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Circulating CD4 and CD8 T Cells Have Little Impact on Host Defense against Experimental Vaginal Candidiasis

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The etiology of recurrent vulvovaginal candidiasis in otherwise healthy women of child-bearing age remains an enigma. To date, results from both clinical studies and a murine model of vaginal candidiasis indicate that *Candida* vaginitis can occur in the presence of *Candida*-specific Th1-type cell-mediated immunity expressed in the peripheral circulation. The present study was designed to determine the role of circulating CD4 and CD8 cells in primary and secondary vaginal infections with *Candida albicans*. Vaginal fungal burden, *Candida*-specific delayed-type hypersensitivity (DTH), and lymph node cell Th1/Th2 cytokine production were monitored in CD4 and/or CD8 cell-depleted mice during persistent primary vaginal infections and secondary vaginal infections against which partial protection was observed. Treatment of mice with anti-CD4 or anti-CD8 antibodies resulted in 90% or greater depletion of the respective cell populations. Mice depleted of CD4 cells had significantly reduced *Candida*-specific DTH and lymph node cell Th1-type cytokine production during a primary vaginal infection, as well as reduced anamnestic DTH during a secondary vaginal infection. In contrast, mice depleted of CD8 cells showed only reduced gamma interferon production during a primary infection; no alterations in DTH were observed. Despite reductions in DTH and cytokine production, however, CD4 and/or CD8 cell depletion had no effect on vaginal *C. albicans* burden in mice after a primary or secondary vaginal inoculation. Taken together, these results suggest that while circulating CD4 and CD8 cells contribute to systemic *Candida*-specific cell-mediated immunity in vaginally infected mice, neither CD4 nor CD8 circulating T cells appear to provide significant host defenses against *C. albicans* at the vaginal mucosa.

Recurrent vulvovaginal candidiasis (RVVC) is an opportunistic mucosal infection of the vagina that is most often caused by *Candida albicans* (25). While an estimated 75% of all women will experience an episode of acute vulvovaginal candidiasis at least once during their lifetime, up to 5% will experience RVVC (15–17). Women with RVVC avoid exogenous factors which predispose to vulvovaginal candidiasis, such as antibiotic and oral contraceptive usage, but continue to have intractable episodes of *Candida* vaginitis.

Since the incidence of mucosal candidiasis is higher in individuals with reduced cell-mediated immunity (CMI), including patients with AIDS (18, 20) or lymphoma (12), transplantation patients (2), or those treated with corticosteroids (19), it has been postulated that susceptibility to RVVC is also associated with some form of reduced CMI. It is unclear, however, whether this putative reduction in CMI involves that expressed in the peripheral circulation, or alternatively, in the local vaginal mucosal environment. In support of the latter view, it has been shown that women with RVVC are rarely susceptible to oral thrush or esophageal candidiasis (25) and that women with chronic mucocutaneous candidiasis are generally not susceptible to vaginitis (22). Although systemic CMI reactivity in women with RVVC determined by in vivo skin test reactivity and/or in vitro peripheral blood lymphocyte proliferation has been examined in several studies (9, 13, 26, 28, 29), the inconsistent and often contradictory results due to differences in experimental conditions have done little to increase our understanding of susceptibility to RVVC. Recently, however, a comprehensive immunological analysis including skin test reactivity, peripheral blood lymphocyte proliferation, and cytokine production was performed in a large number of RVVC patients. The results of this study showed that women with RVVC had no detectable reduction in systemic CMI responsiveness during clinical vaginitis or in periods of remission (8) and provided the first solid evidence that systemic *Candida*-specific CMI may not represent a major host defense factor at the vaginal mucosa.

To study potential host defense mechanisms which provide protection against *C. albicans* vaginitis more closely, we used an estrogen-dependent murine model of experimental vaginal candidiasis. To date, our studies have shown (i) that a local vaginal *C. albicans* infection stimulates *Candida*-specific systemic Th1-type CMI

MATERIALS AND METHODS

Mice. Female CBA/J ($H\text{-}2^k$) mice, 8 to 10 weeks of age, purchased from Jackson Laboratory, Bar Harbor, Maine, were used throughout these studies.

Antigens. *C. albicans* culture filtrate antigen(s) was prepared as previously described (5), using *C. albicans* 3153A. Briefly, supernatant from a 3-day *C. albicans* blastoconidial culture grown in , 12,000-molecular-weight phytone-peptone-glucose dialysate medium was concentrated 10-fold on a 10,000-molecular-weight-exclusion membrane (Amicon Corp., Danvers, Mass.) while being washed with 1 to 2 volumes of phosphate-buffered saline (PBS). The final filtered preparation had a protein concentration of 0.2 to 0.5 mg/ml as measured by a Lowry trichloroacetic acid precipitation kit (Sigma Chemical Co., St. Louis, Mo.). The *C. albicans* soluble cytoplasmic substance preparation (SCS) (3) was a kind gift of Judith Domer, Tulane University School of Medicine, New Orleans, La.

Antibodies. Complement-fixing antibodies specific for CD4 (L3T4) and CD8 (Lyt 2) cells were obtained from the hybridoma cell lines GK 1.5 and HB129 (American Type Culture Collection, Rockville, Md.), respectively. The cell lines were grown in bulk culture, and the antibodies were collected by ammonium sulfate precipitation. Fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies specific for CD4, CD8 (both of which had epitope specificities that were different from that of the hybridoma lines), a/b T-cell receptor (TCR), g/d TCR, or interleukin 2 (IL-2) receptor (IL-2R) (CD25) were purchased from Pharmingen Corp., San Diego, Calif.

Vaginal infections and DTH reactivity. Primary vaginal infections were initiated in both estrogen-treated and untreated mice, while secondary vaginal infections were initiated in only estrogen-treated mice as previously described (4–7). Mice evaluated during a primary vaginal infection were treated with estrogen weekly by injecting 0.5 mg of estradiol valerate in 0.1 ml of sesame oil subcutaneously (5). Seventy-two hours after the first estrogen treatment, mice were inoculated vaginally with 5×10^4 stationary-phase *C. albicans* blastoconidia in 20 ml of PBS. At weekly intervals, groups of four to five mice were footpad challenged with approximately 10 mg of *C. albicans* culture filtrate antigen as previously described (5, 6). The footpad swelling was measured 18 to 24 h later, and the mice were sacrificed thereafter. A vaginal lavage with 100 ml of PBS was performed after sacrifice, and vaginal *C. albicans* was enumerated by quantitative culture of lavage fluid as previously described (5, 6). Additionally, vaginal lavage fluid was analyzed microscopically in a blinded manner and scored (0 to 1 1 1 1) for the presence of hyphae.

For secondary vaginal infections, non-estrogen-treated mice were first given a primary vaginal infection that was allowed to clear spontaneously. For this, mice were given 5×10^5 *C. albicans* blastoconidia intravaginally in 20 ml of PBS in the absence of estrogen. At biweekly intervals beginning at week 2, groups of four to five mice were monitored for DTH as described above and sacrificed, lavage was performed, and the vaginal lavage fluid was examined microscopically and cultured. Non-estrogen-treated mice showed complete and spontaneous clearance of the vaginal infection by week 3 following a primary infection, as indicated by sterile lavage fluid and no microscopic evidence of hyphae (4). During the third week following a primary infection, mice began receiving estrogen. They were then given a second vaginal infection at week 4 with 5×10^4 *C. albicans* cells as described above. DTH, quantitative lavage cultures, and lavage fluid hyphal scores were monitored during the subsequent week. Estrogen-treated mice were used for the reinfection studies, since we have shown previously that candidal recovery in non-estrogen-treated reinfected mice was too low for reliable assessment of vaginal protection (4).

Antibody treatment. Three days prior to the primary or secondary vaginal inoculation, mice were randomized to receive intravenous injections of an anti-CD4 or anti-CD8 antibody (0.5 mg per injection) in 0.5 ml of PBS. Control mice received PBS alone. Injections were given every 3 days until completion of the study.

Cytokine analysis of infected mice. Following vaginal lavages, inguinal, mesenteric, and lumbar draining lymph node cells from infected mice treated with complement-fixing antibodies or PBS were collected and cultured as previously described (6). Briefly, single-cell suspensions (4×10^6 cells per ml) were cultured with *C. albicans* SCS (125 mg/ml) or in complete medium only (RPMI 1640 medium supplemented with penicillin [100 U/ml], streptomycin [100 mg/ml], L-glutamine [2 mM], 2-mercaptoethanol [5×10^{25} M], sodium pyruvate [2 mM], *N*-2-hydroxyethylpiperazine-*N*9-2-ethanesulfonic acid [HEPES] buffer [20 mM], and 5% heat-inactivated fetal bovine serum [all from GIBCO, Grand Island, N.Y.]). Culture supernatants were collected after 48 h and stored at 2 708C. IL-2 in the culture supernatants was quantitated by bioassay with the IL-2-dependent CTLL (11) cell line (American Type Culture Collection). Concentrations of IL-2 produced in response to *C. albicans* SCS were determined by using known concentrations of recombinant human IL-2 (Cetus Corp., Emeryville, Calif.) (6). Gamma interferon (IFN-g) in the culture supernatants was quantitated by a sensitive and specific enzyme-linked immunosorbent assay (Genzyme Corp., Cambridge, Mass.) as previously described (6). IL-4 in the culture supernatants was quantitated by bioassay by using the IL-4-dependent cell line CT.4S (14), kindly provided by William Paul, National Institutes of Health, Bethesda, Md. Concentrations of IL-4 produced by lymphocytes in response to *C. albicans* SCS were determined by using known concentrations of recombinant murine IL-4 (6), kindly provided by Immunex Corp., Seattle, Wash.

Flow cytometry. Percentages of $CD4^1$, $CD8^1$, a/b or g/d TCR^1, or $IL\text{-}2R^1$ cells in the lymph nodes of mice were determined with a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) and three-color analysis software. Analyses were performed at the analytical flow cytometry facility at the Michigan Cancer Foundation, Wayne State University School of Medicine.

Statistical analysis. The unpaired Student's *t* test was used to analyze the data. Significant differences were defined as a confidence level where *P* is , 0.05 by a one-tailed test.

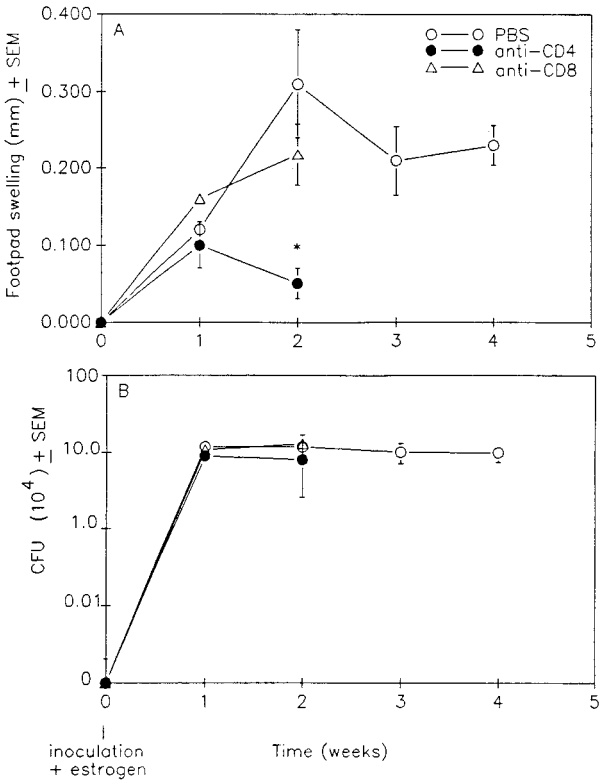

FIG. 1. Effects of circulating CD4 and CD8 cells on primary *C. albicans* vaginal infections. Groups of randomly selected mice were injected intravenously with an anti-CD4 (GK 1.5) or anti-CD8 (HB129) complement-fixing antibody (0.5 mg per injection) beginning 72 h prior to vaginal inoculation and continuing every 3 days for 2 weeks. Positive control mice were injected with 0.5 ml of PBS. Mice were estrogen treated weekly (0.5 mg per injection) beginning 72 h prior to vaginal inoculation. Mice were inoculated with 5×10^5 stationary-phase blastoconidia. Antibody- and PBS-treated infected mice were monitored for 2 to 4 weeks for DTH (A) and vaginal *C. albicans* burden (B). Results are expressed as means 6 standard errors of the means (SEM) for four experiments. Asterisks indicate where significant differences were found.

RESULTS

Effects of circulating CD4 and CD8 T cells on a primary vaginal *C. albicans* infection. Groups of estrogen-treated mice were injected intravenously with an anti-CD4 or anti-CD8 complement-fixing antibody, or with PBS as a control, and then inoculated with viable *C. albicans* blastoconidia intravaginally. DTH analysis and vaginal lavages were performed for 2 weeks in antibody-treated mice and 4 weeks in PBS-treated mice. Mice treated with the anti-CD4 antibody had significantly lower DTH 2 weeks after infection compared with PBS-treated mice (P , 0.001) (Fig. 1A). In contrast, DTH in anti-CD8 antibody-treated mice was lower but not significantly different from that in PBS-treated mice. In spite of a reduction in DTH, neither anti-CD4 nor anti-CD8 antibody treatment affected the vaginal *C. albicans* burden during the first 2 weeks of infection in comparison with PBS-treated control mice (Fig.

1B). These results were supported by microscopic observations of hyphae in the vaginal lavage fluid. All three groups of mice had similar scores (data not shown). Since anti-CD4 and anti-CD8 antibody treatments did not modulate the vaginal *C. albicans* burden through 2 weeks, antibody treatment was terminated. However, PBS-treated mice were monitored for an additional 2 weeks to confirm that the pattern of infection (persistent vaginitis) and immune reactivity (Th1 type) in subsequent weeks was not different from that found in previous studies (5, 6).

Effects of anti-CD4 and anti-CD8 antibody treatments on Th1/Th2 lymphokine production during primary vaginal *C. albicans* infection. Lymph node cells collected from PBS- or antibody-treated mice were analyzed by flow cytometry to confirm cellular depletion and then cultured with *Candida* antigen to characterize the cytokine responses. The cytokines analyzed in the culture supernatants included IL-2, IFN-g (Th1 type), and IL-4 (Th2 type). The results are summarized in Table 1. Treatment of mice with anti-CD4 antibodies reduced the CD4 cells in the lymph nodes from 41 to 3% and from 43 to 4% when tested during weeks 1 and 2, respectively, following vaginal inoculation. While the absolute number of CD8 cells in anti-CD4 antibody-treated mice was unchanged (data not shown), the relative percentage of CD8 cells was increased twofold, presumably as a result of the analysis of a finite number of cells (10^6) containing fewer CD4 cells. Anti-CD8 antibody treatments reduced the percentage of CD8 cells in mice from 21 to 5% and from 16 to 4% during weeks 1 and 2, respectively. As with anti-CD4 antibody-treated mice, while the absolute numbers of CD4 cells did not change (data not shown), flow cytometric analysis of CD8 cell-depleted mice showed a 1.3-fold increase in the relative percentage of CD4 cells.

During weeks 1 and 2 after vaginal inoculation, anti-CD4 antibody treatments resulted in a significant reduction in *C. albicans* antigen-stimulated IL-2 and IFN-g production by lymph node cells compared with similar cultures from mice given PBS (P , 0.01 and P , 0.04, respectively). Anti-CD8 antibody treatments had no effect on IL-2 production but did lead to reduced IFN-g production compared with PBS-treated mice during week 1 (P , 0.05). In contrast, IL-4 production was not affected by treatment with either antibody and remained low during the 2-week infection period.

Effects of circulating CD4 and CD8 T cells on primary vaginal infection in the absence of pseudoestrus. To examine the potential role of circulating CD4 and CD8 cells on the spontaneous clearance of a primary vaginal infection in the absence of estrogen, groups of mice were randomized to receive the anti-CD4 or anti-CD8 antibody or PBS as a control and then vaginally inoculated with *C. albicans*

on DTH. With respect to vaginal fungal burden in primary-infected mice in the absence of estrogen, both anti-CD4 and anti-CD8 antibody-treated mice showed a low-grade infection at weeks 1 and 2 after vaginal inoculation similar to that in PBS-treated mice during the same time period (Fig. 2B). Although antibody-treated mice were not monitored beyond 2 weeks, the vaginal infection in PBS-treated positive control mice was cleared at week 3, as determined by quantitative culture (Fig. 2B), but continued to show positive DTH (Fig. 2A) as found previously (6).

Effects of systemic circulating CD4 and CD8 T cells on secondary vaginal *C. albicans* infection. For experiments investigating the effects of circulating CD4 and CD8 cells on secondary vaginal infections, animals were first given a primary vaginal inoculation in the absence of estrogen treatment (4). At consecutive weeks following inoculation, randomly selected mice were monitored for DTH and vaginal fungal burden in order to establish that DTH had been induced and that the vaginal infection had cleared spontaneously as shown previously (4). These mice are represented by the PBS-treated, non-estrogen-treated animals described above (Fig. 2), since the two experiments were performed simultaneously.

Non-estrogen-treated mice which had recovered from a primary infection but which continued to have positive DTH in the absence of a positive vaginal culture were given estrogen on day 25 and then reinfected on day 28 (week 4). Injections of anti-CD4 and/or anti-CD8 antibodies, or PBS, began on day 25 and continued every 3 days until completion of the study. DTH and vaginal fungal burden were determined 1 week following reinfection. The results are illustrated in Fig. 2 (weeks 4 and 5). One week after reinfection (week 5), PBS-treated mice showed a characteristic anamnestic DTH response as shown previously under these conditions (4). DTH was significantly reduced (P , 0.01) in reinfected mice treated with the anti-CD4 antibody

DISCUSSION

To date, our studies in women with RVVC and an animal model of *C. albicans* vaginitis indicate that systemic CMI may not play a prominent role in protection against *C. albicans* infections of the vagina (4, 7, 8). In animals, the confirmation of in vivo *Candida*-specific systemic CMI has relied heavily on the presence or absence of DTH. Accordingly, the effects of systemic CMI on vaginal candidiasis are often interpreted by the correlation of *Candida*-specific DTH with vaginal fungal burden. However, recent studies by Garner and Domer showed that the absence of *Candida*-specific DTH had no effect on protection against candidal infections (10), suggesting that mechanisms other than or in addition to those associated with DTH play a role in protective immunity against *C. albicans*. Therefore, we chose to examine the role of systemic CMI at the vaginal mucosa on a broader level by monitoring primary and secondary vaginal infections in mice depleted of CD4 and/or CD8 T cells.

In mice with a primary vaginal infection, 90% depletion of CD4 cells resulted in abrogation of both *Candida*-specific DTH and lymph node cell production of IL-2 and IFN-g, in confirmation of the fact that CD4 cells were the predominant population mediating those reactivities. In contrast, following 90% depletion of CD8 cells, primary-infected mice continued to show *Candida*-specific DTH and showed no change in in vitro cytokine production under most experimental conditions, although IFN-g was reduced during the first but not the second week postinfection under pseudoestrus conditions. Thus, while we recognize that CD8 cells are capable of producing IFN-g (24, 27), the lack of reduced IFN-g production in CD8 cell-depleted mice under all experimental conditions suggests that CD8 cells are, at most, minor contributors to the Th1-type cytokine production in primary-infected mice. Nevertheless, in spite of the major and minor contributions of CD4 and CD8 cells to systemic Th1-type reactivity, depletion of circulating CD4 and CD8 systemic T cells had no effect on vaginal fungal burden during a primary vaginal *C. albicans* infection. This important observation provides further support to our contention that systemic CMI does not represent a dominant host defense factor in the vaginal mucosa.

The results of reinfection in anti-CD4 or anti-CD8 antibody-treated mice indicated that CD4 cells were also responsible for anamnestic DTH. Surprisingly, and in contrast to what we observed during the primary infection, anti-CD4 antibody treatment had no effect on Th1-type reactivity (IL-2 and IFN-g production) in reinfected mice (Table 2). These results suggested either (i) that both CD4 and CD8 T cells express Th1-type reactivity during a secondary vaginal infection, (ii) that cells other than CD4 and CD8 T cells (possibly $CD4^2$ $CD8^2$ g/d T cells) express Th1-type reactivity during a secondary infection, or (iii) that the remaining CD4 cells in anti-CD4 antibody-treated mice were responsible for the Th1-type reactivity. The fact that Th1-type reactivity was also retained in mice depleted of both CD4 and CD8 cells reduces the possibility that both CD4 and CD8 cells expressed Th1-type reactivity. Similarly, the lack of any change with respect to percentages of $CD4^1$, $CD4^2$, $CD8^1$, or $CD8^2$ a/b or g/d T cells, or changes in the cellular expression of CD25 on these cell populations, provided no evidence that cells other than CD4 and CD8 cells were activated and expressed Th1-type reactivity.

Although it would appear unlikely that CD4 cells which escaped depletion by the anti-CD4 antibody (10%) could retain such high levels of Th1-type reactivity, a recent report showed that in vivo treatment of mice with an anti-CD4 (GK 1.5 epitope) antibody eliminated high percentages of resting, naive CD4 cells but was much less effective on memory CD4 cells (1). Thus, it remains possible that the observed Th1-type reactivity was due to $CD4^1$ effector cells induced during the primary infection that escaped the anti-CD4 antibody depletion following the second inoculation of *C. albicans*. The reduced yet positive DTH in reinfected anti-CD4 antibody-treated mice lends support to this interesting observation.

Despite the effects (or lack thereof) of CD4 or CD8 T-cell depletion on immune reactivity during reinfection, depletion of 90% or greater of CD4 and/or CD8 cells with indiscriminate antigenic specificities has no effect on vaginal fungal burden after reinfection. Since the number of yeast cells in PBS- or antibody-treated mice was similar to that which is characteristic of partial protection against a second vaginal infection (4), we contend that circulating CD4 and CD8 cells do not play a major role in partial protection against *C. albicans* vaginitis. The results of this study are also in agreement with our previous studies in which *Candida*-specific suppression of the anamnestic DTH similarly had no effect on the local protective effect (4). Thus, the present study provides additional evidence that systemic CMI is not protective at the vaginal mucosa. We recognize, however, in light of this accumulating evidence for a limited role of systemic CMI in the vaginal mucosa, that the local protection could be mediated in part by non-DTH-associated systemic $CD4^1$ effector T cells that escape either *Candida*-specific suppressor mechanisms (4, 7) or nonspecific antibody-mediated lysis. However, the likelihood that both forms of immunoregulation (antigen specific and nonspecific) affect only DTH-associated cells, sparing non-DTH-associated cells, is remote. Thus, while our results do not refute the concept of non-DTH-associated protective immunity (10), it does not appear that such mechanisms apply to this model of *C. albicans* vaginitis.

In conclusion, the results of this study along with those previously reported (4, 7) provide convincing evidence that CMI expressed systemically does not represent a major host defense mechanism at the vaginal mucosa. Accordingly, studies to examine the possibility of compartmentalized CMI at the vaginal mucosa are needed. It is interesting to envision a local network of acquired and innate host defense mechanisms functioning at the vaginal mucosa. Recognizing that T cells are present in the vaginal mucosa (21), and the fact that anti-*Candida* protective antibodies can be induced locally by intravaginal immunization (23), continued efforts to study locally acquired CMI and humoral immune host defense mechanisms in appropriate animal models may be useful in reducing episodes of RVVC and may also be applied to vaginal infections caused by other pathogens.

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