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Flow Cytometric and Immunohistochemical Characterization of the γ / δ T-Lymphocyte Population in Normal Human Lymphoid Tissue and Peripheral Blood

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We determined the quantitative and topographic distribution of γ/δ lymphocytes in normal human lymphoid tissue and peripheral blood using a monoclonal antibody that detects a framework determinant on δ molecules and delineated the immunophenotypic characteristics of the γ/δ lymphocyte population by one- and/or two-color immunohistochemical and two- and/or three-color flow cytometric analysis. Variable, but generally small, numbers of γ/δ lymphocytes are present in peripheral blood and in all lymphoid tissues. The vast majority, ≥90%, of lymphoid tissue δ lymphocytes reside in interfollicular (T-cell) zones. Approximately 90% of δ thymocytes are present in the thymic medulla. The percentage of CD3-positive T cells that express δ are: spleen 12.5 ± 8.1%, peripheral blood 4.0 ± 3.1%, appendix 2.9 ± 1%, lymph node 2.2 ± 1%, thymus 1.4 ± 0.5%, and tonsil 0.7 ± 0.5%. We further demonstrated that 1) γ/δ-thymocytes and γ/δ peripheral lymphocytes express T-cell lineage restricted antigens CD3 and CD2 but only a variable subset, 30% to 90%, express T-cell lineage associated antigens CD5 and/or CD8; (2) approximately 60% of γ/δ thymocytes express low-density CD4 while all γ/δ peripheral lymphocytes lack detectable CD4; 3) γ/δ lymphocytes lack natural killer (NK), macrophage, and B-cell associated antigens CD16, CD14, and CD20, respectively, but ≥70% of γ/δ T lymphocytes express CD11b, Leu7, and NKH-1, antigens, which are also expressed by suppressor/cytotoxic and NK cells; and 4) a large subpopulation, approximately 25%, of γ/δ thymocytes are in S_1-G_2 phase, while ≥98% of γ/δ peripheral lymphocytes are small lymphocytes in G_0-G_1 phase and lack activation/proliferation markers. Together these results indicate that γ/δ lymphocytes are resting, mature T cells that probably play a primary role in suppressor/cytotoxic phenomena. They also indicate that γ/δ lymphocytes variably express multiple-cell surface antigens associated with various cell lineages, suggesting that γ/δ lymphocytes represent a considerably more heterogeneous cell population than previously appreciated and that they may actually subserve multiple functions. (Am J Pathol 1990, 136:357-367)

B and T lymphocytes recognize antigens through structurally similar molecules, immunoglobulin, and T-cell receptors (TCR), respectively.[1] Like immunoglobulin receptors, TCRs consist of two polypeptide chains, designated α and β, which consist of variable (V), joining (J), and constant (C) regions.[1] α and β molecules are disulfide bond-linked glycosylated polypeptide chains of 49 and 43 kilodaltons (kd), respectively.[2,3] The diversity of amino acid sequences in the N-terminal domain of their variable regions determines TCR antigen specificity. However, in contrast to B lymphocytes, T lymphocytes always bind target antigens in association with cell-surface molecules encoded by the major histocompatibility complex (MHC)[4] and TCR molecules are only expressed in association with the invariant molecular protein complex termed T3.[2,5]

In addition to the TCR α and β genes, two new TCR genes termed γ and δ have been identified recently in mice and humans.[6-12] γ and δ chains are disulfide or non-disulfide bond-linked glycosylated polypeptide chains of 55 kd and 40 kd, respectively.[13,14] The rearrangement and

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expression of all of these genes appear to be related to different stages of T-cell development.[15,16] The γ and δ genes are principally and highly expressed in immature thymocytes while the α and β genes are transcribed in more mature thymocytes and in peripheral T cells.[15,16] However, whether α/β and γ/δ-lymphocytes derive from independent and distinct T-cell subpopulations or represent different stages of maturation of the same T-lymphocyte population, remains unclear.

Several monoclonal antibodies recognizing common determinants of the human TCR β, γ, and δ chains have been produced recently and the distribution and role of α/β-lymphocytes has been intensively investigated.[17–20] However, relatively little is known concerning the origin, nature, distribution and function of γ/δ-lymphocytes. We have determined the quantitative and topographic distribution of γ/δ-lymphocytes in normal human tissue and peripheral blood using a monoclonal antibody that detects a framework determinant on δ molecules and have delineated the immunophenotypic characteristics of the γ/δ-lymphocyte population by one- and/or two-color immunohistochemical and two- and/or three-color flow cytometric analysis. Our results indicate that γ/δ lymphocytes are present in all lymphoid tissues and peripheral blood and variably express multiple cell-surface antigens but lack activation/proliferation-associated antigens. These findings suggest that γ/δ lymphocytes are a heterogeneous subpopulation of mature, resting CD3-antigen-positive T lymphocytes that may subserve multiple functions.

Materials and Methods

Specimens

Representative portions of multiple nonpathologic specimens, (lymph node 13, tonsils 13, thymus 9, spleen 9, ileum 4, descending colon 3, appendix 3, and stomach 1) were collected from random patients during the course of routine diagnosis. In addition, heparinized peripheral venous blood samples were collected from 11 healthy normal volunteers after informed consent. Cell suspensions were prepared by teasing a representative portion of each tissue specimen apart in RPMI 1640. A cell suspension containing ≥95% viable mononuclear cells and devoid of erythrocytes, dead cells, and debris was prepared from each tissue and peripheral blood sample by Ficoll-hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. An additional representative portion of each tissue specimen was snap frozen in OCT-embedding compound on circular cork disks in a mixture of isopentane and dry ice and cryopreserved at −70 C.

Monoclonal Antibodies

γ/δ lymphocytes were identified with a monoclonal antibody recognizing the framework determinant portion of δ molecules.[20] The other monoclonal antibodies used in this study were: OKT3 (CD3), OKT4 (CD4), OKT6 (CD1), OKT8 (CD8), OKT11 (CD2) (Ortho Pharmaceutical, Raritan, NJ), Leu1, Leu 14 (CD22), Leu15 (CD11b), B73.1 (CD16), WT31 (TCRβ) (Becton-Dickinson, Mountain View, CA), B1 (CD20), NKH-1 (Coulter, Hialeah, FL), Ki-67 (DAKO, Santa Barbara, CA) and anti-IL-2R (CD25) (courtesy of Dr. T. Waldmann).

Immunohistochemical Staining

Serial 4-μ thick frozen sections were cut from the cryopreserved tissue blocks, fixed in a cold 1:1 acetone-chloroform mixture for 7 minutes at 4 C, and washed with phosphate-buffered saline (PBS) at room temperature for 20 minutes. The sections were subsequently placed in a humidified chamber where they were overlayered with appropriately titered monoclonal antibodies and incubated for 35 minutes at 37 C. The sections were washed in PBS three times at room temperature, overlayered with $F(ab^1)_2$ goat anti-mouse IgG (Fc γ specific, 1:200, Organon Technike, Malvern, PA), and incubated for 20 minutes at 37 C. The sections were washed in PBS three times at room temperature, incubated with alkaline phosphatase-anti-alkaline phosphatase (APAAP) complex (DAKO, Santa Barbara, CA) for 20 minutes at 37 C, washed in PBS three times at room temperature, and developed for 20 minutes at room temperature on a shaker using New Fuschin and B-napthol-AS-Bi-phosphate as substrate.

Two-color immunohistochemical staining was also performed in cryostat tissue sections. Briefly, after fixation as described above, sections were overlayered with monoclonal antibody Ki-67, anti-IL-2R, or OKT6 for 1 hour at room temperature, washed in PBS, and reacted with biotinylated sheep anti-mouse IgG_1 (1:200) or IgG_{2b} (1:50) immunoglobulin (The Binding Site, San Diego, CA). Peroxidase or glucose oxidase conjugated avidin-biotin complex was applied and developed with diaminobenzidine (DAB) or nitro blue tetrazolium (NBT) as previously described.[21,22] The tissues were subsequently stained with monoclonal anti-δ antibody followed by secondary and tertiary antibodies as described above.

Unrelated monoclonal antibodies (MOPC21 (IgG_1) and MOPC141 (IgG_{2b}) (Sigma Chemical Co., St. Louis, MO) were used as negative controls for the peroxidase, glucose oxidase, and alkaline phosphatase techniques. Negative controls for two-color immunohistochemical staining consisted of serially incubating sections with unrelated monoclonal antibody MOPC21 or MOPC141, sec-

ondary antibody and peroxidase or glucose oxidase complex, developing them with DAB or NBT, and then staining them to detect δ-positive lymphocytes as described above. Similarly, cryostat tissue sections stained for Ki-67, CD1, or CD25 positive cells were reacted with MOPC21 followed by appropriate secondary and tertiary antibodies and then developed with APAAP substrate. These controls allowed us to exclude false-positive staining in both our one- and two-color immunohistochemical staining.

Flow Cytometric Analyses

Flow cytometric analysis was performed using the FACScan fluorescent activated cell sorter (Becton-Dickinson) equipped with an air-cooled Argon laser. At least 2.5×10^4 events were collected in list mode fashion, stored, and analyzed using the CD30 Hewlett Packard (Becton-Dickinson) system. Dead cells and debris were excluded by conventional scatter gating and propidium iodide (PI) staining. Fluoroisothiocyanate, phycoerythrin, and duochrome (DC) emission signals were collected using appropriate filters at 530, 575, and 625 nm, respectively, after logarithmic amplification. Two- and three-color analyses were depicted by graphic (contour) representation of three-dimensional surfaces in which fluorescent intensities of individual cells define the location of cells on 10^4-by-10^4 grids and the frequency of cells of each location define the elevation of that location.

For one-color flow cytometric analysis, 1×10^6 cells were incubated in PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA) in a total volume of 100 μl with predetermined appropriately diluted monoclonal antibody for 30 minutes at 4 C. Following three PBS-BSA washes, the cells were incubated with affinity-purified, solid-phase human immunoglobulin-absorbed, fluoroscein isothiocyanate (FITC)-conjugated $F(ab^1)_2$ fragments of goat anti-mouse immunoglobulin (FITC-$F(ab^1)_2$ fragment GAM immunoglobulin (1:200; Organon Technike, Malvern, PA).

For two- and three-color flow cytometric analysis, 1×10^6 cells were incubated with monoclonal anti-δ antibody followed by FITC-$F(ab^1)_2$ fragment GAM immunoglobulin as described above. After washing, the cells were incubated with mouse serum (diluted 1:10) (Sigma Chemical) for 20 minutes at 4 C and then without washing, phycoerythin- and/or biotin-conjugated antibodies were added. Biotin-conjugated monoclonal antibodies were developed with duochrome streptavidin (DC-avidin). Analogous cells were incubated with purified-, biotin- or phycoerythrin-conjugated isotype-matched monoclonal antibodies followed by FITC-$F(ab^1)_2$ fragment GAM immunoglobulin or DC-avidin, respectively. Multiple double and triple combinations of these isotype-matched monoclonal antibodies with irrelevant specificity were also used as negative controls. Cells stained with single positive fluorescence emission were used as single positive controls to perform a manual electronic compensation.

Cell-cycle analysis was performed as previously described.[23] Briefly, 1×10^6 cells were stained as described above and then fixed with 70% ethanol for 20 minutes at room temperature. After two washes with PBS, the cells were incubated with RNAase, DNAase free (1 mg/ml, Sigma) for 30 minutes at 37 C, washed twice in PBS, and then stained with propidium iodide (PI, 25 μg/ml) for 1 hour. Positive cells were electronically gated and 5×10^3 events were collected. Cell-cycle analysis was performed using Becton Dickinson cell-cycle analysis software.

Results

Immunohistochemical Staining

One-color immunohistochemical staining was performed in cryostat tissue sections prepared from multiple nonpathologic specimens using monoclonal antibodies that detect TCR β and δ chains, T-lymphocyte-associated antigens CD3, CD4, and CD8, and B-lymphocyte-restricted antigen CD22, as described above. δ lymphocytes were identified in all of the sections prepared from all lymphoid tissues studied. However, the proportion of δ lymphocytes varied greatly among the different organs studied. The largest number of δ lymphocytes appeared to be present in spleen and the smallest number in tonsil. δ lymphocytes were always individually dispersed and appeared as small round cells without obvious cytoplasmic projections in these sections. Approximately 90% of δ-thymocytes were located in the thymic medulla but they did not exhibit an apparent relationship with Hassall's Corpuscles. The remaining δ-thymocytes were dispersed throughout the thymic cortex. The vast majority, ≥90%, of lymph node and tonsil δ lymphocytes were present in interfollicular (T-cell) areas and ≤5% were present in germinal centers and their mantle zones. More than 95% of splenic δ lymphocytes were evenly dispersed throughout the red pulp; rare δ lymphocytes were identified in the white pulp. δ lymphocytes were evenly dispersed throughout the gastrointestinal tract. δ lymphocytes were located within the epithelium and lamina propria, without any specific topographic localization within the gastrointestinal wall (Figure 1).

Two-color immunohistochemical staining was performed using monoclonal anti-δ antibody in combination with monoclonal anti-IL-2R (CD25) or Ki-67 to identify activated and proliferating (S_1G_2 phase) δ thymocytes, respectively, and also with OKT6 (CD1) to identify the stage

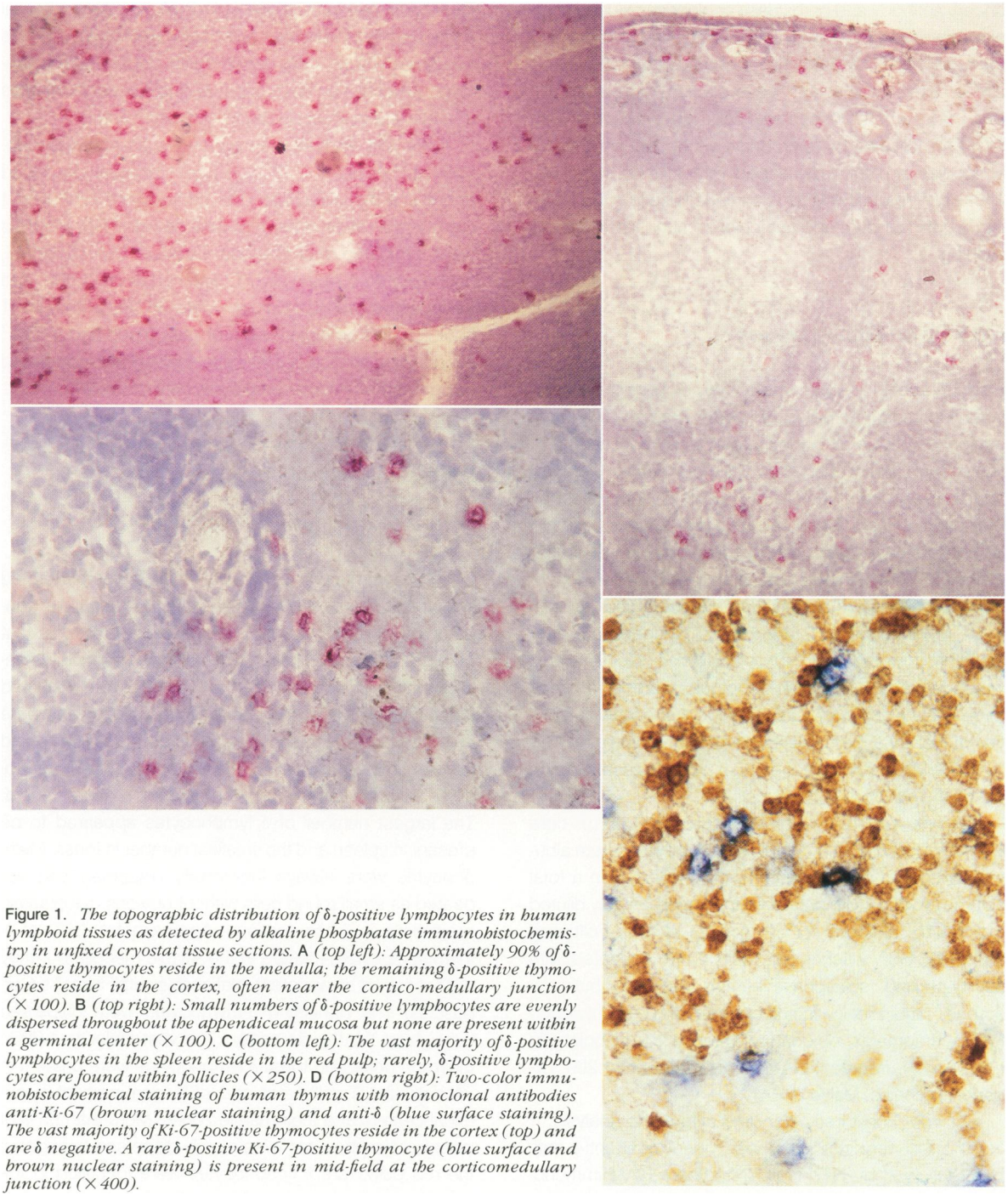


Figure 1. *The topographic distribution of δ-positive lymphocytes in human lymphoid tissues as detected by alkaline phosphatase immunohistochemistry in unfixed cryostat tissue sections.* A *(top left): Approximately 90% of δ-positive thymocytes reside in the medulla; the remaining δ-positive thymocytes reside in the cortex, often near the cortico-medullary junction (× 100).* B *(top right): Small numbers of δ-positive lymphocytes are evenly dispersed throughout the appendiceal mucosa but none are present within a germinal center (× 100).* C *(bottom left): The vast majority of δ-positive lymphocytes in the spleen reside in the red pulp; rarely, δ-positive lymphocytes are found within follicles (× 250).* D *(bottom right): Two-color immunohistochemical staining of human thymus with monoclonal antibodies anti-Ki-67 (brown nuclear staining) and anti-δ (blue surface staining). The vast majority of Ki-67-positive thymocytes reside in the cortex (top) and are δ negative. A rare δ-positive Ki-67-positive thymocyte (blue surface and brown nuclear staining) is present in mid-field at the corticomedullary junction (× 400).*

of maturation of the δ thymocytes. We found that ≥95% of Ki-67-positive thymocytes reside in the cortex, while ≥90% of IL-2R (CD25)- positive thymocytes reside in the medulla. We found that ≤10% of all δ thymocytes express Ki-67 or IL2-R. The Ki-67-positive δ thymocytes exclusively reside in the cortex, while the majority of IL2–R-positive δ thymocytes reside in the medulla. Comparable studies in several spleen and lymph node samples also failed to reveal a significant population of Ki-67- or IL2–R-positive δ lymphocytes. These results suggest that the vast majority of δ lymphocytes are resting cells; only a very small number of δ lymphocytes in the thymus exhibit activation/proliferation antigens (Figure 1). Approximately 10% of δ-thymocytes weakly express CD1 and ≥90% of

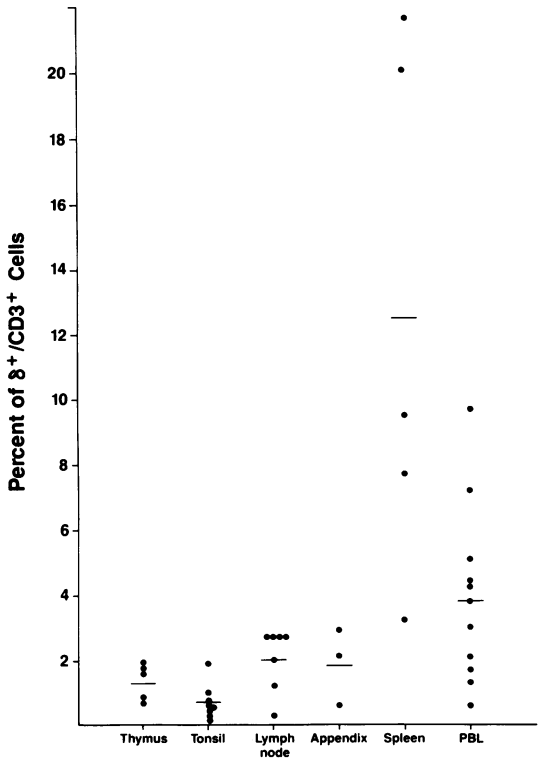


Figure 2. *Percentage of δ-positive CD3 (T) lymphocytes in various lymphoid tissues and peripheral blood based on indirect immunofluorescent flow cytometric analysis of mononuclear cells in suspension. The percentage of δ-positive CD3 (T) lymphocytes is indicated on the y axis. The horizontal bars represent the mean percentage of δ-positive cells for each lymphoid tissue.*

these CD1-positive δ thymocytes reside in the thymic cortex.

Cytofluorometric Analysis

We performed one-color cytofluorometric analysis of mononuclear cells obtained from multiple peripheral blood and lymphoid tissue specimens using a panel of monoclonal antibodies that detect TCR β and δ chains, T-lymphocyte-associated antigens CD3, CD4, and CD8, and B-cell-restricted antigen CD20, as described above. δ lymphocytes were present in all samples analyzed; the spleen contained the highest number and the tonsil the lowest number of δ lymphocytes. The average number of δ lymphocytes in each lymphoid tissue analyzed was as follows: spleen 12.5 ± 8.1%, peripheral blood 4.0 ± 3.1%, appendix 2.9 ± 1%, lymph node 2.2 ± 1%, thymus 1.4 ± 0.5%, and tonsil 0.7 ± 0.5%. The widest range of distribution of δ lymphocytes was in the peripheral blood and spleen, 4 ± 3.1 and 12.5 ± 8.1, respectively. δ-lymphocytes had a considerably smaller range of distribution in the other lymphoid tissues (Figure 2).

We performed two-color cytofluorometric analysis of 10 samples, using an expanded panel of monoclonal antibodies, to further characterize the δ-lymphocyte population (Table 1, Figures 3, 4). We demonstrated that all δ lymphocytes express CD2 and CD3 antigens but that CD5-antigen expression among δ cells is heterogeneous. The majority of δ lymphocytes identified in peripheral blood (Figure 3, Panel F), thymus (Figure 4, Panel I-E′), and tonsil (Figure 4, Panel III-E′) express CD5. However, peripheral blood and tonsil δ lymphocytes are weakly stained for CD5, indicating that they express lesser numbers of CD5 molecules on the membrane per cell. In addition, a distinct subpopulation of δ lymphocytes derived from lymph node (Figure 4, Panel II-E′) and spleen (Figure 4, Panel IV-E′) does not express detectable CD5 molecules (Quadrant 4). The majority of δ-peripheral lymphocytes (57.7 ± 15%) express CD8. In contrast, all peripheral blood and lymphoid tissue δ lymphocytes lack CD4 while 52.5 ± 6.3% of δ-thymocytes express low density CD4. We next performed three-color cytofluorometric analysis to investigate whether CD4 positive δ thymocytes also express CD8 and therefore represent an immature thymocyte population (Figure 5). δ thymocytes were gated and analyzed for both CD4 and CD8 (quadrant 1). The majority of δ-positive thymocytes do not express either CD4 or CD8 molecules. However 30% of all δ thymocytes express low-density CD4 and lack CD8 (quadrant 4), and the remaining δ thymocytes are evenly distributed among $CD4^+$ $CD8^+$ (20%) and $CD4^-$ $CD8^+$ (<10%) subsets (quadrants 2 and 1, respectively). δ-negative thymocytes were also analyzed for CD8 expression (Figure 5, panel B). In this case, the two major populations were $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ (quadrants 2 and 4, respectively). These findings were confirmed in three other thymic samples (data not shown).

The majority of δ-peripheral lymphocytes (≥60%) also express CD11b, Leu7, and NKH-1 (Table 1), antigens that are expressed by suppressor/cytotoxic T cells and natural killer (NK) cells. In contrast, δ lymphocytes lack NK, B-cell, and macrophage–lineage-restricted antigens CD16, CD20, and CD13, respectively. Two-color flow cytometric analysis with monoclonal anti-TRC β (WT31) and anti-TRC δ showed that γ/δ lymphocytes are always weakly positive with WT31, (Figure 3, Panel D). This finding was confirmed in all the assays performed using δ-positive cells derived from both the central and peripheral lymphoid organs.

Cells freshly isolated from three peripheral blood and three thymus samples were stained with monoclonal antibodies that detect TCR β and δ chains and the CD4 and CD8 antigens. After fixation, the cells were incubated with propidium iodide and equal numbers of positive cells were analyzed for their DNA context using positive electronic gating. The large majority of peripheral blood δ-posi-

Table 1. *Delta (δ)-Positive T Lymphocytes in Normal Human Lymphoid Tissues and Peripheral Blood*

	Thymus		Spleen		Lymph node		Tonsil		Peripheral blood	
Antigen	Total %	A	Total %	A	Total %	A	Total %	A	Total %	A
δ	3.2		10.5		1.3		0.3		5.9	
CD3	75	100	42	100	54	100	61	100	85	100
CD2	98	100	47	100	55	100	63	100	85	100
CD5	97	100	42	~70	53	~50	63	100	87	~100
CD4	79	~50	24	0	25	0	58	0	56	0
CD8	60	~30	16	~55	20	80	11	50	32	~60
CD1	75	~70	—	—	—	—	—	—	—	—
CD25	7	<10	3	0	3	<5	—	—	18	<5
CD11b	2	<10	22	~70	—	—	—	—	18.5	~85
Leu7	1	<5	43	~80	—	—	—	—	22	75
NKH-1	0.8	<5	13	~70	0	0	0.7	15	10.5	65
CD16	—	0	4	0	12.7	0	1.5	0	4.0	0
CD20	2	0	51	0	45	0	23	0	13.5	0
CD14	2	0	8	0	5	0	2.5	0	17	0

Mononuclear cells were isolated from representative portions of freshly obtained lymphoid tissues and peripheral blood. The cells were stained with monoclonal anti-δ antibody and developed with $F(ab^1)_2$ fragments of goat anti-mouse immunoglobulin conjugated to fluorocein isothiocyanate. Multiple subpopulations of cells were analyzed using specific monoclonal antibodies directly conjugated with phycoerythrin or biotinylated monoclonal antibodies developed with phycoerythrin-avidin. The total percentage given for each tissue represents the total percentage of cells in that tissue expressing the antigen indicated in the left-hand column. Column A represents the total percentage of δ cells in that tissue expressing the antigen indicated in the left-hand column. All numbers represent percentages.

tive cells are in G_0-G_1 phase (93.5 ± 2.5%) and their cell-cycle distribution is similar to that of peripheral blood α/β cells (93.5 ± 2%). In contrast, a distinct subpopulation, corresponding to approximately 25%, of δ-thymocytes is in S_1-G_2 phase.

Finally, we performed multiple three-color flow cytometric analyses of lymphocytes obtained from two peripheral blood donors to further characterize the multiple subpopulations of δ lymphocytes. These analyses confirm that δ lymphocytes are always CD3 positive but that a small subpopulation of CD5-negative δ lymphocytes exists. Moreover, we found that not all $CD3^+$ $CD5^-$ lymphocytes (only 2% of all $CD3^+$ lymphocytes) belong to the δ-lymphocyte population. In fact, $CD5^-$ δ lymphocytes rep-

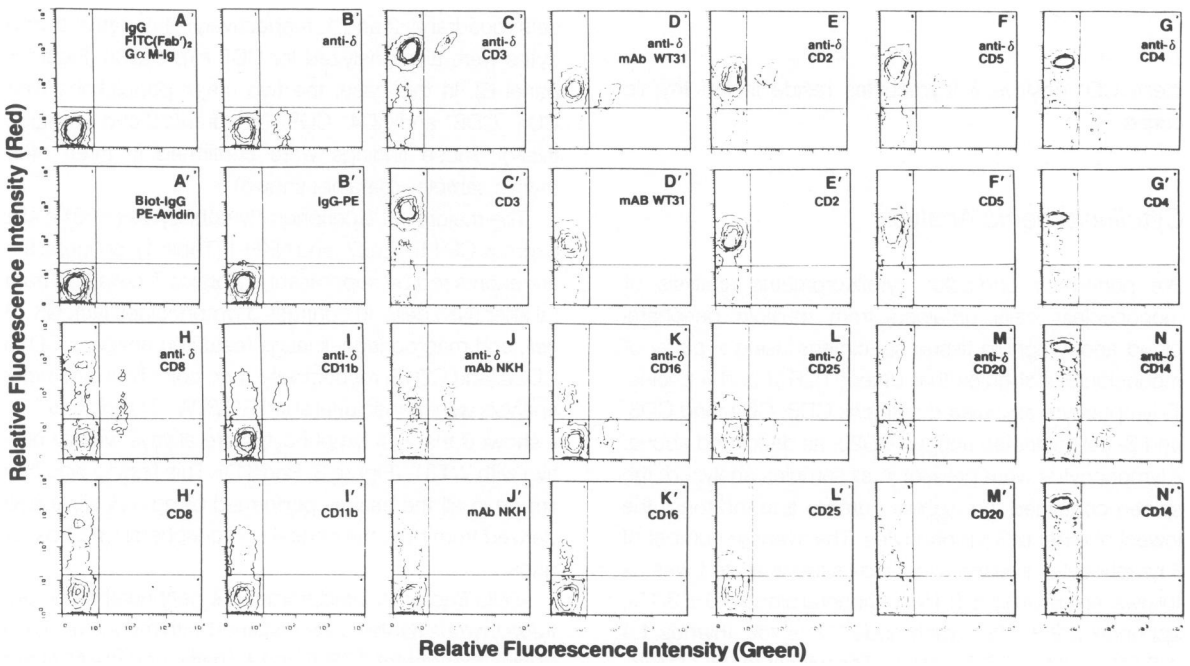


Figure 3. *Identification of peripheral blood mononuclear cells expressing γ/δ receptors based on indirect immunofluorescent flow cytometric analysis. Peripheral blood mononuclear cells were incubated with monoclonal anti-δ antibody and/or monoclonal antibodies specific for antigens associated with various lymphoid cell subpopulations. Green fluorescence (δ) and red fluorescence (other monoclonal antibodies) intensities were determined and contour maps were derived. The thresholds of positivity for green (vertical line) and red (horizontal line) fluorescence intensity were established on the basis of negative control samples incubated with unrelated monoclonal antibodies.*

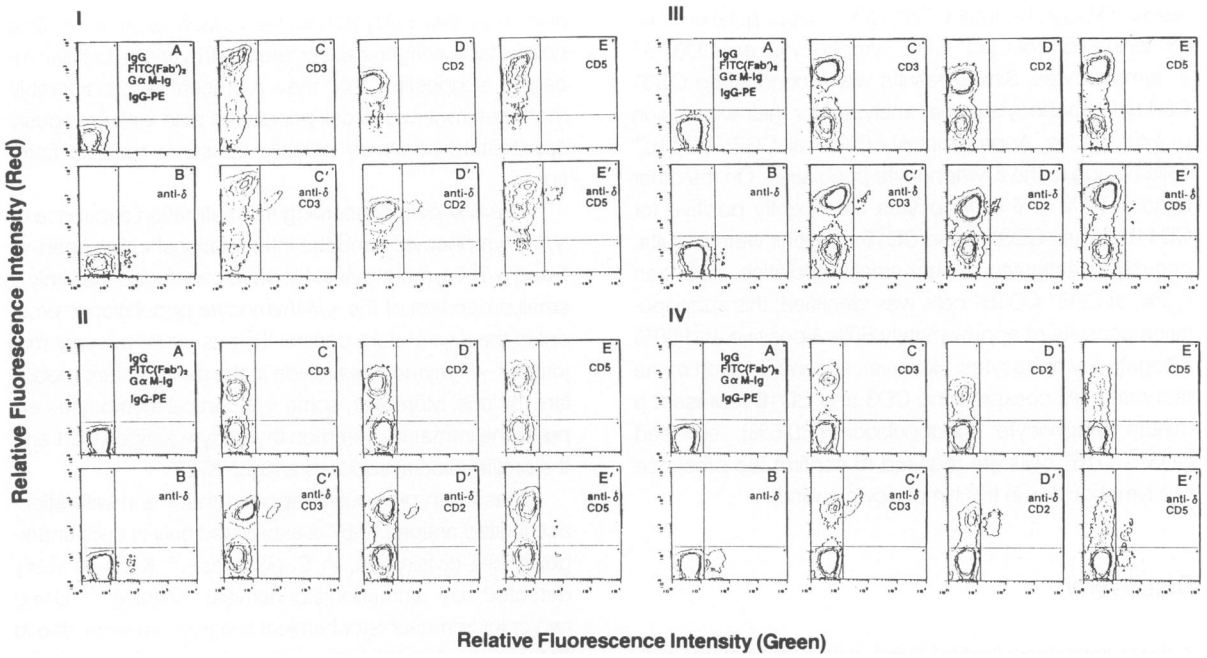
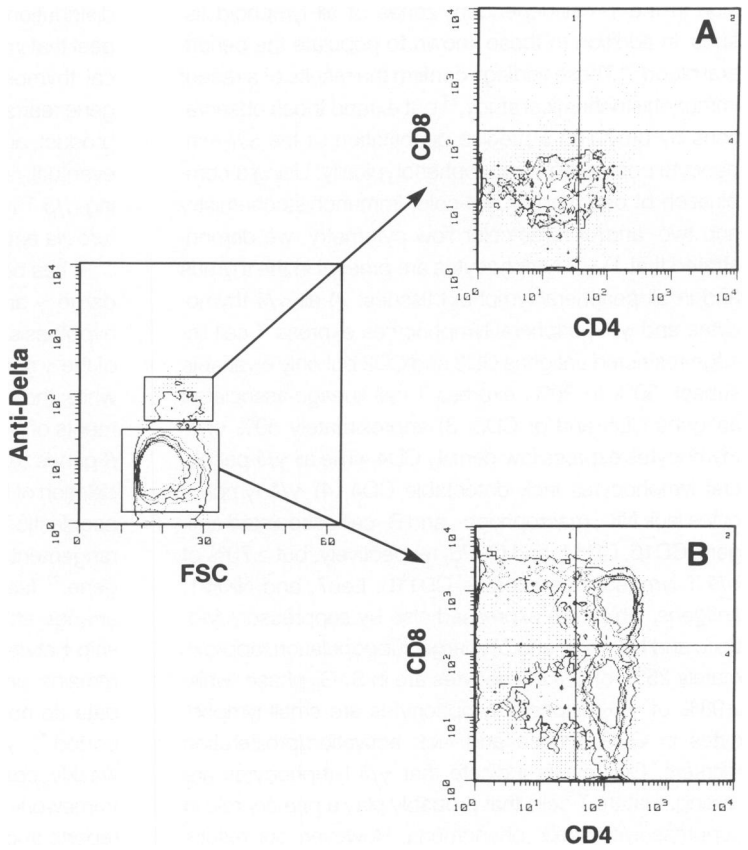


Figure 4. *Immunofluorescent flow cytometric analysis of pan-T-cell antigen expression by γ/δ T lymphocytes. Lymphoid cells derived from normal thymus (panel I), lymph node (panel II), tonsil (panel III) and spleen (panel IV) were incubated with monoclonal anti-δ antibody and/or monoclonal antibodies specific for CD3, CD2, and CD5 pan-T-cell antigens. Green fluorescence (δ) and red fluorescence (CD3, CD2, and CD5) intensities, depicted on the x and y axes, respectively, were determined and contour maps were derived. The thresholds of positivity for green (vertical line) and red (horizontal line) fluorescence intensities were established in each tissue sample on the basis of negative control samples stained with unrelated, isotype-matched, labeled monoclonal antibodies (quadrants A).*

Figure 5. *Presence of δ-positive CD4 thymocytes and absence of δ-positive CD8 thymocytes based on indirect immunofluorescent flow cytometric analysis. Thymocytes were stained with monoclonal anti-δ, anti-CD4 and anti-CD8 antibodies. The thresholds of positivity were established on the basis of negative control samples of cells incubated with isotype-matched monoclonal antibodies. Forward angle light scatter (linear scale) is plotted against green fluorescence (anti-δ) in the left quadrant. Electronic gates on δ-positive and δ-negative cells are represented by inserts A and B, respectively. The correlation between CD4 and CD8 and δ-positive and δ-negative cells are displayed in the right quadrants.*



resent 35% of the total $CD3^+$ $CD5^-$ subpopulation. The remaining 65% of $CD3^+$ $CD5^-$ lymphocytes are $CD3^+$ β^+ δ^- lymphocytes. Similar results were found when $CD3^+$ $CD11b^+$ lymphocytes were analyzed for their expression of δ molecules. Approximately 50% of all $CD3^+$ $CD11b^+$ cells belong to the δ lymphocyte population. On the other hand, ≥70% of δ lymphocytes are brightly positive for CD11b. When CD3, δ, and CD16 antigens were simultaneously investigated, a very small population, less than 0.5%, of $CD3^+$ $CD16^+$ cells was identified; this subpopulation consists of approximately 50% δ-positive and 50% δ-negative lymphocytes. However, in consideration of the fact that cells coexpressing CD3 and CD16 represent a minute lymphocyte subpopulation (<0.5%), enriched $CD3^+$ $CD16^+$ cells are required to confirm the presence of δ lymphocytes in this lymphocyte subset.

Discussion

In the studies described here, we used a monoclonal antibody recognizing a framework determinant of the TCR δ chain in conjunction with flow cytometry and immunohistochemistry to determine the quantitative and topographic distribution of γ/δ lymphocytes in normal lymphoid tissue. Our results demonstrate that variable, but generally small, numbers of γ/δ lymphocytes normally reside in the T-cell-dependent zones of all lymphoid tissues, in addition to those known to populate the peripheral blood.[14] These findings confirm the results of a recent immunohistochemical study,[24] but extend those observations by providing a precise quantitation of the γ/δ-lymphocyte population immunophenotypically. Using a combination of one- and/or two-color immunohistochemistry and two- and/or three-color flow cytometry, we demonstrated that 1) γ/δ lymphocytes are present in the thymus and in all peripheral lymphoid tissues; 2) all γ/δ thymocytes and γ/δ-peripheral lymphocytes express T-cell lineage-restricted antigens CD3 and CD2 but only a variable subset, 30% to 90%, express T-cell lineage-associated antigens CD5 and/or CD8; 3) approximately 50% of/δ-thymocytes express low-density CD4 while all γ/δ-peripheral lymphocytes lack detectable CD4; 4) γ/δ lymphocytes lack NK-, macrophage-, and B-cell-associated antigens CD16, CD14, and CD20, respectively, but ≥70% of γ/δ T lymphocytes express CD11b, Leu7, and NKH-1, antigens, which are expressed also by suppressor/cytotoxic and NK cells; and 5) a large subpopulation, approximately 25%, of γ/δ-thymocytes are in S_1-G_2 phase, while ≥98% of γ/δ-peripheral lymphocytes are small lymphocytes in G_0-G_1 phase and lack activation/proliferation markers. Our results indicate that γ/δ lymphocytes are resting, mature T cells that probably play a primary role in suppressor/cytotoxic phenomena. However, our results also show that γ/δ lymphocytes variably express multiple cell-surface antigens associated with various cellular lineages, suggesting that they represent a considerably more heterogeneous cell population than was previously appreciated and may actually subserve multiple functions.

Little is known concerning the maturation sequence of γ/δ T lymphocytes and the inter-relationship between γ/δ and α/β T lymphocytes. However, we found that only a small proportion of the γ/δ-thymocyte population is present in the less mature cortical thymus, while the vast majority of γ/δ thymocytes reside in the more mature medullary thymus. Moreover, some γ/δ cortical thymocytes express the immature common thymocyte antigen CD1 and the proliferation-associated antigen Ki-67.

It has been previously reported that the proliferation-associated antigen Ki-67 is expressed only in cells undergoing cell division, ie, in S_1-G_2 phase.[25] Ki-67 is easily detected by immunohistochemical staining.[21] Using two-color immunohistochemical analysis, we were able to detect a small but definite subpopulation of δ thymocytes preferentially localized to the thymic cortex that express CD1 and Ki-67. We obtained similar results when CD1-positive δ thymocytes were investigated by flow cytometric analysis to determine the percentage of δ cells in S_1-G_2 phase (data not shown). Interestingly, proliferating α/β thymocytes and γ/δ thymocytes are present in the same distribution with the thymus. Together these findings suggest that immature γ/δ T lymphocytes residing in the cortical thymus probably first undergo productive γ and δ gene rearrangement, leading to an efficient TCR γ/δ gene product, and then proceed into the medullary thymus and eventually into the peripheral blood, analogous to maturing α/β T lymphocytes. Thus, γ/δ lymphocytes may mature *via* pathways similar to those of α/β T lymphocytes.

It has been postulated that any T lymphocyte may undergo γ and δ gene rearrangement.[13] According to this hypothesis, cells undergoing productive rearrangements of the γ or the δ gene become mature γ/δ T lymphocytes, while those that fail to undergo productive rearrangements of both the γ and the δ genes rearrange the α and β genes to become mature α/β T lymphocytes,[11,26] with deletion of the δ gene during the process. However, some neoplastic T cells exhibiting TCR α and β chain gene rearrangements do not display structural changes of the δ gene,[27] leading to speculation that the δ gene may rearrange after the α and β genes rearrange. The relationship between α/β and γ/δ cells is not yet resolved and remains under active investigation. Immunophenotypic data do not help in this regard. In fact, as previously reported,[14] γ/δ lymphocytes were always proved to be weakly positive with antibodies specific for the TCRβ framework determinant. Our data confirm these previous reports and leave the controversy unresolved. However,

one reasonable explanation is that monoclonal antibodies against TCR β and δ framework determinants recognize highly homologous amino acid sequences. Obviously, only RNA and immunoprecipitation assays will resolve this crucial point.

Although we were unable to detect a CD4 antigen-positive peripheral γ/δ T-lymphocyte subpopulation, we were able to demonstrate the existence of a small population of low-density $CD4^+$ $CD8^-$ γ/δ thymocytes. Our finding of a human $CD4^+$ $CD8^-$ δ-thymocyte population is supported by the recent demonstration of $CD4^+$ $CD8^-$ δ thymocytes in the thymus of neonatal and young mice.[28] At this time we do not have any information regarding the stage of differentiation and the possible role played by $CD4^+$ $CD8^-$ δ thymocytes. It could be postulated that these cells represent the immature developmental stage of an extremely small subpopulation of peripheral CD4-positive γ/δ thymocytes or alternatively, that CD4-positive γ/δ T lymphocytes either do not leave the thymus or die immediately on leaving the thymus. The first hypothesis is supported by recent findings.[28,29] However, this issue is still a matter of controversy because many other investigators have failed to identify human peripheral δ lymphocytes bearing CD4 molecules.[14,19]

Within the peripheral lymphoid tissues, γ/δ lymphocytes are preferentially distributed within T-cell-dependent zones and are rarely present in the germinal centers. However, γ/δ-lymphocyte distribution within the gastrointestinal tract is of particular interest. The vast majority of murine lymphocytes homing to the gut are γ/δ lymphocytes,[30] and in the avian species these γ/δ lymphocytes are preferentially localized to the epithelium.[31] Our findings confirm that γ/δ lymphocytes are also present in the human gastrointestinal tract. However, there are two major differences. First, δ lymphocytes represent a small subpopulation of the CD3-positive T cells in the human gut. Second, δ lymphocytes are randomly distributed within both the epithelium and the lamina propria. These data are in total agreement with recently reported results.[29]

The recent identification of a small population, less than 1%, of CD3-positive T lymphocytes that bear Fc receptors for IgG (CD16)[32] prompted us to investigate γ/δ T lymphocytes for the expression of CD16, but we were unable to conclusively identify a CD16 positive γ/δ T-lymphocyte subset. However, because γ/δ lymphocytes represent a small subset of CD3-positive T lymphocytes, and CD16-positive lymphocytes are a minute subpopulation of CD3-positive cells, we cannot exclude the possibility that some γ/δ T lymphocytes with dim fluorescent intensity for CD16, equating with low numbers of receptors, exist. Also, we cannot exclude the possibility that some γ/δ lymphocytes will express the CD16 antigen on stimulation; for example, culturing in the presence of interleukin-2. Alternatively, γ/δ T lymphocytes may express a different Fc γ receptor.

The functional role(s) of the γ/δ-lymphocyte population is incompletely understood at this time. δ lymphocytes are involved in MHC-restricted cytotoxicity[33,34] and their TCR does not play a crucial role in antigen recognition.[35] However, the majority of γ/δ lymphocytes express antigens associated with cytotoxic/suppressor functions when expressed on α/β lymphocytes. This strongly suggests that γ/δ lymphocytes are primarily involved in cytotoxic/suppressor phenomena. It is particularly interesting in this regard that a large proportion of γ/δ lymphocytes express CD11b, the C3bi receptor.[36] This receptor is also expressed on mature T cells exhibiting suppressor function and on macrophages[37] and has been postulated to play an important role in opsinization.[38] Thus, it is possible that this molecule is involved with the γ/δ TCR in the recognition of target antigens. Obviously, the functional analysis of purified γ/δ lymphocytes and their various subsets will be necessary to substantiate these hypotheses.

Finally, based on the concept that malignant transformation results in freezing a target cell at a given stage in its differentiation pathway, our immunophenotypic data concerning γ/δ lymphocytes may prove useful in recognizing and characterizing the neoplastic counterpart of the benign, normal γ/δ-lymphocyte population. It has been shown recently that while peripheral T-cell lymphomas do not express γ/δ receptors, approximately 10% of thymic lymphomas do express γ/δ receptors.[24] However, because γ/δ lymphocytes represent a small T-lymphocyte subpopulation, more extensive studies of larger numbers of cases are necessary to confirm these findings. Furthermore, virtually nothing is known concerning γ/δ receptor expression by T-cell leukemias. Nonetheless, it does not seem unreasonable to speculate that some cases of Tγ-lymphoproliferative disease (Tγ-LPD) probably represent the malignant counterpart of the γ/δ-lymphocyte population. Tγ-LPD represents a heterogeneous group of neoplasms consisting of a minority of cases of true NK cell origin (CD3 antigen negative, TCR β germline) and a majority of cases exhibiting a combination of immunophenotypic, functional, and immunogenotypic properties seen in T cells displaying cytotoxic properties. Extensive immunophenotypic, functional, and molecular genetic analysis of neoplasms derived from the γ/δ-lymphocyte population should allow us to better understand the origin, nature, and normal physiologic functional roles of the γ/δ T-lymphocyte population and its subsets.

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