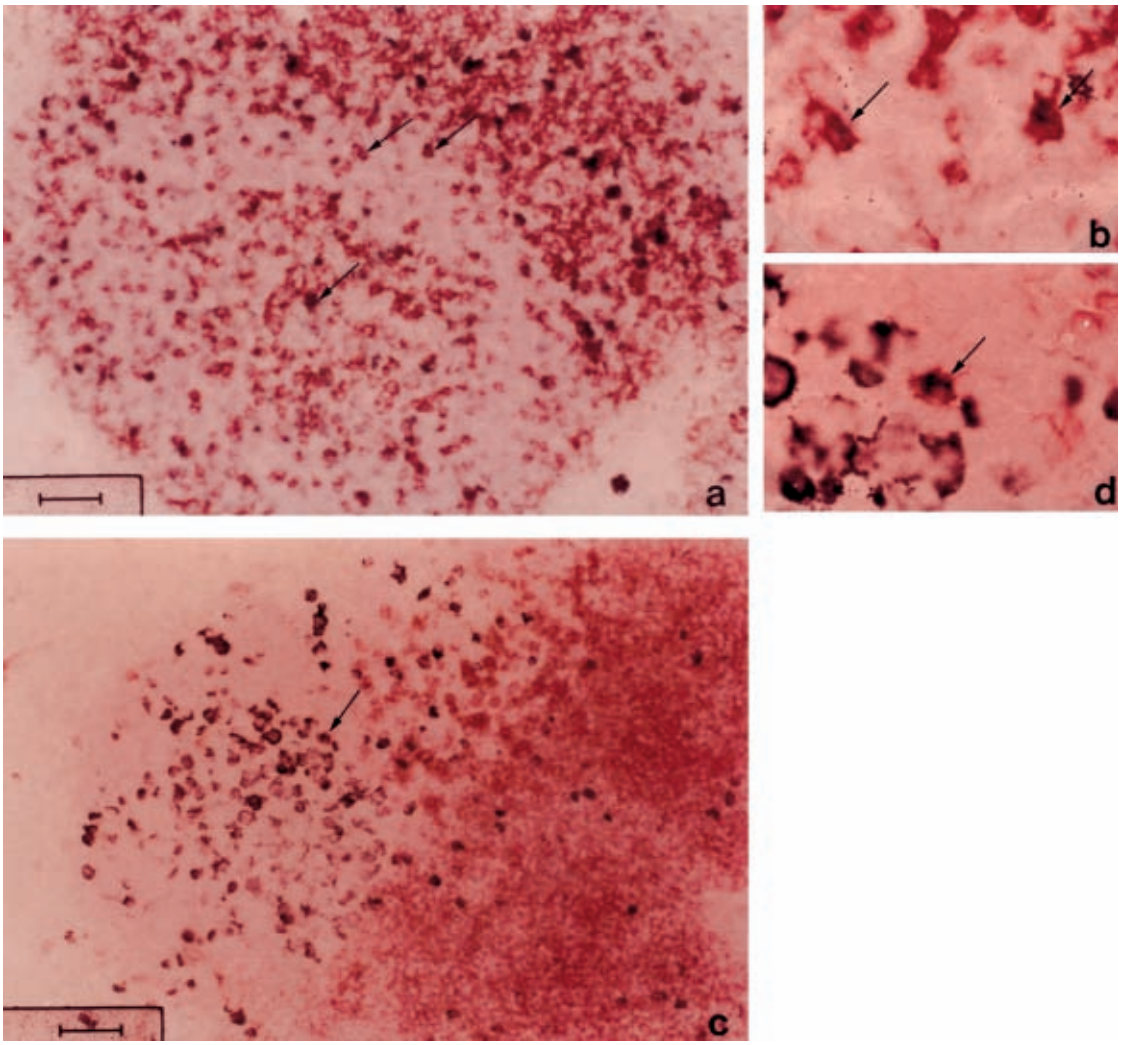

Fig. 2. ^{3}H-thymidine-labeled T cells in lymph follicles. ^{3}H-thymidine-labeled CD4^{+} (arrows in a, b) or CD8^{+} (arrows in c, d) T cells are found in the lymph follicles. (ABC method and autoradiography, bar : 40 μm.)

H-thymidine-labeled T cells was approximately 4 : 1.

Location of ^{3}H-thymidine-labeled T cells

Secondary lymph follicles were divided into four zones by double staining with anti-CD3 and autoradiography : a dark zone (DZ), where ^{3}H-thymidine-labeled cells were rich ; a light zone (LZ), where many CD3^{+} T cells were found ; a mantle zone (MZ), comprising the area surrounding the germinal center and containing many small, resting B cells ; and an outer zone (OZ), that existed between the T-dependent zone and the germinal center (Fig. 1). However, occasionally ^{3}H-thymidine-labeled cells could not be located precisely because the polarity of the germinal center was not always clearly recognizable. Most T cells in the S phase were located in the light zone, but some were also detected in the mantle and other zones, as shown in Table 1.

For phenotype expression, most proliferating CD4^{+} T cells were located in the light and mantle zones. Although most proliferating CD8^{+} T cells were detected in the outer zones, they were also found in the dark and light zones. In other words, CD4^{+} T cells appeared to divide in the light and mantle zones, whereas CD8^{+} T cells did so in the light, dark, and outer zones.

Outside the follicles, many T cells covered with radioactive grains were encountered in the T-dependent zones. Some ^{3}H-thymidine-labeled

Table 1. The location and phenotype of ^{3}H-thymidine labeled T cells inside follicles

Location	Total	Phenotype		
		$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Mantle zone	35 (19.9)	32 (22.5)	3 (8.8)	2.6
Light zone	62 (35.2)	54 (38.0)	8 (23.5)	1.6
Dark zone	27 (15.3)	19 (13.4)	8 (23.5)	0.6
Outer zone	32 (18.2)	21 (14.8)	11 (32.4)	0.5
Undetermined†	20 (11.4)	16 (11.3)	4 (11.8)	1.0
Total	176(100.0)	142(100.0)	34(100.0)	

†Number and percentages of cells could not be located precisely.

Table 2. Comparison of the phenotype of ^{3}H-thymidine labeled T cells in the lymph follicle and T-zone

	$CD4^+$ T cells	$CD8^+$ T cells
	^{3}H-labeled/total $CD4^+$ cells (%)	^{3}H-labeled/total $CD8^+$ cells (%)
Lymph follicle	30/4518(0.66)*	6/603(0.99)*
T-zone	275/17716(1.55)	52/5952(0.87)

* The difference between these ratios was statistically significant ($p < 0.05$).

cells were found in the marginal and medullary sinuses and high endothelial venules (HEV) of lymph nodes. Most could have been macrophages or dendritic cells, given their size and their nuclear and cytoplasmic aspects; but a few cells were CD3-positive.

Phenotype of ^{3}H-thymidine-labeled T cells

As mentioned above, more $CD4^+$ T cells than $CD8^+$ T cells appeared to proliferate in the lymph follicles. We consequently undertook to compare ratios of proliferating to total $CD4^+$ and $CD8^+$ cells counted in lymph follicles and T-dependent zones. The results are shown in Table 2. Inside the follicles, ^{3}H-thymidine-labeled $CD4^+$ cells were found more frequently than ^{3}H-thymidine-labeled $CD8^+$ cells, but the proportion of dividing cells to total cells was actually higher in the $CD8^+$ population. The difference between the proportions of $CD4^+$ and $CD8^+$ dividing cells was statistically significant ($p < 0.05$). In the T-dependent zones, where the ratio of $CD4^+$ to $CD8^+$ cells was 1.78, both the number and proportion of dividing cells were larger in the $CD4^+$ population, but there was no statistically significant difference.

Distribution of ^{3}H-thymidine-labeled T cells in the T-dependent zones

Next, we examined the distribution of ^{3}H-thymidine-labeled T cells in the T-dependent zones. T cells in the S phase in T-dependent zones seemed to be distributed closer to the lymph follicles (Fig. 1). This was found among both the $CD4^+$ and the $CD8^+$ cells.

DISCUSSION

Do T cells divide in lymph follicles? The answer was clearly yes. After a one-hour pulse with ^{3}H-thymidine, we found that T cells in the secondary lymph follicles of mice, immunized or not, incorporated this precursor into their DNA.

Mitosis of follicular T cells has been suspected since the work of Fuller, et al.,[6] who administered bromodeoxyuridine to immunized mice over a period of several days. Follicular T cells were labeled by this procedure, but whether the labeled cells derived from division cycles occurring inside or outside the follicles remained uncertain. Here, we clearly demonstrated that inside the follicles some T cells could incorporate ^{3}H-thymidine. These cells are thus engaged in a cell cycle.

The ^{3}H-thymidine grain density was generally higher in the T-cell nuclei than in the B-cell nuclei. This may have been due to the fact that germinal center T cells were generally medium-sized[7,8] and thus smaller than centroblasts. But it might also have reflected T cells dividing at a slower rate than B cells.

^{3}H-thymidine-labeled T cells were found in every part of the follicles. They were most numerous in the light zones, followed by the mantle and dark zones. This did not mean, however, that these cells remained immobile; apparently cells in the G1, S, and G2 phases could

move. We do not know whether the final stage of mitosis occurred in one particular zone or in all parts of the follicle. Since many ^{3}H-thymidine-labeled T cells were found in the outer zone, which is considered a transitional zone,[9] many T cells may have been actively migrating through the follicles. The fact that some ^{3}H-thymidine-labeled T cells were detected in the lymph node sinuses further supported the idea that migrating T cells could be engaged in a cell cycle.

Both $CD4^+$ and $CD8^+$ cells in the follicles incorporated ^{3}H-thymidine. Globally, $CD4^+$ cells were more frequently labeled than $CD8^+$ cells. This was the first demonstration of $CD8^+$ cell division inside the follicles of a healthy animal. In cases of human immunodeficiency virus (HIV) infection, however, intrafollicular $CD8^+$ cells containing TIA-1, a granule-associated protein, have been seen within mitosis, indicating clonal expansion of cytotoxic cells in the follicles.[10] Another finding of the present study was that in the dark and outer zones there appeared to be more $CD8^+$ cells than $CD4^+$ cells in the S phase.

Most GC T cells displayed the $CD4^+CD57^+$ phenotype and were distributed mainly in the light zones. These GC T cells are believed to exert helper functions in aiding B cells to proliferate and transform into memory cells.[3,4] However, they do not increase the proliferation of B cells as much as do the classical T cells ($CD4^+$ $CD57^-$).[7,8,11] On the other hand, Ruco, et al., reported that they observed a relative increase in OKT-8^+ T cells in reactive and neoplastic B-cell proliferation in humans, and that suppressor/cytotoxic T cells may regulate the proliferative activity of B-lymphocytes.[5] Such differences in the functions that $CD4^+$ or $CD8^+$ T cells produce against B cells may decide the location of T cells, and might have been responsible for our results.

We found that dividing T cells were more numerous in the T-dependent zone than in lymph follicles. We also detected more S phase cells in the T-dependent zones very near the follicles than further away. These findings suggested that T cells presenting antigen (Ag) from interdigitating cells (IDC) were activated and proliferated in the T-dependent zones, and that a portion of these dividing T cells may have been CD4-positive, and may have migrated to lymph follicles, making contact with B cells. This finding also suggested that memory B cells migrating from the follicles presented to T cells the Ag received from follicular dendritic cells and induced T-cell proliferation.[12]

We demonstrated that, in mice, $CD4^+$ and $CD8^+$ cells could incorporate ^{3}H-thymidine in every part of the lymph follicles. We also examined human tonsil cryosections after double immunostaining with anti-CD3 and anti-Ki67 antibodies and found cycling T cells in the same locations as secondary follicles (personal observation). Berman, et al.,[13] observed, after injecting ^{3}H-thymidine into normal mice, the incorporation of the label into primary-follicle T cells but not into secondary-follicle T cells. We cannot explain this discrepancy.

We are currently analyzing the different T cell division patterns observed in secondary follicles at various times after antigenic stimulation.

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