THE MOUSE GUT T LYMPHOCYTE, A NOVEL TYPE OF T CELL

Nature, Origin, and Traffic in Mice in Normal and Graft-Versus-Host Conditions

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The normal intestinal mucosa contains, between the Peyer's patches (PP),[1] a large number of lymphocytes present both within the lamina propria and between the epithelial cells (for review on the gut intraepithelial (IE) lymphocytes, see reference 1). In the mouse, it has previously been shown, using immunofluorescent staining to detect T lymphocytes on tissue sections, that most of these lymphocytes are of T nature (2). In addition, cell-transfer experiments showed that T lymphoblasts from thoracic duct lymph (TDL) or mesenteric lymph nodes (MLN), but not from peripheral lymph nodes (PLN), have the property of homing in the recipient's gut mucosa (2).

In the present work, the conditions of appearance, nature, origin, and cell traffic of the mouse gut T lymphocytes (GTL) were studied, either in normal or germ-free mice, or by making use of two pathologic conditions which amplify some of the characteristics of the GTL system: (*a*) a model of graft-versus-host (GVH) reaction in which lethally irradiated F_1 mice are injected with parental thymocytes (3): after 3–4 days during which the mucosa is strongly depleted in GTL as the result of the irradiation, there is a rapid accumulation of T lymphocytes within the mucosa. Because the new GTL were found to have the same characteristics as normal GTL, this accelerated repopulation of the mucosa represents a favorable situation to study the origin and traffic of these cells; (*b*) study of beige mice, which are an equivalent of the Chediak-Higashi syndrome in man and which show giant granules in various cell types, among which polymorphonuclear leukocytes and mast cells (4): because it was found that in normal mice numerous GTL contain granules of various sizes, the possibility was explored that these GTL granules might be enlarged in beige mice and that their frequency and nature might thus be easier to study.

These experiments led to the conclusion that GTL are a special type of T lymphocytes, generated in PP by antigenic stimulation of T cells to divide, endowed

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; GTL, gut T lymphocytes; GVH, graft-versus-host; [^{3}H]TdR, [^{3}H]thymidine; IE, intraepithelial; MLN, mesenteric lymph nodes; MTLA, mouse T lymphocyte antigen; PHA, phytohemagglutinin; PLN, peripheral lymph nodes; PP, Peyer's patches; PVC, polyvinyl chloride; TDL, thoracic duct lymph.

with special traffic properties leading to their gut homing, and related to mucosal mast cells.

Materials and Methods

Animals. Balb/c, C3H, C57Bl$_6$, DBA$_2$, and (C57 × DBA$_2$)F$_1$ mice were obtained from Charles River (St Aubin-les-Elbeuf, France). Beige (C57Bl-B$_g^j$B$_g^j$) mice were kindly provided by Dr. Guenet, Pasteur Institute, Paris) and axenic mice by Dr. Ducluzeau (Institut National de la Recherche Agronomique-Centre National de la Recherche Zoologique, Jouy en Josas, France). GVH reactions were performed as described by Sprent and Miller (3), by the intravenous injections into lethally irradiated (900 rads) adult (C57 × DBA)F$_1$ mice of 2 × 10^8 C57 thymocytes. In some experiments, the same protocol was used with syngeneic mice injected with allogenic thymocytes, with similar results. Animals bearing graft of fetal intestine (5) were grafted subcutaneously 20 days before the GVH reaction.

Preparation of Cell Suspension. Cell suspensions from lymphoid organs and thoracic duct cannulation were performed as described previously (2). For isolation of gut IE lymphocytes, the small bowel was washed with phosphate-buffered saline, and after gentle excision of all the PP, opened on a wet linen square. The mucosa were then scraped, and dissociated by stirring with a magnetic stirrer for 5 min in medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing dithioerythritol, 1 mM. The cells released by this procedure were passed through a small glass-wool column (1.8 g packed in a 20-ml syringe) in Hank's balanced salt solution (HBSS) with 10% fetal calf serum (Grand Island Biological Co.), followed by purification on a Ficoll-Isopaque gradient (6). This procedure usually yields per mouse 2–8 × 10^6 lymphocytes in a rather pure suspension (Fig. 2). Peritoneal mast cells were obtained by washing the peritoneal cavity with 5–10 ml of HBSS containing 20 μg/ml of heparin (Liquemine Roche, Hoffmann La Roche, Basel, Switzerland) and removal of adherent cells by 20-min incubation at 37°C in plastic bottles.

Cell Cultures and In Vitro Cell Labeling. Cells were cultured at a concentration of 1 × 10^6/ml in medium RPMI 1640 (Grand Island Biological Co.) containing 5% fetal calf serum (Grand Island Biological Co.) with either concanavalin A (con A) (Miles Lab. Inc., Elkhart, Indiana), 1 μg/ml, or phytohemagglutinin P (PHA) (Difco Laboratories, Detroit, Mich.), 8 μg/ml. When con A-stimulated cells were used for transfer, they were washed in the presence of 0.1 M α-methyl mannoside before transfer, to remove surface bound con A. Mixed lymphocyte cultures with irradiated allogeneic cells were performed as described by Cerottini et al. (7). [^{3}H]-thymidine ([^{3}H]TdR) (Commissarriat à L'Energie Atomique, Saclay, France, 5 C/mM) incorporation was measured as described elsewhere (8). Labeling of cells used for transfer was by in vitro incubation for 1–4 h in the presence of [^{3}H]TdR, 1 μC/ml, or of [^{3}H]uridine (Commissarriat à L'Energie Atomique, Saclay, France, 20–30 C/mM), 10 μC/ml.

In Vivo Labeling with Radioactive Precursors. The amounts injected intravenously or intraperitoneally were: for [^{3}H]TdR, 0.5 μC/g of body weight; for $^{35}SO_4$ (Radiochemical Centre, Amersham, England, >5 mC/mg), 4 mC; for [^{3}H]serotonin (Amersham, 5 hydroxy[G-^{3}H]-tryptamine creatinine sulfate, 15,2 C/mM), 1 mC; for [^{3}H]dopa (Amersham, 3,4-dihydroxyphenyl[2-3^{3}H]alanine, 2.5 C/mM), 1 mC; for [^{3}H-5]hydroxytryptophan (Amersham, DL-5-hydroxy[G-^{3}H]tryptophane, 4.7 C/mM), 1 mC. Topical labeling of the PP was according to Joel et al. (9): filter papers with 0.025–0.05 μC[^{3}H]TdR were placed for 1 min on each of the PP, then 2 μmol of [^{1}H]TdR was injected intraperitoneally.

Selective irradiation of lymphoid organs with ^{32}P followed the principle described by Hollingworth et al. (10). Pieces of ^{32}P containing polyvinyl chloride (PVC) (CEA, Saclay, France, 6–15 rads/mm) were cut to the size and shape of the organs to be irradiated (PP, MLN, or spleen), covered with lead paper on one face and glued on the other face with Ethicon bucrylat glue (Ethnor, Paris, France) on the structures to be irradiated.

Radioautography and Immunofluorescent or Histochemical Procedures. Radioautography and immunofluorescence, separately or in combination, on cell suspensions or tissue sections, were performed as described previously (2) with the same types of antisera. Rabbit anti-mouse T lymphocyte antigen (MTLA) IgG and rhodamine-conjugated rabbit Fab$_2$ anti-MTLA, were kind gifts of Dr. Bron, Institut de Biochimie, University of Lausanne (11). This antiserum recognizes a single membrane protein, identical to that recognized by mouse anti-Thy 1 antisera

(as shown also by double immunofluorescence) (12); MTLA corresponds to the "T 25" T lymphocyte antigen described by Trowbridge (12 A), which also carries the Thy 1 antigen. This antiserum does not stain B lymphocytes, macrophages, mast cells, or any other lymphoid cell type not killed by treatment with anti-Thy 1 + C′. Mouse anti-Thy 1.2 antiserum was from Bionetics (Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, Md.) and used for immunofluorescence (revealed by fluorescein rabbit anti-mouse Ig) or for cytotoxicity at 1:25 dilution in the presence of agar-absorbed rabbit serum as a source of C′ (13). Balb/c anti-C3H and C3H anti-Balb/c alloantisera were prepared and used for immunofluorescence staining as described elswhere (14). For intracellular histamine detection, cell smears were treated with 1% ortho-phthaldialdehyde (Calbiochem A.G., Lucerne, Switzerland) in xylene in wet chambers at 37°C for 20 s, then mounted in tetrahydrofurfuryl alcohol and examined with UV light (UG 1 excitation filter) (15) with a Leitz Orthoplan microscope with an Opak-Fluor vertical illuminator (E. Leitz, Wetzlar, Federal Republic of Germany). For monoamine detection, cell smears were treated for 2–20 h with formaldehyde gas, then examined with UV light (16). Alcian blue and toluidine blue stainings were performed as described by Miller and Walshaw (17).

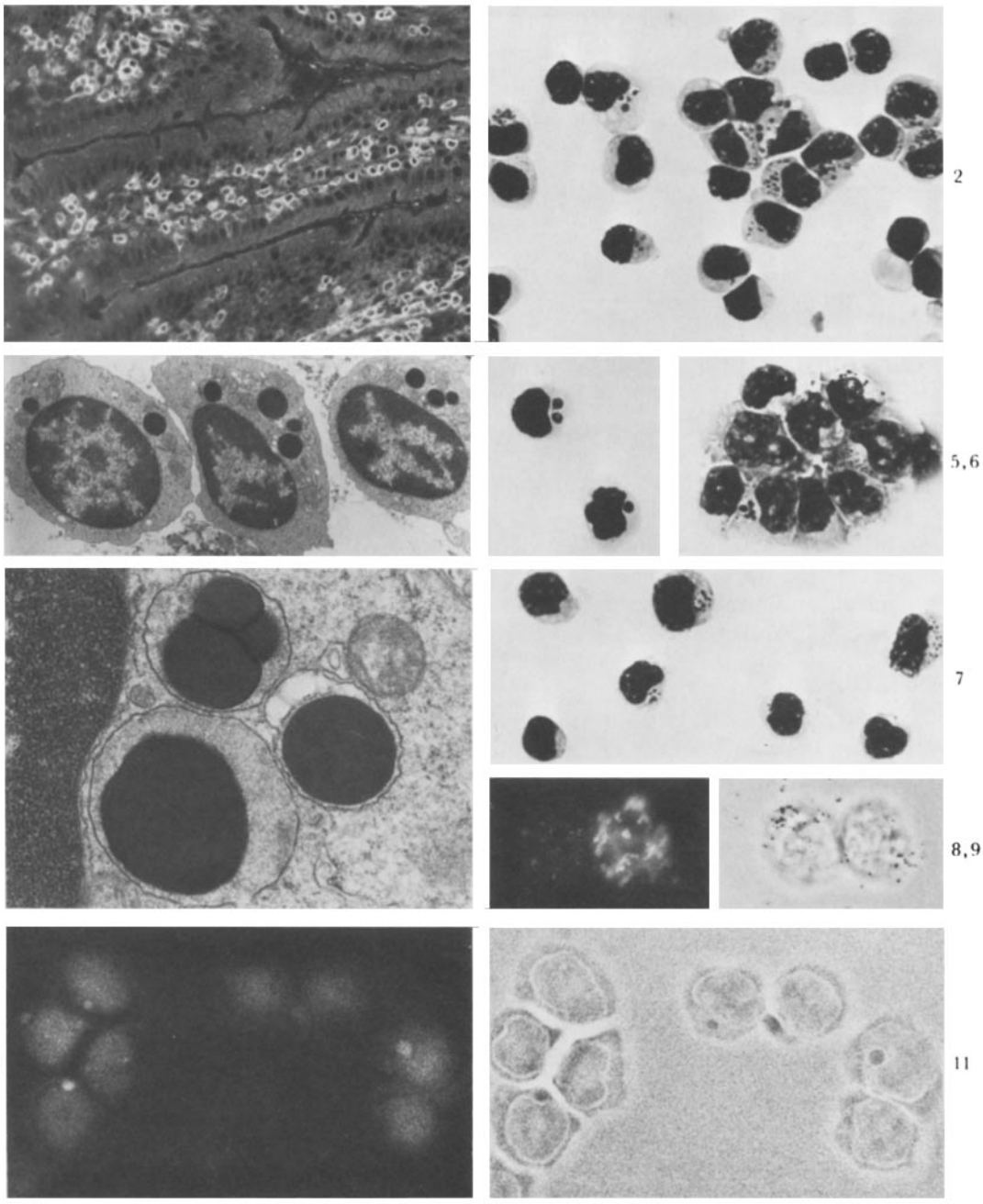
Results

Gut T Lymphocytes of Adult Mice in Normal Conditions

IDENTIFICATION, DESCRIPTION, AND BEHAVIOUR IN CULTURE. GTL were detected and counted on tissue sections by immunofluorescence using an anti-MTLA antiserum as previously described (Fig. 1). In all mouse strains explored (Balb/c, C3H, $C57Bl_6$, $(DBA_2 \times C57Bl_6)F_1$, and beige mice) the average density of T lymphocytes in adult mice is ≅30–50 cells/250 × microscopic field at all levels of the small bowel; they are markedly less numerous in the large bowel. This density, which remains constant during adult life, reaches adult level later (around the 50th day) than that of gut mucosa IgA plasma cells; T lymphocytes are practically absent during the first 3 wk, and still 3–4 times less numerous than in adult between the 21st and 35th day. GTL are detected within the lamina propria and between the epithelial cells in roughly equivalent proportions (Fig. 1). IE lymphocytes can be more precisely studied because they can be isolated from the mucosa and obtained in suspension in a rather pure form (see methods and Fig. 2): ≅80–85% are medium-sized and 20% or less large lymphocytes.

Immunofluorescent studies of these lymphocyte suspensions show, in all strains studied, including beige mice, that 80–90% are stained with rabbit anti-MTLA or mouse anti-Thy 1.2 antiserum, or are killed after incubation in this last antiserum in the presence of rabbit C′. Surface Ig-bearing cells are very rare (≅1%) in these suspensions, which contain no more than 0.1% IgA plasma cells indicating that the isolation procedure selects almost exclusively the IE lymphocytes, because IgA plasma cells are very numerous in the lamina propria. On Giemsa-stained smears, ≅30–45% of the cells contain several intracytoplasmic granules of various sizes (Fig. 2). In beige mice, however, there is most often a single granule of much larger size (Fig. 5), present in ≅80% of the cells; this greater percentage of granulated lymphocytes appears to reflect the easier detection of the granules. On electron microscopic sections, the granules are very osmiophilic, or contain a very dark center surrounded by a less osmiophilic, membrane limited material (Figs. 3, 4) and resemble mast cell granules. The cells contain few ribosomes. In normal mice, the IE lymphocytes are only weakly (and inconsistently, i.e., with variations from one preparation to another) stained by alcian blue at pH 0.3 or 2.2, or by toluidine blue (with metachromasy). In the beige mice, however, the large granules are clearly and consistently alcian blue positive.

When mice are injected with ^{35}S-labeled sulphate 3–48 h before sacrifice, a procedure which leads to strong labeling of peritoneal mast cells on radioautographs, the presence of $^{35}SO_4$ is easily detectable in the IE lymphocyte granules, but a combination of radioautography and immunofluorescence shows that labeled granules are also present in cells weakly or not stained by the anti-MTLA serum, where the granules



appear to be more numerous and larger (Figs. 8, 9). Granules of similar type are not found in lymphocytes of other sources, except for TDL lymphocytes, where ≅1% of the cells contain granules.

Because gut IE lymphocyte granules resemble those of mast cells, their possible histamine content was explored by the *o*-phthaldialdehyde staining reaction (15). No or only equivocal staining is observed with the cells of normal mice. On the other hand, about one-half of the giant granules of beige mice IE lymphocytes show an unequivocal yellow fluorescence (Figs. 10, 11), only slightly weaker than that of the granules of peritoneal mast cells of beige or normal mice, the only other cell type where fluorescence is observed. However, in contrast to the granules of peritoneal mast cells, IE T-lymphocyte granules, even in beige mice, do not contain detectable amounts of serotonin and dopamine, as judged by their lack of fluorescence using the formaldehyde-fixation gas method, even after precursor injections (18), nor are they labeled on radioautographs after injection of [^{3}H]serotonin, [^{3}H]5-hydroxytryptophan, or [^{3}H]L-dopa, as peritoneal mast cells are (see Methods).

Due to their relatively small size, normal lymphocyte granules are difficult to identify on tissue sections, and thus, in normal animals, it cannot be ascertained whether granules are restricted to IE T lymphocytes or also present in T lymphocytes located within the lamina propria. In beige mice, however, lamina propria T lymphocytes with granules can be easily identified on frozen tissue sections stained with alcian blue.

Finally, the capacity of IE T lymphocytes to proliferate in culture in response to con A and PHA (after 3 days), or to allogeneic irradiated lymph node cells (after 6 days), was explored and compared to that of lymph node cells. No proliferation was observed with con A (even in presence of irradiated syngeneic lymph node cells) nor in mixed lymphocyte cultures. PHA induced a weak response (≅10 times weaker in [^{3}H]TdR incorporation than that of lymph node cells); with ≅50% of the blasts containing granules (Fig. 6).

T LYMPHOCYTES APPEAR IN THE GUT MUCOSA AS THE RESULT OF ANTIGENIC STIMULA-

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FIG. 1. Duodenum of an adult Balb/c mouse stained with rabbit anti-MTLA antiserum, revealed with a rhodamine-conjugated sheep IgG fraction anti-rabbit Ig. T cells are seen in the lamina propria and within the epithelium, mostly at its basis.

FIG. 2. Smear of isolated IE lymphocytes of a normal mouse. Giemsa staining. Note the variable size and number of the granules.

FIG. 3. Ultrastructural appearance of isolated IE lymphocytes. The granules are variable in size.

FIG. 4. Ultrastructural detail of three granules within a lymphocyte. The dark mass on the left is the nucleus.

FIG. 5. Smear of isolated IE lymphocytes of a beige mouse. One cell contains one and the other two giant-sized granules.

FIG. 6. Smear of isolated IE lymphocytes of a normal mouse stimulated by PHA in culture. Some of the blasts have granules of various sizes.

FIG. 7. Smear of isolated IE lymphocytes of a mouse with a GVH reaction. Granules are clearly seen in 3 lymphocytes.

FIG. 8. and 9. Smears of isolated IE cells of a normal mouse 20 h after injection of [^{35}S]SO_4; immunofluorescence with anti-MTLA on the left, radioautography on the right. The two lymphocytes contain [^{35}S]SO_4 grains, but only the one on the left is stained with anti-MTLA.

FIG. 10. and 11. Smears of isolated IE lymphocytes of a beige mouse seen with UV fluorescence after OPT treatment (Fig. 10), and by phase-contrast microscopy (Fig. 11). There are four dots clearly stained yellow on the blue background of nuclei. Not all the giant granules are clearly seen on the phase-contrast picture because some are out of the plane of focus. Not all of the granules are yellow with the UV light.

TION. Gut T lymphocytes are practically absent in all the adult axenic mice of the various strains explored (Balb/c, C3H, CBA/J, and DBA_2), and appear in about normal concentration within 1 or 2 mo when these mice are placed in nonsterile conditions.

THE GUT T LYMPHOCYTES ARE MOSTLY RECENTLY DIVIDED CELLS BUT HAVE ONLY A LOW OR INSIGNIFICANT RATE OF REPLICATION IN THE INTESTINAL WALL. It has been reported that the majority of intestinal lymphocytes observed in tissue sections are labeled after multiple injections of [^{3}H]TdR (19, 20). After 5 days of three daily [^{3}H]TdR injections, suspensions of IE T lymphocytes contained by far the highest proportion of labeled T cells (up to 60%) when compared to the T-cell populations of various peripheral lymphoid sources (PP, 12.5%; spleen, 7.6%; MLN and PLN, 6%; TDL, 3%), as judged by combined immunofluorescence and autoradiography on a pool of lymphoid cells of three Balb/c mice. In contrast, after a single pulse of [^{3}H]-TdR, either by incubation of IE T lymphocytes for 1 h in vitro, or by isolation of these lymphocytes from an animal sacrificed 40 min after [^{3}H]TdR injection, only, respectively, 0.1 and 0.3% of cells were labeled.

CELL-TRANSFER EXPERIMENTS USING A VARIETY OF LYMPHOCYTES SHOW THAT ONLY T BLASTS PRESENT IN TDL OR MLN HAVE THE PROPERTY OF HOMING IN HIGH PERCENTAGE IN THE GUT WALL. It has already been shown, using transfer of cells labeled in vitro by a short pulse of [^{3}H]TdR (and thus defined as blasts) followed by radioautography of the recipient's tissues, that T blasts obtained from TDL, and to a lesser extent from MLN, home strikingly in the gut lamina propria, in contrast to blasts obtained from PLN or PP (2). Further transfer experiments were performed, using a variety of labeled T cells to appraise, from their relative tissue distribution on radioautographs, their gut-homing tendency. These experiments, not reported in detail, showed that the following cells display no appreciable gut-homing tendency 1 day after transfer (compared to results reported on Table IV and V of reference 2): (*a*) blasts from spleen (containing ≅30% T cells as judged by immunofluorescence) and from thymus, as well as blasts obtained from cultures of MLN, PLN, or PP lymphocytes stimulated by PHA or con A, or by allogeneic cells; (*b*) recently divided TDL lymphocytes, labeled in vivo after 5 days of three daily injections of [^{3}H]TdR; (*c*) [^{3}H]uridine labeled total lymphocyte populations obtained from PLN, MLN, TDL, and PP (in this last case enriched in T cells by passage on nylon wool): Table I.

These experiments suggest that T blasts normally present in the MLN and released in the TDL are the main progenitors of GTL. It is possible, however, that a minor population, among MLN and TDL nondividing lymphocytes, has a gut-homing tendency because, after transfer of [^{3}H]uridine-labeled lymphocytes, the density of labeled cells observed in the gut wall, although very low, is reproducibly higher in the case of transfered MLN and TDL lymphocytes than after transfer of PLN lymphocytes (Table I). It seems difficult to ascribe this difference simply to the gut-homing T blasts present in the MLN and TDL [^{3}H]uridine-labeled lymphocyte populations, because their proportion is very small (0.5–1.7% of the cells are pulse-labeled with [^{3}H]TdR, vs. almost 100% with [^{3}H]uridine). The question may thus be asked whether some GTL can reach the gut lymph and then recirculate to home in the gut, thus representing a small subpopulation of nondividing lymphocytes with gut-homing properties, present in MLN and TDL.

TABLE I

Homing Pattern of [^{3}H]Uridine-Labeled Cells Obtained from Various Tissues, after Transfer into Syngeneic Mice

Source of transferred cells	Time between transfer and sacrifice	Spleen (labeled cells per microscopic field × 400)	Other recipient tissues* (in percentage of labeled cell density in spleen)				
			Liver	PLN	MLN	PP	Gut
	h						
PLN	20	21	1	207	195	85	0.1
PP‡	20	88	Not done	70	57	54	0.6
MLN	20	30	0.2	200	180	26	2 (10)§
TDL	20	56	0	263	179	93	3 (8)
Gut IE lymphocytes	20	10.5	25	2	3	7	1 (19)
	48	8.5	11	1.5	4	14	2 (29)
	96	11	13	1	4	15	7 (41)

* These are average results of two experiments.

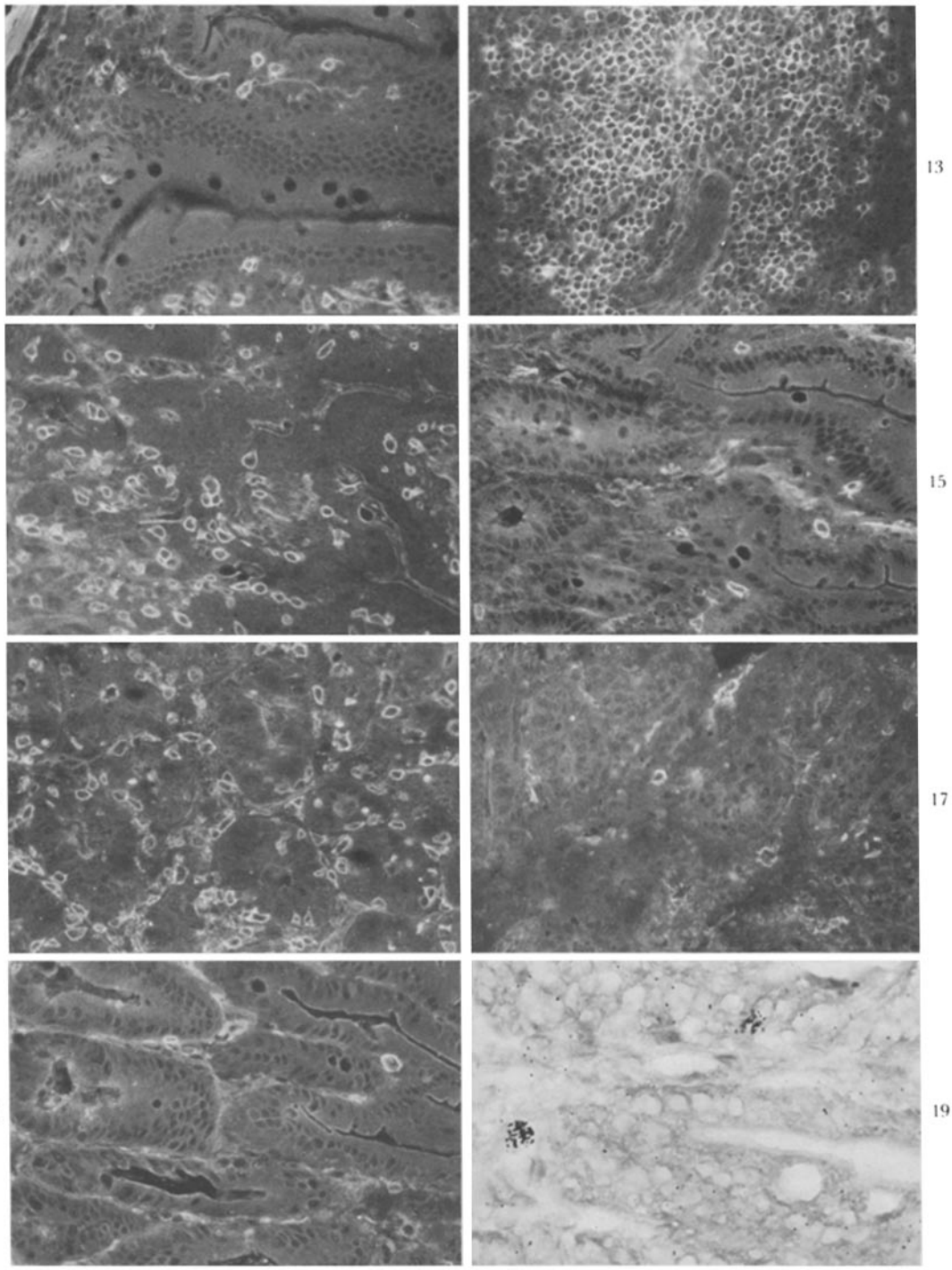
‡ Nylon wool passaged cells (65% T).

§ Percent of IE cells among labeled cells (established by counting, per microscopic field, on one hand all the labeled cells, on the other hand only the labeled cells located within the epithelium).

To explore this possibility, transfer experiments were performed with [^{3}H]uridine-labeled gut IE lymphocytes. In contrast to normal lymphocytes, these cells remain well labeled for several days after transfer, indicating that their RNA turnover is especially low, which is in keeping with their low ribosomal content. Except for the spleen, these cells home very little in the peripheral lymphoid organs, but they are recovered in higher density in the PP than in PLN and MLN, and their homing in the gut mucosa and epithelium, although initially very low, increases strikingly between day 1 and 4 after transfer (Table I).

EVIDENCE FOR A GUT T-LYMPHOCYTE TRAFFIC CYCLE. Experiments were devised to explore the possibility that T blasts arising in the PP, then migrating to MLN and TDL are the precursors of GTL. As mentioned above, cell transfers with PP blasts do not show evidence of gut homing of these blasts, but this might be due to the fact that only ≅6% of the in vitro [^{3}H]TdR-labeled PP blasts were of T nature (2). Two types of experiments were performed: (*a*) selective *in situ* labeling of PP blasts by topical applications of [^{3}H]TdR, to explore whether PP T blasts are able to circulate and to home in the gut; (*b*) selective irradiation of PP or MLN to explore whether selective destruction of lymphocytes in these organs would selectively induce a T-cell depletion in the gut.

Mice were killed 2 days, or their thoracic duct cannulated (for 24 h) 1 day after topical application of [^{3}H]TdR on their PP. Radioautographs of lymphoid cell smears (8–10,000 cells counted) or tissue sections were examined with combined immunofluorescence to detect T cells. The intestinal cells were found to be labeled on the villus immediately adjacent to the PP, but not elsewhere, indicating the absence of [^{3}H]-TdR available for detectable DNA labeling outside the site of topical application of the label. In the TDL, 0.25% of the cells were labeled, ≅ 25% being IgA-containing cells; among the TDL T cells, 0.17% were labeled, two-thirds of these labeled cells being large cells. In the MLN, spleen- and PLN-cell suspensions, respectively, 0.28, 0.05, and no labeled T cells were found. A few labeled lymphoid cells were found in



the gut mucosa at a distance from the PP, both in the lamina propria and within the epithelium (illustration of the result of a similar experimental procedure, but performed on a mouse undergoing GVH, is shown on Fig. 19).

For selective irradiation of PP and MLN, ^{32}P-containing small disks or strips of PVC were glued on the patches or on the nodes (see Methods). Mice wearing PVC disks on their PP could not be kept in good health more than a few days, because the disks interfered with the normal intestinal peristaltism and led to intestinal occlusion. On the other hand, strips on MLN did not result in intestinal damage. Balb/c mice bearing ^{32}P strips were compared to normal Balb/c mice, which were found to have a very constant density of IE T lymphocytes (23 ± 3%/250 × microscopic field) (IE lymphocyte counts were used because they are more prone to precise quantitations). Only one mouse bearing ^{32}P-disks on its PP for 5 days was found with a morphologically unaltered gut allowing a good comparison to controls: the density of IE T lymphocytes was 6.8 cells/field, with a corresponding decrease of T cells within the lamina propria. For mice bearing ^{32}P-strips on their MLN, a progressive decrease in GTL was observed with time: after 4 days, 22 IE T cells/microscopic field; after 7 days, 12 cells; 10 days, 6 cells; 17 days, 2.3 (Fig. 12). At this last time, the MLN contained very few lymphoid cells and showed a marked sclerosis. By contrast, PLN, PP, and spleens showed no histologic damage and unaltered T lymphocyte areas (as judged by immunofluorescence: Fig. 13). For controlling the specificity of the radiation effect, mice bearing ^{32}P-strips on their spleens were studied at the same times. Only a moderate decrease of gut IE T lymphocytes was observed: after 5 days, 18 cells; 7 days, 17.4; 10 days, 14.7; 17 days, 17. The areas of PP remained normal during this whole period, although the spleen became progressively very small and fibrous.

Gut T Lymphocytes in Mice with a GVH Reaction

Lethally irradiated mice (Balb/c or (C57 × DBA)F_1) which, as the result of irradiation have a profound depletion of their GTL, were used to produce a GVH reaction by injection of allogeneic (C3H) or parental (C57) thymocytes (3).

IDENTIFICATION AND DESCRIPTION OF THE GTL IN THIS TYPE OF GVH REACTION. Starting on the 4th day after irradiation and injection of foreign or parental thymocytes, the gut wall becomes massively infiltrated by T lymphocytes, in the lamina propria as well as in the epithelium (Fig. 14). Isolation of IE lymphocytes in suspension shows cells similar in cytologic and ultrastructural appearance to those of the normal gut

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FIG. 12. and 13. Balb/c mouse bearing [^{32}P]PVC strip on MLN for 17 days. Immunofluorescence with anti-MTLA on the duodenum (Fig. 12) showing profound depletion in GTL, and on the spleen (Fig. 13) showing a normal density in T lymphocytes of a T-cell area of the white pulp.

FIG. 14. and 15. Immunofluorescence with anti-MTLA of the duodenum of a lethally irradiated (C57 × DBA)F_1 mouse injected with C57 thymocytes (Fig. 14), showing the strong T lymphocyte infiltration characteristic of the GVH reaction, and of a mouse of the same group injected with syngeneic F_1 thymocytes (Fig. 15), where there is no repopulation of the mucosa in T cells.

FIG. 16. and 17. Immunofluorescence with anti-MTLA of a subcutaneous fetal gut graft in an F_1 mouse injected with parental thymocytes 6 days earlier (Fig. 16), showing a strong infiltration of T cells, whereas the graft of a mouse of the same group, but with a thoracic duct drainage from day 4 to 6 (Fig. 17) is practically devoid of T lymphocytes.

FIG. 18. Immunofluorescence with anti-MTLA of the duodenum of an F_1 mouse injected with parental thymocytes 6 days earlier, such as in Fig. 14, but with ^{32}P-pieces on its PP from day 4 to 6. The mucosa is practically devoid of T lymphocytes.

FIG. 19. Autoradiography of an F_1 mouse 6 days after the induction of a GVH reaction, having received topical application of [^3H]TdR on its PP on day 4. Two [^3H]TdR-labeled lymphocytes are seen, one clearly intraepithelial. 5 Mo exposure, methylgreen pyronin staining.

(although somewhat larger and more basophilic), with a similar percentage of granulated cells (Fig. 7). Granulated lymphocytes are not found elsewhere, except for TDL (≅2–3% of the cells). These cells are T lymphocytes of donor origin, as shown by immunofluorescence with anti-MTLA and with specific anti-H2 antisera. They do not proliferate when cultured for 3 days with irradiated spleen cells of the host strain, in contrast to TDL (see below).

GUT T LYMPHOCYTES ACCUMULATING DURING GVH REACTION RESULT FROM THE ALLOANTIGENIC STIMULUS BUT ARE NOT ATTRACTED TO THE GUT BY AN ANTIGEN-SPECIFIC RECOGNITION MECHANISM. Irradiated mice injected with syngeneic thymocytes show a GTL depletion as strong as that of noninjected mice (Fig. 15), indicating that the infiltration observed in the GVH reaction results from the alloantigenic stimulation. On the other hand, this infiltration does not result from an antigen-specific recognition mechanism, because when GVH is produced by C57 thymocytes in irradiated (C57 × DBA)F_1 mice bearing a graft of fetal intestine of either C57 or DBA origin, both grafts, syngeneic or allogeneic to donor thymocytes, show a similar degree of T-cell accumulation, comparable to that of the recipient gut itself (Fig. 16).

TRANSFER EXPERIMENTS WITH CELLS OF MICE UNDERGOING GVH REACTION SHOW THAT T BLASTS OF TDL OR MLN HAVE A MARKEDLY HIGHER TENDENCY TO HOME IN THE GUT THAN T CELLS FROM OTHER SOURCES. Cells obtained from the TDL, MLN, PNL, and spleen of mice on the 5th day of GVH reaction, labeled in vitro with [^{3}H]TdR, are all T blasts of graft origin. They were transferred into syngeneic normal mice killed 20 h later to explore their gut-homing tendency, as described above and previously (2). Table II shows that TDL blasts home in the gut, in the epithelium as well as in the lamina propria, at least 10 times more, and MLN blasts 4 times more than blasts from the spleen or PLN; homing of TDL and MLN blasts is also higher in PP and MLN. Two further observations deserve emphasis: (*a*) the gut-homing tendency of the total population of TDL cells (labeled with [^{3}H]uridine) is much weaker than that of the T blasts of the same origin (Table II), although these cells are all recently divided T cells of donor origin (100% of labeled cells after 5 days of [^{3}H]TdR injection); (*b*) these TDL lymphocytes display a strong proliferative response when cultured for 3 days with irradiated spleen cells of the host strain, but blasts from these cultures, labeled with [^{3}H]TdR, show a much weaker gut homing than the circulating blasts of the same source (Table II).

THE GTL TRAFFIC CYCLE IN GVH REACTION. Experiments using selective irradiation and selective [^{3}H]TdR labeling of the PP were carried out as described above for normal mice. In addition, the effect of thoracic duct drainage on the postulated traffic cycle was explored. (*a*) Selective irradiation of the PP between day 4 and 6 of the GVH reaction resulted in a very strong decrease or total depletion of the GTL on the 6th day, on the whole length of the gut (Fig. 18) as well as in subcutaneous grafts of fetal gut. Sham-operated animals bearing nonradioactive PVC pieces on the PP showed the same T-cell infiltration of the gut as nonoperated animals. GTL depletion was selective, because the spleen, MLN, and PLN were as rich in T blasts (detected by radioautography after in vivo [^{3}H]TdR injection) as in nonoperated mice. Selective irradiation of the spleen from day 4 to 6 led to only a slight decrease of the GTL. (*b*) Selective in vivo labeling of PP blasts by topical application of [^{3}H]TdR on day 4 was followed by the appearance, in animals killed on day 5, of labeled cells in MLN (5.5% on cell smears) and to a more limited extent, in the gut wall, whereas no labeled cells among thousands screened were detected in PLN and spleen cell smears. In sections

TABLE II

*Homing Pattern of Labeled T Cells Obtained from Various Tissues of Mice Presenting a GVH Reaction, after Transfer into Recipient Syngeneic to the GVH-Inducing Cells**

Labeling and source of transferred cells	Percentage of labeled cells/transferred cells	Spleen (labeled cells per microscopic field × 400)	Other recipient tissues‡ (in percentage of labeled cell density in spleen)				
			Liver	PLN	MLN	PP	Gut
– ^{3}H TdR in vitro							
PLN	8	15	2	38	11.5	2.5	<1
Spleen	25	17	5	16	23	9	2
MLN	12	8	8	10.5	21	18.5	12.5
TDL	13.5	8.5	3	11	18	38	39.5
– ^{3}H uridine in vitro							
TDL	100	50	2	58	65	29	3
– ^{3}H TdR in vitro alloantigen-stimulated							
TDL§	30	16.5	12	2	5	4	1

* All the animals (C57 strain) were sacrificed 20 h after transfer of cells obtained from mice on the 5th day of a GVH reaction (resulting from the injection of 2 × 10^8 C57 thymocytes into lethally irradiated (C57 × DBA)F_1 mice, as indicated in methods). After in vitro labeling (see methods), 15–45 × 10^6 cells were injected intravenously into normal C57 recipients.

‡ Expressed in percentage of the labeled density observed in spleen. These are average results of 2 experiments.

§ TDL cells from (C57 × DBA)F_1 irradiated mouse injected with C57 thymocytes, collected between the 4th and 5th day of the reaction, and cultured for 3 days with irradiated DBA spleen cells.

of the gut, the epithelium covering the PP was labeled, and some labeled cells were also present within the patches. At a distance from the PP, as described above for normal mice, the epithelial cells were not labeled, although some labeled lymphocytes were found, both within the epithelium and in the lamina propria (Fig. 19). (*c*) Thoracic duct drainage from day 4 to 6 resulted in almost total depletion of GTL in the gut itself as well as in fetal gut grafts (used to avoid possible intestinal alterations resulting from prolonged TD cannulation) (Fig. 17 compared to 16).

Discussion

The present experiments show that the GTL, which are found in about equal proportions in the mucosa lamina propria and within the epithelium itself (Fig. 1), are a special type of T lymphocytes, peculiar not only by their localization, but also by their structure and probably function, as well as by their origin and the traffic properties of their progenitors. Their T nature is shown by their surface markers; in addition, in the GVH model studied, in which lethally irradiated F_1 mice are injected with parental thymocytes, almost all the GTL observed are the progeny of the parental thymocytes injected 5 to 6 days earlier.

The peculiarity of the gut T lymphocytes which makes them unique is the presence of cytoplasmic granules. Characteristics of the granulated lymphocytes will be discussed first, then the nature of the granules.

The granules are clearly seen only on smears of purified IE lymphocytes (see Methods), as first described by Rudzik and Bienenstock (21) in the rabbit. While almost none of these lymphocytes bear surface Ig, ≅80–90% bear the T markers, and ≅30–45% of the lymphocytes contain granules, whether in normal mice (Fig. 2) or in GVH conditions (Fig. 7). However, because the size and number of the granules are

variable, granulated cells may not always be clearly identifiable. In beige mice, the gut IE lymphocytes have giant granules (Fig. 5), making the granulated cells easier to identify and to count. Because, in this case, up to 80% of the gut IE lymphocytes are granulated, it is likely that in fact the majority of these cells are granulated even in normal mice. Three points deserve special emphasis: (*a*) granulated T cells are observed only in the gut (to the exception of 1–3% of granulated lymphocytes in the TDL), whether the mice are normal, of the beige strain, or presenting a GVH reaction. The abundance of granulated T cells and their restriction to the gut wall is all the more striking in this last case, where the T cells found in the gut or in the peripheral lymphoid organs all have a common origin, the parental thymocytes injected a few days earlier; (*b*) not all the IE granulated lymphocytes bear the T markers. This is best appraised by a combination of immunofluorescence and radioautography, because the granules take up injected [^{35}S]SO_4 (see below). The cells containing most silver grains are sometime weakly or not stained with the anti-MTLA antiserum (Fig. 8, 9). This suggests a process of differentiation, where the T cells acquiring more granules (or perhaps more mature granules, see below) lose their membrane protein characteristics of the T-cell lineage. (*c*) The granulated T cells described so far are clearly observed only on isolated IE lymphocytes. The granulations are difficult to see on tissue sections, and thus the question may be raised whether the T lymphocytes present in the lamina propria represent a different subpopulation of T lymphocytes. In beige mice, however, the lymphocyte granules, because of their size and strong alcianophily, are easy to see on tissue sections, and granulated lymphocytes are equally found within the lamina propria. Although it is not possible to evaluate the percentage of lamina propria T lymphocytes which are granulated, this last observation, as well as the evidence for a common origin for all GTL discussed below, strongly suggests that lamina propria and IE gut lymphocytes represent the same T-cell subpopulation, perhaps at different stages of differentiation.

Ultrastructurally and histochemically, the GTL granules resemble mast cell granules, and, like them, become labeled with [^{35}S]SO_4 after intravenous injection, although not as strongly. The most sensitive and specific method for histamine detection, the *o*-phthaldialdehyde staining reaction (15), gives at best equivocal results, except with the gut IE lymphocytes of beige mice, where about one-half of the giant granules unequivocally show specific staining (Fig. 10, 11), although not as intense as in mast cell granules. This suggests that the gut IE lymphocyte granules of normal mice may contain histamine, which, however, because of the smaller size and lower histamine concentration of the granules compared to those of mast cells, cannot be clearly identified, a difficulty which is overcome in the case of beige mice lymphocytes by the giant size of the granules. Among the mast cells of rats and mice, serosal mast cells, such as peritoneal mast cells, appear to somewhat differ in their nature from the mucosal mast cells, such as those found in the small bowel mucosa (18). We propose, as further discussed below, that granulated GTL are related to mucosal mast cells.

Gut IE T cells differ from T cells of lymphoid organs in other respects than the presence of granules. They do not proliferate in cultures in response to con A or allogeneic lymphoid cells in the conditions studied, a failure which is not explained by the lack of macrophages (see Results). They have not, however, entirely lost the capacity to respond to PHA, and show a weak proliferative response, giving raise to granule-containing blasts (Fig. 6).

Concerning the origin of the GTL, the present results offer conclusive evidence that

they are the progeny of T blasts arising in the PP as the result of antigenic stimulation, leaving the patches to reach the MLN, then the TDL still as rapidly replicating cells, and finally disseminating from the blood all along the intestinal mucosa because of their peculiar gut-homing property, i.e., after a traffic cycle comparable to that of the gut IgA plasma cell precursors (2, 22, 23). The evidence can be summarized as follows: (*a*) kinetics of [^{3}H]TdR labeling show that gut IE T lymphocytes derive mostly from rapidly replicating cells, and that the rate of cell replication within the mucosa is very low. Thus, the majority of these cells have to derive from cells reaching the gut mucosa as T blasts or as recently divided T cells. (*b*) Transfer experiments show that, in normal conditions as well as in GVH, TDL (2, 24), and to a lesser extent MLN, T blasts have a marked gut-homing tendency, a property which is shared neither by T blasts from other sources (spleen, PLN, thymus) nor by recently divided T cells circulating in the TDL. This suggests that the progenitors of GTL are T blasts present in TDL and MLN. (*c*) If some MLN and TDL T blasts represent migrating cells originating in the PP and making a cycle to colonize the gut mucosa, one should expect to observe that: (*a*) selective labeling of PP blasts (by topical application of [^{3}H]TdR) leads to the appearance of labeled T cells in the MLN, the TDL and finally the gut mucosa, but not in other peripheral lymphoid tissues; (*b*) selective irradiation of PP, or of MLN leads to progressive disappearance (or, in GVH, lack of appearance) of GTL, without altering the T-lymphocyte populations of other lymphoid organs, although selective irradiation of the spleen for the same period of time has little or no effect on the amount of GTL. This is indeed what is observed, in normal mice and still more strikingly in GVH condition, because in this last case the process of colonization of the gut mucosa is considerably accelerated. In addition, thoracic duct drainage from the 4th day of the GVH reaction totally prevents accumulation of T lymphocytes in the recipient gut and in a graft of fetal gut on day 5 and 6, as might be expected from an interruption of a GTL cycle.

These observations do not imply that all GTL are the immediate progeny of PP blasts. Kinetics of [^{3}H]TdR labeling show that not all GTL are rapidly labeled, and there is evidence for the existence of long-lived gut lymphocytes (19, 20). Transfer experiments with [^{3}H]uridine labeled cells suggest that a minor subpopulation of nondividing cells among TDL and MLN might have gut-homing properties. This may be expected if some GTL reach the gut lymph and recirculate, keeping their gut-homing tendency. This possibility is consistent with the observation of a few granulated lymphocytes in TDL, and with transfer experiments using [^{3}H]uridine-labeled gut IE lymphocytes, showing that they can slowly home back to the gut (and to the PP) (Table I). Thus, although all GTL may be the progeny of T cells stimulated to divide in the PP, this is not incompatible with the existence of a small nondividing population of recirculating T lymphocytes with a gut-homing specificity, more distant from the last cell division than the majority of GTL, and able to home back in the gut after more than one cycle to and from the gut wall. This possibility, previously discussed (2), but more strongly suggested by the present experiments, is in agreement with the gut-homing tendency and thoracic duct recirculation observed after transfer of lymphocytes obtained from the sheep mesenteric duct (25).

The presence of T lymphocytes within the gut mucosa is the result of antigenic stimulation, as clearly shown by the observations made in axenic mice, consistent with previous observations on the gut IE lymphocytes in germ-free conditions (26, 27). However, the gut-homing mechanism responsible for the GTL accumulation is

entirely independent of the presence of antigen in the gut, as seen with animals bearing a graft of fetal intestine, in which T lymphocyte homing is identical to that observed in the recipient's own gut (2): in the present GVH experiments the T-cell infiltration of fetal gut grafts was identical whether the grafts were syngeneic to host or to the thymocytes of the donor. Therefore, it appears that it is the generation of T blasts within the PP which confers by itself to these blasts their gut-homing properties. Two types of signals should thus be received by a T lymphocyte to become a gut-homing T cell: the antigenic stimulation and a signal provided by the PP environment. This would explain why T blasts generated in vitro by stimulation with mitogens or allogeneic cells do not, whatever the source of T lymphocytes used for culture, display a gut-homing tendency. In the GVH reaction, the intestinal infiltration by parental lymphocytes does not correspond to a mechanism basically different from that operating in normal mice: only the parental thymocytes (or their progeny) proliferating in the PP will become GTL. This clearly demonstrates that the thymic influence and antigenic stimulation are not the only mechanisms involved in T-lymphocyte differentiation, and that further differentiation can be under the control of peripheral lymphoid organs.

What can be the role of GTL? As discussed above, their granules seem to relate them to mucosal mast cells. Athymic nude mice, which have a marked decrease in IE lymphocytes (27) and GTL in general (28) (although not a complete absence, as also seen for T cells in other lymphoid organs, 28) have very few intestinal mast cells (but abundant tissue mast cells elsewhere), and do not show an intestinal mast cell response after nematode infection, their response being restored after a thymic graft (29). Direct evidence linking GTL and mucosal mast cells, to be reported elsewhere,[2] has been obtained in rats infected with *Nippostrongylus brasiliensis*. After several infections followed by worm rejection, numerous mast cells are present all along the small bowel mucosa. When such previously infected rats receive TDL blasts from normal rats, a high percentage of the transferred cells found 24 h later in the gut wall of the recipient are mast cells. This shows: (*a*) that TDL blasts can be transformed not only into gut granulated T lymphocytes, but also into fully differentiated mucosal mast cells; (*b*) that this transformation is probably under the influence of some elements of the mucosa itself. That thymocytes can transform into mast cells has already been proposed by Burnet (30), and the emergence of clones of mast cells in cultures of thymocytes or TDL lymphocytes is a well documented observation (31, 32). It would thus appear that the gut homing T lymphocytes, which have already received in the PP an instruction conferring on them their gut-homing property, receive on reaching the gut mucosa a second instruction allowing their differentiation along the mast cell pathway. Depending upon the state of the mucosa, the differentiation would be only partial (granulated lymphocytes) or complete (mucosal mast cells). Whether the only functional role of GTL is to serve as potential precursors of mucosal mast cells (precursors which may be in part eliminated through the epithelium when the conditions favoring and perhaps requiring their full differentiation do not exist), has to be further studied. The role of mucosal mast cells, even in conditions rather extensively studied such as the rejection of some parasites, is not fully understood. It seems likely that a similar scheme of events involving mucosal T lymphocytes and

[2] D. Guy-Grand, G. Luffau, C. Griscelli, and P. Vassalli. Manuscript in preparation.

mast cells may be part of an immune defense system common to all the mucosa, notably the bronchial mucosa, where IE "basophils" appear to be common (33).

Summary

Lymphocytes of the mouse intestinal mucosa, identified in tissue sections or purified suspensions of intraepithelial lymphocytes as T cells (gut T lymphocytes [GTL]), were studied in normal mice or in beige mice (the equivalent of the Chediak-Higashi syndrome in man, characterized by giant granules in various cell types, including mast cells). Mice were studied in normal or in germ-free conditions, or during a graft versus host (GVH) reaction resulting from the injection of parental thymocytes into lethally irradiated F_1 mice, a condition leading to massive accumulation of T lymphocytes of donor origin in the host gut mucosa. In normal as well as in GVH conditions, a high percentage of the gut IE lymphocytes contain granules (up to 80% in the beige mouse). These granules have ultrastructural, histochemical and other features resembling those of mast cell granules; in beige mice, up to 50% of them can be shown to contain histamine. Granulated T cells are also found in the lamina propria. It appears that the GTL may progressively lose their surface T antigens when the granules become more developed.

Kinetics of [^{3}H]TdR labeling of the GTL, transfer experiments with T cells of various origins, selective [^{3}H]TdR labeling and selective irradiation of the Peyer's patches (PP), and effect of thoracic duct (TD) drainage led to the conclusion that GTL are the progeny of T cells stimulated to divide in the PP microenvironment, which endows them with a gut-homing tendency. From the PP, these cells follow a cycle, migrating to the TD and to the blood to colonize the whole intestinal mucosa, the majority of them as dividing cells undergoing a single round of traffic, with some probably able to recirculate and becoming a more long-lived variety. Antigenic stimulation within the PP is necessary for the emergence of GTL progenitors, but their gut-homing property is unrelated to the antigen as shown with fetal gut grafts, notably in GVH where grafts syngeneic to the host or donor become similarly infiltrated by GTL.

On the basis of their properties and of further evidence to be reported elsewhere, it is proposed that GTL belong to a special class of T lymphocytes, related to the immune defenses of the mucosal systems in general, and capable of acting as progenitors of mucosal mast cells.

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