The Importance of Local Mucosal HIV-Specific CD8$^+$ Cytotoxic T Lymphocytes for Resistance to Mucosal Viral Transmission in Mice and Enhancement of Resistance by Local Administration of IL-12

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Abstract

Although crucial to mucosal vaccine development, the mechanisms of defense against mucosal viral infection are still poorly understood. Protection, cytotoxic T lymphocytes (CTL), and neutralizing antibodies have all been observed, but cause and effect have been difficult to determine. The ability of CTL in the mucosa to mediate protection against mucosal viral transmission has never been proven. Here, we use an HIV peptide immunogen and an HIV-1 gp160-expressing recombinant vaccinia viral intrarectal murine challenge system, in which neutralizing antibodies do not play a role, to demonstrate for the first time that long-lasting immune resistance to mucosal viral transmission can be accomplished by CD8$^+$ CTL that must be present in the mucosal site of exposure. The resistance is ablated by depleting CD8$^+$ cells in vivo and requires CTL in the mucosa, whereas systemic (splenic) CTL are shown to be unable to protect against mucosal challenge. Furthermore, the resistance as well as the CTL response can be increased by local mucosal delivery of IL-12 with the vaccine. These results imply that induction of local mucosal CTL may be critical for success of a vaccine against viruses transmitted through a mucosal route, such as HIV. (*J. Clin. Invest.* 1998. 102: 2072–2081.) Key words: immunity • mucosal • immunity • cellular • AIDS vaccines • T lymphocytes • cytotoxic • Peyer's patches

Introduction

Vaccines capable of protecting against HIV must be capable of inducing long-term mucosal immune responses, given the fact that most natural transmission is via a mucosal route. In addition, it has recently been found that even after intravenous inoculation of simian immunodeficiency virus (SIV)[1] in monkeys, the mucosal lymphoid tissue is a major initial area of viral proliferation (1), and it is possible that mucosal immunity would help reduce this proliferation. However, it remains unclear how to best achieve mucosal protection and what mechanisms contribute to this protection. The latter is important in determining which immune response to aim for in developing a vaccine.

Previous studies demonstrating protection against mucosal challenge with virus have generally not determined the immune mechanism of protection (2–8). Other studies have demonstrated induction of cytotoxic T lymphocytes (CTL) in the mucosa but have not shown a role for these in protection (9–12). Likewise, previous studies of mucosal immune responses elicited by mucosal challenge with viruses have disclosed that the latter induces antiviral antibody responses and in some cases CTL responses in the intraepithelial lymphoid populations (13, 14). It is not clear if either of such responses is relevant to protection against viral infection in general or HIV infection in particular. Finally, some studies have shown a role for CTL in protection against infections that involve the pulmonary mucosa, such as influenza or respiratory syncytial virus (15–17), but these studies have not addressed the question whether the CTL must be present at the mucosal site of infection, or act systemically. To our knowledge, no previous study has demonstrated protection against mucosal challenge with virus that is mediated by CTL that must be present locally at the mucosal site of challenge. Similarly, in our own previous study (18), we showed induction of CTL and of protection with an engineered peptide vaccine, but we also could not tie the two together to determine the mechanism of protection against mucosal transmission of virus.

In the present work, we have addressed this question of mechanism and tested the hypothesis that protection against mucosal challenge with virus can be mediated by CTL, and indeed by CTL located in the local mucosal environment. Prior studies of protection have been confounded by the fact that multiple immune responses are present, and one cannot determine which is responsible for protection. Thus, to conduct the present mechanistic study, we took advantage of a novel approach we developed to evaluate protection in mice by mucosal CTL responses against HIV-1 antigens, in the absence of the ability to infect mice with HIV-1, under circumstances in which neutralizing anti-HIV antibodies would not confound

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; CTM, complete T cell medium; IR, intrarectal or intrarectally; LP, lamina propria; LPL, lamina propria lymphocyte(s); pfu, plaque-forming units; PP, Peyer's patch; SIV, simian immunodeficiency virus; SP, spleen.

the interpretation of the results. Mice were immunized intrarectally with an HIV-1 peptide vaccine construct consisting of a multideterminant helper portion ("cluster peptide") from the CD4 binding domain of gp160, called PCLUS3, colinearly synthesized with a CTL epitope and neutralizing antibody portion of the V3 loop from gp160 called P18, which elicits neutralizing antibodies, T cell help and CTL in BALB/c mice as well as other strains (19–21). However, we challenged the mice intrarectally with a recombinant vaccinia virus expressing the HIV-1 envelope protein gp160 (22) and measured titers of virus in the ovary, where this virus preferentially replicates and the highest titers are achieved. This virus has the important property that it expresses gp160 in the cells it infects but does not incorporate gp160 into the virus particle, so it is not susceptible to neutralization by neutralizing anti-gp160 antibodies (23). Thus, any protection achieved would have to be by an immune response directed against infected cells. CTL specific for gp160 would be expected to limit replication of the recombinant vaccinia virus as they would be expected to limit actual HIV infection, by targeting infected cells. Thus, the challenge model developed here allows us to determine the ability of CTL in mucosal sites to protect against a mucosal viral challenge.

An additional goal was to determine the optimal conditions for development of such mucosal CTL responses and protection. Of particular interest was whether IL-12 administration would enhance CTL induction and resistance against mucosal challenge. We focused on this cytokine and hypothesized that this might be the case, because our previous work (18) showed that induction of mucosal CTL was dependent on endogenous IL-12, in that treatment of mice with anti–IL-12 antibodies at the time of immunization abrogated the response. Furthermore, there was precedent from nonmucosal immunization studies from our lab (24) and others (25–27) that delivery of certain cytokines, including IL-12, with the antigen, can enhance systemic CTL responses. Therefore, in the current study we hypothesized that endogenous IL-12 might be limiting, and therefore the mucosal CTL response, and possibly protection against challenge as well, would also be further enhanced by mucosal delivery of exogenous IL-12 with the antigen.

Methods

Animals. Female BALB/c mice were purchased from Frederick Cancer Research Center (Frederick, MD). IFN-$\gamma^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice used in this study were 6–12 wk old. The IFN-$\gamma^{-/-}$ mice were maintained in a specific pathogen-free microisolator environment.

Immunization. Mice were immunized with four doses of the synthetic HIV peptide vaccine construct PCLUS3-18IIIB (19) (50 μg per mouse for each immunization) on days 0, 7, 14, and 21 in combination with cholera toxin (10 μg/mouse; List Biological Laboratories, Campbell, CA) by intrarectal administration. For subcutaneous immunization, incomplete Freund's adjuvant was used. rmIL-12 (a generous gift of Genetics Institute, Inc., Cambridge, MA) was delivered either intraperitoneally (1 μg) or intrarectally (1 μg) mixed with DOTAP (Boehringer Mannheim, Indianapolis, IN), a cationic lipofection agent, along with the peptide vaccine.

Cell purification. 5 wk to 6 mo after the first dose, antigen-specific T cells were isolated from Peyer's patches (PP), lamina propria (LP), and the spleen (SP). The PPs were carefully excised from the large and small intestinal wall and dissociated into single cells by use of collagenase type VIII, 300 U/ml (Sigma Chemical Co., St. Louis, MO) as described (28). It should be noted that we have combined lymphoid tissue from the large and small intestine on the assumption that the CTL activity is similar because of sharing of the common mucosal lymphoid circulation in this area, since the number of cells from the large intestine alone is not sufficient to perform the CTL studies. Our data showed that most PP $CD3^+$ T cells isolated from normal mice were $CD4^+$, while $CD3^+CD8^+$ T cells were less frequent. Furthermore, collagenase did not alter expression of CD3, CD4, or CD8 on splenic T cells treated with this enzyme. LP lymphocyte (LPL) isolation was performed as described (28). The small and large intestines were dissected from individual mice, and the mesenteric and connective tissues were carefully removed. Fecal material was flushed from the lumen with unsupplemented medium (RPMI 1640). After the PP were identified and removed from the intestinal wall, the intestines were opened longitudinally, cut into short segments, and washed extensively in RPMI containing 2% FBS. To remove the epithelial cell layer, tissues were placed into 100 ml of 1 mM EDTA and incubated twice (first for 40 min and then for 20 min) at 37°C with stirring. After the EDTA treatment, tissues were washed in complete RPMI medium for 10 min at room temperature and then placed into 50 ml of RPMI containing 10% FCS and incubated for 15 min at 37°C with stirring. The tissues and medium were transferred to a 50-ml tube and shaken vigorously for 15 s, and then the medium containing epithelial cells was removed. This mechanical removal of cells was repeated twice more, by using fresh medium each time, to completely remove the epithelial cell layer. Histologic examination revealed that the structure of the villi and lamina propria were preserved. To isolate LPL, tissues were cut into small pieces and incubated in RPMI 1640 containing collagenase type VIII, 300 U/ml (Sigma) for 50 min at 37°C with stirring. Supernatants containing cells were collected, washed, and then resuspended in complete RPMI 1640. This collagenase dissociation procedure was repeated two times, and the isolated cells were pooled and washed again. Cells were passed through a cotton–glass wool column to remove dead cells and tissue debris and then layered onto a discontinuous gradient containing 75% and 40% Percoll (Amersham Pharmacia Biotech, Inc., Stockholm, Sweden). After centrifugation (4°C, 600 *g*, 20 min), the interface layer between the 75% and 40% Percoll was carefully removed and washed with incomplete medium. This procedure provided > 90% viable lymphocytes with a cell yield of $1.5–2 \times 10^6$ lymphocytes per mouse. The SP were aseptically removed and single-cell suspensions prepared by gently teasing them through sterile screens. The erythrocytes were lysed in Tris-buffered ammonium chloride, and the remaining cells were washed extensively in RPMI 1640 containing 2% FBS.

Cytotoxic T lymphocyte assay. Immune cells from SP, PP, and LP (in the case of LP pooled from five animals) were cultured at 5×10^6/ml in 24-well culture plates in complete T cell medium (CTM): RPMI 1640 containing 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5×10^{-5} M 2-mercaptoethanol. 3 d later, we added 10% concanavalin A supernatant–containing medium as a source of IL-2. LPL were studied after 7 d stimulation with 1 μM P18IIIB-I10 peptide together with 4×10^6 of 3,300-rad irradiated syngeneic spleen cells. SP and PP cells were stimulated in vitro similarly for one or two 7-d culture periods before assay. Cytolytic activity of CTL lines was measured by a 4-h assay with ^{51}Cr-labeled targets. Two different cell lines were used as target cells: 1) 15–12 cells (29) (BALB/c 3T3 fibroblasts transfected with HIV-1IIIB gp160 and endogenously expressing HIV gp160), compared with 18 Neo BALB/c 3T3 fibroblasts transfected with Neo^R alone as a control, and 2) P815 targets tested in the presence or absense of I10 peptide (1 μM). For testing the peptide specificity of CTL, ^{51}Cr-labeled P815 targets were pulsed for 2 h with peptide at the beginning of the assay. The percentage of specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. Spontaneous release was determined from target cells incubated without added effector cells.

Recombinant vaccinia virus used for challenge studies. Recombinant vaccinia virus vPE16 expresses the HIV-1 gp160 gene from isolate IIIB (BH8) (22). Expression is directed by the compound early/late P7.5 promoter. Two copies of the sequence T5NT, which serves as a transcription termination signal for early vaccinia virus genes, are present in the IIIB gp160 gene. Both of these have been altered in vPE16 so as to retain the original coding sequence and allow early transcription of the gene. The virus, vSC8, is used as a negative control without gp160 (30). Both vPE16 and vSC8 express beta-galactosidase.

Study of the virus titer in the ovary. On day 35 or 6 mo after cluster peptide HIV vaccine immunization, mice were challenged intrarectally with 2.5×10^7 or 5×10^7 plaque-forming units (pfu) of vaccinia virus expressing gp160IIIB (vPE16) or vSC8 control virus. 6 d (or 2 d as indicated) after the challenge with recombinant vaccinia virus, the mice were killed, and ovaries were removed, homogenized, sonicated, and assayed for vPE16 or vSC8 titer by plating serial 10-fold dilutions on a plate of BSC-2 indicator cells, staining with crystal violet and counting plaques at each dilution. The minimal detectable level of virus was 100 pfu.

Results

Mucosal immunization of mice with cluster peptide construct provides long-lasting resistance to infection with recombinant vaccinia virus expressing HIV-1 gp160. We have recently observed the ability of the mucosal immune responses induced by the HIV cluster peptide vaccine PCLUS3-18 IIIB to protect against virus challenge via a mucosal route (18). However, in that study, we could not determine the mechanism of protection. First, to determine the specificity of this protection for recombinant protein HIV-1 IIIB gp160, we immunized mice intrarectally (IR) with PCLUS3-18IIIB mixed with cholera toxin adjuvant on days 0, 7, 14, and 21 and challenged the mice on day 35 by IR infusion with vaccinia virus expressing HIV-1 IIIB gp160 (vPE16) or with control vaccinia virus expressing β-galactosidase (vSC8) (Fig. 1). Unimmunized animals challenged with vPE16 or vSC8 served as controls. 6 d after the challenge, mice were killed, and the ovaries were removed and assayed for vaccinia titer (6 d after infection with vaccinia, the ovaries contain the highest titer of virus). We found that IR immunization with the synthetic HIV peptide vaccine protected mice against an IR challenge with vaccinia virus expressing HIV-1 IIIB gp160 compared with unimmunized controls but did not protect against IR challenge with vaccinia virus expressing only an unrelated protein, β-galactosidase (Fig. 1). Thus, the protection was specific for virus expressing HIV-1 gp160, and any nonspecific inflammatory response induced by the peptide infusion intrarectally was not sufficent to protect against viral challenge 2 wk after the last dose of the immunization.

Although we have observed the presence of mucosal memory CTL precursors, requiring restimulation in vitro for activity, 6 mo after IR immunization (18), it was not clear if these would be sufficient for protection (31, 32). To address this question, we challenged the IR immunized mice 6 mo after the start of immunization with PCLUS3-18IIIB, by IR administration with vaccinia virus expressing HIV-1 IIIB (vPE16). We found that even 6 mo after HIV cluster peptide immunization, BALB/c mice exhibited similar resistance against recombinant HIV–vaccinia challenge (Fig. 2).

Resistance of mice against mucosal viral challenge is mediated by CD8⁺ CTL in the mucosal site. To determine the immune mechanism responsible for the resistance against mucosal challenge with virus expressing HIV gp160, we first treated mice IP with 0.5 mg monoclonal anti-CD8 antibody (clone 2.43, National Institutes of Health, Frederick, MD) 1 d before and after each of the four immunizations and also 2 d before and 3 d after the challenge with vPE16. We found that such treatment led to a significant inhibition of the protection against mucosal challenge with vPE16 (Fig. 3 *A*). This experiment implies that $CD8^+$ cells are necessary but does not distinguish whether they are required in the induction phase or the effector phase of the response. We expected the latter, since the main function of $CD8^+$ CTL is effector rather than regula-

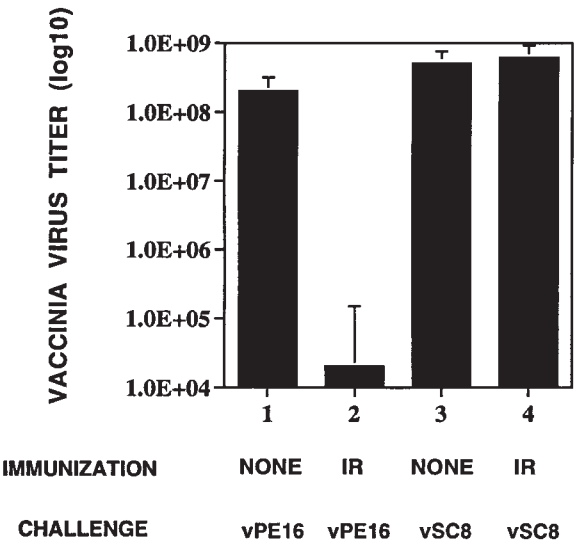

Figure 1. Protection induced by mucosal immunization with HIV-1 peptide vaccine is specific. On day 35, mice were challenged intrarectally with 2.5×10^7 pfu of vaccinia virus expressing gp 160IIIB (*vPE16*) or with 2.5×10^7 pfu of vaccinia virus expressing β-galactosidase (*vSC8*). Bars = SEM of five mice per group. The difference is significant at $P < 0.01$ by Student's *t* test.

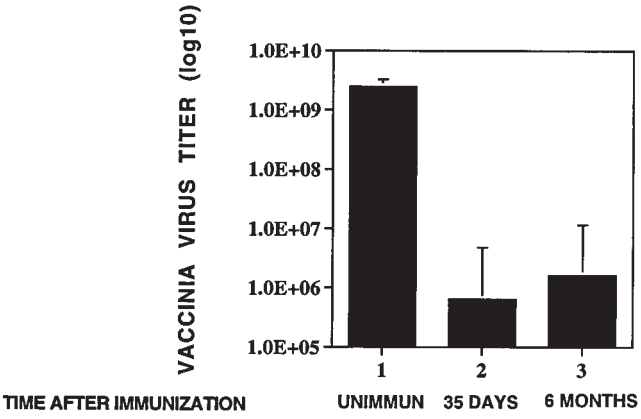

Figure 2. Protection induced by mucosal immunization with HIV-1 peptide vaccine is long lasting. On day 35 or 6 mo after the start of the immunization, mice were challenged intrarectally with 2.5×10^7 pfu of vaccinia virus expressing gp 160IIIB. Bars = SEM of five mice per group. The difference is significant at $P < 0.01$ by Student's *t* test.

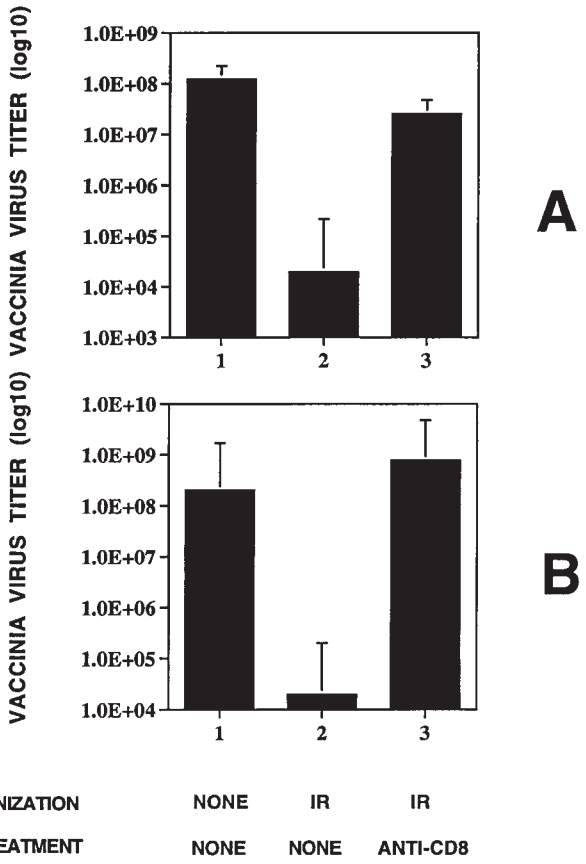

Figure 3. Protection induced by mucosal immunization with HIV-1 peptide is dependent on CD8 positive T cells. (*A*) BALB/c mice were treated IP with 0.5 mg monoclonal anti-CD8 antibody (clone 2.43, NIH, Frederick, MD) 1 d before and after each immunization and also 2 d before and 3 d after the challenge with vPE16. Mice were challenged intrarectally with 2×10^7 pfu of vPE16 vaccinia virus expressing gp 160IIIB. The difference between groups 2 and 3 is significant at $P < 0.01$ by Student's *t* test. (*B*) A similar experiment was carried out except that the anti-CD8 antibodies were administered only 2 days before and 3 days after vPE16 challenge, not before or after immunization. The difference between groups 2 and 3 is significant at $P < 0.01$ by Student's *t* test.

tory, but to test this, we repeated the experiment with anti-CD8 treatment only at the time of viral challenge, not at the time of immunization. The same result was obtained (Fig. 3 *B*). Thus, protection in the mucosal site against the virus expressing HIV-1 gp160 is mediated by $CD8^+$ lymphocytes.

Because our peptide construct elicits both strong mucosal and systemic MHC class I restricted $CD8^+$ CTL responses (18), we wanted to know which of these was responsible for resistance to mucosal challenge. Since we have previously found (18) that SC immunization with the peptide vaccine elicits splenic but not mucosal CTL, whereas IR immunization elicits both (Fig. 4 *A*), we could thus compare SC and IR immunizations to ask whether systemic CTL were sufficient to protect against mucosal challenge or whether local mucosal CTL were necessary. Accordingly, we immunized mice with PCLUS3-18IIIB plus incomplete Freund's adjuvant by the SC route or with PCLUS3-18IIIB and CT by the IR route on days 0, 7, 14, and 21 and compared these. On day 35 after the start of immunization, these groups of mice as well as unimmunized control mice were challenged by IR administration of vaccinia virus expressing HIV-1 gp160 (vPE16). Finally, 6 d after the challenge, mice were killed, and their ovaries were assayed for viral titer. SC immunization with PCLUS3-18IIIB did not protect mice against mucosal challenge with vPE16, whereas IR immunization with the same peptide did protect (Fig. 4 *B*). Thus, resistance against mucosal challenge with virus expressing HIV-1 gp160 can be induced only by mucosal immunization of mice and correlates with local mucosal CTL activity, not with splenic CTL activity. We conclude that the $CD8^+$ CTL-mediated protection from mucosal challenge with recombinant vaccinia expressing HIV-1 gp160 requires local mucosal $CD8^+$ CTL, whereas a systemic CTL response is not sufficient.

To confirm that the reduced viral titer in the ovary was due to limitation of replication at the site of mucosal transmission rather than in the ovary, we assessed the virus titer in the ovary 2 d after challenge, when the ovaries would be expected to be seeded and before significant viral replication in the ovary would be expected to have taken place. Although the viral titers were substantially lower at this time, the titer was almost two logs lower in the immunized compared with unimmmunized mice (Fig. 4 *C*). We conclude that the reduction in titer in the ovary really reflects a reduction in the amount of virus that can escape the initial mucosal site of infection and therefore represents the activity the CTL in the mucosal tissues.

Further support for this conclusion comes from attempts to measure virus titer in colorectal tissue 2 d after intrarectal inoculation. Although the numbers of viral pfu at this site, compared with the ovary, were too low to analyze in detail, they ranged from $\sim$ 6,000 to $\sim$ 14,000 pfu per organ in unimmunized mice but were below the limits of detection (100 pfu) in the intrarectally immunized mice. Thus, although we could not conduct most studies by using pfu in the colorectal tissue rather than the ovary, the limited data obtainable support the conclusion that the reduction in titer in the ovary was due to action of CTL in the mucosa at the site of entry and initial replication of virus.

Enhancement of resistance by local administration of IL-12 with the vaccine. Another goal was to determine whether the level of resistance to viral challenge could be enhanced by the use of cytokines as an adjuvant. In previous studies (18), we found that induction of mucosal CTL by the peptide vaccine was dependent on endogenous IL-12, in that it could be blocked by in vivo treatment of the mice with anti–IL-12. In the current study, we hypothesized that if endogenous IL-12 was necessary but limiting, additional IL-12 might increase the CTL response and enhance protection as well. To test this hypothesis, we first treated BALB/c ($H\text{-}2D^d$) mice by the IP route with 1 μg of the rmIL-12 each day of the IR immunization with PCLUS3-18IIIB (50 μg/mice). We found that this treatment did not lead to the significant changes in the HIV-specific CTL activity in either mucosal or systemic sites (Fig. 5). However, when we treated the mice with the rmIL-12 (1 μg) + DOTAP intrarectally together with peptide, we found a significant increase in the CTL level in both mucosal and systemic sites 35 d after the start of immunization compared with peptide alone in DOTAP (Fig. 5).

In view of the above results, we asked whether rmIL-12 administration at the local site and time of mucosal immunization can increase the protection against mucosal challenge with vaccinia virus expressing HIV-1 gp160. To address this

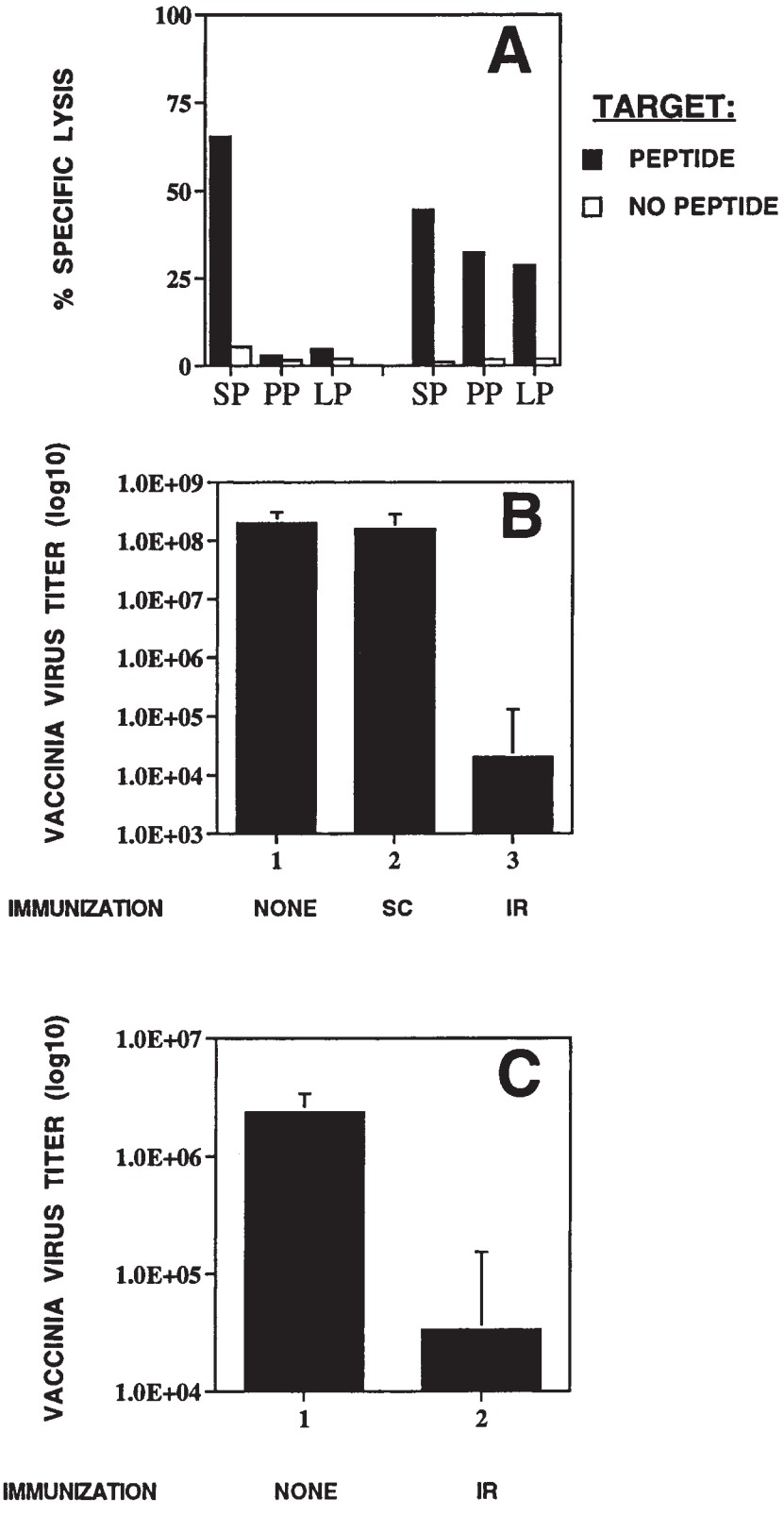

Figure 4. Mucosal immunization with HIV-1 peptide induces mucosal CTL responses (*A*) and protective immunity against intrarectal recombinant HIV-1 vaccinia challenge (*B*). (*A*) Induction of the mucosal and systemic CTL responses by different routes of immunization with synthetic peptide HIV vaccine (left, SC; right, IR immunization). CTL activity was measured in lymphocytes isolated from the spleen (*SP*), Peyer's patches (*PP*), or lamina propria (*LP*). Killing of peptide-pulsed targets (*closed bars*) is compared with killing of unpulsed targets (*open bars*) at an effector-to-target ratio of 50:1. (*B*) On day 35, IR (bar *3*) or SC (bar *2*) immunized BALB/c mice were challenged intrarectally with 2.5×10^7 pfu of vaccinia virus expressing gp 160IIIB and compared with unimmunized mice (bar *1*). The difference between group 3 and either of the others is significant at $P < 0.01$ by Student's *t* test. (*C*) Reduction in viral titer in the ovary in IR-immunized (*right*) compared with unimmunized (*left*) mice is detected as early as 2 d after viral challenge with 2.5×10^7 pfu of vPE16 ($P < 0.05$).

question, we immunized BALB/c mice with the rmIL-12 + DOTAP intrarectally together with peptide. We then challenged the immunized mice on day 35 after the start of immunization by IR administration of vaccinia virus expressing HIV-1IIIB gp160. In this experiment, we used twice the dose of challenge virus, allowing a bigger window since the unimmunized mice had a titer of several times 10^{10} rather than several times 10^8 seen in earlier experiments with a lower chal-

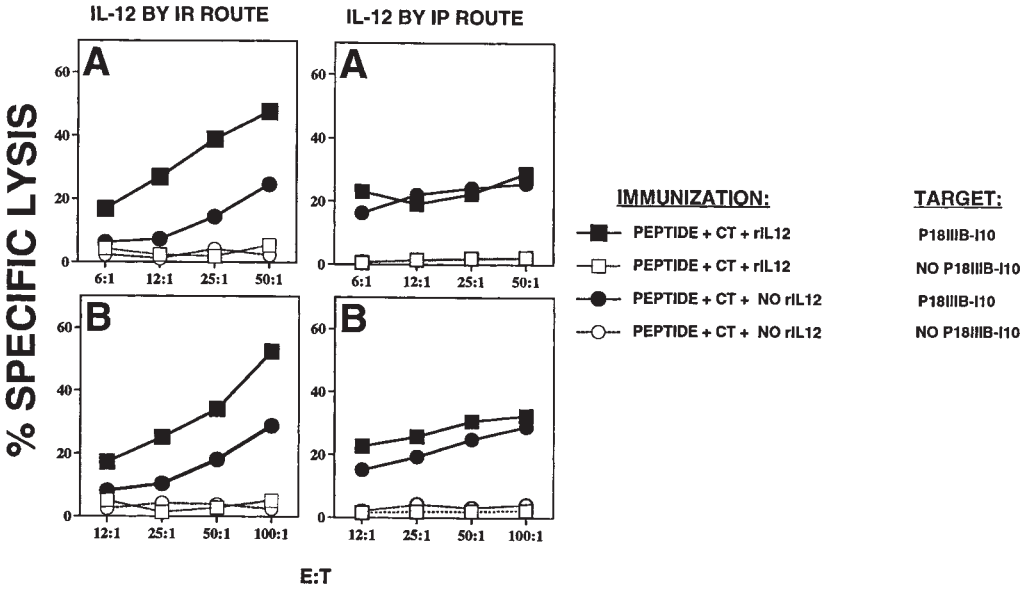

Figure 5. Enhancement of the mucosal (*A*) and systemic (*B*) CTL responses to HIV-1 peptide by the mucosal (not systemic) treatment with rmIL-12. BALB/c mice were treated by the IP route (*right column*) or IR route (*left column*) with 1 μg of the rmIL-12 each day of the IR immunization with PCLUS3-18IIIB (50 μg/mice). For intrarectal administration, both peptide alone and peptide plus IL-12 were administered in DOTAP to allow comparison. On day 35 HIV-specific Peyer's patch CTL (*A*) and spleen CTL (*B*) were studied.

lenge dose. Nevertheless, the immunized mice showed a reduction of > 4 logs in virus titer, as had been seen in the earlier experiments (Fig. 6, bar *2*). Importantly, IR immunization with the synthetic HIV peptide vaccine plus rmIL-12 protected mice against an IR challenge with this gp160–recombinant vaccinia virus even more effectively than after the IR immunization with peptide alone (6-log reduction in viral pfu vs. 4-log reduction, $P < 0.05$) (Fig. 6, bar *3* vs. bar *2*). The correlation between enhanced CTL response and enhanced resistance adds further correlative support to the conclusion that the protection is mediated by CTL.

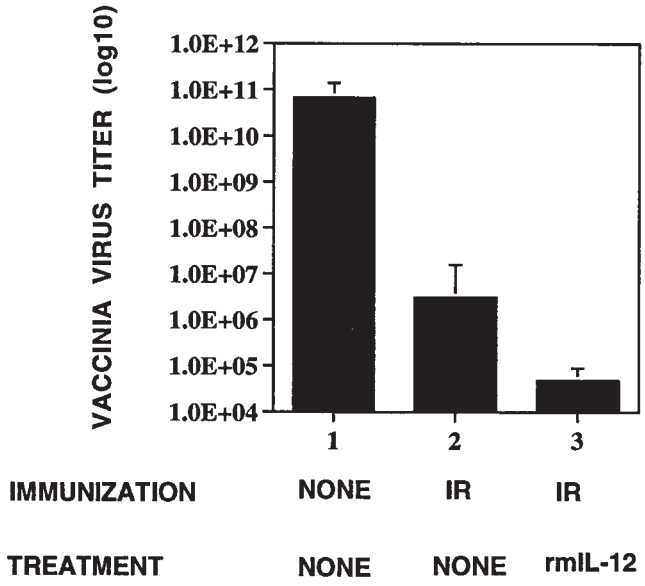

Figure 6. Mucosal treatment with rmIL-12 in DOTAP along with HIV peptide vaccine enhances protection against mucosal challenge with vaccinia virus expressing gp 160IIIB (vPE16). Five mice per group were immunized IR on days 0, 7, 14, and 21 with nothing, with 50 μg PCLUS3-18IIIB alone in DOTAP, or with peptide plus 1 μg rmIL-12 in DOTAP, and challenged on day 35 intrarectally with 5×10^7 pfu of vaccinia virus expressing gp 160IIIB. Viral pfu in the ovaries were determined 6 d later. The difference between groups 2 and 3 is significant at $P < 0.05$ by Student's *t* test.

Since we previously found that induction of mucosal $CD8^+$ CTL is strongly dependent on IL-12 and IFN-γ (18), we asked which cytokine acts directly in generating mucosal CTL and which acts through a secondary mechanism. To address this question, we treated IFN-$\gamma^{-/-}$ mice (BALB/c background) and conventional BALB/c mice with the rmIL-12 (1 μg/mouse) + DOTAP IR together with peptide. We found that mucosal treatment of IR-immunized IFN-$\gamma^{-/-}$ mice with rmIL-12 did not lead to the induction of mucosal or systemic CTL (Fig. 7). It thus appears that IL-12 cannot act directly in the induction of mucosal $CD8^+$ CTL in the absence of IFN-γ.

Discussion

In our earlier studies, we developed a synthetic HIV peptide vaccine construct consisting of clusters of overlapping helper T cell epitopes (33) attached to a peptide portion (P18) of the V3 loop of gp160 known to induce neutralizing antibodies (34–36) that we showed also elicited a strong immunodominant $CD8^+$ CTL response in BALB/c mice (29, 37, 38). These vaccine constructs were multifunctional so that when administered SC in complete or incomplete Freund's adjuvant in mice, they elicited high titer neutralizing antibodies and helper T cells (19, 21) and splenic CTL (20). The relevance of these constructs to human HIV infection was recently shown by the fact that they elicit CTL-, helper T cell-, and neutralizing antibody responses in HIV-infected humans (39), when given SC in montanide ISA 51, an incomplete Freund's-like emulsion adjuvant. Recently, we found that this synthetic HIV peptide vaccine administered IR can induce both mucosal and systemic P18-specific CTL responses in mice (18). We also observed that mucosally immunized mice were protected against mucosal challenge (18), but we could not address the mechanism or specificity of protection or demonstrate that the CTL response observed had anything to do with the protection.

In the current study, we took up the question of the mechanism of protection, and in particular, whether the protection was mediated by CTL, and if so, whether it required local mu-

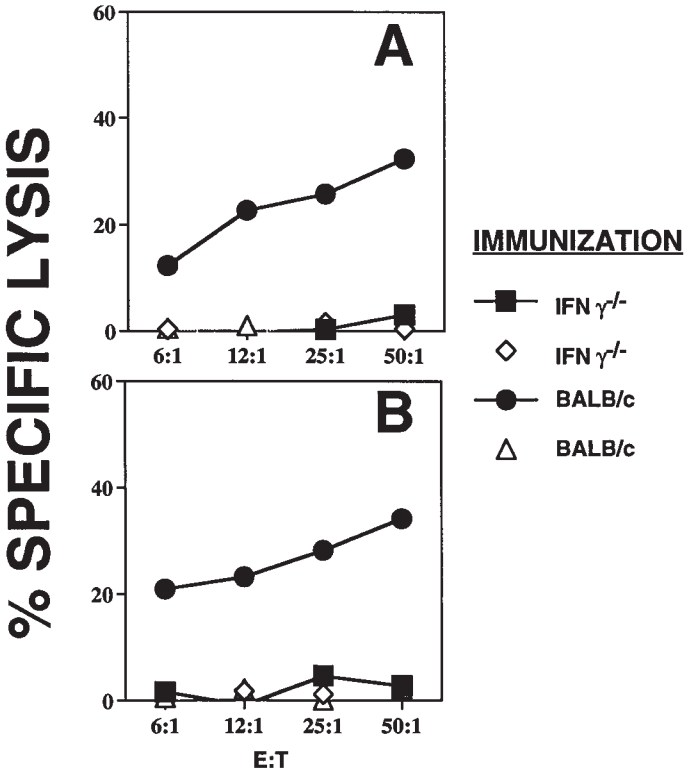

Figure 7. IL-12 cannot act directly in the induction of mucosal $CD8^+$ CTL in the absence of IFN-γ. IFN-$\gamma^{-/-}$ mice (BALB/c background) were immunized IR with the rmIL-12 (1 μg/mouse) + DOTAP together with peptide. On day 35 HIV-specific Peyer's patch CTL (*A*) and spleen CTL (*B*) were studied. Killing of P18IIIB-I10-pulsed targets by effector cells from immunized IFN-$\gamma^{-/-}$ mice (*closed squares*) or conventional BALB/c mice (*closed circles*) is compared with killing of unpulsed targets (*open squares* or *circles*).

cosal CTL. The role of CTL in protection against mucosal infections has been of interest for decades, especially in the case of influenza virus (9, 15, 16). However, in each system only a part of the question could be addressed. Thus, while a number of studies have shown a role for CTL in protection against infections such as influenza that have a mucosal component (15–17), these have not established whether the CTL need to be in the local mucosal site to protect. Conversely, while other studies have shown the induction of CTL in the mucosa, they have not established that these cells have a role in protection (9–12). Yet, other studies have shown the induction by vaccines of protective immunity in the mucosa but, in the face of multiple immune responses including neutralizing antibodies, have not been able to sort out which responses are responsible for protection (3–8). Thus, studies have either looked at immune responses or looked at protection, but it has been hard to prove that a particular immune response is the one mediating protection. In this regard, because mucosal infection by virus induces a local IgA response, it has been often assumed that this response and not a concomitant CTL response was responsible for protection against viral infection through the mucosal route. However, the role of secretory IgA in neutralizing and protecting against mucosal HIV challenge is not clear. We are not aware of any previous studies that demonstrate protection against viral infection through a mucosal route to be mediated by CTL that must be present in the local mucosal site of exposure to virus. The present system lends itself to addressing this issue.

To approach this problem, we developed a novel viral challenge system in which recombinant vaccinia virus expressing HIV-1 gp160 is used as a surrogate for HIV-1 since we cannot infect the mice with HIV-1. Importantly, in this system, neutralizing antibodies to gp160 cannot protect against the recombinant vaccinia expressing gp160 because the virus does not incorporate gp160 in the virus particle but expresses it only in the infected cell (23). Thus, the protective immune response must be directed at the infected cell. It is not likely to be mediated by a noncomplement-fixing antibody such as IgA. This system allowed us to ask whether CTL alone are capable of protection against mucosal challenge with virus in the absence of the confounding effects of a neutralizing antibody response. We now show that the resistance to transmission in this system is completely dependent on $CD8^+$ cells, by the abrogation of protection after in vivo depletion of $CD8^+$ cells. Thus, the results show unequivocally that it is $CD8^+$ CTL (whether via lytic activity or via secretion of cytokines or other soluble factors) that protect. Since it has been shown (40) that protection against vaccinia infection can be mediated by interferon-γ, which is secreted by $CD8^+$ CTL in response to antigen stimulation, it is quite possible that the mechanism involves local secretion of this cytokine by the CTL rather than lysis of infected cells, but by either mechanism, the CTL are the cells mediating protection.

However, since the mucosal immunization induces CTL in both the local mucosal site and the spleen, this result does not distinguish which CTL are responsible for resistance to viral transmission. To address this second part of the question, we took advantage of the fact that SC immunization with the peptide induces systemic CTL in the spleen at a level at least as high as that induced by mucosal immunization but does not induce mucosal CTL. Splenic CTL resulting from both immunization routes killed target cells endogenously expressing HIV-1 gp160 (reference 18 and data not shown). Thus, if systemic CTL against this epitope protected against mucosal challenge, then the subcutaneously immunized mice would have been expected to be protected. However, the SC-immunized mice showed no evidence of protection against mucosal challenge.

Thus, the protection correlated with CTL activity in the local mucosal sites, not with CTL activity in the spleen. We conclude that the protection not only was mediated by CTL but also required CTL in the local mucosal site of challenge. Systemic CTL were not sufficient. Determining whether or not local mucosal CTL are also sufficient, as well as necessary, would require studies in which CTL could be generated only in the mucosa and not concurrently in the spleen. Although the levels of virus that we could measure in the mucosal tissues themselves were too low to analyze as the primary output for the experiments rather than pfu in the ovary, the low colorectal viral levels seen in controls were reduced to below the limit of detection in the immunized animals. Additional support for the resistance occurring at the mucosal site comes from the observation that we observed a 2-log reduction in viral pfu in the ovary even at day 2 after mucosal viral challenge (from 2.37×10^6 pfu in unimmunized mice to 3.34×10^4 pfu in IR immunized mice), before much replication could have occurred in the ovary, suggesting that the reduction in titer in the ovary really reflects a reduction in the amount of virus that can escape the initial mucosal site of infection. In addition, the enhancement of both CTL activity and protection by rmIL-12 depended on local mucosal administration of the cytokine, not systemic administration. We conclude that protection against GI mucosal challenge not only is CTL mediated but also requires local mucosal CTL at the site of challenge (either in the colorectal mucosa and/or possibly the lymphoid tissue draining this area). These results have important implications for the development of protective vaccines against mucosal exposure to viruses.

We also found surprising persistence not only of memory CTL in the mucosa but also of protective immunity against mucosal viral challenge. Factors controlling CTL memory, and the role of persistent antigen in maintaining memory CTL, represent another issue that has been of interest for some time (31, 32, 41) but has been little studied in the context of mucosal immune responses and protection. In one study of mucosal CTL memory, it was shown that memory CTL remained at the mucosal site longer if the immunization was via the mucosal route (10), but the duration of protection by such mucosal CTL was not studied. The ability of mucosal memory CTL to protect will depend in part on the rapidity by which they can expand and be activated after virus exposure. For these reasons, it was important to know the duration of mucosal protection dependent on local mucosal $CD8^+$ CTL. In a previous study, we found mucosal $CD8^+$ CTL precursors at least 6 mo after IR immunization of mice with PCLUS3-18IIIB. This study did not address the longevity of protection, which is believed to require activated CTL. Here, we show that the persistence of mucosal CTL even 6 mo after mucosal immunization with the peptide vaccine construct is accompanied by resistance to mucosal challenge with vaccinia virus expressing gp160. This result is particularly striking because the immunogen is just a peptide administered without any depot form of adjuvant that would maintain the presence of antigen for extended periods. It would be expected that free peptide delivered to the lumen of the gut, or even after transport by mucosal cells, would have a very short half-life. Therefore, either memory CTL can persist in the mucosa at levels sufficient to protect in the absence of persistent antigen, or antigen must persist locally in some cell-bound form, perhaps on MHC molecules of dendritic cells or as a crossreactive microbial antigen.

Several pieces of evidence indicate that the resistance to viral challenge is mediated by memory CTL induced by the vaccine and not CTL induced by the viral challenge. First, resistance was detected as early as 2 d after challenge (Fig. 4 *C*), before a primary antivaccinia CTL response could be mounted. Second, the resistance is specific for the gp160, in that unimmunized mice did not show protection against the recombinant vaccinia virus expressing gp160, and immunized mice did not show protection against a control recombinant vaccinia expressing only beta-galactosidase in addition to the vaccinia antigens (Fig. 1). Thus, the resistance is clearly vaccine mediated and vaccine specific.

Another goal of this study was to optimize the induction of CTL response and protective immunity. We (24) and others (25–27) have found that delivery of certain cytokines such as IL-12 at the site of antigen immunization systemically can enhance systemic CTL responses, but comparable studies have not been done for mucosal CTL responses. In addition, we found that the induction of mucosal CTL was dependent on endogenous production of IL-12 by the mouse, because it could be inhibited by anti–IL-12 antibody given in vivo before and after each immunization (18). In the current study, we reasoned that if endogenous IL-12 was necessary and perhaps limiting (because of the ease of inhibition), we might be able to increase the CTL response by delivering additional IL-12 with the antigen. Indeed, as shown in Fig. 5, IL-12 given with the antigen intrarectally, significantly enhanced CTL induction and also, most importantly, increased protection against intrarectal vaccinia viral challenge (Fig. 6). However, it was striking that IL-12 delivered systemically intraperitoneally did not enhance CTL induction either in the spleen or in the mucosa. This difference may be due to the short half-life of IL-12, preventing it from surviving long enough to get to the sites of CTL induction. Therefore, we conclude that for mucosal CTL induction as well as for protection, it is important to deliver the cytokine directly to the site of antigen administration and CTL induction. Furthermore, enhancement of CTL induction in the mucosa with recombinant IL-12 may be a valuable strategy for mucosal vaccine development. In this context, small doses given locally in the mucosal sites are not likely to have the toxicity that has been associated with systemic administration of this cytokine.

Our results further show that enhancement of the CTL response in vivo by rmIL-12 is dependent on IFN-γ, as no enhancement was observed in IFN-$\gamma^{-/-}$ mice. However, at least two mechanisms can explain this result. First, IL-12 may be acting through its well-defined ability to induce production of IFN-γ (42), which then acts directly on CTL precursors. Alternatively, since IFN-γ is important for expression of the IL-12 receptor (43), IL-12 may act directly on CTL but may not be able to act in IFN-$\gamma^{-/-}$ mice because of the lack of IL-12R expression. Thus, whether IL-12 acts directly or indirectly to enhance protective CTL responses remains a chicken–egg problem that has plagued all studies of the interaction between IL-12 and IFN-γ.

There is circumstantial evidence in humans to suggest that CTL activity may play a role in protective immunity against HIV-1 (reviewed in 44–46). While these HIV and SIV studies were not directed at mucosal immunity, the combination with our current model study in mice suggests that CTL immunity at the local site of mucosal exposure to virus should be a critical goal of vaccine development to prevent HIV infection or

disease in humans as well. Because the gastrointestinal tract is a major site of early SIV (and presumably HIV) replication (1), and because this and other mucosal sites are frequent sites of entry of virus, it is particularly critical to achieve protection at mucosal sites. While the current study of pox virus infection in mice is not an exact model of HIV infection in humans, it is a model that has allowed demonstration of proof of principle of the requirement for local mucosal CTL for controlling mucosal viral transmission. It provides a scientific basis for now attempting to translate this result to primate and human studies. We are currently initiating studies in primates to test the possibility that such peptide vaccines administered intrarectally might protect against SIV infection through a mucosal route.

In summary, these studies demonstrate that resistance against mucosal viral challenge can be mediated by CTL, which must be present in the local mucosa, and provide a way of enhancing the induction of such local mucosal CTL and also increasing protection by a peptide vaccine given together with recombinant IL-12 at the mucosal site.

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