Long-lived Cytotoxic T Lymphocyte Memory in Mucosal Tissues After Mucosal but Not Systemic Immunization

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Summary

The induction and maintenance of long-term CTL memory at mucosal surfaces may be a critical component of protection against mucosal pathogens and is one goal towards development of effective mucosal vaccines. In these studies we have functionally evaluated short and long-term CTL memory in systemic and respiratory or genital-associated lymphoid tissues following mucosal or systemic routes of immunization. Our results indicate that shortly after immunizing mice with a recombinant adenovirus vector expressing glycoprotein B (gB) of herpes simplex virus (AdgB8), gB-specific CTL memory responses were observed in systemic and mucosal immune compartments regardless of the route of inoculation. In contrast, several months after immunization, anamnestic CTL responses compartmentalized exclusively to mucosal or systemic lymphoid tissues after mucosal or systemic immunization, respectively. Furthermore, the compartmentalized CTL memory responses in mucosal tissues were functionally observed for longer than 1.5 yr after intranasal immunization, and CTL precursor frequencies one year after immunization were comparable to those seen shortly after immunization. Therefore, to our knowledge, this is the first functional demonstration that the maintenance of anti-viral memory CTL in mucosal tissues is dependent on the route of immunization and the time of assessment. These results have important implications for our understanding of the development, maintenance, and compartmentalization of functional T cell memory and the development and evaluation of vaccines for mucosal pathogens, such as HSV and HIV.

Memory is a hallmark of both humoral and T cell–mediated immune responses and is typified by a more rapid and intense immune response on re-exposure to the same or closely related antigen. Indeed, a desirable property of vaccines is the ability to generate long-term immunological memory capable of preventing infection or limiting disease. Our current understanding of T cell memory stems from studies of systemic immune responses, however little is known concerning the induction and maintenance of T cell memory responses in mucosal tissues. Numerous pathogens, including respiratory, gastrointestinal and sexually transmitted agents, such as HSV and human immunodeficiency virus (HIV), initiate infection at mucosal surfaces. Since the mucosal immune system is somewhat separate and distinct from systemic immunity (1–3), a better understanding of specific immunologic T cell memory in mucosal tissues should contribute to the development of effective mucosal vaccines and improved control of infections at these sites.

Mucosal surfaces are largely protected by secretory IgA as well as transudated IgG antibodies (4). The induction of B cells in mucosal tissues following antigen exposure or infection results in the migration of B lymphocytes to mucosal tissues in the common mucosal immune system, thus ensuring the maintenance of secretory immunity at mucosal surfaces (1–3). T cell–mediated immunity is also a critical component of protection against mucosal pathogens. In addition to the detection of cell-mediated cytotoxicity in mucosa-associated tissues (5, 6), the passive transfer of CTL is associated with the clearance of virus at mucosal surfaces and may reduce virus-related pathology (7–12). Furthermore, as with B cells, mucosally derived T cells migrate to mucosal tissues and this homing is even more pronounced during re-exposure to the same pathogen (2, 12).

The maintenance of T cell memory to viruses may be the result of long-lived antigen-specific lymphocytes (13–15). Alternatively, T cell memory may be maintained by constant stimulation as a result of antigen persistence (16, 17), idiotypic networks (18) or cross-reactions with other antigens (19). Of interest to us, and an often ignored issue, is that the development of immune responses within the unique environments of secondary lymphoid tissues (20, 21) and the homing of effector and memory lymphocytes to the tissues in which antigen exposure originally occurred (12, 22, 23) may restrict the maintenance and observation of T cell memory to distinct immune compartments. In-

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deed, in our previous studies involving mice immunized intranasally (i.n.)[1] against HSV, we observed a decline in the levels of splenic CTL such that one year after immunization we were unable to detect any anti-HSV CTL in the spleen. Interestingly, these mice were protected from an intranasal challenge with HSV-2 (24). We suspected that the lack of detectable splenic CTL at this time was a reflection of the recruitment, retention, and recirculation of specific CTL within the common mucosal immune system after intranasal immunization. In support of this theory was the concurrent observation that although systemically immunized animals displayed short-term protection from mucosal HSV-2 challenge, protection was not long-lived despite the fact that CTL specific for HSV-2 were readily detectable in the spleen (24).

Therefore, in this study we have examined the development and the long-term maintenance of CTL memory within the distinct immune compartments of the mucosal and systemic immune systems after antigen exposure in either compartment. To induce immunity to HSV, mice were immunized with a recombinant adenovirus vector expressing the immunodominant CTL antigen of HSV, glycoprotein B (AdgB8). The CTL immune responses that developed were evaluated in the spleen and mucosal tissues of the respiratory and genital tracts.

Cytotoxic T lymphocyte memory has been functionally observed as an enhanced secondary CTL response in vitro (25–27) and in vivo (13, 28–33) after re-exposure to antigen. We functionally examined HSV-specific memory CTL in the spleen after restimulation and expansion with antigen in vitro. CTL memory within the mucosal immune compartments of AdgB8 immunized mice was examined by detecting the rapid recall response after re-exposure to antigen in vivo. Our results indicate that the functional presentation of short-term CTL memory is independent of the route of antigen exposure, whereas long-term CTL memory compartmentalizes over time and is dependent on the route of immunization.

Materials and Methods

Animals and Cell Cultures. Inbred female C57BL/6 (H-2^b) mice (purchased from Charles River Canada, St. Constant, Quebec, Canada) were used for these studies. 293 and 293-N2S cells were grown in α-MEM (GIBCO Laboratories, Burlington, Canada), supplemented with 10% FCS (GIBCO), and 1% penicillin-streptomycin and L-glutamine (GIBCO). MC57 (H-2^b) and SVBalb (H-2^d) fibroblasts served as targets in the CTL assays.

Virus Strains and Inoculations. The construction of replication-competent recombinant adenovirus vectors, AdgB8 and AdE3$^-$, is reported elsewhere (33, 34). In brief, AdgB8 contains the glycoprotein B (gB) gene from HSV-1 coupled to the SV40 promoter and inserted into the E3 region of human adenovirus type 5. AdE3$^-$ contains a deletion in the E3 region and served as a control. The recombinant adenoviruses were grown in 293-N2S cells, purified twice on CsCl gradients, and titered on 293 cells (35). The vaccinia vector expressing gB of HSV-1 (VacgB11) was kindly provided by B. Moss (NIH, Bethesda, MD) and its construction is reported elsewhere (36). HSV type 2 (HSV-2) strain 333 was propagated and virus titres were determined on Vero cells. Each mouse was immunized with a total of 10^8 PFU of AdgB8 or AdE3$^-$ in the given volumes of PBS pH 7.4. Mice immunized i.n. were anaesthetized with Halothane, inverted, and 10–20 μl of virus in PBS was introduced into the nares by means of a micropipet (24). i.p. and hind foot pad (h.f.p.) immunizations were performed by inoculation of virus in 0.2 ml and 50 μl of PBS, respectively. Mice were challenged intravaginally (ivag) or i.n. with 10^7 PFU of HSV-2 in 10 or 20 μl of PBS, respectively. Intravaginally challenged mice were first inoculated subcutaneously with 2 mg of progestin/mouse (Depo-Provera, Upjohn, Don Mills, Ontario) 5 d before challenge, and then anesthetized using Halothane, swabbed with a cotton applicator, placed on their backs and infected for 1 h while under anesthetic.

CTL Assays. Spleen and lymph node effector cells were prepared by teasing the tissues through a stainless steel grid. Splenic CTL were examined following secondary in vitro stimulation as follows; isolated spleen cells were incubated for 6 d with gamma-irradiated (5,000 rad), AdgB8-infected, syngeneic MC57 cells, at an effector/stimulator ratio of 1:166 in RPMI 1640 medium with 10% FCS, 50 μM 2-mercaptoethanol, 1% L-glutamine, penicillin, and streptomycin. After stimulation splenic effector cells were incubated with uninfected and HSV-2-infected (multiplicity of infection [1] MOI = 10, 6-h infection period) syngeneic (MC57) and allogeneic (SVBalb) targets at effector to target ratios of 80:1, 40:1, 20:1, and 10:1 in a 6-h ^{51}Cr-release assay. Inhibition of cytotoxicity was determined by adding 100 μl of monoclonal antibody 145-2C11 (anti-CD3) to wells containing effector cells 1 h before addition of target cells at the 40:1 ratio. Data are expressed as percent-specific lysis = 100 × [(experimental cpm − spontaneous cpm)/(maximum release cpm − spontaneous release cpm)]. Cytotoxic T lymphocytes from lymph nodes draining the site of infection were examined in a primary CTL assay by the protocol of Pfizenmaier et al. (37) with modifications (38). In brief, in the primary CTL assay lymphocytes from lymph nodes draining the site of infection were harvested from mice 2 or 3 d post HSV-2 challenge and incubated for 3 d (without antigen stimulation) in RPMI 1640 medium (supplemented as above). In vitro incubation (without antigen) has been shown to be necessary in the herpes system for CTL to become fully cytolytic (37). After incubation, the lymph node effectors were incubated with targets in a 6 h ^{51}Cr release assay as described above. VacgB11 (MOI = 10, 16-h infection period) as well as HSV-2 infected (as above) and uninfected fibroblasts served as targets.

Determination of CTL Precursor Frequencies and Lytic Units. For determining the CTL precursor frequencies of HSV-2-specific splenocytes limiting dilution analysis was performed. In brief, splenocytes were isolated by Ficol gradient and titrated into round bottom, 96-well plates (NUNC, Roskilde, Denmark) with 12 replicates at each dilution. Feeders (irradiated splenocytes: 2,000 rads) were isolated by Ficol gradient and added at 2 × 10^5 mononuclear cells per well. Stimulators were irradiated (2,000 rads) with HSV-2-infected splenocytes set at 2 × 10^5/well. 5 d after incubation, the contents of each well was transferred to a corresponding well in V-bottomed NUNC plates and 5,000 ^{51}Cr labeled and HSV-2- or VacgB-infected syngeneic fibroblasts were added to each well. Plates were pulse spun up to 1,500 rpm and

[1] *Abbreviations used in this paper:* CI, confidence interval; h.f.p., hind foot pad; HSV-2, HSV type 2; ILN, iliac lymph nodes; i.n., intranasally; ivag, intravaginally; LU, lytic units; MLN, mediastinal lymph nodes; MOI, multiplicity of infection.

incubated for 6 h in a ^{51}Cr release assay. Positive wells were defined as those wells whose ^{51}Cr release exceeded the mean spontaneous release from control cultures, containing feeder cells but no responder cells, by at least three standard deviations.

The assay used to determine CTL precursor frequencies of HSV-2-specific lymphocytes from the draining lymph nodes of immunized mice was similar to that reported by Nugent et al. (31, 33). This method involved expansion of CTL in the presence of exogenous cytokines but in the absence of added antigen, and results in analysis of effector CTL expanded from CTLp exclusively activated in vivo after HSV-2 challenge. Lymphocytes from iliac lymph nodes of mice infected ivag with HSV-2 were titrated into round-bottom, 96-well plates, in 100 μl of supplemented RPMI with 16 replicates for each lymphocyte concentration. Feeders (irradiated splenocytes, 2,000 rads) were isolated by Ficol gradient and added at 2×10^5 mononuclear cells per well in 100 μl of 5 U of rIL-2 (Genzyme, Cambridge, MA), 10% (vol/vol) Rat T-Stim (Collaborative Biomedical Research Products, Cambridge, MA), and 50 mM α-methyl mannoside (Sigma). 5 d after incubation cultures were assessed as above for CTL precursor frequencies.

The lytic activity of CTL in the draining lymph nodes of HSV-2-infected tissues was estimated by determining the lytic units (LU). One LU is defined as the number of mononuclear cells required to obtain 10% specific lysis of 5,000 infected targets. The 10% level of lysis was chosen to accommodate the low levels of killing experienced by certain experimental groups. We have expressed the lytic activity of the mononuclear cells recovered from the draining lymph nodes as the number of lytic units per 10^7 cells. In short, mononuclear cells were harvested from the lymph nodes draining the sites of HSV-2 infection and incubated for 3 d (37) in RPMI 1640 medium with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 1% L-glutamine, penicillin, and streptomycin. The lymph node effectors were then plated in twofold serial dilutions in 96-well V-bottomed plates (NUNC, Roskilde, Denmark) with 12 replicates at each dilution. 5,000 HSV-2-infected targets were added to each well and plates were briefly spun at 1,500 rpm and incubated for 6 h at 37°C in a ^{51}Cr release assay. One lytic unit was determined by estimating the number of lymph node effectors required to cause 10% ^{51}Cr release by fitting a curve to the data using the equation described by Clark et al. (39) and a program generously supplied by D.A. Clark (McMaster University, Hamilton, ON).

Statistics. Frequency estimates of CTL_p were calculated using χ^2 analysis as described by Taswell (40), by using a computer program kindly provided by Richard Miller (University of Michigan, Ann Arbor). Estimates of CTL_p frequencies were considered valid only if the plot of the logarithm of the fraction of negative cultures against the number of responder cells on a linear scale obeyed single-order kinetics with a probability greater than 0.05 (41).

The lytic activity for each mouse is expressed as the mean LU/10^7 cells ± SEM. Statistical analysis of LU was carried out using analysis of variance (ANOVA) based on the error associated with the line fitted to the lytic values of at least three effector to target dilutions of a given sample.

Results

Dissipation of Splenic CTL Memory After Intranasal AdgB8 Immunization. The maintenance of systemic anti-HSV-2 CTL was examined in C57BL/6 mice after i.n. or i.p. immunization with AdgB8 at various time points over a 19-wk period (Fig. 1). To determine whether memory CTL were present within the systemic immune system, HSVgB-specific splenocytes were first expanded in vitro after bulk stimulation with antigen. The expansion of the memory population permits qualitative evaluation of the extent of CTL memory in the spleen. Both i.p. and i.n. AdgB8 immunization resulted in the presence of splenic effectors at early time points post immunization as evidenced by the high levels of CTL-mediated killing of HSV-2-infected syngeneic targets (Fig. 1, *A* and *B*). The lysis was T cell–mediated since anti-CD3 treatment markedly inhibited killing (Fig. 1, *A* and *B*). Further, CTL-mediated lysis was MHC-restricted and virus-specific because uninfected syngeneic and allogeneic HSV-infected targets were not killed (data not shown). In mice immunized i.p. with AdgB8 the level of lysis of HSV-2-infected targets was maintained over the 19-wk period (Fig. 1 *A*). In contrast, in i.n. immunized mice the lysis of HSV-2-infected targets decreased

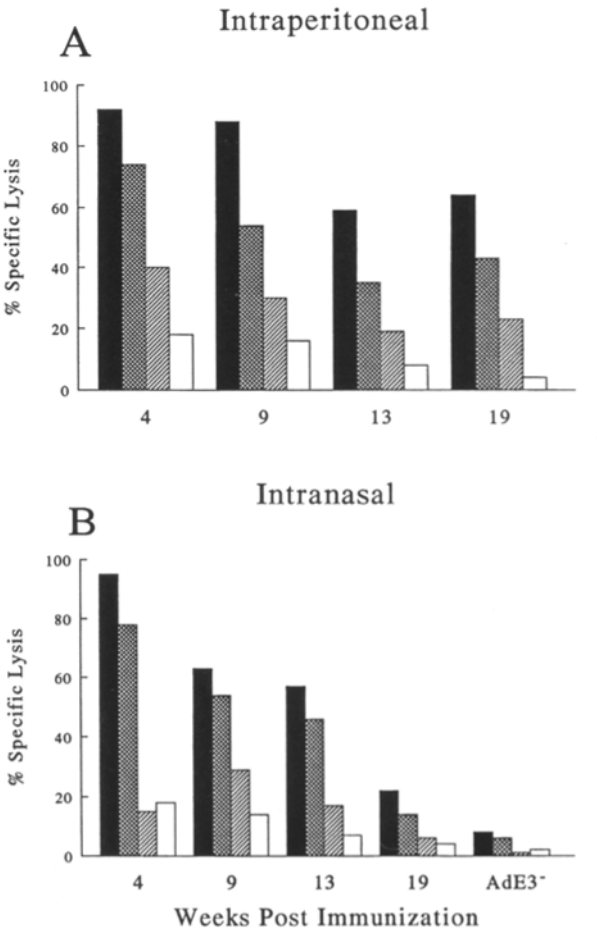


Figure 1. Maintenance of systemic CTL specific for HSV-2 in mice immunized i.p. (*A*) but not i.n. (*B*) with AdgB8. Three mice were immunized at each time point over the course of 19 wk at ~5 wk intervals. As a control group three mice were i.n. immunized at 9 wk with $AdE3^-$ (*B*). After 6 d of in vitro culture with irradiated, AdgB8-infected stimulator cells the pooled splenocytes in each group were examined for CTL activity against HSV-2-infected MC57 targets. Effector to target ratios reported are 40:1 (■), 20:1 (▩), 10:1 (▨), and 40:1 + anti-CD3 (□).

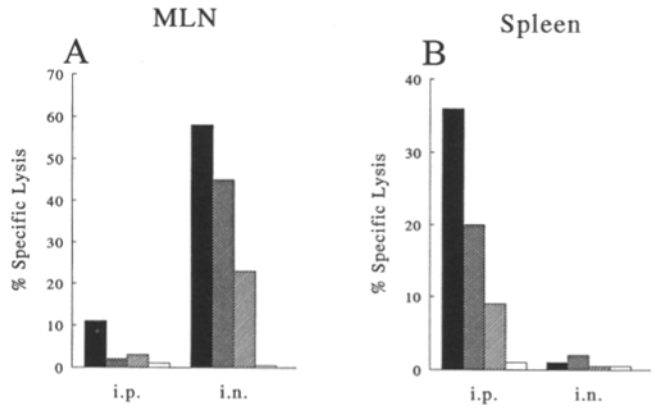


Figure 2. Systemic and mucosal CTL memory in the respiratory-associated lymphoid tissue (*A*) and spleens (*B*) of AdgB8 immunized mice. Mice were immunized i.n. or i.p. and 7 mo later challenged i.n. with HSV-2. The spleens and MLN were isolated 3 d later and combined within groups. The spleens (*B*) were stimulated for 6 d with irradiated AdgB8-infected stimulators before being analyzed in a CTL assay. The MLNs (*A*) were cultured without antigen stimulation for 3 d before being assayed. CTL activity is reported at E/T ratios of 40:1 (■), 20:1 (▒), 10:1 (▨), and 40:1 + anti-CD3 (□).

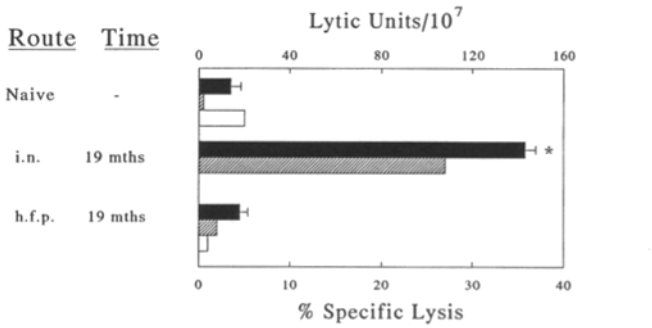


Figure 3. Long-term CTL memory in the respiratory-associated lymphoid tissue of mice immunized i.n. with AdgB8. Two mice immunized either i.n. or in the h.f.p. with AdgB8 were i.n. challenged 19 mo later with HSV-2. A naive (unimmunized) mouse was also challenged and served as a control representing the magnitude of a primary response. 2 d after i.n. challenge the MLN were cultured without antigen stimulation for 3 d before being examined for CTL activity. MC57 targets were infected with HSV-2 and the 40:1 (▨) and 40:1 + anti-CD3 (□) E/T ratios are reported as well as the lytic activity (■) in mean LU per 10^7 mononuclear cells ± SEM. * Significantly different from naive and h.f.p. immunized mice ($P < 0.0001$).

to levels similar to that of control mice over the 19-wk period (Fig. 1 *B*). Moreover, in separate experiments, splenic CTL precursor frequencies were estimated at 1 in 21,280 (95% confidence interval (CI), 12,658 to 35,714) and 1 in 500,000 (95% CI, 250,000 to 10^6) mononuclear cells 14 mo post i.p. or i.n. immunization, respectively. In addition, limiting dilution analysis using fibroblasts infected with AdgB8 as stimulators gave similar results (data not shown). These results indicate that while both routes of AdgB8 immunization initially resulted in the presence of anti-HSV CTL in the spleen, only i.p. immunized mice maintained long-term splenic anti-HSV memory CTL.

CTL Memory Responses in the Draining Lymph Nodes of the Respiratory Tract After AdgB8 Immunization and Intranasal HSV-2 Challenge. To investigate antigen-specific CTL memory within the respiratory tract, we examined the levels of CTL activity in the draining lymph nodes after an HSV-2 challenge. We first determined that primary anti-HSV CTL responses in the draining lymph nodes of HSV-2-infected mucosal tissues of naive mice appeared at low levels on days 2–3 and peaked by day 5 post infection (data not shown). Therefore, in assessing memory CTL responses in the respiratory tract of AdgB8 immunized mice, mice immunized 7 mo previously with AdgB8 were challenged i.n. with HSV-2, and 3 d later the mediastinal lymph nodes (MLN) which drain the respiratory tract were examined for CTL activity. Fig. 2 *A* shows that only lymphocytes from the MLNs of mice immunized i.n. 7 mo previously with AdgB8 contained a strong anti-HSV-2 CTL recall response. In contrast, MLN cells from i.p. AdgB8 immunized mice failed to appreciably lyse HSV-2-infected targets (Fig. 2 *A*). This indicates that long-term CTL memory, as observed through a functional recall response, existed within the local mucosal immune compartment of the respiratory tract after i.n. but not i.p. AdgB8 immunization. Interestingly, when the spleens of these same mice were examined for anti-HSV CTL, only mice immunized i.p. demonstrated splenocytes that recognized and killed HSV-2-infected targets (Fig. 2 *B*).

To further investigate the maintenance of long-term T cell memory in the respiratory tract, we examined individual mice immunized with AdgB8 and compared them to unimmunized (naive) animals 2 d after i.n. HSV-2 challenge. Fig. 3 demonstrates that at 2 d post i.n. HSV-2 challenge, only MLN lymphocytes from the mouse immunized i.n. with AdgB8 19 mo previously demonstrated the ability to lyse HSV-2-infected syngeneic targets. In contrast, the lymphocytes from the MLN of the naive or h.f.p. immunized mouse failed to appreciably lyse HSV-2-infected targets (Fig. 3). Furthermore, when the lytic activity in the MLNs of the three mice were examined, the lymphocytes in the i.n. AdgB8 immunized mouse contained significantly ($P \leq 0.0001$) and more than eight times the lytic activity than that found in either the naive or i.p. immunized mouse (Fig. 3). These results are representative of several experiments in which individual mice were examined at late time points post immunization and at no time did we observe killing from the MLNs of i.p. or h.f.p. immunized mice. HSV-2-specific lysis was T cell–mediated since it was completely inhibited with anti-CD3 antibody (Fig. 3). Killing was also virus-specific and MHC-restricted in the i.n. immunized mouse because uninfected and allo-infected targets were not lysed (data not shown). Figs. 2 and 3 demonstrate that long-term T cell memory responses, as observed in the draining lymph nodes, are maintained in the respiratory tract following local (i.n.) but not systemic immunization.

In Fig. 4 the presence of short-term CTL memory in the respiratory tract after i.n. or h.f.p. AdgB8 immunization was evaluated. 3 wk post immunization both these groups demonstrated a CTL recall response that was greater than

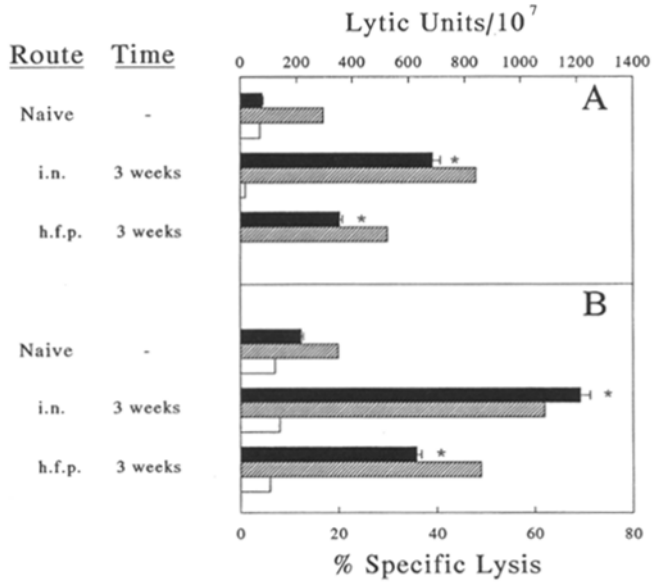


Figure 4. Short-term CTL memory in the respiratory-associated lymphoid tissue of mice immunized systemically or mucosally with AdgB8. Two mice immunized either i.n. or in the h.f.p. with AdgB8 were i.n. challenged 3 wk later with HSV-2. Naive (unimmunized) mice were also challenged and served as controls representing the magnitude of a primary response. 2 d after i.n. challenge the MLN were isolated and incubated for 3 d without antigen stimulation before being examined for CTL activity. MC57 targets were infected with HSV-2 (*A*) or VacgB11 (*B*) and the 40:1 (▨) and 40:1 + anti-CD3 (□) E/T ratios are reported as well as the lytic activity (■) in mean LU per 10^7 mononuclear cells ± SEM * Significantly different from the naive mouse ($P < 0.0001$).

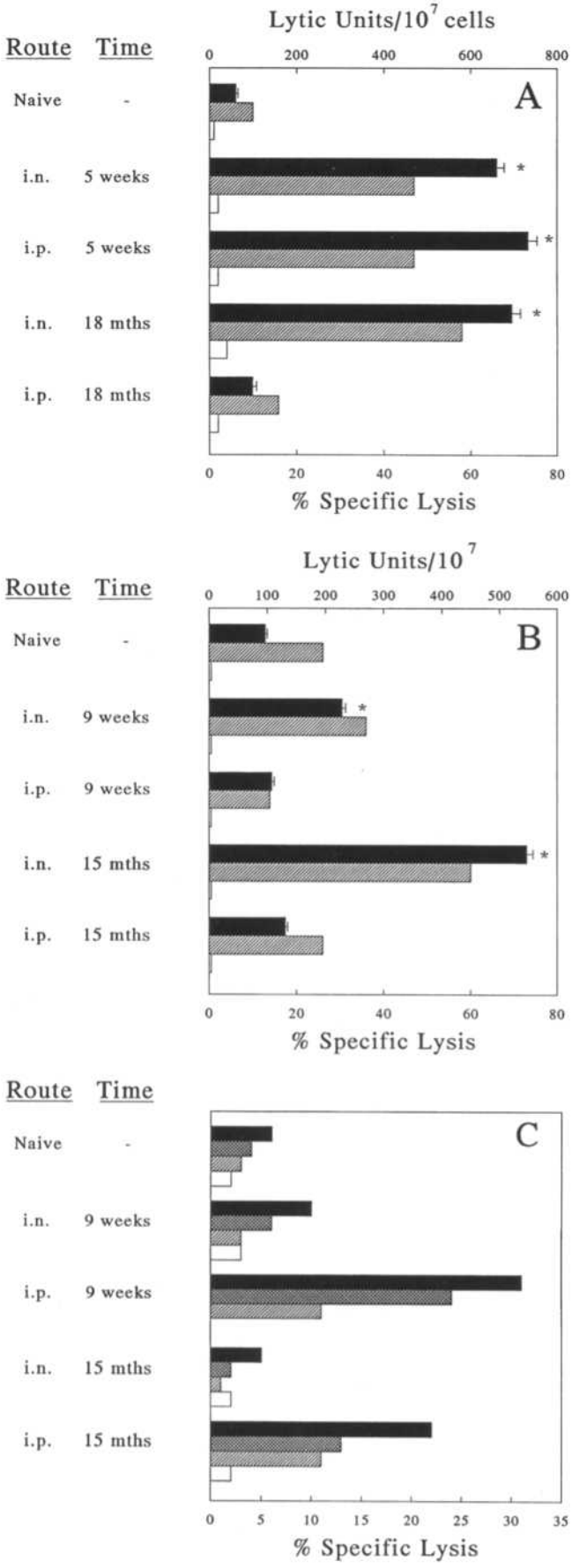
that of the naive mouse and was completely inhibitable with anti-CD3 (Fig. 4 *A*). As well, the lytic activity in the MLNs of i.n. or h.f.p. immunized mice was significantly greater than in the naive mouse ($P \leq 0.0001$). In fact, there was more than eight and four times the number of lytic units in i.n. or h.f.p. immunized mice, respectively, demonstrating that shortly after either mucosal or systemic AdgB8 immunization mice were able to mount anti-HSV-2 CTL memory responses in the mucosal-associated lymphoid tissue of the respiratory tract (Fig. 4 *A*).

To determine whether the short-term CTL memory response to HSV-2 challenge was specific for gB of HSV, targets were infected with a vaccinia virus vector expressing gB of HSV (VacgB11). The lysis of VacgB11-infected targets was similar to that of HSV-2-infected targets (Fig. 4 *B*), confirming the presence of short-term CTL memory responses in both i.n. and h.f.p. immunized mice. Moreover, since the lysis and lytic activity against VacgB11 infected targets was as high as against HSV-2-infected targets, the CTL memory response to HSV-2 challenge was for the most part directed against gB of HSV (Fig. 4 *B*).

CTL Memory Responses in the Draining Lymph Nodes of the Genital Tract After AdgB8 Immunization and Intravaginal Challenge with HSV-2. We next addressed the question of whether the maintenance of CTL memory in mucosal tissues was purely a local phenomena (i.e., due to local immunization) or was also present at distant mucosal sites. Moreover, since HSV-2 is a sexually transmitted virus and since one of our main interests has been the development and characterization of anti-HSV-2 immune responses in the genital tract, we investigated CTL memory responses at this site after AdgB8 immunization. Fig. 5 consists of two representative experiments showing CTL recall responses in individual mice at early (5–9 wk) and late (15–18 mo) time points post immunization (Fig. 5, *A* and *B*). The magnitude of the primary response is indicated by the CTL activity in the naive (unimmunized) mice. In these experiments, lymphocytes were isolated from the iliac lymph nodes (ILN), which drain the genital tract, 3 d after ivag HSV-2 challenge and examined for their ability to lyse HSV-2-infected targets. Fig. 5 *A* demonstrates that at early time points post immunization (i.e., 5 wk) both i.n. and i.p. AdgB8 immunized mice demonstrated lysis of HSV-2-infected targets that exceeded primary responses observed in naive mice. The killing was TCR-mediated as indicated by the inhibition of lysis with anti-CD3 antibody. The lytic activity in both groups was not only similar but was significantly ($P \leq 0.0001$) and more than 11 times greater than that observed in the naive mouse. Thus, as observed in the respiratory tract, shortly after immunization there exists a phase of CTL memory in the genital tract which is not dependent on the route of immunization. In the second experiment (Fig. 5 *B*) mice were examined 9 wk post immunization and again ILN lymphocytes from the mouse immunized i.n. maintained short-term CTL memory. However, at this time point the i.p. immunized mouse lacked a significant memory response when the lysis of targets or the lytic activity was compared to that observed in the naive mouse (Fig. 5 *B*). Therefore, systemic immunization induced a phase of short-term memory in the genital tract, which we observed in the draining lymph nodes and which was absent by 9 wk post immunization.

To examine the long-term maintenance of mucosal CTL memory, mice were challenged with HSV-2 intravaginally 15 or 18 mo after AdgB8 immunization (Fig. 5, *A* and *B*). At 18 and 15 mo, i.n. immunized mice demonstrated much higher as well as anti-CD3 inhibitable levels of killing when compared to i.p. immunized mice. Indeed, the level of lysis and the lytic activity in i.n. immunized mice at 18 or 15 mo post immunization was at least as high as that occurring at 5 or 9 wk post i.n. immunization, respectively (Fig. 5, *A* and *B*). Furthermore, in both experiments the lytic activity in ILN cells of i.n. immunized mice was significantly ($P \leq 0.0001$) and more than four times greater than that observed in i.p. immunized mice. In contrast, mice immunized i.p. 18 or 15 mo before ivag HSV-2 challenge lacked a memory response and their lytic activity was not significantly different from that of the naive mice (Fig. 5, *A* and *B*). These results indicate that only i.n. immunized mice maintained long-term anti-HSV CTL memory responses in the genital tract and the strength of these responses did not appear to decrease with time.

Splenic CTL Responses in AdgB8 Immunized Mice After Intravaginal HSV-2 Challenge. The maintenance of splenic CTL memory in AdgB8 immunized and ivag challenged



mice in Fig. 5 *B* was examined (Fig. 5 *C*). 3 d after ivag HSV-2 challenge, the splenocytes from mice immunized i.p. with AdgB8 (9 wk and 15 mo previously) demonstrated anti-HSV-2 CTL activity (after in vitro stimulation) (Fig. 5 *C*). This is in agreement with our previous results (Fig. 1 *A*) which demonstrated that i.p. AdgB8 immunization induced long-term splenic CTL memory. Interestingly, neither of these mice demonstrated a CTL memory response in their ILNs after ivag HSV-2 challenge (Fig. 5 *B*). In contrast, both mice immunized i.n. (i.e., 9 wk or 15 mo previously) mounted CTL memory responses in their ILNs (Fig. 5 *B*) yet failed to display high levels of anti-HSV-2 CTL in their spleens (Fig. 5 *C*). In fact, the mouse immunized i.n. 15 mo previously displayed a level of killing from the spleen similar to the naive mouse. These results suggest that immunological T cell memory can be observed functionally in mucosal tissues without being observed in the systemic system and vice-versa.

Specificity of the CTL Memory Responses in the Draining Lymph Nodes of the Genital Tract After AdgB8 Immunization and Intravaginal Challenge with HSV-2. To examine the specificity and further characterize the long-term memory response in the genital tracts of i.n. immunized mice, the anti-HSV CTL activity in ILNs from mice immunized 5 mo previously were examined 2 d post HSV-2 challenge using targets infected with VacgB11 (Fig. 6). In addition, two systemic routes of immunization (i.p. and h.f.p.) were assessed and a naive mouse was included to represent the primary response to gB of HSV. Fig. 6 demonstrates that 2 d after ivag HSV-2 challenge the ILN cells in the i.n. immunized mouse lysed the VacgB11 infected targets and this lysis was completely inhibitable with anti-CD3 antibody. Furthermore, the lytic activity in the i.n. immunized mouse was significantly ($P \leq 0.0005$) and more than four times greater than in the naive or systemically immunized mice. In contrast, the level of lysis of VacgB11 targets in naive and systemically immunized mice were very low and not always inhibitable with anti-CD3. The lytic activity was also very low and not significantly different between the naive and systemically immunized mice (Fig. 6). These results confirm that long-term immunological T cell memory is maintained in the genital tracts of mice immunized i.n., but not

Figure 5. Short- and long-term CTL memory in the genital-associated lymphoid tissues (*A* and *B*) and spleens (*C*) of AdgB8 immunized mice. Individual mice in two separate experiments (*A* and *B*) were i.n. or i.p. immunized with AdgB8. At 5 wk and 18 mo (*A*) or 9 wk and 15 mo (*B*) post immunization mice were challenged ivag with HSV-2. 3 d later the ILN (*A* and *B*) draining the genital tract were isolated from individual mice, cultured for 3 d without antigen stimulation, and examined for CTL activity against HSV-2-infected MC57 targets at E/T ratios of 40:1 (▨) and 40:1 + anti-CD3 (□) (*A* and *B*). In addition, the lytic activity (■) was determined and expressed in mean lytic units per 10^7 mononuclear cells ± SEM. * Significantly different from the naive and 18 mo i.p. immunized mouse ($P < 0.0001$) (*A*) and from the naive and i.p. immunized mice at 9 wk and 15 mo ($P < 0.0001$) (*B*). *C* shows the CTL activity against HSV-2-infected targets from the spleens of mice in *B*. Splenocytes were stimulated as described in the Materials and Methods and incubated with targets at E/T ratios of 40:1 (■), 20:1 (▩), 10:1 (▨), 40:1 + anti-CD3 (□).

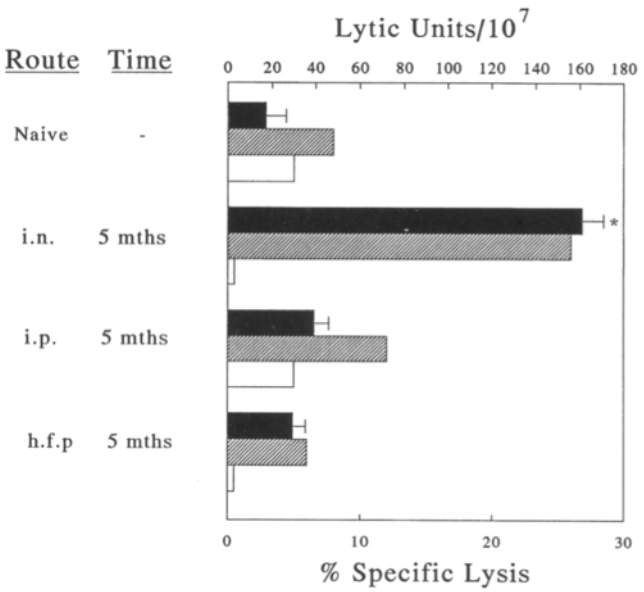


Figure 6. Specificity of long-term memory CTL in the genital-associated lymphoid tissue of intranasally immunized mice. Mice immunized 5 mo previously with AdgB8 by the i.n., i.p., or h.f.p. routes were challenged ivag with HSV-2. A naive (unimmunized) mouse was also challenged and served as a control representing the magnitude of a primary response. 2 d later the ILNs were isolated from individual mice, incubated for 3 d without antigen stimulation, and examined for CTL activity against VacgB11-infected targets. The 40:1 (▨), and 40:1 + anti-CD3 (□) E/T ratios are shown as well as the lytic activity (■) expressed in mean lytic units per 10^7 mononuclear cells ± SEM. * Significantly different from the naive, i.p. and h.f.p. immunized mice ($P < 0.0005$).

systemically, with AdgB8 and demonstrates that these memory CTL are specific for gB of HSV.

Limiting Dilution Analysis of CTLp Frequencies in Mice One Year After AdgB8 Immunization. To further examine the compartmentalization of memory CTL in AdgB8 immunized mice, we determined the precursor frequencies of gB-specific CTL in the ILNs of mice immunized 1 yr previously and 60 h after an intravaginal HSV-2 challenge (Table 1). Unlike splenic memory CTL which typically require antigen to expand into effectors, gB-specific CTLp in the draining lymph nodes of HSV-2-infected tissues were activated in vivo and therefore, functional analysis of the CTLp frequency is possible after expansion of effectors in the absence of antigen. Analysis of the frequencies of gB-specific CTLp expanded under limiting dilution conditions showed that the ILNs of i.n. immunized mice (ranging from 1 in 3411-5946) contained significantly and several-fold more gB-specific CTLp than the ILNs of control (unimmunized; ranging from 1 in 23899-35350) or systemically (i.p. or h.f.p.; ranging from 1 in 15643-31006) immunized mice (Table 1). Moreover, the CTL frequencies demonstrated

Table 1. *Cytotoxic T Lymphocyte Memory Responses in the Spleen and Iliac Lymph Nodes of Mice Immunized One Year Previously With AdgB8*

	ILN		Spleen		
Route of AdgB8 immunization*	Reciprocal CTLp frequency (95% CI)‡	Probability§	CTL lysis at 80:1 (SD)‖	Reciprocal CTLp frequency (95% CI)	Probability
		P			*P*
Intranasal	3411 (2645-4924)	0.99	3 (0.6)	667609 (421812-1599898)	0.82
	5946 (4497-8774)	0.41	2 (2.6)	325543 (325543-1006668)	0.52
Intraperitoneal	23607 (18271-33346)	0.84	52 (1.0)	66265 (51694-92273)	0.99
	30391 (23431-43232)	0.87	50 (3.4)	55068 (43388-75351)	0.54
	31006 (23790-445050	0.76	37 (2.6)	72671 (55262-106092)	0.55
Hind Foot Pad	23555 (17586-35655)	0.70	75 (6.3)	95959 (67974-163100)	0.19
	15643 (12002-22453)	0.55	72 (6.8)	94844 (70597-144460)	0.72
Unimmunized	26106 (20118-37169)	0.71	–	–	
	23899 (18365-34208)	0.51	–	–	
	35350 (26590-52717)	0.99	–	–	

*1 yr after AdgB8 immunization mice were challenged ivag with 2×10^7 PFU of HSV-2 and 60 h later the lymphocytes from the spleens and ILN were isolated and the CTLp frequencies were determined in a limiting dilution assay. ILNs were cultured under expansion conditions without exogenous antigen.

‡Reciprocal frequency of culture wells exhibiting positive cytolytic activity (three standard deviations above the mean values obtained from cultures without responders) were determined by minimal χ^2 analysis. Each frequency and 95% CI represents an individual mouse (2–3 per group) and all animals were analyzed together in one experiment against VacgB11 infected targets.

§Probability of obeying single-order kinetics, based upon χ^2 analysis for n-1 degrees of freedom, where n was always greater than 5 for ILNs and 4 for splenocytes and represents the number of responder cell dilutions tested.

‖CTL-mediated lysis was determined after bulk stimulation of splenocytes as described in the Materials and Methods.

Table 2. *Cytotoxic T Lymphocyte Memory Responses in the Spleen and Iliac Lymph Nodes of Mice Recently Immunized with AdgB8*

Route of AdgB8 immunization*	Time after immunization	ILN		Spleen	
		Reciprocal CTLp Frequency (95% CI)‡	Probability§	Reciprocal CTLp frequency (95% CI)	Probability
			P		*P*
Intranasal	2 wk	4680 (3510-7000)	0.72	145600 (98510-278900)	0.10
		4030 (3030-5990)	0.44	95020 (64570-179900)	0.21
		6850 (5300-9650)	0.94	86470 (61670-144600)	0.61
	12 wk	2520 (1980-3490)	0.33	127560 (91100-212800)	0.16
		5150 (3860-7760)	0.40	101890 (72000-174100)	0.26
		3590 (2850-4860)	0.88	142150 (100100-245100)	0.52
Intraperitoneal	2 wk	13820 (10590-19900)	0.60	46130 (32180-81410)	0.32
		15310 (11530-22790)	0.97	40230 (27600-74170)	0.20
		13140 (10140-18630)	0.74	47900 (32530-90800)	0.64
	12 wk	29290 (22090-43450)	0.64	29050 (19970-53260)	0.17
		17830 (13390-26680)	0.80	49880 (35360-84620)	0.08
		27170 (20920-38720)	0.84	45590 (31300-83890)	0.05

*2 or 12 wk after AdgB8 immunization mice were challenged ivag with 2×10^7 PFU of HSV-2 and 60 h later the lymphocytes from the spleens and ILN were isolated and the CTLp frequencies were determined in a limiting dilution assay. ILNs were cultured under expansion conditions without exogenous antigen.

‡Reciprocal frequency of culture wells exhibiting positive cytolytic activity (three standard deviations above the mean values obtained from cultures without responders) were determined by minimal χ^2 analysis. Each frequency and 95% CI represents an individual mouse (2–3 per group) and all animals were analyzed together in one experiment against VacgB11 infected targets

§Probability of obeying single-order kinetics, based upon χ^2 analysis for n-1 degrees of freedom, where n was always greater than 5 for ILNs and 4 for splenocytes and represents the number of responder cell dilutions tested.

that the recall responses in systemically immunized mice were no different than the primary responses occurring in control animals, indicating that long-term mucosal T cell memory is maintained after mucosal but not systemic immunization.

Analysis of the splenocytes of these same animals expanded with antigen under limiting dilution conditions demonstrated that the precursor frequencies of gB-specific CTL in i.n. immunized mice were extremely low (ranging from 1 in 325543-667609). In fact, we were unable to detect any gB-specific CTL-mediated lysis after bulk stimulation of splenocytes (Table 1). In contrast, mice immunized systemically maintained relatively high frequencies of CTLp (ranging from 1 in 55068-95959) and CTL-mediated lysis was readily detectable after bulk stimulation.

Limiting Dilution Analysis of CTLp Frequencies in Mice Shortly After AdgB8 Immunization. To evaluate the early development of mucosal T cell memory in AdgB8 immunized mice, we examined the CTLp frequencies in the ILNs of mice 2 or 12 wk after AdgB8 immunization. The results in Table 2 demonstrate that 60 h after an intravaginal HSV-2 challenge, the gB-specific CTLp frequencies of ILN cells in i.n. immunized mice (ranging from 1 in 2523-6845) were significantly higher than in i.p. immunized mice (at either time point; ranging from 1 in 13135-29292). Interestingly, the CTLp frequencies were similar in mice immunized i.n. at 2, 12, or 52 wk previously (ranging from 1 in 2523-6845) (Tables 1 and 2). Although the gB-specific CTLp frequencies in the ILNs of i.p. immunized mice at 2 wk (ranging from 1 in 13135-15311) were not as high as in i.n. immunized mice, they were higher than at 12 wk (ranging from 1 in 17833-29292). Moreover, by 12 wk the CTLp frequencies in the ILNs of i.p. immunized mice were similar to those observed in unimmunized mice in Table 1, suggesting that systemic immunization can provide mucosal T cell recall responses, however, the maintenance of this mucosal T cell memory is short-lived.

The gB-specific splenic CTLp frequencies were also determined in these mice and demonstrate that both i.n. and i.p. immunization successfully induced short-term levels of T cell memory within the systemic immune system. However, the frequencies were generally two- to threefold higher in i.p. immunized mice. In addition, the splenic CTLp frequencies in i.p. immunized mice only slightly decreased from 2 wk (ranging from 1 in 40233-47904) to 1 yr (ranging from 1 in 55068-72671), whereas, in i.n. immunized mice there was a substantial decrease in CTLp frequencies during this period (two- to sevenfold) (Tables 1 and 2).

Discussion

T cell memory has been functionally viewed as the more rapid generation of recall responses following a second exposure to antigen (13, 28, 29, 31). Increased numbers of

antigen-specific T cell precursors as well as qualitative differences in memory T cell activation requirements likely contribute to the magnitude of recall responses (42–46). The ability of memory T cells to persist for long periods of time may depend on several factors, including the persistence or complexity of antigen (16, 17), regulatory networks (18), cross-stimulation (19) or long-lived T cells (13–15). In addition, the maintenance of T cell memory may depend on the tissues in which exposure to antigen first occurred. Indeed, the analysis of surface markers on T cells has demonstrated that the recirculation of activated and memory lymphocytes is selective and depends on the tissues and lymph nodes from which the lymphocytes originated (47). By using recall responses we have investigated CTL memory in mucosal and systemic immune compartments after various routes of immunization with an adenovirus vector expressing gB of HSV. Our results indicate that CTL memory, when examined as a functional recall response, has both an early and a late phase which manifests in the biphasic expression of memory CTL within a given tissue depending on the site of initial antigen exposure. More specifically, the presence of systemic CTL was observed to be short-lived after i.n. immunization, however, mucosally, CTL memory was long-lived. Similarly, systemic immunization resulted in short-lived mucosal memory CTL, but long-lived systemic CTL. In addition, intranasal immunization resulted not only in long-term CTL memory in the local mucosal tissues of the respiratory tract, but also distantly in the mucosal tissues of the genital tract. This would imply that the genital tract is an implicit effector site within the common mucosal immune system. Therefore, our results demonstrate functionally the phenomenon of selective recirculation of memory lymphocytes to the tissue compartments in which sensitization to antigen first occurred. In addition, there is a short-lived period in which T cell memory can be functionally observed within the opposing immune compartment (i.e., mucosal versus systemic).

Although we have established that AdgB8 immunization results in long-lived CTL, we have not explored the underlying mechanism of this memory. However, recent evidence (48, 49) suggests that infection with similar replication-competent recombinant adenoviruses (E3 inserts) results in only short-term expression of inserted antigen, suggesting that persistent antigen production is not the underlying mechanism responsible for the long-term maintenance of CTL memory within our system. Indeed, these studies also demonstrated that initially, vector derived antigen was present in both systemic and mucosal immune compartments regardless of the route of inoculation (48, 49). This would suggest that the persistence of antigen is unlikely driving the compartmentalized maintenance of CTL memory.

The primary observations that led to this study included the early detection of anti-HSV CTL in the spleens of i.n. or i.p. AdgB8 immunized mice, but the absence of long-term memory from the spleens of i.n. immunized mice (24). As well, mice immunized i.n. with AdgB8 were protected against heterologous intranasal challenge with HSV-2, and this protection lasted longer than in i.p. immunized mice (24). Moreover, we recently observed that i.n. AdgB8 immunization protected mice from an intravaginal HSV-2 challenge, and this protection was long-lived and significantly better than after i.p. immunization (Gallichan, W.G., and K.L. Rosenthal, manuscript submitted for publication). By investigating these phenomena further, we show here that i.n. and i.p. routes of AdgB8 immunization initially induced high anti-gB CTLp frequencies and following bulk stimulation, similar levels of splenic anti-HSV CTL. However, several months later, bulk stimulation of splenocytes demonstrated that mice immunized i.n. had barely detectable CTL specific for HSV. Limiting dilution analysis of mice 12–14 mo following i.n. immunization confirmed that the CTLp frequencies had decreased and had remained permanently low or undetectable. In contrast, i.p. immunization resulted in relatively high CTLp frequencies for longer than 14 mo. Others have also shown the persistence of splenic CTL following systemic exposure to gB of HSV (50). However, the transient nature of splenic CTL following i.n. immunization may be due to the selective migration of memory T cells to mucosal sites within the common mucosal immune system (1–3, 11, 12, 20, 23, 47).

Thus, to address these seemingly conflicting observations of long-lived mucosal protection in the absence of systemic CTL, we examined short and long-term CTL memory in mucosal tissues by assessing recall responses within mucosal associated lymph nodes after local HSV-2 challenge. At less than 9 wk after either systemic (i.p. or h.f.p.) or mucosal (i.n.) routes of AdgB8 immunization, we observed rapid recall responses in the draining lymph nodes of the lungs and genital tract. In addition, CTLp frequencies indicated that mucosal memory responses were present in animals two wk after either systemic or mucosal immunization. Thus, short-term CTL memory in mucosal tissues is not dependent on the route of immunization. In a similar manner, we examined short-term recall responses in the systemic compartment and observed that mice immunized either i.n. or systemically developed memory responses to h.f.p. HSV-2 challenge (data not shown). These results suggest that functionally there exists within a population of recently activated lymphocytes, T cells that are capable of recirculating indiscriminately throughout the body. This is interesting since recently activated lymphocytes or blasts derived from skin or mucosal tissues have been shown to preferentially but not exclusively migrate back to the tissues in which antigen exposure originally occurred (1–3, 11, 12, 20, 23, 47). Our findings may then reflect the persistence of a population of recently activated lymphocytes that are capable of responding to sites of inflammation. This is quite possible in light of work demonstrating the increased penetration of memory or activated lymphocytes into sites of antigen challenge (2, 12). Alternatively, the routes of immunization used here may not reflect tissue restricted infection, resulting in some overlap of immune induction within opposing immune compartments. However, our observation of distinctively compartmentalized long-term memory responses suggests that this mechanism is unlikely to ac-

count for the observed short-term phase of T-cell memory. Moreover, it is interesting that as for short-term CTL memory, short-term protection from mucosal HSV-2 challenge (24) is not dependent on the route of immunization.

In our initial evaluation of long-term mucosal CTL memory, we examined recall responses in the respiratory-associated lymphoid tissue and observed a dissociation of memory responses in i.n. and i.p. immunized animals. 7–19 mo after AdgB8 administration only mice immunized i.n. demonstrated CTL memory responses in the draining mediastinal lymph nodes of the respiratory tract. Interestingly, in the mice immunized 7 mo previously, only i.p. immunization resulted in the presence of long-term anti-HSV-2 CTL in the spleen. These results suggest that mucosally induced memory lymphocytes are not maintained within the spleen but perhaps within the lymphoid or extralymphoid tissues of the respiratory mucosa.

Our results, as well as our previous observations of IgA induction in the respiratory tract (24), suggest that i.n. immunization with AdgB8 stimulates the induction of immune responses in the bronchus-associated lymphoid tissue (BALT) (51). The BALT serves as part of the mucosa-associated lymphoid tissues (MALT) and as such shares organizational as well as functional similarities with other mucosal surfaces as part of the common mucosal immune system. Lymphocytes derived from one mucosal tissue can recirculate through and localize selectively within other mucosal surfaces, including the respiratory tract and uterus (1–3, 52). In agreement with this, we recently demonstrated that i.n. AdgB8 immunization results in the presence of secretory anti-HSVgB IgA in the genital tract (53) and protection from intravaginal HSV-2 challenge (54).

To investigate the extent of mucosal CTL memory in distant mucosal tissues after i.n. AdgB8 immunization we evaluated long-term recall responses in the genital tract. Our results demonstrate that mice immunized i.n. with AdgB8 and challenged ivag up to 18 mo later with heterologous HSV-2 developed strong anti-HSV-2 CTL memory responses. Furthermore, limiting dilution analysis of the recall responses confirmed that i.n. but not systemically immunized animals maintained memory CTL for as long as one year either within the genital tract or its associated lymphoid tissues or capable of entering these sites after infection. To our knowledge, this is the first functional demonstration of long-lived CTL in local and distant mucosal tissues despite low or undetectable levels of systemic CTL.

Interestingly, the population of HSV-specific CTL that are maintained within the systemic immune system after i.p. immunization with AdgB8 are unable to mount memory responses to i.n. or ivag infection with HSV-2. This suggests that the population of lymphocytes present shortly after systemic immunization that contained T cells capable of mounting short-term mucosal memory responses were no longer present. This leaves us to suggest that during the early stages of an immune response there are populations of functionally active T cells that have no tissue-specific homing pattern or are able to respond or enter sites of inflammation and that either do not represent the long-term population of memory T cells or phenotypically change into that population.

Our results also demonstrate that the memory responses were predominantly specific for gB of HSV since the lytic activities and the lysis of targets infected with recombinant vaccinia virus expressing gB of HSV (VacgB11) were at least as high as for HSV-2-infected targets. Moreover, analysis of CTLp frequencies specific for gB of HSV occurring in the recall response demonstrated that the memory CTL were in fact predominantly gB-specific. Cytotoxic T lymphocyte frequencies were also assessed against HSV-infected targets and the results confirmed that i.n. but not i.p. immunization induced long-term mucosal CTL memory but the absence of long-term splenic CTL memory. These results demonstrate that part of the primary CTL response to AdgB8 immunization and the recall response to HSV-2 challenge are directed towards gB of HSV. This is in contrast to recent reports by Nugent et al. (31, 33) where it was shown that although a large portion of the primary response to HSV-1 inoculation involves gB-specific CTL, early secondary responses did not. Unlike in our system, the lack of an early gB-specific recall response in their model is most likely due to the fact that HSV-1 was used for both sensitization and challenge and likely reflects the interactions and complexities of numerous neutralizing anti-HSV and CTL epitopes during both periods.

Currently, many vaccines or immunotherapeutics are given systemically with the objective of providing mucosal immune functions. Our studies demonstrate that mucosal (i.n.) administration of recombinant adenovirus vectors induced long-lived antigen-specific CTL in mucosal-associated lymphoid tissues, whereas systemic administration of this vaccine induced long-lived CTL systemically but not mucosally. Development of successful vaccines against mucosal pathogens, such as HSV and HIV, will require the induction of long-lived mucosal immune responses. Recently, we showed that intranasal immunization with AdgB8 induced secretory IgA specific for gB of HSV in both the respiratory (24) and genital tracts (53). The ability of recombinant adenoviruses to induce specific mucosal humoral responses and the long-term maintenance of anti-HSV-2 CTL in respiratory and genital tissues suggest that these vectors may serve as excellent mucosal vaccines.

Our results also show that short-term mucosal CTL memory was present after both i.n. and systemic immunization and only after several months did the memory CTL responses compartmentalize to mucosal or systemic tissues. These results have important implications with regard to the evaluation of vaccines since the time of assessment after vaccination may affect detection of CTL activity. Furthermore, the functional evaluation of memory CTL specific for mucosal pathogens should be based on assessment of CTL in mucosal-associated lymphoid tissues and not in the spleen.

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