

Simian Immunodeficiency Virus-Specific CD8⁺ Lymphocyte Response in Acutely Infected Rhesus Monkeys

YASUHIRO YASUTOMI, KEITH A. REIMANN, CAROL I. LORD, MICHAEL D. MILLER,
AND NORMAN L. LETVIN*

*Harvard Medical School, New England Regional Primate Research Center,
Southborough, Massachusetts 01772-9102*

Received 8 September 1992/Accepted 14 December 1992

To assess the possible role of cytotoxic T lymphocytes (CTLs) in containing the spread of human immunodeficiency virus in acutely infected individuals, the temporal evolution of the virus-specific CD8⁺ lymphocyte response was defined in simian immunodeficiency virus of macaques (SIV_{mac})-infected rhesus monkeys. A brief period of SIV_{mac} plasma antigenemia was seen 9 to 16 days following intravenous infection with SIV_{mac}, ending as the absolute number of CD8⁺ peripheral blood lymphocytes (PBLs) increased. In a prospective assessment of the ability of CD8⁺ lymphocytes of these monkeys to suppress SIV_{mac} replication in autologous PBLs, inhibitory activity was detected as early as 4 days, with a more pronounced effect 12 to 16 days following infection. SIV_{mac} Gag- and Nef-specific CD8⁺ effector cell activities were demonstrable in PBLs of animals by 2 weeks following virus inoculation. In fact, SIV_{mac}-specific CTL precursors were documented in the PBLs of rhesus monkeys 4 to 6 days after SIV_{mac} infection. These studies indicate that AIDS virus-specific CD8⁺ CTLs are present in PBLs within days of infection and may play an important role in containing the early spread of virus.

Since human immunodeficiency virus (HIV) transmission is likely to occur frequently with cell-associated virus, antiviral antibody may not play an important role in containing the spread of the virus. Cytotoxic T lymphocytes (CTLs) have been shown to block the early dissemination of virus in a number of viral infections (18, 20, 30). The contribution of virus-specific CTLs to the containment of a chronic HIV infection is currently being explored (3, 6, 19, 17, 24, 25). It will be important to gain an understanding of the virus-specific CD8⁺ cell response early following HIV infection to define the natural history of AIDS. Moreover, the definition of the HIV-specific CTL response in the setting of an acute infection will be critical for developing therapeutic and prophylactic strategies to enhance the protective immune response (7).

A definition of the emergence of an HIV-specific CTL response following acute infection cannot be readily accomplished with humans because infected individuals do not come to medical attention until a number of weeks following infection. Studies of this response can, however, be conducted by employing an animal model for AIDS. Simian immunodeficiency virus of macaques (SIV_{mac}) has extensive sequence homology to HIV and a similar tropism for CD4⁺ lymphocytes and macrophages (2, 5, 21, 27). Importantly, this virus induces an AIDS-like disease in macaque monkeys (10). The SIV_{mac}-macaque model, therefore, provides a powerful system for exploring the immunopathogenesis of AIDS (8). We sought to clarify the role played by CTLs in acute AIDS virus infection by defining the temporal evolution of virus-specific CD8⁺ cell responses in SIV_{mac}-infected monkeys.

Viral antigenemia and peripheral blood lymphocyte (PBL) phenotypic changes in rhesus monkeys were assessed for a 3-week period following SIV_{mac} infection. Three monkeys received three animal infectious doses of the 251 isolate of

SIV_{mac} by intravenous inoculation. The concentration of SIV_{mac} p27 in plasma was determined every 3 to 4 days after virus inoculation (SIV Core Antigen Assay; Coulter Corp., Hialeah, Fla.). Antigenemia was detected on days 9, 12, and 16 for animals Mm 246-91 and Mm 253-90 and only on day 112 for Mm 250-91. Absolute circulating CD4⁺ and CD8⁺ lymphocyte counts were assessed by monoclonal antibody staining and flow cytometric analysis. Relative proportions of CD4⁺ and CD8⁺ cells remained constant until serum antigenemia was observed (Fig. 1A). After this point, there was an inversion of the PBL CD4/CD8 ratio. We have observed this pattern of CD4/CD8 inversion coincident with SIV antigenemia for other acutely infected animals (data not shown). Interestingly, the change in the CD4/CD8 ratio was due primarily to an increase in circulating CD8⁺ PBLs (Fig. 1B).

Since the increase in circulating CD8⁺ lymphocytes correlated with the disappearance of SIV_{mac} antigenemia, it was of interest to determine whether these cells play a role in suppressing viremia in the monkeys. CD8⁺ lymphocytes of SIV_{mac}- and HIV-infected individuals can suppress AIDS virus replication in autologous PBLs (4, 23, 26). We determined the time course for the generation of this virus-inhibitory response in PBLs from the three prospectively studied monkeys (Table 1). PBLs were obtained from the animals 2, 4, 6, 9, 12, and 16 days following infection. Lymphocytes were cultivated *in vitro* with 5 μg of concanavalin A per ml for 1 day. An aliquot of these activated cells from each animal was then depleted of CD8⁺ lymphocytes by using an anti-CD8 monoclonal antibody and immunomagnetic beads. The percent CD4⁺ cells in both the unfractionated and the CD8⁺ lymphocyte-depleted cell populations of each monkey were determined immunophenotypically by flow cytometric analysis. On the basis of these analyses, unfractionated and CD8⁺ lymphocyte-depleted PBLs were placed in culture at cell concentrations established to yield 10⁶ CD4⁺ cells per well in 24-well plates in

* Corresponding author.

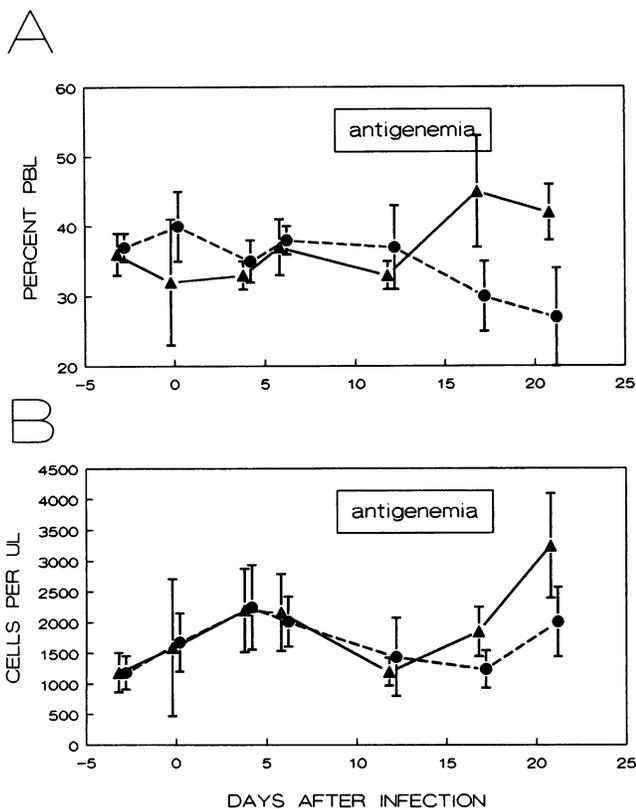


FIG. 1. Change in immunophenotype of PBLs during acute infection of monkeys with SIV_{mac}. PBLs were immunophenotyped for CD4 (●) (OKT4-fluorescein isothiocyanate; Ortho Diagnostic Systems, Raritan, N.J.) and CD8 (▲) (T8-fluorescein isothiocyanate; Dako, Inc., Carpinteria, Calif.), as determined by flow cytometry using a whole-blood lysis technique. The period of time during which these animals exhibited plasma antigenemia is indicated. (A) Relative percentage; (B) absolute number of cells per microliter.

medium supplemented with 20 U of recombinant human interleukin-2 (IL-2) (Hoffmann-La Roche, Nutley, N.J.) per ml. Supernatants were assessed every 2 to 3 days for SIV_{mac} p27 antigen (Coulter Corp.) as a measure of virus replication.

Peak SIV_{mac} replication was observed with both the unfractionated and the CD8⁺ lymphocyte-depleted PBLs obtained from these monkeys between days 6 and 12 following virus inoculation. Interestingly, the ability of the CD8⁺ cells to inhibit SIV_{mac} replication was detectable as early as

day 4 following inoculation. This effect became much more pronounced 12 to 16 days following infection, a time point coincident with both the inversion of the normal PBL CD4/CD8 ratio and the increase in absolute numbers of circulating CD8⁺ lymphocytes. Since the CD8⁺ lymphocytes which suppress AIDS virus replication have phenotypic characteristics of CTLs (23), these observations suggested that CTLs may be demonstrable in rhesus monkeys quite early following SIV_{mac} infection.

The time course for the generation SIV_{mac}-specific CTLs in rhesus monkeys was then determined. Five monkeys received the 251 isolate of SIV_{mac} intravenously, 0.3 to 3 animal infectious doses of virus. The quantity of virus in the inoculum did not correlate with any of the parameters subsequently assessed for the animals. All five monkeys became transiently antigenemic between 7 and 14 days following virus inoculation (data not shown). Moreover, while no monkey had serum antibodies reactive with SIV_{mac} by 2 weeks, all monkeys had seroconverted by 4 weeks following virus inoculation (data not shown).

These five monkeys, as well as two uninfected control animals, were monitored prospectively for the development of SIV_{mac}-specific CTLs. This was done by assessing the ability of their concanavalin A-stimulated, IL-2-expanded PBLs to lyse autologous B-lymphoblastoid cell lines infected with recombinant vaccinia viruses expressing SIV_{mac} Gag, Env, or Nef (Fig. 2). This assay is employed routinely to detect the presence of SIV_{mac}-specific CTLs in PBLs (15). These concanavalin A-stimulated, IL-2-expanded cells were also assessed for their ability to lyse the natural killer cell-sensitive cell line K562. No appreciable lysis of these target cells by effector cells from the animals was demonstrable prior to the inoculation of virus. Even lysis of Env-expressing autologous target cells by PBLs of these animals, found for many uninfected rhesus monkeys (29), was less than 15% prior to the inoculation of the animals with SIV_{mac}. Significant lysis of Env-expressing targets by the effector cells from these monkeys was seen by 1 week following infection. Significant killing of Gag-expressing target cells by PBLs of one monkey and Nef-specific killing by PBLs of another monkey were seen by 2 weeks following infection. By 4 weeks following the infection, Nef-specific killing by the PBLs of three monkeys, Gag-specific killing by the PBLs of four monkeys, and Env-specific killing by the PBLs of all these animals were seen. PBLs of the uninfected animals never demonstrated lysis of SIV_{mac} protein-expressing autologous target cells.

The observation that virus-specific cytolytic function was demonstrable as early as 1 week following infection suggested that SIV_{mac}-specific CTL precursors may expand to

TABLE 1. Time course for the generation of CD8⁺ SIV_{mac}-inhibitory response following virus inoculation^a

Animal	Day after infection ^b :				
	4	6	9	12	16
Mm 246-91	7.4/4.2 (1.8)	8.5/4.1 (2.1)	7.2/2.0 (3.6)	26.5/1.9 (13.9)	5.2/0.4 (13.0)
Mm 250-91	0.9/0.6 (1.5)	21.1/7.5 (2.8)	11.4/4.2 (2.7)	23.5/6.4 (3.7)	7.9/0.05 (158.0)
Mm 253-90	40.1/ND	23.9/6.4 (3.7)	42.2/21.4 (2.0)	13.4/5.7 (2.4)	15.6/0.9 (17.3)

^a Rhesus monkeys were inoculated intravenously with three animal infectious doses of SIV_{mac} 251. PBLs were obtained on the indicated days following infection, lectin stimulated, and cultured in IL-2-supplemented medium as unfractionated cells or following CD8⁺ lymphocyte depletion. The unfractionated lymphocytes were composed of 45% ± 10% CD4⁺ and 45% ± 10% CD8⁺ lymphocytes. Following CD8⁺ lymphocyte depletion, these populations consisted of <1% CD8⁺ and >85% CD4⁺ lymphocytes.

^b Results are given as peak antigen concentrations (ng/ml) in CD8⁺ cell-depleted culture supernatants/peak antigen concentrations (ng/ml) in unfractionated PBL culture supernatants. The ratios are given in parentheses. ND, not determined. All results were negative on day 2.

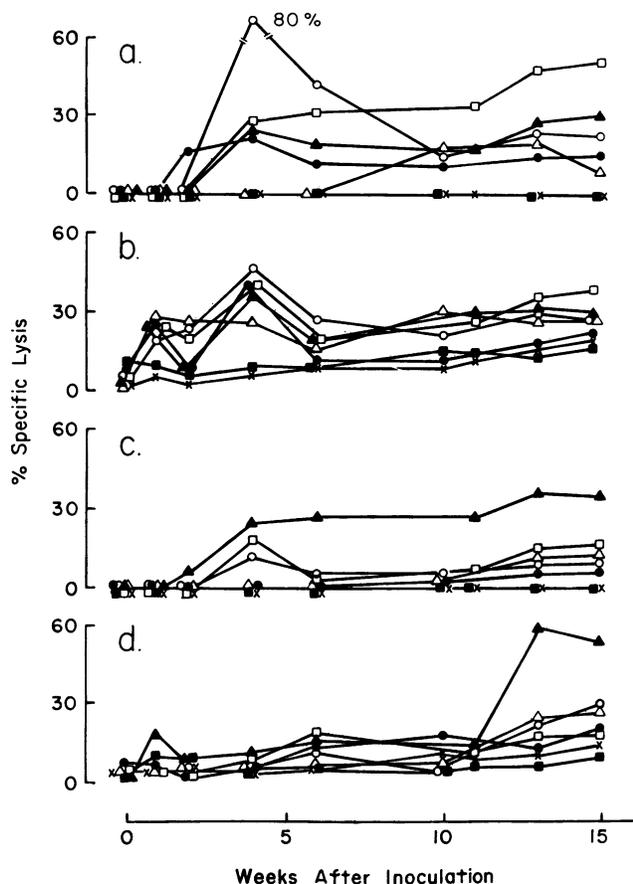


FIG. 2. Generation of SIV_{mac}-specific effector T lymphocytes in five virus-infected rhesus monkeys. PBLs of five SIV_{mac}-infected (164-88 [○], 210-88 [●], 385-89 [△], 247-90 [▲], and 239-89 [□]) and two uninfected (81-87 [■] and 253-90 [×]) rhesus monkeys were assessed for their capacities to lyse Gag- (a), Env- (b), and Nef- (c) expressing autologous target cells as well as K562 cells (d). Rhesus monkey B-lymphoblastoid cell lines immortalized with herpesvirus papio served as target cells (15). These B-lymphoblastoid cell lines were infected with recombinant vaccinia viruses carrying the SIV_{mac} gag, env, and nef genes and a control (equine herpesvirus 1 gH) gene (15). K562 cell lysis was assessed as a reflection of natural killer cell activity. Effector cells were Ficoll-diatrizoate-isolated PBLs obtained prior to and following SIV_{mac} infection of the monkeys. PBLs were cultured for 3 days at 10⁶/ml with concanavalin A (5 μg/ml) (Sigma, St. Louis, Mo.), washed, and then maintained for another 3 days in medium supplemented with human recombinant IL-2 (20 U/ml) (provided by Hoffmann-La Roche). ⁵¹Cr-labeled target cells were incubated for 5 h with effector cells at an effector/target ratio of 100:1. Spontaneous release varied from 10 to 20%. Specific release was calculated as [(experimental release - spontaneous release)/(100% release - spontaneous release)] × 100. The values shown for percent specific lysis represent the lysis of target cells infected with a recombinant vaccinia virus expressing one of the three SIV_{mac} genes minus lysis of target cells infected with a recombinant vaccinia virus expressing the equine herpesvirus 1 gH gene. The control target cell lysis never exceeded 5%.

detectable levels quite early in the course of the virus infection. We sought to determine the time following SIV_{mac} infection at which virus-specific CTL precursors are present in PBLs of rhesus monkeys. To address this question, we made use of our previous observation that SIV_{mac} Gag recognition is limited to a single peptide fragment of the Gag

TABLE 2. Detection of precursors of SIV_{mac} Gag peptide-specific effector cells in PBLs of SIV_{mac}-infected rhesus monkeys^a

Day	Gag peptide		% Specific lysis ^b										
	Stimulation ^c	Target ^d	Mm 238-85				Mm 467-91 ^e						
			20:1	10:1	5:1	3:1	20:1	10:1	5:1	3:1			
0	6	6	0	0	0	0	0	0	0	0	0	0	0
		11	1	0	1	0	0	1	0	0			
	11	6	0	0	0	0	1	2	0	0			
		11	3	0	0	0	2	0	1	0			
2	6	6	0	0	0	0	0	1	0	0			
		11	0	1	0	0	1	1	0	1			
	11	6	5	1	1	0	1	0	0	0			
		11	2	1	1	1	0	0	0	0			
4	6	6	1	0	0	0	1	0	1	0			
		11	4	2	0	0	3	0	0	0			
	11	6	6	2	1	0	3	0	1	0			
		11	29	20	15	8	3	1	0	0			
6	6	6	1	1	0	0	6	5	4	1			
		11	0	0	0	0	4	3	1	1			
	11	6	4	1	1	0	11	8	6	3			
		11	18	5	6	1	69	66	58	55			

^a PBLs were obtained from a Mamu-A*01-positive rhesus monkey before and at 2, 4, and 6 days after SIV_{mac} infection.

^b Target cells were labeled with ⁵¹Cr and incubated for 5 h with effector cells at the indicated effector/target ratios.

^c Effector cells were generated from density gradient-isolated PBLs. Cells were cultured for 3 days at 2.5 × 10⁶/ml with SIV_{mac} Gag peptide 11 (VPGFQUALSEGCTYPYDINQMLNCVGD) or peptide 6 (WCHI-HAEEKVKHTEAKQIVORHLVV) (5 μg/ml). IL-2 (400 U/ml) was added to the culture 3 days later. Peptide-pulsed autologous PBLs were added as feeder and stimulator cells on day 6 of culture. Cytolytic assays were performed on day 11 of culture.

^d Target cells were autologous B-lymphoblastoid cell lines incubated with peptide 6 or 11 at a final concentration of 50 μg/ml for 16 h at 37°C.

^e Monkey Mm 467-91 was serum antigenemic from day 8 through day 16 following infection. Its absolute CD4⁺ lymphocyte count fell from 1,490 at the time of virus inoculation to a low of 402 on day 8 following infection. The absolute CD8⁺ lymphocyte count fell during the same time interval from 969 to 163 and rose during the ensuing period of antigenemia to 888 by day 19. Corresponding values for Mm 238-85 were not determined.

protein (residues 182 to 190, contained in Gag peptide 11) in those SIV_{mac}-infected rhesus monkeys that express the major histocompatibility complex class I gene product Mamu-A*01 (16). Two rhesus monkeys positive for Mamu-A*01 were assessed prior to and every 2 days following inoculation with 10 animal infectious doses of SIV_{mac} for SIV_{mac} Gag peptide 11-specific CTL precursors in their PBLs. PBLs were cultured in IL-2-supplemented medium with mitomycin-treated autologous PBLs which had been pulsed with peptide 11 or an irrelevant SIV_{mac} Gag peptide (peptide 6). After 11 days in culture, PBLs were assessed for peptide-specific killing activity (Table 2). Peptide stimulation did not expand peptide 11-specific killing activity by PBLs from these monkeys before or 2 days following SIV_{mac} inoculation. However, by 4 days after virus inoculation in one monkey and 6 days after virus inoculation in the other monkey, peptide 11-stimulated PBL lysis of peptide 11-pulsed autologous target cells was readily demonstrable. Therefore, virus-specific CTL precursors are present in the PBLs of these rhesus monkeys as early as 4 to 6 days following virus infection.

We have, therefore, demonstrated that SIV_{mac}-specific CTLs are present in PBLs of rhesus monkeys well before a

virus-specific antibody response can be detected. This response emerges at the time of peak early SIV_{mac} replication in PBLs, and its expansion appears to coincide with the damping of this burst of viral replication. This temporal sequence of events suggests that CTLs may play a role in the early containment of SIV_{mac} replication in rhesus monkeys. However, this temporal relationship does not prove that CTLs block early spread of SIV_{mac} in rhesus monkeys.

In mice, the ability of CTLs to contain the early spread of some viruses in the absence of humoral immunity has been shown by assessing virus replication in animals depleted of CD4⁺ lymphocytes through monoclonal antibody infusions (1, 13). Cloned virus-specific CTLs have also been demonstrated to inhibit virus replication *in vivo* in mice (14, 22). We have been unable to employ these approaches in studying SIV_{mac}-specific CTLs in rhesus monkeys. We have previously shown that monoclonal antibodies can clear circulating T-lymphocyte populations from the peripheral blood for only a few hours in monkeys (12). These antibodies also do not eliminate these cells from lymph nodes or spleens (9, 11). Moreover, although we have shown that we can clone SIV_{mac}-specific CTLs from PBLs of virus-infected monkeys (28), we have not been successful in expanding any of these cellular clones to numbers large enough to employ in adoptive-transfer experiments. Therefore, we cannot definitely prove a role for CTLs in blocking the spread of the AIDS virus.

Nevertheless, in the present study we showed that SIV_{mac}-specific CTL precursors are present in PBLs of rhesus monkeys within a few days of infection and their increase in number is temporally associated with a decrease in virus replication. The expansion of a preexisting AIDS virus-specific CTL precursor pool through vaccination may significantly enhance the capacity of CTLs to contain virus replication during the first days following infection.

We thank S. Kotlikoff for the preparation of the manuscript.

This work was funded by National Institutes of Health grants AI20729, CA50139, AI26507, DK43351, RR00055, and RR00168.

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