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T Lymphocytes in the Murine Vaginal Mucosa Are Phenotypically Distinct from Those in the Periphery

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The results from both clinical studies of women with recurrent vulvovaginal candidiasis and a murine model of experimental vaginitis indicate that systemic cell-mediated immunity may not represent a dominant host defense mechanism against vaginal infections by *Candida albicans*. Recent experimental evidence indicates the presence of local vaginal immune reactivity against *C. albicans*. The present study was designed to examine T-lymphocyte subpopulations in the vaginal mucosae of naive CBA/J mice. Vaginal lymphocytes (VL) were isolated by collagenase digestion of whole vaginal tissues. Cell populations were identified by flow cytometry, and the results were compared with those for both lymph node cells (LNC) and peripheral blood lymphocytes (PBL). The results of flow cytometry showed that 45% ± 10% of lymphocytes in the vaginal mucosa are $CD3^+$ compared with 75% ± 5% in LNC and 50% ± 5% in PBL. The majority (85%) of $CD3^+$ VL are $CD4^+$ and express the α/β T-cell receptor (TCR), similar to the results for LNC and PBL. In contrast to LNC and PBL, VL contain a significantly higher percentage (15 to 20%) of γ/δ TCR^+ cells, 80% or more of which appear to express CD4. In addition, while CD4-CD8 cell ratios in LNC and PBL were 3:1 and 6:1, respectively, only 1% of VL expressed CD8, resulting in a CD4-CD8 cell ratio of >100:1. Finally, while LNC and PBL recognized two epitope-distinct (GK 1.5 and 2B6) anti-CD4 antibodies, VL recognized only 2B6 anti-CD4 antibodies. Further analysis of VL showed that Thy-1 cells, but not CD4 cells, were reduced after intravaginal injection of complement-fixing anti-Thy-1.2 and GK 1.5 anti-CD4 antibodies, respectively. Taken together, these data suggest that T lymphocytes in the vaginal mucosae of mice are phenotypically distinct from those in the periphery and that $CD4^+$ VL have an uncharacteristic or atypical expression of the CD4 receptor.

Recurrent vulvovaginal candidiasis (RVVC) is an opportunistic mucosal infection caused by *Candida albicans* that affects up to 5% of otherwise healthy women of child-bearing age (26). While the use of antibiotics and oral contraceptives predisposes healthy women to acute VVC (26), the underlying factors contributing to RVVC are largely unknown. The high incidence of mucosal candidiasis in patients with reduced cell-mediated immunity (CMI), (i.e., AIDS [16, 21]), transplantation [3], and corticosteroid therapy [17]) strongly suggests that deficiencies in CMI play a role in the etiology of RVVC. However, studies examining systemic CMI in the peripheral blood of women with RVVC have not shown a distinct pattern of reduced activity (7, 12, 27). Moreover, although reductions in systemic *Candida*-specific responsiveness have been observed in some instances (7, 12, 13, 29, 30), these changes are likely to be the consequences and not the causes of RVVC (7). Since systemic CMI in women with RVVC is usually within normal levels, it is logical to investigate local vaginal CMI reactivity. This is important in light of the fact that clinical experience shows that women with RVVC are not susceptible to oral or esophageal candidiasis (26), while women with chronic mucocutaneous candidiasis are generally not susceptible to vaginal candidiasis (23). We therefore postulate that changes in local vaginal immune host defenses predispose to RVVC. This hypothesis is supported by increasing evidence for compartmentalized CMI reactivity in other mucosal tissues, including the gastrointestinal tract (5, 25, 31), oral cavity (20), and reproductive tract (14, 15, 22, 24).

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Our laboratory has been studying the host defense mechanisms important for protection against vaginal *C. albicans* infections through an estrogen-dependent murine model of vaginal candidiasis. The results indicate that a localized vaginal *C. albicans* infection stimulates *Candida*-specific systemic Th1-type CMI (8, 9), but preinduced *Candida*-specific systemic CMI is not protective against vaginal candidiasis (10). In contrast, mice given a primary vaginal *C. albicans* infection that spontaneously resolves (in the absence of estrogen) are partially protected against a second vaginal *C. albicans* infection under conditions of pseudoestrus (6), but this is not systemically derived. Furthermore, whereas in vivo depletion of peripheral CD4 and CD8 T cells with complement-fixing antibodies had no effect on the natural history of primary or secondary vaginal *C. albicans* infections, depletion of CD4 T cells significantly abrogated systemic *Candida*-specific Th1-type reactivity in infected mice (11). These results support the clinical observations and suggest that systemic CMI is not a major host defense mechanism at the vaginal mucosa and that a local (compartmentalized) acquired mucosal immune response is the critical protective mechanism against vaginal *C. albicans* infections.

Except for protection experiments in animals, there is a paucity of information regarding CMI at a local cellular level within the vaginal mucosa. The present study focused on the identification of the T-cell populations present in the vaginal mucosae of naive mice to determine and realize the potential for a local CMI response against *C. albicans*. While a limited number of studies have examined some aspects of vaginal T lymphocytes in mice (15, 22), a definitive survey or profile of specific T-cell subpopulations has not been performed for vaginal tissue. To address this, vaginal lymphocytes (VL) isolated by collagenase digestion of vaginae were examined by flow

cytometry for the expression of CD3, CD4, CD8, and the T-cell receptor (TCR) (α/β or γ/δ).

MATERIALS AND METHODS

Mice. Female CBA/J (*H-2*^{k}) mice, 8 to 10 weeks of age and purchased from Jackson Laboratories, Bar Harbor, Maine, were used throughout these studies. All animals were housed and handled according to institutionally recommended guidelines. The mice from Jackson Laboratories were not colonized by *C. albicans*, as detected by quantitative culture of stool samples (6) and vaginal lavage fluids (8) or by positive delayed-type hypersensitivity after challenge with *C. albicans* culture filtrate antigens (8).

Antibodies. Phycoerythrin (PE)- and biotin-conjugated antibodies specific for mouse CD3 (pan-T), CD4 (L3T4), CD8α (Lyt 2), CD8β (Lyt 3), Thy-1.2, and the α/β and γ/δ TCR were purchased from Pharmingen Corp., San Diego, Calif. Biotin-conjugated antibodies were identified with cy-chrome-conjugated streptavidin (Pharmingen). The anti-CD4 antibodies were from two different clones with different epitope specificities (RM-4.5, which blocks GK 1.5 antibody epitope specificity, and RM-4.4, which blocks 2B6 antibody epitope specificity). Control antibodies corresponding to the source of murine antibodies included rat immunoglobulin G2a (IgG2a), rat IgG2b, and hamster IgG (Pharmingen). Complement-fixing antibodies specific for CD4 (IgG2b) and Thy-1.2 (IgG2b) were obtained from the GK 1.5 and 30-H12 hybridoma cell lines (American Type Culture Collection, Rockville, Md.), respectively. The cell lines were grown in bulk culture, and the antibodies were collected by ammonium sulfate precipitation. Antibodies were concentrated 20-fold in phosphate-buffered saline (PBS). Fluorochrome-conjugated anti-Thy-1.2 antibodies used to detect Thy-1$^+$ cells by flow cytometry had an epitope specificity different than that from the hybridoma cell line used for in vivo injection.

Isolation of peripheral cell populations. Lymph node cells (LNC) (inguinal, mesenteric, and pelvic) and peripheral blood lymphocytes (PBL) were used as control cell populations for comparisons of cell surface antigen expression. Lymph nodes were made into single-cell suspensions by passage through a sterile mesh screen as previously described (9). Cells were resuspended in Hanks' balanced salt solution (HBSS) and counted by trypan blue dye exclusion. Blood samples from mice were collected by orbital bleeding in a heparinized tube. PBL were isolated by differential centrifugation using lymphocyte Accu Prep (Accurate Chemical and Scientific Co., Westbury, N.J.), washed, resuspended in HBSS, and counted as described above. For each experiment, LNC were generally pooled from 5 mice, while 10 mice were used for the isolation of PBL.

Isolation of VL. The vagina was excised, the cervix was removed, and the remaining vaginal tissue was cut longitudinally and minced with a sterile scalpel in complete tissue culture medium consisting of RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (2 mM), HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 20 mM), and 5% heat-inactivated fetal calf serum (FCS) (all from GIBCO, Grand Island, N.Y.). Minced tissues (epithelium and lamina propria) were digested in complete medium with sterile 0.25% collagenase type IV (Sigma Chemical Co., St. Louis, Mo.). Digestion was accomplished with shaking incubation at 37°C for 30 min. Before, during, and immediately after the incubation period, samples were subjected to 10-s intervals of more severe agitation in a stomacher homogenizer (Tekmar Inc., Cincinnati, Ohio). After digestion, tissues and cells were filtered through a sterile gauze mesh and washed with RPMI 1640 medium, and additional tissue debris was excluded by slow-speed centrifugation ($200 \times g$) for 1 min. Cells were collected from the supernatant by centrifugation at $800 \times g$, resuspended in HBSS, and counted by trypan blue dye exclusion. Approximately 10^5 VL were collected per mouse. Ten mice were usually employed per experiment. Random selection of mice did not distinguish between stages of the menstrual cycle, and thus the data presented represent those for a pool of cells from all stages of the menstrual cycle. The reproducibility of results showed that appropriate sampling was obtained, and phenotypic results did not differ in isolated experiments in which the majority of randomly selected animals were either in estrus or not in estrus.

In specific experiments, LNC were used as controls for the effects of collagenase. These included analyses of LNC after the incubation of 2×10^7 LNC in 0.25% collagenase with shaking and homogenization at 37°C and the addition of 2×10^7 LNC to vaginal tissue that was subsequently incubated and homogenized in 0.25% collagenase. A third control examined the potential for antigen reexpression on VL in case surface antigens on vaginal cells were affected during the isolation process. For these experiments, collagenase-treated vaginal cells were incubated for 18 h at 4 or 37°C before phenotypic analyses were performed.

Flow cytometry. Standard methodology was employed for direct and indirect immunofluorescence of VL. Briefly, 10^5 VL, 1×10^5 to 2×10^5 PBL, or 10^6 LNC were pelleted in Eppendorf tubes and then incubated for 30 min on ice with 1 μg of fluorochrome- or biotin-conjugated antibodies diluted in 100 μl of PBS–2% FCS (PBS-FCS). The cells were washed twice with 250 μl of PBS-FCS after incubation. Biotin-labeled cells were incubated for an additional 30 min on ice with cy-chrome-conjugated streptavidin (0.05 μg) and similarly washed with PBS-FCS. The samples were analyzed by a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) by trained personnel at MLPCCCMD Analytical Cytometry Facility at Wayne State University. Cells incubated with rat IgG2a, rat IgG2b, or hamster IgG isotype control antibodies were used to determine the background fluorescence. Compensation for each fluorochrome was determined by parallel single-color analysis of cells labeled with one of each fluorochrome-conjugated antibody. The gating limits for the analysis of VL were determined from an analysis of positive-staining cells recovered after the addition of 2×10^7 LNC to the VL isolation protocol. Data are expressed in histograms of fluorescence intensity versus the number of fluorescing cells, indicating the percentage of the cell population positive for a specific cell surface antigen. Whereas the background fluorescence for LNC was generally 2 to 4%, VL consistently fluoresced at 8 to 10% but without resolution of any defined cell population similar to those obtained with antibodies to specific antigenic markers. Therefore, the isotype control antibodies included in each experiment were considered the true baseline fluorescence used to evaluate and illustrate the results for the T-cell-specific antigen markers.

In vivo cellular depletion. Mice were randomized to receive local intravaginal injections of anti-Thy-1.2 or GK 1.5 anti-CD4 antibodies. Mice were injected in vaginal tissue at each of two sites with 50 μl (100 μg) of antibody in PBS every 2 to 3 days for 1 week (600 μg of antibody). Control animals received injections of PBS. VL and LNC were collected after this 1-week period and analyzed by flow cytometry for surface marker expression as described above. Seven mice were used per antibody treatment.

RESULTS

Identification of VL by flow cytometry. VL isolated from collagenase-digested vaginal tissue were labeled with biotin-conjugated anti-CD3 antibodies and subsequently with cy-chrome-conjugated streptavidin and analyzed by flow cytometry. The results are illustrated in Fig. 1. The results of light scatter analysis (Fig. 1A) are shown, with the location of cells staining positive for cy-chrome-conjugated anti-CD3 antibodies shown in red. CD3$^+$ cells represented 12% of the total vaginal cells isolated. For subsequent analyses, the cell populations monitored for fluorescence were restricted to the region containing the majority of CD3$^+$ cells (circled in Fig. 1A) and defined as the lymphoid-like cell compartment, recognizing that fewer CD3$^+$ cells were sporadically detected outside the gated region. By using these parameters, T cells, as determined by anti-CD3 antibody staining, were found to constitute approximately 50% of the lymphoid cell compartment (Fig. 1B). The background fluorescence (Fig. 1C and D), as determined by cellular staining with isotype control antibodies, was found to be approximately 9 to 12% with rat IgG2a antibodies, 8% with rat IgG2b antibodies, and negligible with hamster IgG antibodies. As shown, specific fluorochromes (cy-chrome and PE) had no effect on background staining since interchanging antibodies and fluorochromes showed similar staining patterns (Fig. 1C and D). By using similar staining procedures, purified LNC were 75% ± 5% CD3$^+$ and PBL were 50% ± 5% CD3$^+$, with isotype control antibodies showing 1 to 2% positive staining with either cell type (data not shown).

TCR expression of VL. LNC, PBL, and vaginal cells were dual labeled with biotin-conjugated anti-CD3 antibodies followed by cy-chrome-streptavidin and either PE-conjugated anti-α/β or PE-conjugated anti-γ/δ TCR antibodies and analyzed by flow cytometry. The fluorescence results for lymphoid-like vaginal cells (defined above) are shown in Fig. 2. Greater than 80% of CD3$^+$ VL expressed the α/β TCR (56% of lymphoid-like cells) (Fig. 2A); the remaining 20% of CD3$^+$ VL (9% of lymphoid-like cells) expressed the γ/δ TCR (Fig. 2B). The CD3$^+$ α/β and γ/δ TCR$^+$ cells in the lymph nodes and PBL were 97 and 3%, respectively (data not shown).

CD4 and CD8 expression on VL. LNC, PBL, and vaginal cells were dual labeled with PE- or biotin-conjugated anti-α/β or anti-γ/δ TCR antibodies together with either anti-CD4 or anti-CD8α (Lyt 2) antibodies. The results for PE- and cy-chrome-fluorescent lymphoid-like vaginal cells are illustrated in Fig. 3. Within the lymphoid-like cell limits, 38% were α/β TCR$^+$ CD4$^+$ (Fig. 3A), 6% were γ/δ TCR$^+$ CD4$^+$ (Fig. 3B), 2% were α/β TCR$^+$ CD8$^+$ (Fig. 3C), and <1% were γ/δ

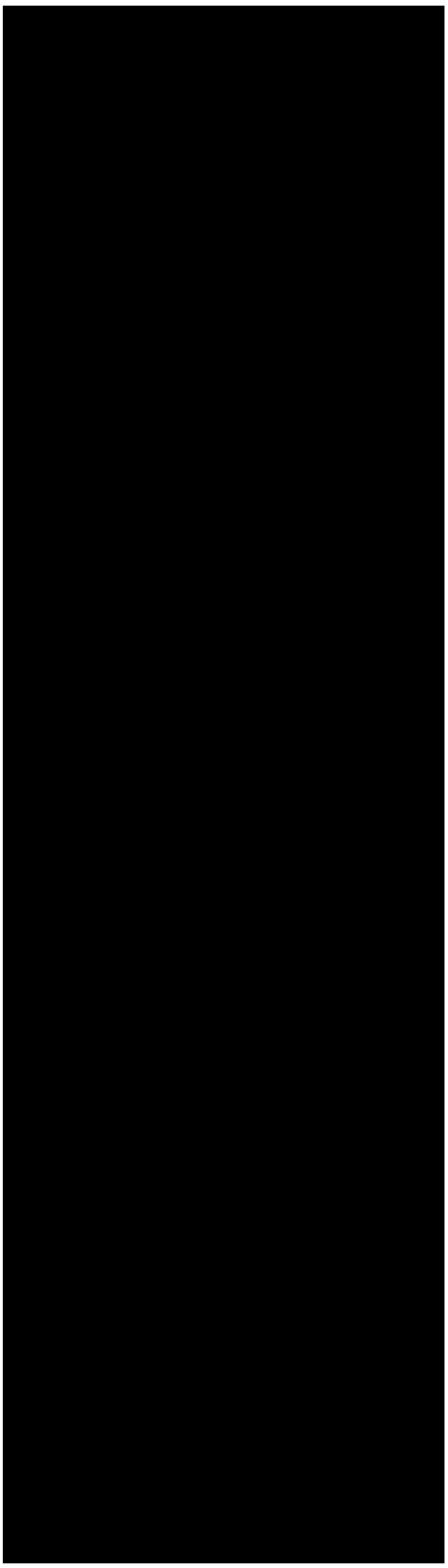

FIG. 1. Identification of VL by flow cytometry. Cells (10^5) isolated from collagenase-digested vaginae were labeled with biotinylated anti-CD3 antibodies and subsequently with cy-chrome (CYC)-conjugated streptavidin. Control samples were labeled with PE-conjugated hamster (HAM) IgG and PE- or CYC-conjugated rat IgG isotype control antibodies. The labeled samples were analyzed by flow cytometry on the basis of gated regions determined by analysis of cells recovered from the isolation of vaginal cells in the presence of LNC. (A) 90° versus forward light scatter analysis (size and granularity); (B) CYC fluorescence, representing $CD3^+$ cells; (C and D) background fluorescence of isotype control antibodies. Percentages represent fluorescent positive cells within the limits circled in panel A (lymphoid-like cells). Data shown are of a representative experiment of no less than five repeats using 10 mice per experiment. FL2, second fluorescent label.

TCR^+ $CD8^+$ (Fig. 3D). The total distribution of CD4 and CD8 cells (Fig. 3E) showed that 40% of VL were $CD4^+$, while <1% expressed CD8, including negligible cells expressing both CD4 and CD8. The results for LNC and PBL, as expected, showed that α/β TCR^+ cells expressed either CD4 or CD8, while γ/δ TCR^+ cells were $CD4^-$ $CD8^-$ (data not shown). In contrast to the CD4-CD8 cell ratios in LNC and PBL (3:1 and 6:1, respectively), the CD4-CD8 cell ratio in the vagina was >100:1. Taken together, these data show that (i) the majority of vaginal cells are $CD4^+$ and express the α/β TCR, (ii) the majority of vaginal γ/δ T cells also express CD4, and (iii) few vaginal cells express CD8.

To further examine the observed lack of expression of CD8 on VL, experiments were designed to assess the potential for atypical expression of the α- and/or β (Lyt 3)-chains of the CD8 receptor on VL. For this, both LNC and vaginal cells were dual labeled with PE- or biotin-conjugated anti-CD8α and anti-CD8β antibodies. The results show that 90% of $CD8^+$ cells in the lymph nodes (10% of all LNC) expressed both the α- and β-chains of the CD8 receptor (Fig. 4A), while VL showed negligible expression of either chain alone and only 1% were positive for both chains (Fig. 4B).

During our preliminary analyses of CD4 expression on vaginal cells, we observed that anti-CD4 antibodies of different epitope specificities recognized lymphoid-like vaginal cells differentially. Specifically, while VL did not bind anti-CD4 antibodies specific for the epitope recognized by GK 1.5 anti-CD4 antibodies, VL did bind anti-CD4 antibodies specific for the epitope recognized by 2B6 anti-CD4 antibodies. To examine this interesting staining pattern more closely, LNC, PBL, and vaginal cells were dual labeled with anti-CD4 antibodies of

FIG. 2. TCR expression on VL. Cells (10^5) isolated from collagenase-digested vaginae were labeled with biotinylated-conjugated anti-CD3 antibodies, cy-chrome (CYC)-conjugated streptavidin, and PE-conjugated anti-α/β or anti-γ/δ TCR antibodies and analyzed by flow cytometry. Gated regions for analysis were determined by positive-staining cells recovered from vaginal cell isolation in the presence of LNC. (A) $CD3^+$ versus α/β TCR^+ cells; (B) $CD3^+$ versus γ/δ TCR^+ cells. The percentage in each quadrant represents the fluorescent positive cells within the lymphoid-like cell limits (Fig. 1A). Data shown are of a representative experiment of no less than five repeats using 10 mice per experiment.

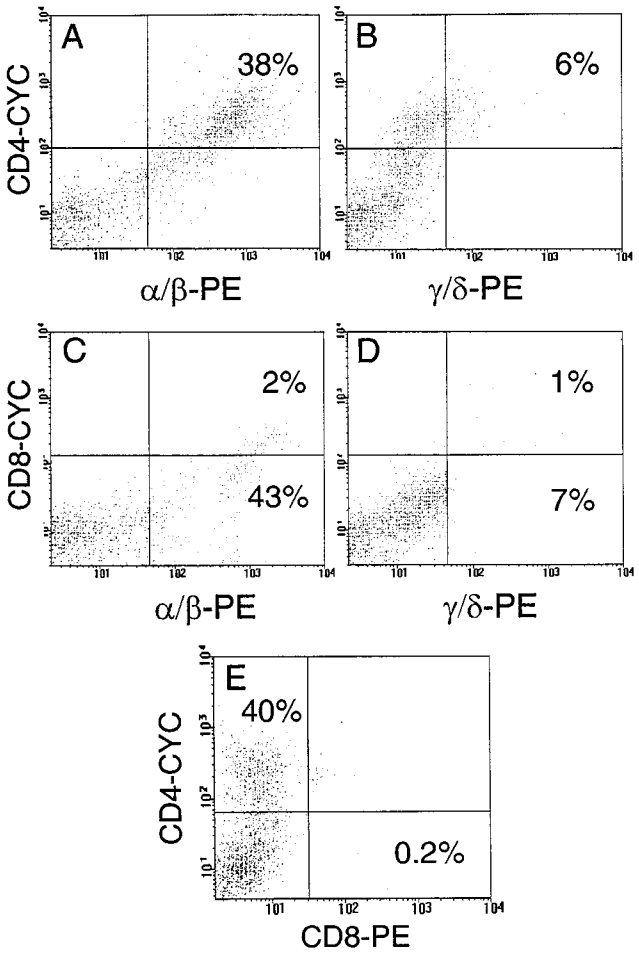

FIG. 3. CD4 and CD8 expression on VL. Cells (10^5) isolated from collagenase-digested vaginae were labeled with biotinylated anti-CD4 or anti-CD8 (α-chain) antibodies followed by cy-chrome (CYC)-conjugated streptavidin, and PE-conjugated anti-α/β or anti-γ/δ TCR antibodies or with biotinylated anti-CD4 antibodies, CYC-streptavidin, and PE-conjugated anti-CD8α antibodies and analyzed by flow cytometry. Gated regions for analysis were determined from positive-staining cells recovered from vaginal cell isolation in the presence of LNC. (A) $CD4^+$ versus α/β TCR^+ cells; (B) $CD4^+$ versus γ/δ TCR^+ cells; (C) $CD8^+$ versus α/β TCR^+ cells; (D) $CD8^+$ versus γ/δ TCR^+ cells; (E) $CD4^+$ versus $CD8^+$ cells. The percentage in each quadrant represents the fluorescent positive cells within the lymphoid-like cell limits (Fig. 1A). Data shown are of a representative experiment of five repeats using 10 mice per experiment.

both epitope specificities and analyzed by flow cytometry. The results are shown in Fig. 5. In the LNC (Fig. 5A) and PBL (Fig. 5B) cell fractions, >90% of $CD4^+$ cells bound antibodies of both epitope specificities, representing 45 and 41% of total LNC and PBL, respectively. Similarly, cells recovered after the addition of 2×10^7 LNC to the vaginal cell isolation preparation recognized both epitope-distinct anti-CD4 antibodies (Fig. 5C), albeit at a different level of resolution compared with that of LNC alone (Fig. 5A) or LNC incubated in collagenase (data not shown). In contrast, 80% of $CD4^+$ VL recognized 2B6 anti-CD4 antibodies, but not GK 1.5 anti-CD4 antibodies (29% of total VL) (Fig. 5D), with <20% of $CD4^+$ VL recognizing both anti-CD4 antibodies (8% of total VL). The results did not differ appreciably when $CD4^+$ cells were analyzed with a single antibody (single label) or in combination with other T-cell-specific antibodies in a triple label (data not shown). In other experiments, VL showed similar staining patterns for both CD4 and CD8 when antibodies with different conjugated fluorochromes were used for labeling, as well as when labeling was performed after an 18-h incubation of isolated cells in complete medium at either 4 or 37°C to examine potential reexpression of surface antigens (data not shown).

In vivo cellular depletions. To examine the putative in situ expression of the CD4 receptor on vaginal cells, mice were injected intravaginally with complement-fixing antibodies (anti-Thy-1.2 or GK 1.5 anti-CD4) over 1 week, after which VL and LNC were collected and analyzed for expression of the respective surface marker by flow cytometry using fluorescence-conjugated antibodies with epitope specificities different from those used to inject the animals. The rationale was that if the CD4 receptor was atypically expressed in situ in vaginal tissue as it is after vaginal cell isolation, vaginal CD4 cells would not be affected by in vivo treatment with GK 1.5 anti-CD4 antibodies while $Thy\text{-}1^+$ vaginal cells would be reduced or eliminated in control mice treated with anti-Thy-1.2 antibodies. The results are summarized in Table 1. Mice treated with anti-Thy-1.2 antibodies showed an 83% reduction in $Thy\text{-}1^+$ cells (56 to 9.2%) in the lymph nodes and a 35% reduction in the vagina (37 to 24%). In contrast, while mice treated with GK 1.5 anti-CD4 antibodies showed a 79% reduction in $CD4^+$ cells in the lymph nodes (analyzed by 2B6 anti-CD4 antibodies), the percentage of $CD4^+$ cells in the vagina remained unchanged at 28%. The lack of a greater reduction in $Thy\text{-}1^+$ cells in the vagina was presumably due to the concentration and access to complement within the mucosal tissue. Interestingly, intravenous administration of these same antibodies had no effect on the expression of $Thy\text{-}1^+$ or $CD4^+$ cells in the vaginal mucosa, despite a greater than 90% reduction in the respective cells in the lymph nodes (unpublished observations).

DISCUSSION

The results of this study provide important evidence that T-cell populations in the vaginal mucosae of naive mice are phenotypically distinct from those in the periphery. Although $CD4^+$ α/β TCR^+ T cells predominate in the vaginal mucosa in a manner similar to that in the lymph nodes and peripheral blood, analysis by flow cytometry provided several distinguishing characteristics of VL. Firstly, the γ/δ TCR^+ population in the vaginal mucosa (15 to 20%) is approximately five- to sixfold

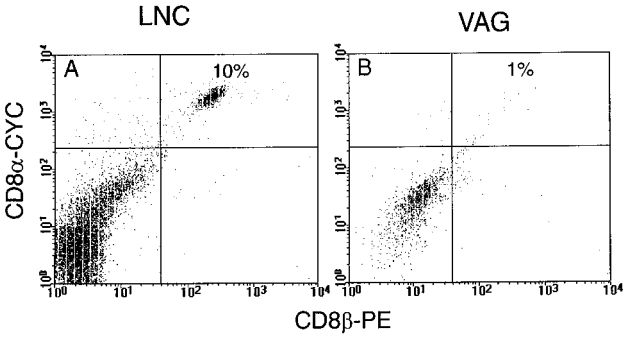

FIG. 4. CD8 expression on VL versus peripheral lymphocytes. LNC (10^6) and cells isolated from collagenase-digested vaginae (10^5) were labeled with biotinylated anti-CD8α, Cy-chrome (CYC)-conjugated streptavidin, and PE-conjugated anti-CD8β antibodies and analyzed by flow cytometry. Gated regions for analysis were determined from positive-staining cells recovered from vaginal cell isolation in the presence of LNC. (A) $CD8\alpha^+$ versus $CD8\beta^+$ LNC; (B) $CD8\alpha^+$ versus $CD8\beta^+$ vaginal (VAG) cells. The percentage in each quadrant represents the fluorescent positive cells within the lymphoid-like cell limits. Data shown are of a representative experiment of two repeats using 10 mice per experiment.

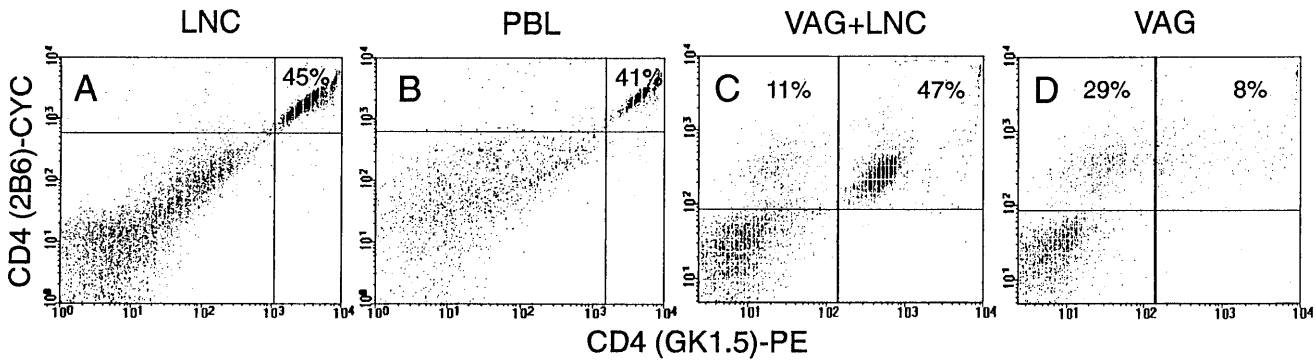

FIG. 5. CD4 receptor expression on VL versus peripheral lymphocytes. LNC (10^6), PBL (2×10^5), and cells from collagenase-digested vaginae (10^5) were labeled with biotinylated (plus cy-chrome [CYC]-conjugated streptavidin) and PE-conjugated anti-CD4 antibodies with distinct epitope specificities for the CD4 receptor. These anti-CD4 antibodies are derived from two distinct clones (RM-4.4 and RM-4.5) and block 2B6 and GK 1.5 anti-CD4 antibodies, respectively. Flow cytometric analyses of the recognition of epitope-distinct 2B6 and GK 1.5-like anti-CD4 antibodies on LNC (A), PBL (B), VL isolated in the presence of LNC (VAG+LNC) (C), and VL (VAG) (D) are shown. The percentage in each quadrant represents the fluorescent positive cells within the lymphoid-like cell limits. Data shown are of a representative experiment of five repeats using seven mice per experiment for analysis of vaginal cells and five mice per experiment for pooled samples of lymph nodes and vaginal tissues.

greater than that in the periphery. Secondly, in contrast to the γ/δ TCR^+ cells in the periphery (which are largely $CD4^-$ $CD8^-$), a high percentage of γ/δ TCR^+ cells in the vaginal mucosa stained positive with anti-CD4 antibodies. Thirdly, neither α/β nor γ/δ TCR^+ vaginal T cells express CD8, which resulted in an enormous CD4-CD8 cell ratio (>100:1) compared with the ratio observed in LNC and PBL. Moreover, there was no evidence for the presence of the gut-associated CD8$\alpha\alpha$ receptor (1) suggestive of extrathymic maturation (18) or an uncharacteristic CD8 receptor (e.g., CD8$\beta\beta$). In each case, although a pool of LNC and vaginal cells was used to identify the limits of analysis (gated regions), the resolution of specific cell populations as detected by fluorescence intensity was more dilute compared with LNC and PBL. We contend that these differences reflect the inherent fact that the vaginal cells were isolated from mucosal rather than lymphoid tissue.

The fourth and most interesting characteristic of VL is the fact that although $CD4^+$ cells were identified in the vaginal mucosa, unlike peripheral lymphocytes, VL recognized 2B6 but not GK 1.5 epitope-distinct anti-CD4 antibodies. In contrast, the control group consisting of cells recovered from a pool of LNC and vaginal tissues subjected to enzymatic digestion stained positive for both epitope-distinct anti-CD4 antibodies (Fig. 5C). This control not only provided evidence for changes and shifts in the flow cytometric resolution of positive-staining LNC subjected to enzymatic digestion but also clearly showed that lymphocytes can retain their phenotypic characteristics during the isolation procedure. The latter is also supported by the increased percentage of GK 1.5-positive cells in the pool of LNC and vaginal cells compared with that for vaginal cells alone (Fig. 5). We cannot, however, exclude the alternative possibility that the CD4 receptor on vaginal and peripheral CD4 cells is identical but that vaginal cells are more vulnerable to enzymatic digestion so that the GK 1.5-specific epitope on vaginal cells but not LNC becomes cleaved or partially digested during isolation. This appears unlikely, though, for several reasons. Firstly, the expression of CD4 (by both 2B6 and GK 1.5 anti-CD4 antibodies) was unchanged on VL after postcollagenase incubation of cells to assess surface antigen reexpression. Secondly, the reduction in $Thy\text{-}1^+$ cells, but not $CD4^+$ cells, in the vagina after intravaginal injection of anti-Thy-1.2 and GK 1.5 anti-CD4 complement-fixing antibodies, respectively, suggests that the distinct phenotype of vaginal CD4 cells similarly occurs in situ. Similar results were observed when VL and LNC collected from naive animals were treated in vitro with anti-Thy-1.2 or GK 1.5 anti-CD4 antibodies and subsequently with complement (data not shown). Taken together, our data suggest that the CD4 receptor on vaginal CD4 cells is atypically expressed compared with expression on peripheral CD4 cells. It is unclear at present whether both α/β and γ/δ $CD4^+$ vaginal T cells share the same property with respect to the expression of the CD4 receptor. We speculate that at least vaginal α/β T cells share this property since they constitute the majority of $CD4^+$ cells in the vaginal mucosa. Further studies are needed to confirm both the CD4 expression on the smaller γ/δ T-cell population and whether it too is atypically expressed.

The putative atypical expression of the CD4 receptor on vaginal CD4 cells may be due to the lack of the epitope that recognizes GK 1.5 anti-CD4 antibodies, or alternatively, the epitope that recognizes GK 1.5 anti-CD4 antibodies may be masked because of a different conformation or glycosylation pattern of the CD4 receptor. Studies that incorporate denaturing agents (immunohistochemistry or Western blot [immunoblot] analysis) are in progress to address this important issue.

Noteworthy in this study was the fact that the fluorescent staining patterns observed for the limited numbers of vaginal cells located outside the defined gated (lymphoid-like) region were not different from those inside the gated region (data not shown). These results indicate that a small percentage of T

TABLE 1. Lack of effect on vaginal $CD4^+$ cells after intravaginal injection of GK 1.5 anti-CD4 complement-fixing antibodies[a]

Treatment	% of cells positive for:			
	CD4 (2B6)		Thy-1.2	
	LNC	VL	LNC	VL
PBS[b]	47	28	56	37
Anti-Thy-1.2	ND[c]	ND	9	24
Anti-CD4 (GK 1.5)	10	28	ND	ND
% Reduction	79	0	83	35

[a] Complement-fixing antibodies (100 μg) were injected into the vaginal wall at each of two sites in a volume of 50 μl every 2 to 3 days for 1 week. The total antibody injected was 600 μg.
[b] Negative control.
[c] ND, not determined.

cells exist outside the arbitrary lymphoid-like region in naive animals but do so without obvious phenotypic divergence, including the recognition of anti-CD4 antibodies. Whereas such cells may be considered larger blasting-type T cells on the basis of light scatter characteristics, we speculate that they instead represent naive cells that remained aggregated to or associated with larger epithelial cells during the isolation process. Nevertheless, while our analysis focused on the majority of T cells in the vaginal preparation defined within a population of cells with lymphoid-like light scatter characteristics, we recognize that during analysis of vaginal cells under specific conditions (i.e., infection), attention should be given to cells outside this defined region, which may include larger blasting antigen-responsive T cells.

There are several points of contrast between the results described here for CBA/J mice and those described for vaginal cells in other murine strains, as well as in relation to T cells at other mucosal sites, including intestinal intraepithelial lymphocytes and gingival T cells of the oral mucosa. Flow cytometric analyses of T cells in the aforementioned mucosal tissues showed that while α/β TCR^+ cells often predominated, the percentages of γ/δ T cells were always considerably higher than that in the periphery (15, 20, 22, 25). Thus, our results add to the accumulating evidence suggesting a role for γ/δ T cells in first-line defense against pathogens invading the skin or mucosal surfaces. In comparison to the limited data available for VL from other murine strains, our results differed in three specific areas, a lower percentage of γ/δ T cells (reported to be as high as 50% in BALB/cJ ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice (15, 22), a predominance of putative $CD4^+$ γ/δ T cells, and negligible numbers of $CD8^+$ cells. These differences may relate to genetic diversity (differences in the class II major histocompatibility complex) or reflect differences in obtaining cells by mechanical disruption (15, 22) versus enzymatic digestion. Additionally, the actual percentages of vaginal cells detected by immunofluorescence were much lower in previous studies (15, 22), presumably because of analyses performed on total cells rather than on gated regions with lymphoid-like light scatter characteristics (Fig. 1). Furthermore, if the CD4 receptor on VL in the other genetic strains is atypically expressed as it is in CBA/J mice, the use of GK 1.5 anti-CD4 antibodies in the named studies would have identified fewer $CD4^+$ cells in the vaginal pool. This might also explain the low-level CD4 expression on γ/δ T cells in previous reports (15, 22) and, together with small numbers of $CD8^+$ cells, would explain the low levels but fairly even distribution of CD4 and CD8 cells in one report (15).

The presence of distinct subpopulations of T cells in the vaginal mucosa adds support for the concept of local CMI as a potential host defense mechanism in the female reproductive tract. In a recent report from our laboratory, we showed that mice given a primary vaginal infection with *C. albicans* can be partially protected against a second vaginal infection in the absence of any detectable participation by systemic CMI (6). Since the in situ location of mucosal cells and changes in respective cell populations can be indicative of potential immune function (24), it will be interesting to determine how the T-cell subpopulations change (numbers and phenotype) after *C. albicans* vaginal infection and to define the in situ location of each T-cell subpopulation in the vaginal tissue before and after infection, as well as during the protective effect. Similarly, in vitro proliferation and/or cytokine production by mucosal cells in response to antigenic stimulation is good evidence for putative local immune reactivity. To date, although considerable data exist for the ability of murine intestinal intraepithelial lymphocytes to produce cytokines (4, 5, 28, 31), the only reports of vaginal cell responses are cytotoxic T-lymphocyte responses by vaginal CD8 cells in macaques (19) and proliferation and/or cytokine production by genital tract cells in response to chlamydial antigens in a murine model of *Chlamydia trachomatis* genital infection (2, 14).

In summary, the present data significantly extend the information regarding distinctive phenotypic properties of VL compared with those in the periphery and provide interesting new evidence for putative atypical expression of the murine CD4 receptor on VL. These results support the concept of some level of independence (compartmentalization) pertaining to mucosal T-cell populations and vaginal immunity.

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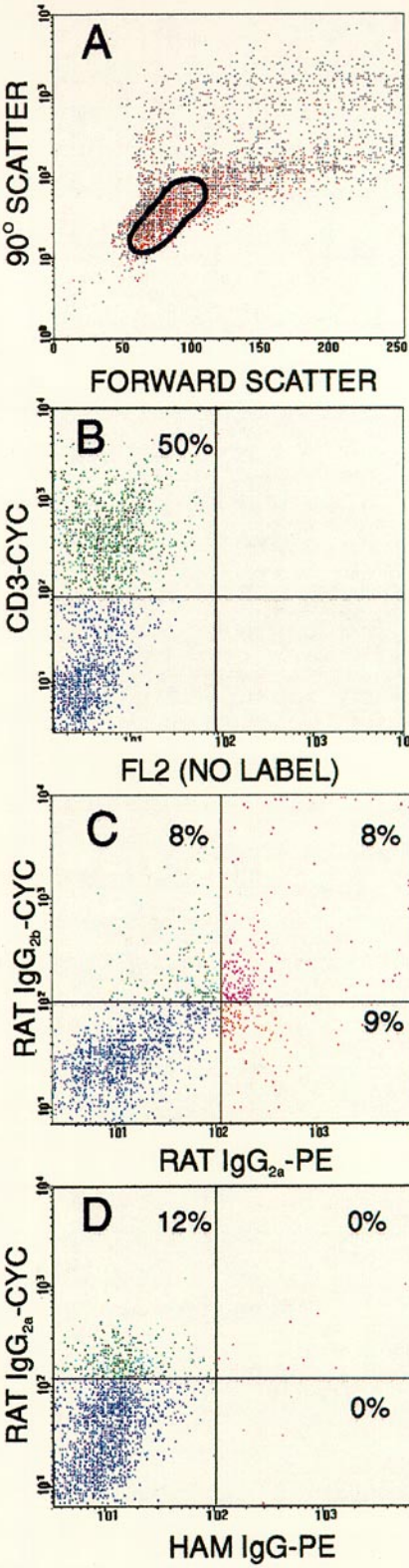
A
90° SCATTER
FORWARD SCATTER
B
50%
CD3-CYC
FL2 (NO LABEL)
C
8%
8%
9%
RAT IgG2b-CYC
RAT IgG2a-PE
D
12%
0%
0%
RAT IgG2a-CYC
HAM IgG-PE