INFECTION AND IMMUNITY, Sept. 1997, p. 3939–3943 Vol. 65, No. 9
0019-9567/97/$04.00+0
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Use of Cellular Depletion Analysis To Examine Circulation of Immune Effector Function between the Vagina and the Periphery

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Received 7 April 1997/Returned for modification 30 April 1997/Accepted 1 July 1997

Results from an animal model of vaginal candidiasis suggest that *Candida*-specific cell-mediated immunity in the systemic circulation does not mediate protection against vaginitis. The present study used cellular depletion analysis to examine the circulation of immune effector function between the vagina and the periphery. Results showed that anti-Thy-1.2 antibodies given intravenously to mice depleted Thy-1$^+$ T lymphocytes in the systemic compartment but not in the vaginal mucosa, while the same antibodies injected intravaginally significantly reduced Thy-1$^+$ T cells in both the vaginal and systemic compartments. These results support a lack or low level of circulation of immune effector function from the periphery to the vaginal mucosa.

Despite the fact that cell-mediated immunity (CMI) is the dominant mechanism of host defense against *Candida albicans* at mucosal surfaces (8, 14, 16), little is known about potential host defense mechanisms acting at the vaginal mucosa. Studies of women with recurrent vulvovaginal candidiasis (3, 9) and an estrogen-dependent murine model of vaginal candidiasis (2, 5, 6) have shown that *Candida*-specific systemic Th1-type CMI (presence of delayed-type hypersensitivity and production of interleukin-2 [IL-2] and gamma interferon, but not IL-4 and IL-10, by lymphocytes in response to *Candida* antigen in vitro) is ineffective against *C. albicans* vaginal infections.

Studies with mice have shown that neither the *Candida*-specific Th1-type systemic CMI induced by an experimental *C. albicans* vaginal infection nor that induced by immunization with *Candida* antigen effectively protects mice against experimental vaginitis (1a, 4, 5). We have therefore concluded that protection against vaginitis (2) is derived from vagina-associated CMI. It is interesting and peculiar that vaginal infection would induce a Th1-type systemic response associated with resistance against *C. albicans* infection (17, 18) that, in turn, has no effect against subsequent vaginal infections. There are several explanations for this. It is possible that *Candida*-specific systemic CMI circulates adequately into the vaginal mucosa but is ineffective or nonfunctional at the mucosal site. Alternatively, *Candida*-specific CMI may circulate into the vaginal mucosa but at levels too low to provide effective host defense. Lastly, the lack of effect may be due to the inability of CMI in the periphery to circulate into the vaginal mucosa. The present study was designed to examine the issue of immune circulation between the systemic and vaginal compartments by using a less discriminate model system. Our approach was to use complement-fixing antibodies against Thy-1 given intravenously or intravaginally to assess depletion of Thy-1$^+$ T lymphocytes in the lymph node and vaginal compartments. Anti-Thy-1 antibodies were chosen because of their capacity to deplete cells in vivo (7), their specificity for T cells, and the fact that Thy-1$^+$ cells have been described in the murine vaginal mucosa (11, 15).

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Effect of systemic administration of anti-Thy-1 antibodies on systemic and vaginal cell populations. Complement-fixing antibodies (immunoglobulin G2b [IgG2b]) directed against the Thy-1.2 surface antigen on T cells were produced by bulk culture of the 30-H12 (TIB 107) hybridoma (American Type Culture Collection, Rockville, Md.) in serum-free AIM V medium (GIBCO, Grand Island, N.Y.) and collected by ammonium sulfate precipitation. The concentration of the antibodies was determined (Sigma Protein Assay kit; Sigma Chemical Co., St. Louis, Mo.), and they were stored in 1-ml aliquots at −70°C until use. The isotype control antibody was rat IgG2b (Zymed, San Francisco, Calif.). Groups of 10 mice were injected intravenously with anti-Thy-1.2 or isotype control antibodies (0.75 mg per injection) in a volume of 0.5 ml every 2 to 3 days for a period of 7 days (a total of three injections). Following the series of injections, peripheral blood, lymph nodes, and vaginal lymphocytes were isolated as previously described (7). Briefly, peripheral blood lymphocytes (PBL) were collected after differential centrifugation by using Ficoll Hypaque. Inguinal, mesenteric, and lumbar lymph nodes were collected and made into single-cell suspensions through a sterile wire mesh. Vaginae were excised, the cervixes were removed, and the tissue was subjected to enzymatic digestion with 0.25% collagenase type IV (Sigma) for 30 min at 37°C with intermittent stomacher (Tekmar, Cinncinnati, Ohio) homogenization. The lymphoid-enriched vaginal cells were clarified ($800 \times g$) from supernatants following two 1-min centrifugations at $200 \times g$ (quick spins). All cells were enumerated by trypan blue dye exclusion. Lymph node cells (10^6), PBL (10^5), and vaginal cells (1×10^5 to 2×10^5) were pelleted and dual labeled with phycoerythrin (PE)-conjugated anti-Thy-1.2 antibodies (53-2.1 clone, with a different epitope specificity than those antibodies produced by the 30-H12 hybridoma; Pharmingen Inc., San Diego, Calif.) and biotin-conjugated anti-CD4 antibodies (RM-4.4; Pharmingen) in 100 μl of phosphate-buffered saline plus 2% heat-inactivated fetal calf serum as previously described (6). Following thorough washing, the biotin-conjugated antibodies were further incubated with avidin-conjugated cy-chrome (CYC) for an additional 30 min on ice.

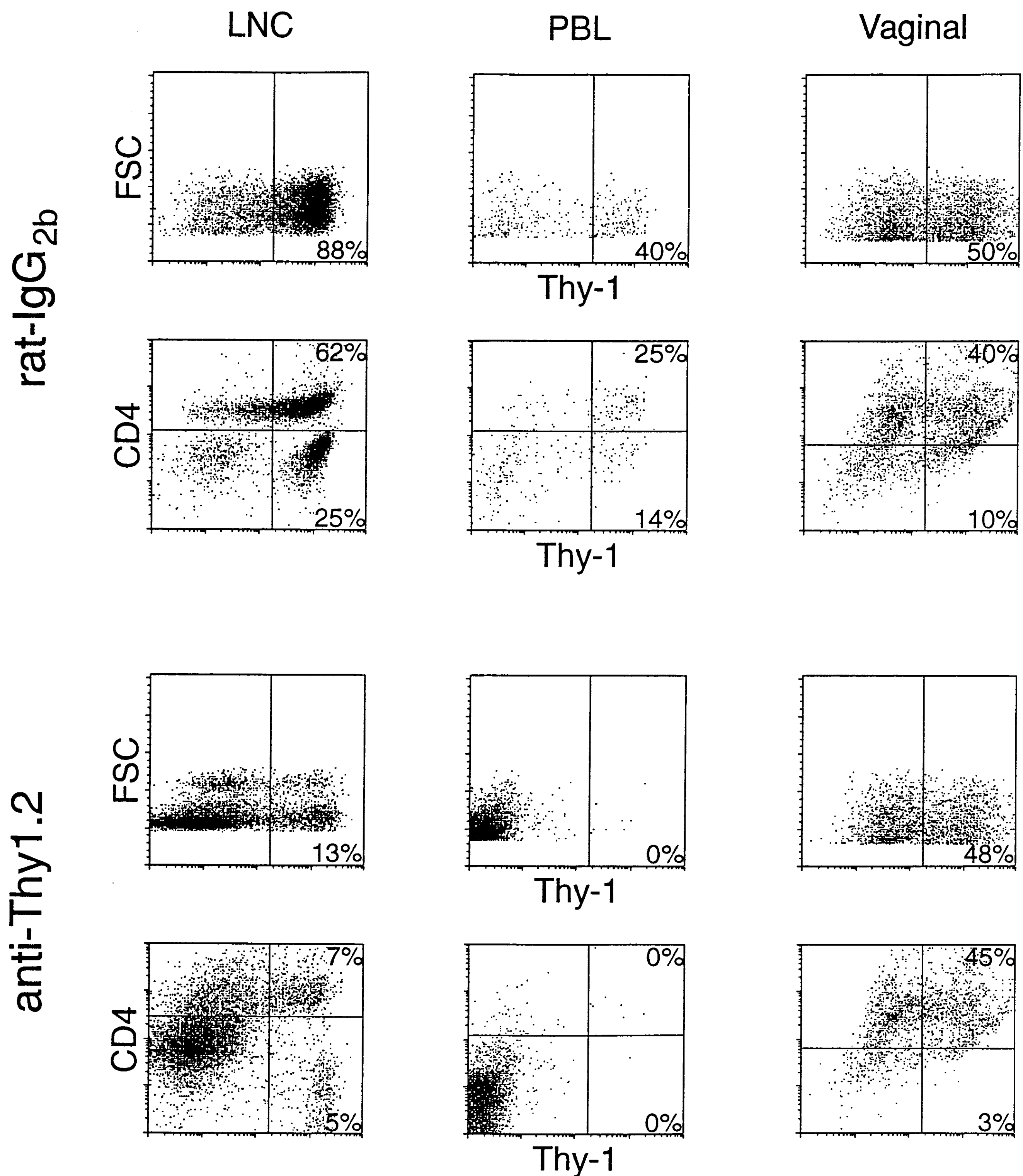

FIG. 1. Effect of intravenous administration of anti-Thy-1.2 antibodies on cells in the systemic and vaginal compartments. Anti-Thy-1.2 complement-fixing antibodies or rat IgG2b (0.75 mg) were given to mice intravenously in a volume of 0.5 ml every 3 days for 1 week. Lymph node cells (LNC), PBL, and vaginal cells were collected and dual labeled with PE-conjugated anti-Thy-1.2 and biotin-conjugated anti-CD4 antibodies. A second incubation with CYC-conjugated streptavidin was used for fluorescence of biotin-labeled cells. Appropriate isotype control antibodies were included but are not shown. All cells were analyzed by flow cytometry. Results are expressed as the percentage of cells with either forward scatter (FSC) versus Thy-1^+ cellular fluorescence or $CD4^+$ versus Thy-1^+ cellular fluorescence. The percentage of positively stained cells is indicated in each plot. Shown are representative experiments of three repeats.

The washed, labeled cells were analyzed by flow cytometry (FACScan [Becton Dickinson, Mountain View, Calif.] or Elite flow cytometer [Coulter, Miami, Fla.]) for the percentages of Thy-1^+ and Thy-1^+ $CD4^+$ cells. The percentage of $CD4^+$ cells was used as an additional marker for T cells. Results from a representative experiment of three repeats are shown in Fig. 1. In anti-Thy-1.2 antibody-treated mice, the percentages of Thy-1^+ and Thy-1^+ $CD4^+$ cells in the lymph nodes were reduced 74 and 78%, respectively, based on the percentages of the respective cells in the isotype control antibody-treated mice (88 to 13% and 62 to 7%). In peripheral blood, the percentage of Thy-1^+ or Thy-1^+ $CD4^+$ cells in anti-Thy-1.2 antibody-treated mice was reduced 100%. In contrast, the percentages of Thy-1^+ and Thy-1^+ $CD4^+$ lymphoid-like vaginal cells remained virtually unchanged in the anti-Thy-1 antibody-treated mice compared to the isotype antibody-treated mice (50 to 48% and 40 to 45%, respectively). Similar results were observed in two additional experiments. These results suggest that intravenous administration of anti-Thy-1 antibodies effectively depletes Thy-1^+ cells in the systemic compartment but not in the vaginal mucosa.

Effect of anti-Thy-1.2 antibodies on depletion of vaginal cells in vitro. To confirm that anti-Thy-1.2 antibodies could effectively recognize lymphoid-enriched vaginal cells for complement-mediated lysis, cells were extracted from the vaginal mucosa and subjected to in vitro mass cytolysis. For this, lymph node cells (10^7) and vaginal cells (0.5×10^6 to 1×10^6) were incubated with either anti-Thy-1.2 antibodies (TIB 207) or rat IgG2b isotype control antibodies (150 μg) in a volume of 100 μl for 30 min on ice. The washed cells were incubated with a 1:10 dilution of rabbit and guinea pig complement (Accurate Chemical Co., Westbury, N.J.) in a volume of 100 μl for 30 min at 37°C. The cells were washed, and those that remained were analyzed for Thy-1^+ cells as described above. Results from a representative experiment of two repeats are shown in Fig. 2. Treatment with anti-Thy-1.2 antibodies reduced the Thy-1^+ lymph node cells from 91 to 9% (90% reduction) and the vaginal Thy-1^+ cells from 27 to 10% (63% reduction). These results indicate that Thy-1^+ vaginal cells can be depleted with anti-Thy-1.2 complement-fixing antibodies. The lower overall percentage of vaginal Thy-1^+ cells in this representative experiment is presumably due to the limits (gated regions) of the lymphoid-like vaginal cell population that unavoidably vary with different experiments.

Effect of intravaginal administration of anti-Thy-1.2 antibodies on systemic and vaginal T-cell expression. To determine whether anti-Thy-1.2 complement-fixing antibodies given intravaginally could reduce the percentage of Thy-1^+ cells in the vaginal mucosa, anti-Thy-1.2 complement-fixing antibodies were injected into the vaginal wall over a period of 1 week. For this, anti-Thy-1.2 or rat IgG2b isotope control antibodies were injected into the left and right walls of ether-anesthetized mice in a volume of 50 μl per side (75 μg per injection) every 2 to 3 days (a total of three injections). On day 7, the lymph node and vaginal cells were isolated and analyzed by flow cytometry for the percentage of Thy-1^+ and Thy-1^+ $CD4^+$ cells. A representative experiment of two repeats is illustrated in Fig. 3. In animals treated with anti-Thy-1.2 antibodies, the percentages of Thy-1^+ and Thy-1^+ $CD4^+$ cells in the lymph nodes were reduced 74 and 65%, respectively, based on the percentages in the isotype antibody-treated mice (74 to 19% and 43 to 15%, respectively). Similarly, the percentages of Thy-1^+ and Thy-1^+ $CD4^+$ vaginal cells in anti-Thy-1.2 antibody-treated mice were reduced 30 and 26%, respectively, compared to that in the isotype antibody-treated mice (46 to 32% and 35 to 26%, respectively). These results suggest that the intravaginal injection of complement-fixing antibodies can reduce or deplete cells in both the vaginal and systemic compartments. The somewhat incomplete depletion of cells in the vaginal mucosa is presumably due to the limited activity or low concentration of complement that bathes the vaginal tissue. Nevertheless, this is the first evidence that cells in the vaginal mucosa can be significantly reduced by in vivo treatment with complement-fixing antibodies under naive conditions. This method may be very useful for identifying or characterizing cell populations important for host defense in the vaginal mucosa, including those involved in protection against *C. albicans* vaginal infection.

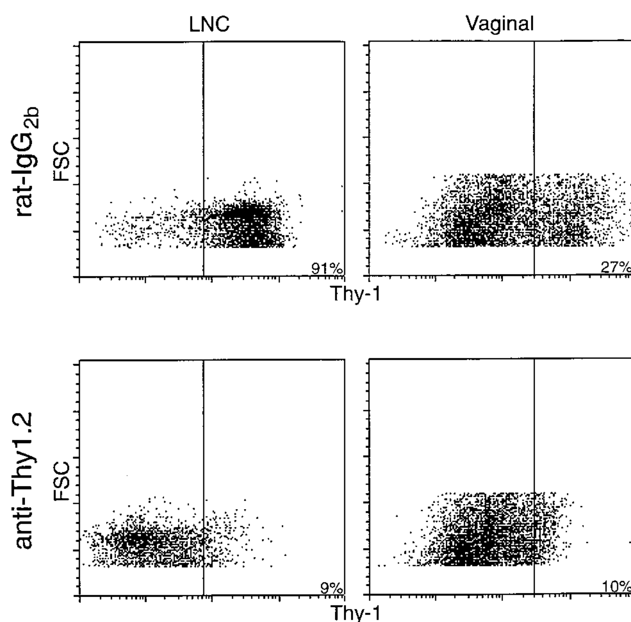

FIG. 2. Effect of anti-Thy-1.2 antibodies on depletion of vaginal cells in vitro. Lymph node cells (LNC) or vaginal cells were collected and incubated with anti-Thy-1.2 antibodies or rat IgG2b (150 μg) in vitro and then with guinea pig complement diluted 1:10. The complement-treated cells were labeled with PE-conjugated anti-Thy-1.2 antibodies and analyzed by flow cytometry. Appropriate isotype control antibodies were included but are not shown. The results are expressed as the percentage of cells with forward scatter (FSC) versus Thy-1 cellular fluorescence. The percentage of positively stained cells is indicated in each plot. Shown are representative experiments of two repeats.

Taken together, our data indicate that the systemic administration of anti-Thy-1.2 complement-fixing antibodies depletes Thy-1^+ cells in the systemic compartment but has no effect on Thy-1^+ cells in the vaginal mucosa. Conversely, intravaginal injection of the antibodies reduced Thy-1^+ cells in the vaginal mucosa, as well as in the systemic compartment. Based on the mass cytolysis experiments, it is clear that vaginal Thy-1^+ cells would have been depleted or reduced if the antibodies had effectively circulated into the vaginal mucosa in high enough concentrations. Recognizing this, it remains unclear whether the antibodies administered systemically did not reach the vaginal mucosa or did not reach the vaginal mucosa in high enough concentrations to be effective. The latter explanation is less likely based on the fact that the total amount of antibody given systemically was fivefold higher than that given intravaginally. We recognize, however, that the antibodies were administered to naive mice and not under conditions of increased vaginal vasodilation that may be expected during infections with profound cellular infiltration. Interestingly, though, we have preliminary evidence that systemic $CD4^+$ T cells do not

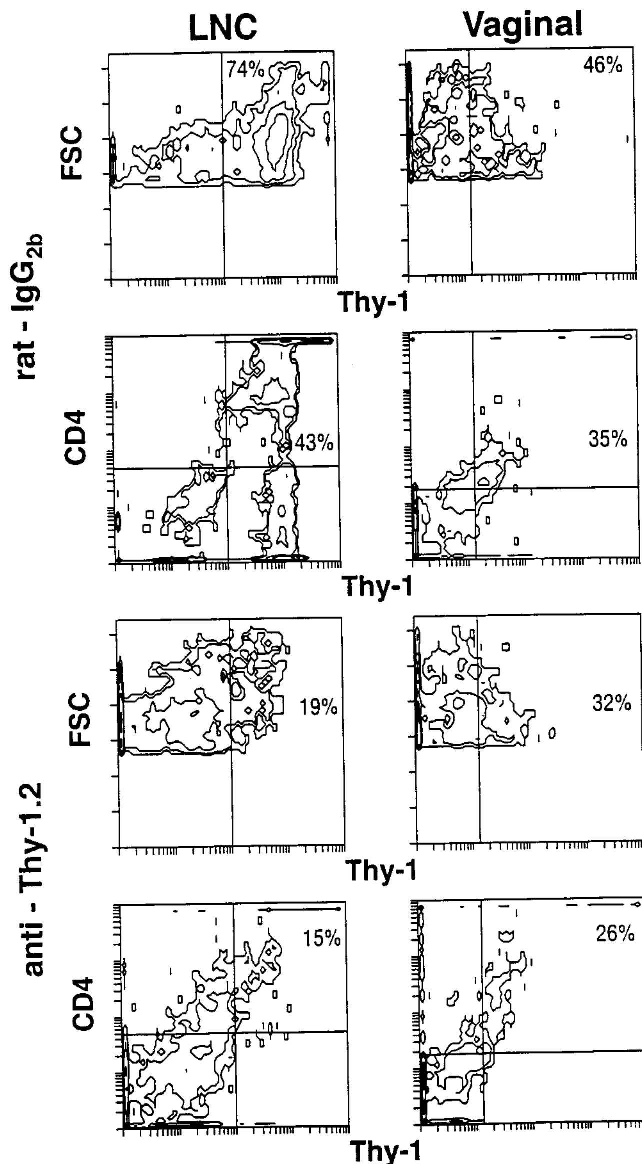

FIG. 3. Effects of intravaginal administration of anti-Thy-1.2 antibodies on cells in the systemic and vaginal compartments. Anti-Thy-1.2 antibodies were given intravaginally (75 μg) in a volume of 50 μl at two sites every 3 days for 1 week. Inguinal, mesenteric, and lumbar lymph node cells (LNC) and vaginal cells were collected and stained with PE-conjugated anti-Thy-1.2 antibodies or biotin-conjugated anti-CD4 antibodies. A second incubation with CYC-conjugated streptavidin was used to detect biotin-positive cells. Appropriate isotype control antibodies were included but are not shown. The cells were analyzed by flow cytometry. Results are expressed as the percentage of cells with either forward scatter (FSC) versus Thy-1^+ fluorescence or Thy-1^+ versus $CD4^+$ fluorescence. The percentage of positively stained cells is indicated in each plot. Shown are representative experiments of two repeats.

physically infiltrate the vaginal mucosa during a vaginal *Candida* infection (1). It should be noted as well that we do not want to imply, based on our results obtained by using anti-Thy-1.2 antibodies, that other complement-fixing antibodies given systemically will not deplete specified cells in the vagina or that systemic administration of immune mediators will not affect vaginal cells. In fact, systemic injection of anti-CD3ε or bacterial superantigens has been reported to increase resident vaginal cells (11). Our results do, however, show the relative inefficiency of circulation by immune mediators from the periphery into the vagina.

The differential circulation of immune effector function, as shown here by cellular depletion analysis, provides a number of parallels to observations from both the murine model of vaginitis and clinical studies. These include (i) the lack of protection against experimental vaginitis by preinduced *Candida*-specific Th1-type CMI detectable in lymph nodes (5), (ii) the intractable episodes of vaginitis in women with recurrent vulvovaginal candidiasis despite the presence of normal Th1-type *Candida*-specific CMI in peripheral blood (3), and (iii) the detection of CMI in draining lymph nodes as a result of experimental vaginal infection (1a, 4).

The present results also lend support to the concept that mucosal tissues have some level of immunological independence. In contrast to a few reports of the presence in vaginal washes of antibodies directed against antigens derived from systemic immunization (10, 19, 20), evidence continues to accumulate that while mucosal immunization, including that which is CMI derived, induces immune responsiveness in both the systemic and mucosal compartments (12, 13, 21, 22), systemic immunization fails to produce mucosal responses despite strong systemic reactivity (22). In each case, although it is unclear whether systemically derived immunity is unable to traverse to mucosal sites or is simply not able to traverse at high enough levels to be effective, there certainly is evidence that differential, or potentially unidirectional, circulation of immune effector function occurs between the vaginal mucosa and the periphery.

This work was supported by Public Health Service grant AI-32556 from the National Institute of Allergy and Infectious Diseases.

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Editor: T. R. Kozel