Effect of Preexisting Immunity to Adenovirus on Transgene Product–Specific Genital T Cell Responses on Vaccination of Mice With a Homologous Vector

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We evaluated changes in global and human immunodeficiency virus (HIV)–specific genital T cells after vaccination of female mice with a replication-defective adenovirus vector of human serotype 5 (AdHu5) expressing Gag protein of HIV-1, in the presence or absence of preexisting immunity to the vaccine carrier. Our data show that preexisting immunity causes a rapid and transient decrease of genital CD4+ T cells without increasing the expression of chemokine (C-C motif) receptor 5. Furthermore, preexposure to AdHu5 affects long-term alterations in the magnitude and quality of vaccine-induced Gag-specific CD8+ T cell responses. AdHu5–specific antibodies interfere with the induction of transgene product–specific CD8+ T cell responses in systemic compartments, whereas some mechanism other than antibodies also seems to affect those that home to the genital tract.

In 2 phase IIb clinical trials (Step and Phambili trials), vaccination with an adenovirus (Ad) vector of human serotype 5 (AdHu5) expressing antigens of human immunodeficiency virus (HIV) induced potent T cell responses but failed to reduce transmission rates or significantly lower viral loads in individuals who became infected [1]. A number of hypotheses were formulated to explain the failure of the potent AdHu5 vaccine and the paradoxical increase in HIV-1 acquisition in male vaccine recipients with preexisting neutralizing antibodies to AdHu5 virus. Lack of protection by the AdHu5 vaccine designed to elicit T cell responses to conserved antigens of HIV-1 was largely attributed to lack of induction of HIV-1 specific antibodies and to the stimulation of a very narrow T cell response [2]. The increase in HIV-1 acquisition in males with circulating neutralizing antibodies to AdHu5 virus was especially worrisome, because it would preclude future use of AdHu5 vectors as vaccine carriers for any pathogen intended for vaccination of adults at risk for HIV-1 acquisition. AdHu5 infects most humans during childhood and up to 90% of humans residing in Sub-Saharan Africa carry neutralizing antibodies to this virus [3, 4]. Specific neutralizing antibodies prevent transduction of cells by AdHu5 vectors and thus expression of the transgene product, which in turn reduces the adaptive immune response to the vaccine antigen [5, 6].

In the Step trial, the increase in HIV-1 acquisition in men who were AdHu5 seropositive before vaccination may have been a reflection of confounding variables, such as circumcision status [7] or coinfections with other genital pathogens [8]. Others have argued that neutralizing antibodies were a surrogate marker for elevated numbers of memory CD4+ T cells to AdHu5.
virus, which expanded after vaccination and on migration to the mucosa of the genital tract or the rectum provided additional targets for HIV-1 infection. This was ruled out, however, because titers of neutralizing antibody to a specific serotype of Ad virus were not predictive of the frequencies of circulating Ad-specific T cells [9].

In a nonhuman primate study we reported elsewhere, pre-existing immunity to AdHu5 virus affected the biodistribution of T cells induced by Ad vaccines [6]. To test this effect in more detail, we compared global and Gag-specific genital T cell responses between groups of female mice that had or had not been preexposed to AdHu5 virus before intramuscular vaccination with an AdHu5 vector expressing HIV-1 Gag. We were particularly interested in changes in genital CD4+ T cells, which might provide targets for HIV infection, and in Gag-specific CD8+ T cells, which might reduce infection rates by eliminating virus-infected cells before HIV-1 replicates, spreads to other tissues, and escapes immune mechanisms through mutations.

MATERIALS AND METHODS

Mice
Female 6–8-week-old BALB/c mice (Ace Animals) were housed at the Animal Facility of the Wistar Institute. Experiments were performed according to institutionally approved protocols.

Viral Vectors
Purified E1-deleted AdHu5 expressing Gag of HIV-1 clade B, glycoprotein of rabies virus (rab.gp), or green fluorescence protein (GFP) was produced according to methods, including quality control, described elsewhere [10, 11].

Vaccination of Mice
For preexposure, groups of BALB/c mice were injected intramuscularly with 10^11 virus particles of AdHu5rab.gp. Two weeks later, serum samples were analyzed for AdHu5 neutralizing antibody titers. Mice with neutralizing antibody titers of ≥1:40 were vaccinated with 10^10 virus particles of AdHu5gag at 3 weeks after intramuscular preexposure.

Isolation of Lymphocytes
Lymphocytes were isolated as described elsewhere [12]. Blood was collected in 4% sodium citrate, and lymphocytes were purified through a Histopaque (Sigma) gradient solution. Spleens and iliac lymph nodes were dissociated against metal screens and washed with L-15 modified medium (Mediatech). For isolation of lymphocytes from the genital tract, the vagina, cervix, uterine horns, and ovaries were removed, cut into fragments, placed into Roswell Park Memorial Institute 1640 medium with 5% fetal bovine serum (Mediatech), and shaken at 130 rpm for 1 h. Cells were recovered, and the remaining fragments were digested with 1.4 mg/mL of collagenase type I (Gibco) for 15 min. Debris was removed by filtration and single cells were pooled. Lymphocytes were purified by a discontinuous Percoll (Sigma) gradient and counted using trypan blue dye (Sigma). Lymphocytes from blood and spleens were tested from individual mice, and cells from iliac lymph nodes and the genital tract were pooled from mice within the same group.

Neutralization Assay
Serum samples from individual mice were tested for AdHu5 neutralizing antibody titers, as described elsewhere [3]. Briefly, samples were inactivated at 56°C for 30 min and then serially diluted in 50 μL of Dulbecco’s modified Eagle medium with 10% fetal bovine serum (Mediatech), added to 50 μL of AdHuGFP vector diluted to 10^6 virus particles/mL, and incubated at room temperature for 1 h. The mixture was then transferred onto 3 × 10^4 HEK 293 cells in 96-well flat-bottomed plates. Plates were incubated at 37°C for 24 h and examined under a fluorescence microscope. Sample dilutions that showed >50% reduction of green fluorescent foci compared with infected controls incubated with naive serum were scored positive for neutralizing antibodies.

Adoptive Transfer of Immune Serum
Mice were injected intraperitoneally with .5 mL of serum derived from naive control mice or from mice preexposed twice with 10^11 virus particles of AdHu5rab.gp given intramuscularly at a 6-week interval.

Tetramer Staining, Phenotypic Analyses and Flow Cytometry
Cells were stained with a Gag peptide (AMQMLKETI)/major histocompatibility class I tetramer (National Institutes of Health), LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) and antibodies to CD8a (Invitrogen); CD4, CD103, chemokine (C-C motif) receptor 5 (CCR5) (BD Pharmingen); CD44, CD62L, PD-1, CD86 (BioLegend); CD69, CD127, NKG2D (eBioscience); and KLRG1 (Southern Biotech). Cells were permeabilized with BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Bioscience) and stained to granzyme B (eBioscience), Ki-67 (BD Pharmingen), and CTLA-4 (RD Systems). Cells were analyzed on a BD LSR II flow cytometer (Becton Dickinson) using FlowJo software (version 8.8; Tree Star) for data analyses [13].

Statistical Analysis
Each experiment was conducted twice with 5–10 mice, and figures show means and standard deviations for samples from independent experiments. Blood and spleens were assessed individually. Cells from iliac lymph nodes and genital tract were pooled from 2–3 or 5 mice, respectively. The statistical significance of differences between groups was calculated by 2-way analysis of variance followed by Bonferroni posttest or Student’s t test using GraphPad Prism software (GraphPad Software).
RESULTS

Genital T Cell Composition after AdHu5 Vaccination

To test whether vaccination with an AdHu5gag vector affects frequencies of T cells within the genital tract and whether such changes are influenced by preexisting immunity to AdHu5, female mice were preexposed intramuscularly with $10^{11}$ virus particles of AdHu5rab.gp. At this high dose of vector, all animals developed neutralizing antibody titers of $\geq 40$ within 14 days. Naive or AdHu5-immune mice were vaccinated intramuscularly with $10^{10}$ virus particles of AdHu5gag. Lymphocytes were isolated from the genital tract, and frequencies of CD4$^+$ and CD8$^+$ T cells were measured over time. Naive mice had stable frequencies of CD4$^+$ T cells within their genital tract throughout the 12-week observation period (Figure 1A). Early after vaccination, AdHu5 preexposed and nonpreexposed mice had reduced frequencies of CD4$^+$ T cells. In AdHu5-seronegative mice, frequencies of CD4$^+$ T cells returned to baseline by week 6, but in the preexposed group they stayed below those of naive mice until week 10 after vaccination. Reduction of frequencies was largely mirrored by a decline in absolute numbers of CD4$^+$ T cells (Figure 1C). Although these data showed significant differences only on days 3 and 7 after vaccination, owing to higher variability, overall they suggest that this decline in CD4$^+$ T cells reflects their egress from the genital tract rather than an influx of other cell types.

Figures and numbers of CD8$^+$ T cells were reduced in vaccinated mice on day 3 after vaccination (Figure 1B and D). In preexposed mice, frequencies of CD8$^+$ T cells remained slightly below those of naive mice for most time points until week 6.

Figure 1. Frequencies and numbers of genital CD4$^+$ and CD8$^+$ T cells. Graphs show percentages of CD4$^+$ (A) and CD8$^+$ (B) T cells, as well as absolute numbers of CD4$^+$ (C) and CD8$^+$ (D) T cells within the genital tract at different intervals after vaccination with AdHu5gag. Graphs show average frequencies or cell counts ± standard deviations (SD) for animals preexposed to adenovirus vector of human serotype 5 (AdHu5) and vaccinated with AdHu5gag 3 weeks later (i.m./i.m.; circles), mice vaccinated with the AdHu5gag vector without preexposure (-/i.m.; closed squares), and naive mice (open squares). Cells were isolated from the female genital tract and stained with a live cell stain and antibodies to CD4 and CD8. Statistical analyses compared 2 individual experiments, using 2-way analysis of variance followed by Bonferroni test. A, Both vaccinated groups had significantly lower frequencies of CD4$^+$ cells over time than did the naive control group ($P < .01$). Frequencies of CD4$^+$ cells for the 2 vaccinated groups differed only at week 8 after vaccination ($P < .05$). B, Frequencies of CD8$^+$ cells were reduced in the 2 vaccinated groups early after vaccination ($P < .01$). Frequencies of CD8$^+$ T cells in the nonpreexposed group were increased between weeks 1 and 8, compared with the naive group ($P < .01$). C, CD4$^+$ cell counts were significantly different on days 3 and 7 between either of the vaccinated groups and the control group ($P < .05$ by t test). D, Cell counts were significantly different for CD8$^+$ T cells only at week 1 when the 2 vaccinated groups were compared with the naive group ($P < .05$).
whereas in nonpreexposed mice they were increased from week 1 to week 8, compared with naive mice.

To test for vaccine-induced differences in phenotypes of genital T cells, lymphocytes isolated at 1, 6, and 12 weeks after vaccination were stained with a panel of antibodies to assess their differentiation status and functions. Lymphocytes were tested for surface expression of CD4, CD44, CD62L, PD-1, CD69, CD127, NKG2D, and KLRG1 and for intracellular granzyme B, Ki-67, and CTLA-4. Most of these markers were expressed at comparable levels on or in genital CD4+ T cells from naive or vaccinated mice. Only CD44, granzyme B, and PD-1 showed significant differences in expression (Figure 2A). A PD-1hiCD4+ population was seen in all vaccinated but not in naive mice by week 1 after vaccination; this population decreased by week 6 and was no longer detectable by week 12. Expression of CCR5 was not increased in either group at any time point (P > .05).

Phenotypic differences were more pronounced for CD8+ T cells from the genital tract of vaccinated mice, compared with unvaccinated mice (Figure 2B). Expression levels of CD44 tended to be higher, whereas those of CD62L were lower on CD8+ T cells from the preexposed group at 1 week after vaccination and from the nonpreexposed group at all time points tested. A subpopulation of CD69hiCD8+ T cells was detected at 1 week after vaccination in nonpreexposed, vaccinated mice; this population disappeared by week 6 and was not seen in any of the other groups at either time point. Levels of Ki-67 were increased on cells from both vaccinated groups at 1 and 6 weeks, and granzyme B levels were also slightly elevated in both groups at 1 week after vaccination. In naive mice, KLRG1 was initially low on most genital CD8+ T cells; however, we observed a population of KLRG1hi cells, which gradually increased over time.

This population may have been a reflection of the young animals’ continued exposure to environmental antigens during the 12-week period of the experiment. PD-1, an early activation marker [14] that may also indicate exhaustion [15], was up-regulated on CD8+ T cells isolated from nonpreexposed mice, compared with naive mice, at 1 week after vaccination. Overall, these results show that after AdHu5 vaccination, T cells appear to initially leave the mucosa and presumably migrate to sites of vaccine-induced inflammation [16, 17], whereas vaccine-induced T cells enter the mucosa after activation.

**Antigen-Specific CD8+ T Cell Responses**

To test whether preexisting immunity to AdHu5 has a differential effect on frequencies of AdHu5gag vector–induced transgene product–specific CD8+ T cells in different tissues, a group of mice was first preexposed intramuscularly with an AdHu5 vector expressing an irrelevant antigen. AdHu5-specific neutralizing antibody titers, measured from serum samples of individual mice 2 weeks later, were ≥1:40 and thus at levels that had previously been shown to reduce transgene product–specific CD8+ T cell responses to AdHu5 vector vaccination [18]. These mice, together with the nonpreexposed group, were vaccinated intramuscularly with AdHu5gag. Lymphocytes were isolated from blood, spleen, iliac lymph nodes, and the genital tract at 1, 4, and 12 weeks after vaccination (Figure 3). Nonvaccinated naive mice were tested in parallel. Gag-specific CD8+ T cells were not detected in naive mice or in mice that had been preexposed but not vaccinated (data shown for naive mice). In vaccinated mice, frequencies of Gag-specific CD8+ T cells from all sites and at all time points tested were significantly lower in preexposed compared with nonpreexposed mice (Figure 3). In blood, preexposure caused a modest reduction (∼50%) in
CD8+ T cell frequencies early after vaccination (ie, by weeks 1 and 4). This reduction became more pronounced by week 12 after vaccination (~80%). Gag-specific CD8+ T cells from the genital tract, although present at higher frequencies, mirrored the pattern seen in cells isolated from blood. In seronegative mice, frequencies of genital Gag-specific CD8+ T cells were highest at 1 week after vaccination, declined slightly by 4 weeks, and remained stable until week 12. In preexposed mice, the decline in frequencies of genital Gag-specific CD8+ T cells was more pronounced between weeks 1 and 12 than observed in cells isolated from blood. In iliac lymph nodes, frequencies were low in nonpreexposed mice and reduced further in preexposed mice; frequencies declined between weeks 1 and 4 and remained stable thereafter for the first group. In spleens, frequencies of Gag-specific CD8+ T cells increased over time in preexposed mice and decreased slightly in nonpreexposed mice, so that the ratio of frequencies between nonpreexposed and preexposed mice was initially high but declined gradually over time.

Preexposure to AdHu5 not only induces antibodies to AdHu5 but also elicits T cell responses to antigens of the Ad vector. We conducted serum transfer studies to test whether the reduction in Gag-specific CD8+ T cell frequencies in different tissues is caused mainly by preexisting antibodies, or whether cellular components of the immune system contribute. Mice were preexposed to AdHu5 or injected with serum from AdHu5-immune or naive mice. On preexposure or immune serum transfer, mice had titers ranging from 1:40 to 1:80. Mice were then vaccinated intramuscularly with AdHu5gag, and lymphocytes were isolated from blood, spleens, iliac lymph nodes, and genital tracts 2 weeks later and analyzed for frequencies of Gag-specific CD8+ T cells (Figure 4). Decreases in frequencies of Gag-specific CD8+ T cells in blood, spleens, and iliac lymph nodes were comparable between preexposed and AdHu5-immune serum-
Table 1. Reduction in Gag-Specific CD8+ T Cell Frequency After Immunization With AdHu5gag in Preexposed or Serum-Transferred Mice

<table>
<thead>
<tr>
<th>Treatment before vaccination</th>
<th>Reduction in Gag-specific CD8+ T cell frequency, mean ± SD, %</th>
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<tbody>
<tr>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>Preexposure to AdHu5</td>
<td>72.75 ± 1.58</td>
</tr>
<tr>
<td>Immune serum transfer</td>
<td>65.81 ± 4.94</td>
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NOTE. Before vaccination with AdHu5gag, one group of mice was preexposed to adenovirus vector of human serotype 5 (AdHu5), and the other group received intraperitoneal transfer of immune serum. Student’s t test was performed to measure differential reductions in frequencies between immunized groups after intramuscular preexposure or serum transfer.

a P < .05.

b P < .001.

Our results show that naive mice have higher frequencies of CD4+ T cell and CD8+ T cells in their genital tract. Unexpectedly, frequencies and absolute numbers of CD4+ T cells and CD8+ T cells initially declined in this tissue after vaccination, later returning to baseline. This effect was dose dependent and was not seen when the vaccine dose was lowered to 10^9 virus particles (data not shown). Higher doses of 10^11 virus particles did not further increase egress of T cells (data not shown), suggesting the presence of different T cell subpopulations that could or could not leave the genital tract once a threshold was reached. Although the decline of either T cell subset was transient, a general exodus of activated T cells from the genital mucosa (and as remains to be investigated, possibly from other peripheral sites) toward an inflammation triggered in response to a vaccine or an infection could potentially affect the host’s resistance to other infections. Phenotypes of CD4+ T cells that remained within the genital tract were similar overall to those observed in unvaccinated mice, except for slight increases in CD44hi and PD-1hi cells, perhaps suggesting the egress of less activated cells or retention of those expressing coinhibitory markers.

Mucosally transmitted HIV-1 isolates are almost uniformly CCR5 tropic. CCR5 is expressed on CD4+ T cells after their activation and expansion [19] and is especially high on CD4+ T cells within the intestinal mucosa [20]. Importantly, expression of CCR5 was not increased on CD4+ T cells within the genital tract at any of the time points analyzed. Overall, provided that T cells act similarly in AdHu5-vaccinated humans, these data suggest that AdHu5 vaccination initially reduces available HIV-1 targets within the female genital tract. Genital CD8+ T cells showed more marked differences between AdHu5-seropositive and seronegative mice after vaccination. Phenotypic analyses suggested that CD8+ T cells infiltrating the genital tract had been recently activated. Further
analyses of Gag-specific CD8$^+$ T cells showed that preexisting immunity to AdHu5 reduced their frequencies in all compartments and at all time points tested, although there were some unexpected quantitative and temporal differences between groups. First, the reduction of specific CD8$^+$ T cells seen at week 1 was more pronounced in spleen and iliac lymph nodes than in blood or the genital tract. Previous studies had indicated that the strength of T cell receptor signaling, which can be related to antigenic load, determines the kinetics of T cell activation [21] and affects CD8$^+$ T cell outcome [22]. The findings at week 12 support this latter notion, because Gag-specific CD8$^+$ T cells from seronegative and seropositive mice continued to show

Figure 5. Phenotypes of Gag-specific CD8$^+$ T cells. Mice preexposed to adenovirus vector of human serotype 5 (AdHu5) (i.m./i.m.) (gray lines) and nonpreexposed mice (-/i.m.) (black lines) were vaccinated with AdHu5gag. Cells from different compartments were analyzed 1, 6, and 12 weeks after vaccination. Cells were stained with a live cell stain, the Gag-specific tetramer, antibodies to CD8a, and the markers indicated. Graphs show expression of markers on tetramer (Tet)$^+$ CD8$^+$ T cells isolated from vaccinated mice. Gray shaded areas show expression of the markers on tetramer −CD8$^+$ T cells from naive mice. Graphs presenting samples from blood and spleens show cells isolated from 5 individual mice, and those from iliac lymph nodes (ILN) show samples pooled from 2 or 3 mice; data from these samples were concatenated using FlowJo software. Graphs of samples derived from the genital tract show results obtained with pooled lymphocytes from 5 mice. Student’s t test was used to compare percentage differences in median fluorescence intensity between samples derived from 2 individual experiments, of which 1 is displayed here. *P < .05 for difference between immunized and naive mice; †P < .05 for difference between the 2 immunized groups. CCR5, chemokine (C-C motif) receptor 5.
marked phenotypic differences. Preexposed mice showed a more pronounced contraction of Gag-specific CD8+ T cells in blood and within the genital tract than did nonpreexposed mice.

Ad vectors persist in a transcriptionally active form [11] and thus maintain primarily more activated CD8+ T cells, which fail to home to lymph nodes. Accordingly, frequencies of Gag-specific CD8+ T cells were consistently low in iliac lymph nodes. In spleens, which contain both highly activated and quiescent antigen-experienced cells, frequencies of Gag-specific CD8+ T cells decreased between weeks 1 and 12 in seronegative mice, but increased during the same period in AdHu5 preexposed mice. Phenotypic analyses showed that a higher proportion of Gag-specific CD8+ T cells from spleen, blood and iliac lymph nodes of Ad-seropositive mice had up-regulated CD62L, compared with those from seronegative mice. This suggests a more pronounced transition into a quiescent stage, although these cells lacked expression of CD127 (not shown), as would be typical for central memory CD8+ T cells.

In addition, cells from spleens and blood expressed elevated levels of CD69, viewed as an early and transient activation marker. CD69hiCD127lo memory T cells have been observed in other studies and represent a population that is maintained independent of cytokines, presumably through T cell receptor stimulation [23], as would be feasible under conditions of continued antigen production by a persisting vector. Notwithstanding, it remains to be investigated if and to what degree CD8+ T cells detected in preexposed mice resemble such cells or represent one of the many transitional stages of T cell differentiation that are being detected in human viral infections [24].

In summary, our results obtained in mice indicate that preexisting immunity to AdHu5 virus does not result in an increase of HIV-1 susceptible targets within the female genital tract, the most common port of HIV-1 entry. Preexposure to the vector changes the magnitude and quality of transgene product-specific CD8+ T cells induced by a homologous vaccine carrier. This reduction, which can be observed in several tissues, including the genital tract, can largely but not exclusively be attributed to a lowering of antigenic load caused by the presence of vaccine vector-specific antibodies. Animal studies can at best approximate what may happen in humans vaccinated with an Ad vector. Clinical trials using Ad vector vaccines, such as those for HIV-1, should be designed to assess not only systemic responses but also immunity at the most common port of entry for the pathogen.

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