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Selective Induction of Protective MHC Class I-Restricted CTL in the Intestinal Lamina Propria of Rhesus Monkeys by Transient SIV Infection of the Colonic Mucosa[1]

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The identification of mucosal immune responses required for protection against sexual transmission of HIV is essential for the development of an efficacious vaccine. To gain a better understanding of these responses, we have characterized the immune responses in the lamina propria (LP) and epithelium of the jejunum, the mesenteric lymph nodes, and peripheral blood (PBMC) of 11 rhesus monkeys following colonic exposure to two molecular clones of SIV. Two monkeys had no signs of infection. Three monkeys became persistently infected. Transient infections, characterized by the sporadic detection of virus in the periphery and/or detection of SIV-specific immune responses in either the gut-associated tissues or PBMC, were induced in six of the monkeys. One persistently infected and three transiently infected monkeys had high levels of SIV env-specific MHC class I restricted CTL in the jejunal LP. Another transiently infected monkey had SIV-specific IgA secreting B cells in the LP. Three or six months postexposure, these animals and four naive controls were challenged intracolonically with the heterologous primary isolate, SIV/DeltaB670. All four monkeys with strong SIV env-specific MHC-restricted CTL in the LP were protected, whereas none of the naive controls or the remaining seven monkeys with little or no CTL in the LP were protected. These experiments provide the first direct evidence that transient mucosal infection can induce SIV-specific immunity that remains localized to the gut-associated tissues. Furthermore, a strong correlation between SIV env-specific MHC-restricted CTL in the LP and protection against colonic mucosal challenge was observed. ***The Journal of Immunology,*** **1999, 162: 540–549.**

An efficacious vaccine for HIV is desperately needed to stop the AIDS epidemic. Selection of the optimal vaccine strategy among the myriad of choices now available would be relatively straightforward if the definition of protective immunity with respect to HIV transmission were only known. A good source for this information may be those individuals who, despite multiple sexual exposures, remain uninfected (1, 2). Identification of protective immunity in these individuals may be difficult, however, because it is likely localized to the vaginal/rectal mucosa and may therefore require invasive procedures difficult to perform in humans.

Recent studies of mucosal immune responses in SIV-infected macaques have demonstrated that this system will be highly useful in identifying protective immunity at the mucosal surface. SIV-specific CTL have been identified in both the vaginal and intestinal mucosae of chronically infected macaques (3, 4). Moreover, macaques chronically infected with attenuated virus have been shown to be protected against rectal challenge (5). Transient infections, whereby monkeys have only sporadic evidence of SIV in the periphery but remain seronegative, have also been demonstrated following mucosal exposure to limiting doses of virus (6–9). These animals may have immune responses that remain localized to the mucosa because several investigators have now observed that these animals are protected from mucosal challenge (7, 8). A direct correlation between mucosal immunity and protection, however, has not been established. Toward this end, we have performed a series of colonic exposures with limiting doses of two molecular clones (SIVmac239 and SIV/17E-Fr) and found an absolute correlation (4 of 4 vs 0 of 7 monkeys) between the selective induction of an MHC class I-restricted CTL response directed against the viral env in the jejunal lamina propria (LP)[6] and protection following colonic challenge with the heterologous primary isolate SIV/DeltaB670.

Materials and Methods

Animals

Twenty-three adult Indian origin rhesus monkeys (*Macaca mulatta*) of either sex were involved in this study. Observation of activity, stool consistency, appetite, and general condition was performed daily. Physical examinations, performed weekly for the first four weeks postinoculation and monthly thereafter, consisted of body temperature and weight measurements, palpation and size grading of lymph nodes and spleen, abdominal

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[6] Abbreviations used in this paper: LP, lamina propria; MNC, mononuclear cells; IEL, intraepithelial lymphocytes; MLN, mesenteric lymph nodes; p.i., postinoculation; ELISPOT, enzyme-linked immunospot; PE, phycoerythrin; PFU, plaque-forming unit; $TCID_{50}$, tissue culture infectious dose 50.

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palpation, and assessment of general condition. Whole blood was collected at the time of physical examination for diagnostic PCR, flow cytometry, viral coculture, ELISA, and p27 antigenemia assays. Appropriate treatment was instituted as necessary, and moribund animals were humanely sacrificed with an i.v. barbiturate overdose.

Viral isolates

Three virus stocks were used to infect the macaques in these studies: SIV/DeltaB670rh, SIV/17E-Fr, and SIVmac239. SIV/DeltaB670rh was obtained by a single passage of SIV/DeltaB670hu stock onto rhesus PHA-stimulated blasts. SIV/DeltaB670hu was obtained by coculturing lymph node tissue from SIV-infected monkey B670 with primary human PHA-stimulated PBMC (PHA blasts). SIVmac239 is an infectious molecular clone that is primarily T cell tropic (10). SIV/17E-Fr is an infectious, molecular congenic clone of SIVmac239 that is macrophage-tropic (kindly provided by J. Clements Johns Hopkins University, Baltimore, MD) (11, 12).

Animal inoculations

Animals were treated with warm water enemas and Golytely (Raintree Laboratories, Raintree, MA) the day prior inoculation. The day of inoculation the animals were chemically restrained with ketamine HCl (10 mg/kg) and treated with Torbutrol (0.05 mg/kg) to minimize pain. An Olympus flexible fiberoptic endoscope (model GIF type PQ20) was used to intracolonically inoculate the animals. The endoscope was introduced rectally and advanced approximately 50 cm in an oral direction to the transverse colon where inoculations of virus stock occurred. Following inoculation, each macaque underwent a series of three ventral abdominal midline celiotomies for the purpose of collecting jejunum and mesenteric lymph nodes. One day before surgery all animals were treated with warm water enemas and Golytely and started on the following treatments: Kefzol (25 mg/kg twice daily for 5 days intramuscularly), lactobacillus paste (1.0 ml per day, mouth), and Torbutrol (0.05 mg/kg three times daily for 3 days i.m.). For surgery, the animals were preanesthetized with glycopyrrolate and acepromazine, induced with Ketamine HCl, and anesthesia was maintained with isoflurane and O_2. One or four months following the last survival surgery, each animal was challenged intracolonically with 1.0 ml containing 10,000 $TCID_{50}$ of SIV/DeltaB670rh. The same routine described for the initial inoculations was followed for the challenge inoculations.

Isolation of lamina propria and mesenteric lymph node mononuclear cells

Mononuclear cells (MNC) from the jejunal LP were isolated using a neutral protease method (13). Briefly, minced 20-cm sections of tissue were treated with 1 mM DTT (Sigma, St. Louis, MO) for 20 min at 37°C followed by incubation in calcium/magnesium-free PBS containing 0.75 mM EDTA and 5% FCS four times for 30 min at 37°C to release intraepithelial lymphocytes (IEL), which were pooled and placed in Iscove's modified Dulbecco's media (Life Technologies, Grand Island, NY), 20% FCS plus Abs, and 0.25 μg/ml Fungizone (Life Technologies) overnight at room temperature. The tissues were sliced into smaller pieces and digested with 1.5 mg/ml Dispase, Grade II (Boehringer Mannheim, Indianapolis, IN) for 30 min. The supernatant containing dissociated cells was collected, and the treatment was repeated four more times. The pooled LP cells were resuspended in Iscove's modified Dulbecco's media overnight at 37°C to reexpress CD8 molecules. Mesenteric lymph nodes (MLN) were teased to release the MNC and placed in Iscove's media (Life Technologies) overnight at room temperature. PBMC were isolated the day of surgery and held overnight as described for MLN. MNC from IEL were passed through glass wool columns followed by discontinuous gradient centrifugation on Percoll (14) (Pharmacia, Piscataway, NJ). Cells from the LP were also purified by Percoll centrifugation. PBMC and MNC from lymph nodes were purified by Ficoll-Paque (Pharmacia) density barrier centrifugation.

Flow cytometry

Lymphocyte subsets and monocyte/macrophages were directly stained per manufacturer's instructions with FITC- or phycoerythrin (PE or RD-1)-conjugated mAbs specific for human subsets, including CD20 (B1-FITC), CD2 (T11-RD1), CD29 (4B4-RD1) (Coulter Immunology, Hialeah, FL); CD4 (OKT4-FITC) (Ortho Diagnostics, Raritan, NJ); CD8 (Leu-2a-FITC or -PE), CD14 (Leu-M3-PE), and CD16 (Lea 11a-FITC) (Becton Dickinson, San Jose, CA). MNC subset compositions of various stained cell preparations were evaluated by flow cytometry using either EPICS 541 (Coulter, Irvine, TX) or FACSCalibur (Becton Dickinson) instruments. Lymphocytes and/or monocyte/macrophages were gated based on light scatter characteristics, and percentages of stained cells were determined relative to labeled isotype controls (Becton Dickinson). For two-color evaluations, the electronic compensation for spectral overlap was set using lymphocyte preparations stained with mutually exclusive OKT4-FITC and Leu-2a-PE.

Quantitation of CTL

Effector cells. Potential effector MNC from blood and mesenteric lymph nodes were purified by Ficoll-Paque (Pharmacia) barrier density centrifugation. MNC from jejunal LP and IEL were purified on discontinuous gradients of 20, 44, and 67% Percoll (Pharmacia) and washed twice with RPMI 1640 (Life Technologies) supplemented with antibiotics, 2 mM L-glutamine, and 2% human AB serum (Irvine Scientific, Santa Anna, CA) before resuspending them at 2×10^6/ml in the RPMI 1640:Click's (Life Technologies) (1:1) medium with 0.04 mM 2-ME (Sigma), 10 ng/ml IL-7 (R&D Systems, Minneapolis, MN), and 2×10^5 PFU/ml (0.1 PFU/cell) of each of the following recombinant vaccinia viruses encoding sequences for SIVmac251 gp160 (env-vaccinia), gag and protease (gag-vaccinia), and pol (pol-vaccinia) (kindly provided by G. P. Mazzara and D. Panicali, Therion Biologic Corp., Cambridge, MA). The cells were incubated in culture tubes or 75-cm^2 flasks at 37°C in 5% CO_2. After 3 days, half of the medium was replaced with the RPMI 1640:Click's medium without IL-7 and the recombinant viruses but including 4 U/ml recombinant IL-2 (Hoffmann-La Roche, Nutley, NJ) for the remaining 4 days of culture.

Target cells. PBMC were isolated by Ficoll-Paque density gradient centrifugation and transformed by exposure to rhesus EBV (15) from a persistently infected cell line. Transformed B cells were maintained on RPMI 1640 supplemented with 2 mM L-glutamine, antibiotics, and 15% FCS. The day before the CTL assay, autologous and MHC class I mismatched B cell lines, as determined by isoelectric focusing (16), were infected with either wild-type vaccinia virus or recombinant viruses env-vaccinia, gag-vaccinia, or pol-vaccinia in 200 μl 2.5×10^8 PFU/ml for each 2×10^6 cells (25 PFU/cell) for 2 h at 37°C, adjusted to 1×10^6/ml, and incubated at 37°C in 5% CO_2 for 16 h. Except for the portion of wild-type vaccinia-infected cells to be used for "cold" targets, aliquots of all infected cells were labeled with Na_2CrO_4 (50 μCi/10^6 cells) (DuPont NEN Research Products, Boston, MA) for 1 h at 37°C. The cell suspensions were then centrifuged through FCS barriers, washed two times, and resuspended to 5×10^5/ml.

CTL assay. Cultured effector cells were pooled, subjected to positive selection of $CD8^+$ cells using Dynabeads M450 with Detachabead (Dynal, Great Neck, NY) as per manufacturer's instructions. Various concentrations of $CD8^+$ effector cells were added to 5×10^3 ^{51}Cr-labeled target cells to achieve E:T ratios of 20:1, 10:1, or 5:1 along with 1×10^5 "cold" targets in 96-well U-bottom plates to a total of 100 μl. Wild-type vaccinia-infected cold targets were used to reduce the effect of any existing anti-EBV or -vaccinia effector cells. However, since fixed recombinant vaccinia-infected EBV-transformed cell lines were not used to stimulate cultures, this effect was dependent on the individual animal and was minimal. Cold targets were also added to the spontaneous ^{51}Cr-release controls. Medium and 0.5% sodium desoxycholate were added in place of effector cells to spontaneous and maximum ^{51}Cr-release controls, respectively. After 1 h incubation at 37°C, an additional 100 μl of medium was added, and the plates were incubated another 4 h. Supernatants were collected with the Skatron Supernatant Collecting System (Skatron, Sterling, VA) and counted on a Packard Cobra II Autoγ solid crystal scintillation system (Packard Instrument, Downers Grove, IL). Spontaneous ^{51}Cr release was always less than 20% of the maximum ^{51}Cr release.

Percent specific ^{51}Cr release (Fig. 1*A* for PBMC and 1*C* for LP) was calculated using the following formula:

$$\%\ \text{Spec.}\ ^{51}\text{Cr release} = \frac{(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{spont}})}{(\text{cpm}_{\text{max}} - \text{cpm}_{\text{spont}})} \times 100.$$

Percent specific ^{51}Cr release for wild-type vaccinia controls was always less than one-half that of positive CTL, with recombinant SIV expressing targets for PBMC and less than one-third for LP and MLN at equivalent E:T ratios.

Percent SIV Ag-specific ^{51}Cr release was calculated by subtracting the nonrecombinant, wild-type vaccinia control percent specific ^{51}Cr release from the recombinant env, gag, or pol vaccinia percent specific ^{51}Cr release (Fig. 1*B* for PBMC and 1*D* for LP). A difference >10% at the 20:1 E:T ratio was considered to be positive. As a means of quantitating relative responses, LUs were obtained by first extrapolating to an estimated E:T ratio that would give 15% SIV Ag-specific lysis (Fig. 1, *B* and *D*). Using the estimated 15% E:T ratio, 15% LUs per 10^6 purified $CD8^+$ effector cells after culture were calculated as follows:

One (1) 15% LU =
number of efector cells to give 15% SIV Ag-Spec. ^{51}Cr release

or

One (1) 15% LU = (est. E:T ratio for 15% SIV Ag-Spec. ^{51}Cr release)
× (No. target cells/well).

Therefore:

No. of 15% LUs/10^6 efector cells =
(1) 15% LU/(Est. E:T ratio for 15% SIV Ag-Spec. ^{51}Cr release)
× (No. target cells/well).

Solving:

No. of 15% LUs (per million cells) =

$$\frac{10^6}{\text{(No. target cells/well)} \times \text{(est.E:T ratio for 15\% SIV Ag-Spec. } ^{51}\text{Cr release}}.$$

SIV-specific serum Ab

SIV-specific gp120 Ab responses were identified in serum by an ELISA using Con A-captured baculovirus-produced SIV/DeltaB670 gp120 as described (17). Recombinant SIV gp120 was kindly provided by Dr. Ronald Montelaro at the University of Pittsburgh School of Medicine.

SIV p27 assay

Antigenemia was determined by measuring the levels of SIV p27 Ag in serum using a commercially available enzyme-linked immunoassay kit specific for SIV (Coulter, Hialeah, FL).

SIV-specific IgA ELISPOT assay

Nitrocellulose HA 96-well plates (Millipore, Bedford, MA) were coated with 0.2 μg SIV envelope recombinant gp140 (kindly provided by Drs. K. Javaherian and G. LaRosa, Repligen, Cambridge, MA) in 100 μl PBS and placed at 4°C overnight. After washing five times with PBS, the wells were blocked with a milk solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and incubated for 4 h at 4°C. Following further washing with PBS, various numbers of MNC from jejunum in RPMI 1640 with 10% human AB serum were added to wells and incubated overnight at 37°C in 5% CO_2. Detection of B lymphocytes secreting SIV envelope-specific IgA Abs was accomplished by incubating the plates 4 h at 37°C with goat anti-monkey IgA conjugated with peroxidase (Nordic Immunological Labs, Capistrano Beach, CA) after first washing with PBS and PBS with 0.05% Tween 20 (Sigma). Following extensive washing with PBS-Tween (10 times), development was accomplished with TMB membrane peroxidase substrate (Kirkegaard & Perry) for about 30 min at room temperature. The plates were then washed with water and dried overnight at 4°C. Spots representing gp140-specific IgA-secreting B lymphocytes were enumerated with a binocular dissecting scope.

Detection of SIV

Identification of SIV in PBMC was performed by nested PCR utilizing conserved primers specific for the viral long terminal repeat (LTR) as described (18). When identification of specific SIV genomes was desired, the first hypervariable region of the viral gp120 was PCR amplified using conserved sequences flanking this region, cloned in a TA cloning vector (Inbitrogen, San Diego, CA), and sequenced as described (18).

Results

To increase our understanding of host:virus interactions associated with mucosal exposure to SIV, 12 adult rhesus monkeys were colonically exposed to either SIVmac239 or SIV/17E-Fr. Limiting amounts of virus were employed because we were particularly interested in those events associated with transient infection since evidence exists in both monkeys (7) and humans (1) that exposure without overt infection may provide protection against subsequent reexposure. Animals were monitored for the presence of virus and virus-specific immunity in the periphery and in the gut-associated tissues so that interactions localized to the site of exposure, if present, could be revealed.

Peripheral blood was sampled at weekly intervals postinoculation (p.i.) to monitor the appearance of SIV and SIV-specific Ab. At 3 and/or 8 wk p.i., CTL responses were also measured in both the peripheral blood and in the gut-associated tissues. In some cases, the presence of SIV-specific IgA-producing B cells was also enumerated in the gut-associated tissues. For analysis of gut-specific immunity, 20-cm sections of jejunum and colonic lymph node(s) were obtained for analysis. Jejunal tissue was selected because it can be surgically excised without adversely affecting the health of the animal. This procedure allowed serial collection of

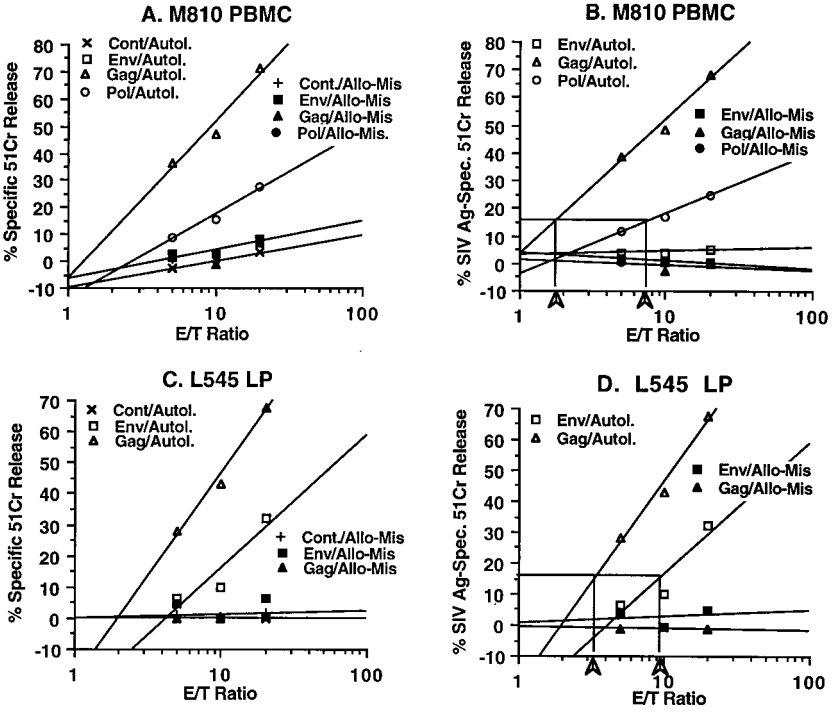

FIGURE 1. Two examples of estimating 15% E:T ratios from CTL assays using PBMC from a monkey infected i.v. with SIV/17E (M810, *A* and *B*) and LP from a monkey infected mucosally with SIV/17E (L545, *C* and *D*). *A* and *C*, Calculated percent specific ^{51}Cr release for control, env, gag, or pol. *B* and *D*, Percent SIV Ag-specific ^{51}Cr release for env, gag, or pol after subtraction of the control. × and open symbols, Autologous targets. + and closed symbols, Allogeneic-mismatched targets. The estimated 15% E:T ratios of gag and pol in *B* are 1.8 and 7.9, respectively.

Table I. *Presence of SIV DNA in PBMC after colonic exposure to limiting doses of SIVmac239 or SIV/17E-Fr*

Expt.	SIV Clone	$TCID_{50}$	Monkey	SIV DNA in PBMC (wk postinoculation)							
				1	2	3	4	8	12	16	20
I	SIVmac239	100	N255	−	−	−	+	−	−	−	−
		100	M224	+	+	+	+	+	+	+	+
		10	M029	−	+	−	NT[a]	−	+	−	−
		10	L870	−	+	−	NT	−	+	−	−
	SIV/17E-Fr	1000	L987	−	−	−	−	−	−	−	−
		100	M259	−	−	−	−	NT	−	−	−
		100	M577	−	−	NT	−	−	−	−	−
II	SIV/17E-Fr	1000	N041	−	−	−	−	−	−	#[b]	#
		1000	N385	+	+	+	+	+	+	#	#
		1000	N138	+	+	+	+	+	+	#	#
		1000	M155	−	−	−	−	−	−	#	#

[a] NT, Not tested.
[b] #, Postchallenge.

tissue so that a causal relationship between gut-specific immunity and protection from experimental challenge could be obtained. The jejunum is appropriate for analysis of effector responses because, once Ag-specific effectors become primed and pass through the regional draining lymph nodes, they are redistributed to the epithelium and lamina propria that line the entire intestine (19–21).

Infection outcome of colonic exposure to limiting doses of SIVmac239 and SIV/17E-Fr

Two doses of each virus preparation were utilized in two separate experiments so that a spectrum of no infection ⇒ transient infection ⇒ disseminated infection could be achieved with each of the clones. The first experiment evaluated the outcome of animals receiving either 10, 100, or 1000 $TCID_{50}$ of either SIVmac239 or SIV/17E-Fr. A second experiment was later initiated to expand the number of monkeys exposed to the higher dose (1000 $TCID_{50}$) of SIV/17E-Fr. Assisted by an endoscope, 1 ml of appropriately diluted cell-free culture supernatant was atraumatically inoculated into the lumen of the transverse colon. This site was chosen to assure accurate delivery of the virus dose, to avoid self-inoculation of leaked virus into other mucosal sites, and to minimize inadvertent i.v. inoculation of virus by exposure of existing rectal abrasions to the inoculum.

One monkey died of causes unrelated to virus exposure; infection outcome in the remaining 11 monkeys is shown in Table I. One of four monkeys exposed to SIVmac239 (monkey M224) and two of seven animals exposed to SIV/17E-Fr (monkeys N385 and N138) developed a disseminated infection as determined by repeated identification of viral sequences in PBMC by PCR amplification. PCR was used to identify virus in the periphery because, in our hands, it is the most sensitive measurement of SIV in the infected animal. Three of the remaining monkeys exposed to SIVmac239 (monkeys N255, M029, and L870) were sporadically PCR positive. No virus was detected by PCR in five of the monkeys exposed to SIV/17E-Fr (L987, M259, M577, N041, and M155).

Immunologic responses to colonic exposure with limiting doses of SIVmac239 and SIV/17E-Fr

MNC were purified from the peripheral blood (PBMC), jejunal LP, and MLN draining the large intestine. Flow cytometric evaluation showed that the composition of MNC found in these tissues was similar, with several minor exceptions (Fig. 2). All three organs had significant populations of T and B lymphocytes and macrophages as measured by surface expression of CD2, CD20, and CD14, respectively, with the exception that a higher percentage of B cells was found in MLN than in the other two organs. All organs had significant populations of both $CD4^+$ (helper) and $CD8^+$ (suppressor-cytotoxic) subsets of T lymphocytes. The ratio of $CD4^+$ to $CD8^+$ lymphocytes, however, was higher in MLN and LP than that found in PBMC. These findings, consistent with previously published data (4), assured us we were not deleteriously affecting these populations during the purification process.

All of the monkeys that were persistently infected had evidence of virus-specific Abs in the peripheral blood by 4 wk p.i. in a manner similar to that observed in monkeys inoculated i.v. (Table II) (22). Conversely, none of the monkeys that were either sporadically PCR positive or PCR negative had detectable Ab in the serum by conventional ELISA.

At 3 and/or 8 wk postexposure, surgeries were also performed to obtain jejunal LP and MLN tissues for analysis of SIV-specific T and B cell function. In samples obtained in experiment I, a portion of the MNC from the jejunal LP was also analyzed for the presence of SIV env-specific IgA-secreting B lymphocytes by ELISPOT. SIV-specific IgA-secreting B cells were detected in the jejunum of only one animal in this group (monkey N255). Positive cells were detected at both time points, at levels of four and six SIV-specific

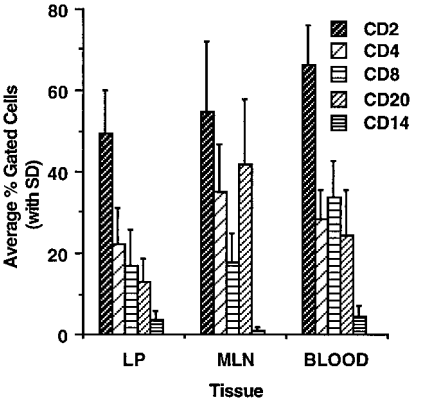

FIGURE 2. Flow cytometry of MNC subsets after isolation from tissues, but before culture for 7 days. Data average of 10 experiments, with 1 SD indicated. LP = lamina propria MNC; MLN = mesenteric lymph node MNC; blood = peripheral blood MNC.

Table II. *Immunologic outcome of colonic exposure to limiting doses of SIVmac239 and SIV/17E-Fr*

Expt.	SIV Clone	Monkey	Serum[a] Ab Titer	LP[b] ELISPOT	CTL[c] 3 wk p.i. PBMC	3 wk p.i. LP	3 wk p.i. MLN	8 wk p.i. PBMC	8 wk p.i. LP	8 wk p.i. MLN
I	SIVmac239	N255	0	6		env [5]			env [4]	
		M224	51,200	0						
		M029	0	0	pol [12]	env [8]		ND	ND	ND
		L870	0	0		env [39]			env [29]	
	SIV/17E-Fr	L987	0	0						
		M259	0	0				gag [5]		
		M577	0	0		env [57]			env [42]	env [8]
II	SIV/17E-Fr	N041	0	NT[d]	NT	NT	NT		env [22]	NT
		N385	3200	NT	NT	NT	NT	env [33] gag [50]	env [17] gag [36]	NT
		N138	3200	NT	NT	NT	NT			NT
		M155	0	NT	NT	NT	NT			NT

[a] Reciprocal dilution; gp120-specific.
[b] Max. No. SIV-specific IgA^+ cells/750,000 total lymphocytes.
[c] Target specificity of SIV gag, pol, and env targets tested (15% LU/10^6 $CD8^+$cells).
[d] NT, not tested.

IgA-producing cells per 750,000 total lymphocytes, respectively (Table II). Interestingly, SIV was identified in the PBMC of this animal at only one time point (4 wk p.i.), and it remained seronegative. Ag-specific T cell proliferative responses were uniformly negative among all LP samples tested (data not shown); these results were not unexpected given the apparent inability of T effectors from the LP to proliferate in response to Ag (23).

Purified $CD8^+$ T lymphocytes from PBMC, LP, and MLN were also evaluated for cytolytic activity using EBV-transformed autologous and allogeneic mismatched targets infected with vaccinia recombinant viruses containing SIV env, gag, and pol at 3 and/or 8 wk postexposure. These data are presented as LU in Table II. Graphic presentation of SIV Ag-specific ^{51}Chromium release at E:T ratios of 20:1, 10:1, and 5:1 are shown for representative animals in Fig. 3.

In PBMC, low levels ($\leq$12 LU) of pol- or gag -specific CTL were observed in monkeys M029 and M259, respectively. Neither of these animals was persistently infected. A higher level ($\geq$33 LU) of CTL activity specific for both env and gag was observed in the PBMC of monkey N385. This result was not surprising, given that this animal was persistently infected with SIV/17E-Fr, an attenuated strain that is a potent inducer of CTL in the periphery (our unpublished observations). CTL activity was not detected in the PBMC of the other two persistently infected monkeys (monkeys N138 and M224). The failure to identify CTL responses in all monkeys infected with SIV is consistent with the observations of others (24) and may be explained by an MHC nonresponder haplotype in these animals.

With respect to the mesenteric lymph nodes, only monkey M577 had demonstrable CTL in this organ among the seven monkeys evaluated (Table II). In contrast, CTL activity was frequently detected in the jejunal LP that, with one exception (monkey N385), was directed solely against viral env determinants. These responses could be divided into two categories: a low level of activity ($\leq$8 LU), as observed in monkeys N255 and M029, and a high level of activity ($\geq$17 LU), as observed in monkeys L870 (Fig. 3, *A* and *B*), M577 (Fig. 3, *C* and *D*), N041 (Fig. 3*E*), and N385 (Fig. 3*F*). This response was a striking finding because three of these animals (monkeys L870, M577, and N041) had little or no evidence of virus in the periphery.

MHC class I restriction of CTL

We routinely employ $CD8^+$ purified effector cells that, by flow cytometric analysis, are >90% $CD2^+$ $CD8^+$ and $CD16^-$ (data not shown). In our hands, cytolysis by PBMC effectors is always MHC class I restricted. The $CD8^+$ population in the intestinal mucosa may differ from that in the blood, however, since mucosal tissue is known to be enriched for TCR $\gamma\delta$, $CD8^+$ T cells that do not require MHC class I recognition for cytolysis (25). To address this issue, CTL activity was assessed in LP taken 3 wk before exposure and compared with the results obtained 8 wk postexposure against autologous and allogeneic-mismatched targets expressing either SIV gag or env. CTL activity detected in PBMC and LP before infection is shown for the representative monkey N385 in Fig. 4. As expected, no cytolysis of either autologous or mismatched targets was observed in the PBMC of the naive animal (Fig. 4*A*). By 8 wk p.i., significant activity against both env and gag targets was observed, but these responses were restricted to autologous targets, a finding that confirms a requirement for MHC class I recognition (Table II). In contrast, a low level of CTL activity against env, but not gag, was detected in the jejunal LP before SIV exposure (Fig. 4*B*). This activity was not MHC class I restricted, however, a finding suggesting the presence of functionally active TCR $\gamma\delta$, $CD8^+$ T lymphocytes in the LP of this animal. At 8 wk postexposure, CTL activity against env was significantly higher in the LP, with activity against gag also apparent (Fig. 3*F* and Table II). In contrast to that observed before virus exposure, this activity required MHC class I recognition. A similar pattern was observed in monkey N041 (data not shown). CTL activity was also analyzed in IEL. One animal (monkey N041) had env-specific CTL in IEL, which mirrored that seen in LPL. The remaining monkeys had no detectable response (data not shown).

Colonic challenge of SIVmac239- and SIV/17E-Fr-immunized animals with SIV/DeltaB670

The relationship of the immune responses observed in animals mucosally exposed to the two SIV clones, particularly those identified in the intestinal LP, with protection against mucosal challenge was further assessed by colonic challenge with a 100% infectious dose (10,000 $TCID_{50}$) of the heterologous primary isolate,

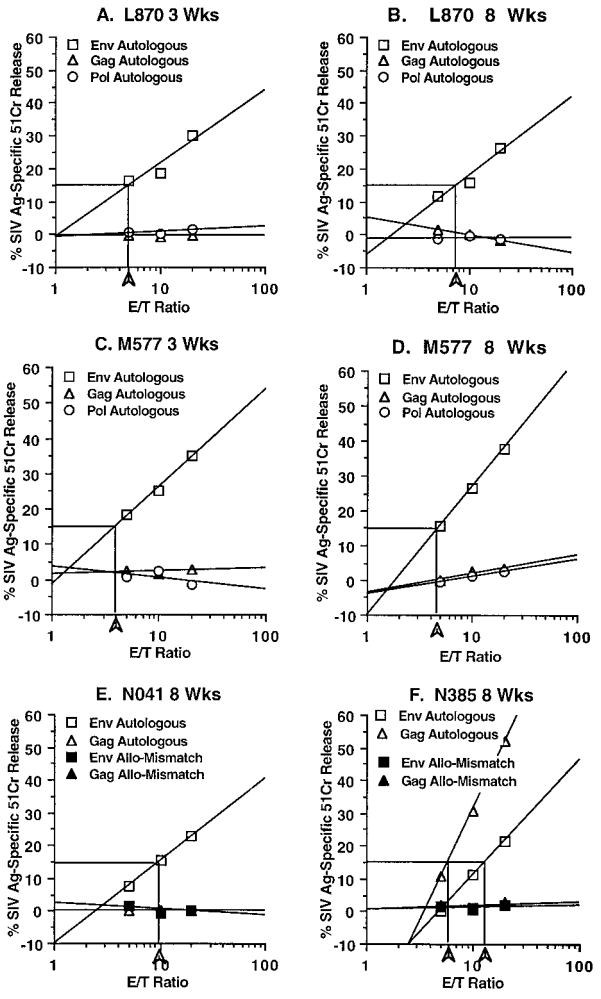

FIGURE 3. Percent SIV Ag-specific cytolysis from the jejunal LP of monkeys at 3 and/or 8 wk following colonic inoculation with SIV clones. *A*, Percent cytolysis (L870) obtained 3 wk post colonic exposure to SIVmac 239. *B*, Percent cytolysis (L870) obtained 8 wk post colonic exposure to SIVmac 239. *C*, Percent cytolysis (M577) obtained 3 wk post colonic exposure to SIV/17E-Fr. *D*, Percent cytolysis (M577) obtained 8 wk post colonic exposure to SIV/17E-Fr. *E*, Percent cytolysis (N041) obtained 8 wk post colonic exposure to SIV/17E-Fr. *F*, Percent cytolysis (N385) obtained 8 wk post colonic exposure to SIV/17E-Fr.

SIV/DeltaB670. Monkeys in experiment I were challenged 6 mo postexposure, whereas monkeys in experiment II were challenged 3 mo postexposure (4 and 1 mo, respectively, from the last evaluation of immune responses in the gut-associated tissues) (Table III). Four naive animals were included as infection controls; all four of these animals became persistently PCR positive by 7 days postchallenge. Three of the infected control animals responded to virus infection with the production of virus-specific Ab in the periphery. One monkey (animal L821) failed to seroconvert, a finding consistent with the rapid and severe disease noted soon after infection (see below).

Eight of the monkeys previously exposed to the SIV clones were PCR negative at challenge. Of these, three animals became virus positive following challenge (monkeys N255, M029, M155) and three remained virus negative (monkeys L870, M577, N041). One of the virus-positive animals (monkey N255) had IgA-secreting B lymphocytes in the LP before challenge. All three of the virus-negative (protected) monkeys had a high level of CTL responses in the LP before challenge whereas none of the virus-positive (unprotected) monkeys had significant CTL in the LP before challenge.

Three monkeys were PCR positive before challenge (monkeys M224, N385, and N138). Two of these animals subsequently developed AIDS in a manner similar to that observed in the challenge controls. One animal (monkey N385), however, failed to develop disease (see below); this animal had significant levels of CTL activity in the LP before challenge. This finding prompted us to analyze the virus found in this animal postchallenge at the sequence level. For this analysis, the V1 region of the viral gp120 from PBMC obtained at 2, 4, and 8 wk postchallenge was PCR amplified, cloned, and sequenced. At 2 wk postchallenge, both the immunizing strain SIV/17E-Fr (seven clones) and the challenge strain SIV/DeltaB670 (three clones) were found. By 8 wk, however, only SIV/17E-Fr could be detected among 17 clones sequenced, a finding that suggests transient infection with the challenge strain (data not shown).

Analysis of SIV-specific CTL activity in the jejunal LP and/or PBMC was repeated at 3 wk postchallenge to determine the persistence of these responses and to relate these responses to the outcome of mucosal challenge. CTL activity was identified in only one control animal (monkey M558). This activity was env specific and detected only in PBMC (Table III). In the two monkeys where LP was analyzed postchallenge (animals L870 and M577), the CTL activity observed before challenge was again observed after challenge. The inclusion of allogeneic-mismatched targets in these assays demonstrated that the responses were MHC class I restricted. Lymphocytes from the LP taken from the other two monkeys that had CTL in LP before challenge (animals N041 and N385) were unavailable for analysis; however, both animals had detectable CTL in PBMC. Like that observed before challenge, analysis of CTL in the LP for MHC class I restriction revealed activity of two types: a larger, MHC class I-restricted component,

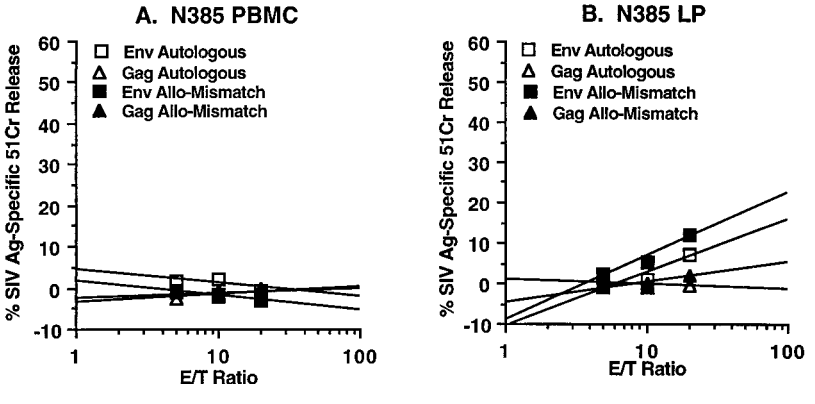

FIGURE 4. Percent SIV Ag-specific cytolysis from naive monkey N385. *A*, Percent cytolysis from PBMC. *B*, Percent cytolysis from LP.

Table III. *Outcome of monkeys colonically exposed to limiting doses of SIVmac239 and SIV/17e-Fr following colonic challenge with 10,000 $TCID_{50}$ SIV/DeltaB670*

Expt.	SIV Clone	Monkey	PCR on PBMC (wk postchallenge) 0	1	2	3	4	8	12	16	20	24	Serum[a] Ab Titer	CTL[b] (3 wk postchallenge) PBMC	LP
	None (controls)	L821	−	+	+	+	+	+	Dead				0	−	−
		L632	−	+	+	+	+	+	+	+	+	+	102,400	−	−
		M558	−	+	+	+	+	+	+	+	+	+	3200	env [15]	−
		M079	−	+	+	+	+	+	+	+	+	+	12,800	−	−
I	SIVmac239	N255	−	+	+	+	+	+	+	+	Dead		25,600	−	−
		M224	+	+	+	+	+	+	+	+	+	+	51,200	−	−
		M029	−	−	+	+	+	+	+	+	+	+	3200	−	−
		L870	−	−	−	−	−	−	−	−	−	−	0	−	env[20/5][c]
	SIV/17E-Fr	L987	−	+	+	+	+	+	+	+	+	Dead	3200	−	−
		M259	−	+	+	+	+	+	+	+	+	+	200	−	−
		M577	−	−	−	−	−	−	−	−	+	−	0	env [7]	env [20/7]
II	SIV/17E-Fr	N041	−	−	−	−	−	−	−	−	−	−	0	env [22]	NT[d]
		N385	+	+	+	+	+	+	+	+	+	+	12,800	env [15], gag [47]	NT
		N138	+	+	+	+	+	+	+	+	+	+	12,800	−	NT
		M155	−	+	+	+	+	+	+	+	+	+	12,800	gag [13]	NT

[a] Reciprocal dilution; gp120-specific.
[b] Target specificity of SIV gag, pol, and env targets tested (LU/10^6 $CD8^+$ cells).
[c] Autologous/allo-mismatched target cytolysis; allo-mismatched LUs indicated only if ≥3, the cut-off value.
[d] NT, Not tested.

and a smaller, MHC class I-unrestricted component. This latter activity, however, was apparently localized to the LP because it was not found in the PBMC of these animals, nor has it been seen in other studies (data not shown). In monkeys M577 and N041, CTL activity was also seen for the first time in the peripheral blood, a finding that may suggest boosting of these responses by challenge, even though the challenge virus was never detected in the periphery.

To confirm our virological assessment of protection following challenge, monkeys were also monitored clinically for signs of disease progression. Flow cytometric enumeration of lymphocyte subsets and SIV-specific p26 antigenemia are shown over time postchallenge for these animals in Figs. 5-7. All 4 control monkeys developed progressive disease characterized by a selective decline in $CD4^+CD29^+$ helper/inducer T lymphocytes (monkeys L821 and M558; Fig. 5, *A* and *C*) or total $CD4^+$ lymphocytes (monkeys L632 and M079; Fig. 5, *B* and *D*) and died of AIDS-associated illness by 450 days postinfection. Like that observed previously (26, 27), the selective decrease in $CD4^+CD29^+$ T lymphocytes observed in these animals was an early indicator of rapid disease progression.

Profiles of monkeys colonically exposed to SIVmac239 are shown in Fig. 6. Monkey M224 (Fig. 6*A*), which became persistently infected after exposure to SIVmac239, had a small peak in antigenemia 14 days postexposure, which coincided with a decline in $CD4^+$ lymphocytes and an increase in $CD8^+$ T lymphocytes. The decline in $CD4^+$ T lymphocytes persisted, with the increase in $CD8^+$ T lymphocytes subsequently declining to preinoculation levels by the time

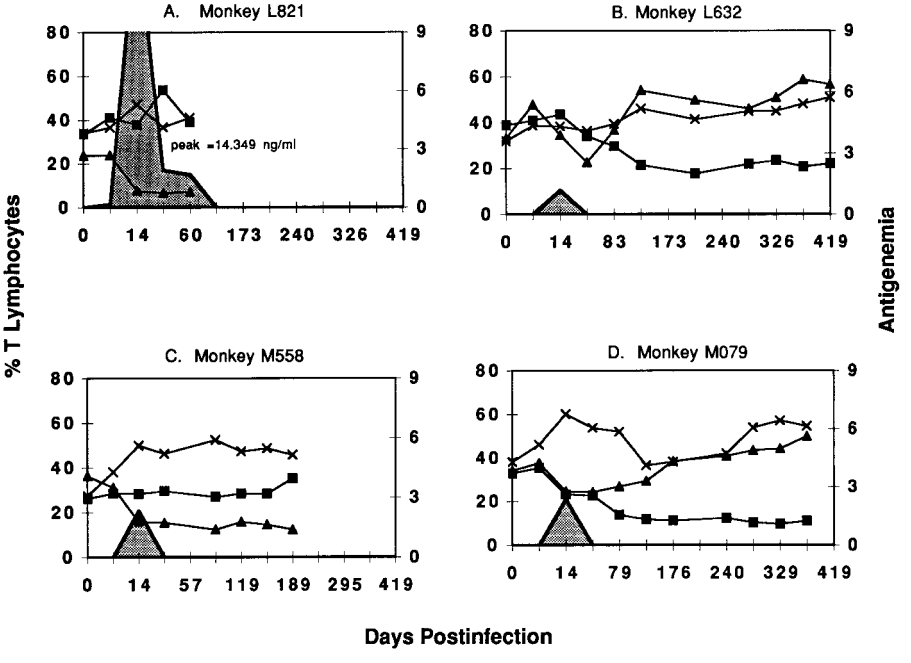

FIGURE 5. T cell subset changes and SIV p27 antigenemia in naive control monkeys challenged intracolonically with SIV/DeltaB670. Shaded areas indicate ng/ml SIV p26 in serum. ♦, Negative antigenemia values. Percent T lymphocyte subsets over time postinfection are indicated as follows: ■, $CD4^+$; ×, $CD8^+$; ▲, $CD4^+CD29^+$.

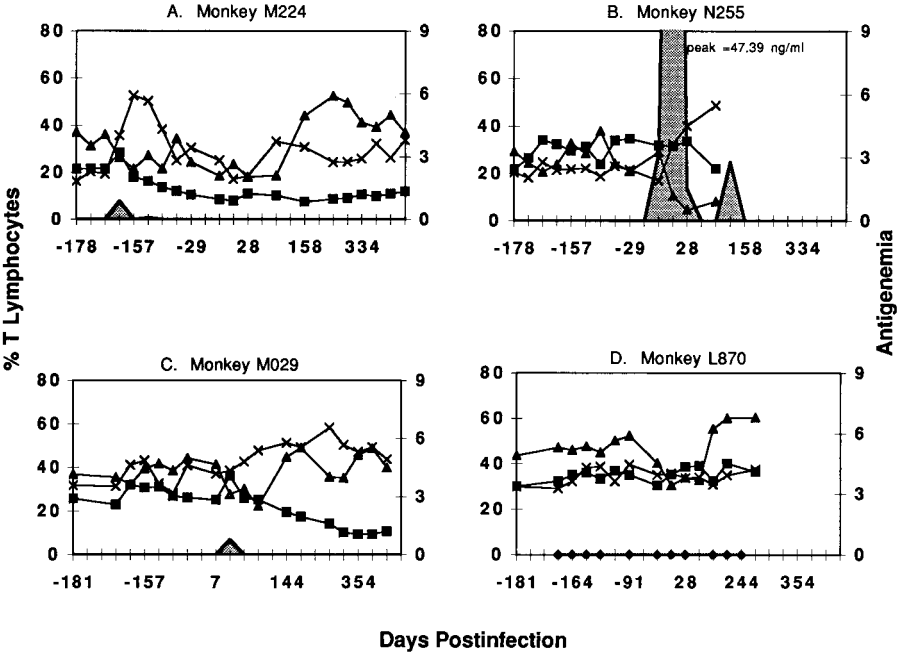

FIGURE 6. T cell subset changes and SIV p27 antigenemia in monkeys colonically exposed to SIVmac239 following intracolonic challenge 6 mo later with SIV/DeltaB670. Shaded areas indicate ng/ml SIV p26 in serum. ◆, Negative antigenemia values. Percent T lymphocyte subsets over time postinfection are indicated as follows: ■, $CD4^+$; ×, $CD8^+$; ▲, $CD4^+CD29^+$.

the animal was challenged with SIV/DeltaB670 6 mo later. As expected, monkeys N255 and M029 showed no change in T lymphocytes until challenge. Following challenge, monkey N255 developed rapid disease characterized by an SIV p26 antigenemic peak of over 47 ng/ml, a selective decline in $CD4^+CD29^+$ helper/inducer T lymphocytes and an increase in $CD8^+$ T suppressor/cytotoxic lymphocytes, and died 119 days later (Fig. 6*B*). Monkey M029 developed disease more slowly and displayed a slow decline in $CD4^+$ T lymphocytes and elevated levels of $CD8^+$ lymphocytes (Fig. 6*C*). This animal was sacrificed at 481 days postinfection for reasons unrelated to disease. In striking contrast to the other animals, the only change noted in monkey L870, which was never virus positive, was a transient decline in $CD4^+CD29^+$ helper-inducer lymphocytes during the time of challenge, but these numbers returned to normal levels thereafter (Fig. 6*D*).

Among the 7 monkeys immunized with SIV/17E-Fr and challenged with SIV/DeltaB670 (Fig. 7), only three monkeys showed no significant changes in T cell subsets in response to either immunization or challenge. These results are in agreement with the virological assessment of infection. Taken together, these data further demonstrate a correlation of the induction of env-specific, MHC class I-restricted CTL in the LP with protection from disease progression following colonic challenge.

Discussion

In this report we describe the atraumatic colonic inoculation of 11 rhesus macaques with limiting doses of two molecular infectious clones (SIVmac239 and SIV/17E-Fr). These studies were performed so that the virus-specific immune responses induced by mucosal infection and the role that these responses play in protection could be determined. Evaluation of immunity in exposed animals was effected by functional analysis of lymphocytes purified from the peripheral blood, mesenteric lymph nodes, and jejunal epithelium and lamina propria. A summary of these findings is outlined in Table IV.

As expected, the utilization of limiting virus dilutions resulted in the failure to infect 100% of the animals, with three monkeys becoming persistently infected and two monkeys showing no signs of infection. Three monkeys (animals N255, M029, and L870) were apparently transiently infected because SIV was sporadically detected in the PBMC in a manner similar to that previously observed by others (7, 9). The definition of transient infection was further expanded by the results obtained in three other monkeys (animals M259, M577, and N041) who, even though virus was never detected in the periphery, had measurable CTL responses in either PBMC or the gut-associated tissues.

Transient infection of the mucosal tissues induced SIV-specific responses that remained localized to the jejunal lamina propria. A persistent level of SIV-specific IgA-secreting B cells was detected in the lamina propria of one animal (monkey N255) and a strong CTL response against viral env determinants in three others (monkeys L870, M577, and N041). A CTL response to both gag and env determinants was also detected in the lamina propria of one persistently infected monkey (animal N385). The incorporation of allogeneic-mismatched targets into the assay further demonstrated that the CTL activity observed in the lamina propria was MHC class I restricted. Although CTL responses have been identified in both the intestinal (4) and vaginal (3) mucosa of chronically infected monkeys, this is the first report to our knowledge of SIV-specific mucosal immunity induced by transient infection. This finding may be analogous to HIV-exposed humans who remain seronegative despite repeated sexual exposure (1, 2).

Colonic challenge with 100% infectious dose of SIV/DeltaB670 resulted in infection and disease in all four naive controls and all of the exposed animals (7 of 11 monkeys tested) that had little or no detectable CTL in the lamina propria before or following infection. Conversely, none of the monkeys (4 of 11 animals tested) that had strong, env-specific CTL in the lamina propria became persistently infected with the challenge virus or developed disease. In three of the protected animals, no other detectable SIV-specific immune responses were identified in either the periphery or the gut-associated tissues. Taken together, these data provide compelling evidence for the requirement of virus-specific MHC class I CTL in mucosal protection.

Although most of the CTL responses observed in the lamina propria in protected animals was MHC class I restricted, a portion of this activity did not require MHC class I recognition. Since this activity was identified only in this tissue, it is possible that $CD8^+$ effectors

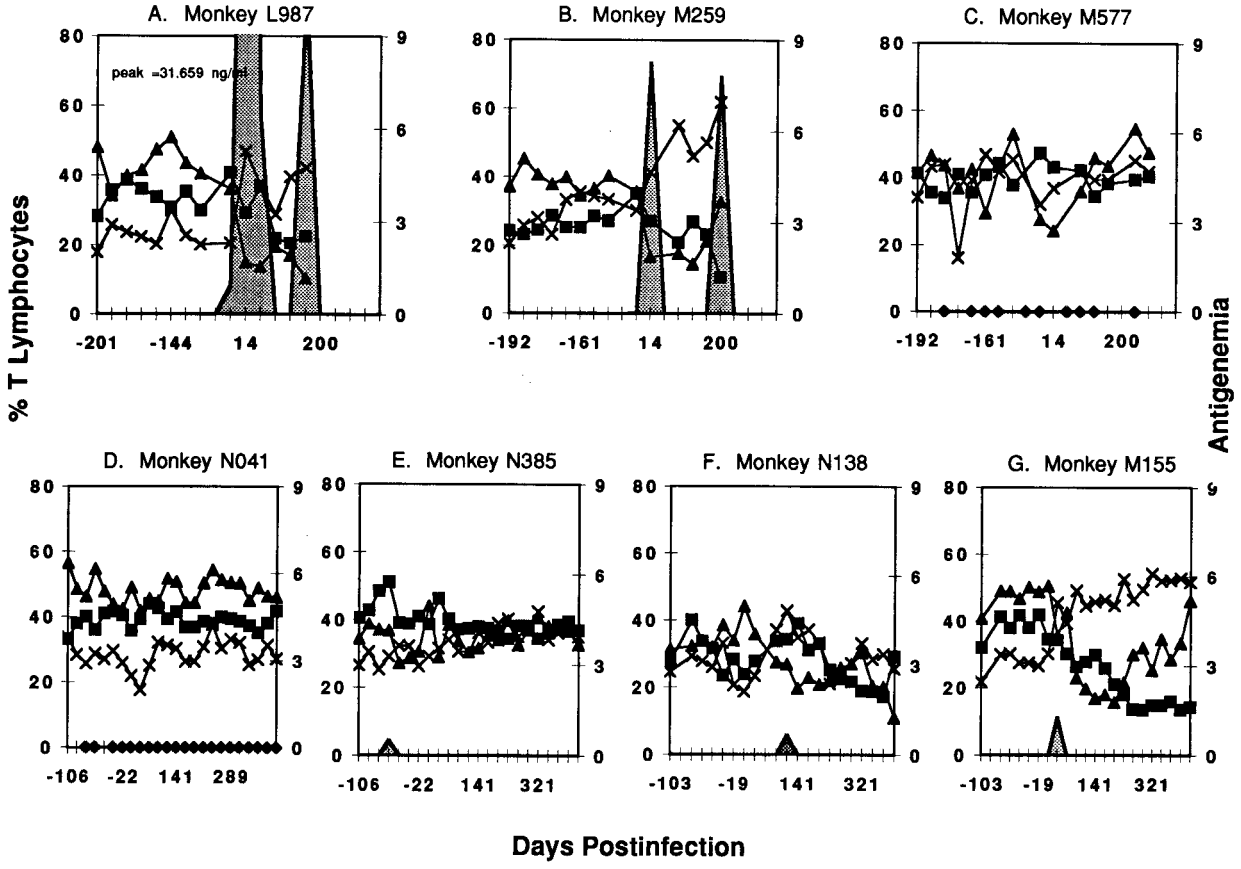

FIGURE 7. T cell subset changes and SIV p27 antigenemia in monkeys colonically exposed to SIV/17E-Fr following intracolonic challenge with SIV/DeltaB670. Monkeys L987, M259, and M577 were challenged 6 mo after exposure to SIV/17E-Fr. Monkeys N041, N385, N138, and M155 were challenged 3 mo after exposure to SIV/17E-Fr. Shaded areas indicate ng/ml SIV p26 in serum. ♦, Negative antigenemia values. Percent T lymphocyte subsets over time postinfection are indicated as follows: ■, $CD4^+$; ×, $CD8^+$; ▲, $CD4^+CD29^+$.

with a $\gamma\delta$, rather than an $\alpha\beta$, TCR phenotype were responsible. Confirmation of this hypothesis will require a more detailed analysis of these populations using mAbs specific for each phenotype. This finding is noteworthy, however, in that, where this activity was detected before SIV exposure, its presence correlated with the subsequent induction of higher levels of MHC class I-restricted CTL after exposure. Whether this is a prerequisite for the induction of TCR $\alpha\beta$ effector cells required for protection is a topic for further investigation.

Table IV. *Summary of immune responses induced by colonic immunization and challenge outcome*

Monkey	Infection (immunizing virus)	Ab		CTL[a]		Infection (challenge virus)	Disease	Protected
		Serum	LP	PBMC	LP			
L821						Persistent	Rapid	No
L632		NA[b]				Persistent	Slow	No
M558						Persistent	Intermediate	No
M079						Persistent	Slow	No
L987	None	−	−	−	−	Persistent	Rapid	No
M155	None	−	NT[c]	−	−	Persistent	Slow	No
M224	Persistent	+	−	−	−	NT	Intermediate	No
N385	Persistent	+	NT	+	+	Transient	None	Yes
N138	Persistent	+	NT	−	−	Persistent	Slow	No
N255	Transient	−	+	−	−	Persistent	Rapid	No
M029	Transient	−	−	+	−	Persistent	Slow	No
L870	Transient	−	−	−	+	None	None	Yes
M259	Transient	−	−	+	−	Persistent	Rapid	No
M577	Transient	−	−	−	+	None	None	Yes
N041	Transient	−	NT	−	+	None	None	Yes

[a] More than 17 LU env-specific MHC Class I-restricted responses.
[b] NA, not applicable.
[c] NT, not tested.

The common denominator for mucosal protection observed in this study was CTL recognition of viral env determinants. The preferential induction of env-specific responses following colonic exposure to limiting virus doses is intriguing. Perhaps the preferential uptake of virus by dendritic cells in the mucosa, coupled with their superior Ag-presenting capabilities, permitted the induction of this response even though only small amounts of Ag were produced. Infection of these cells may not initially be productive, a condition that might promote the preferential expression of env proteins. This finding may be important because, unlike the systemic protection observed following infection with attenuated SIV (17, 28), protection was induced by low dose, transient expression of virus. Furthermore, a correlation with protection and a response to a single virus protein was observed. Taken together, these observations should rapidly facilitate the development and implementation of safer, noninfectious vaccine approaches such as that described in a recent report of mucosal protection in mice by induction of env-specific CTL following immunization with a synthetic multideterminant HIV gp160 peptide (29).

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