Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8638–8642, November 1988
Immunology

Group-specific, major histocompatibility complex class I-restricted cytotoxic responses to human immunodeficiency virus 1 (HIV-1) envelope proteins by cloned peripheral blood T cells from an HIV-1-infected individual

(acquired immunodeficiency syndrome/cytotoxic T cells/vaccinia virus expression vector)

SCOTT KOENIG*†, PATRICIA EARL‡, DOUGLAS POWELL*, GIUSEPPE PANTALEO*§¶, SILVIA MERLI‡¶, BERNARD MOSS‡, AND ANTHONY S. FAUCI*

Laboratories of *Immunoregulation and ‡Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Bernard Moss, August 23, 1988

ABSTRACT **Freshly separated unfractionated peripheral blood mononuclear cells (PBMC) and cloned cell lines from a healthy human immunodeficiency virus 1 (HIV-1)-seropositive individual were examined for cytotoxic responses to HIV proteins expressed by recombinant vaccinia viruses. It was found that freshly isolated PBMC recognize variant envelope proteins of HIV-1 but not a more distantly related envelope protein derived from the simian immunodeficiency virus (SIV_{mac}). Although the effector cells were predominantly $CD8^+$, both MHC-matched and -unmatched target cells were lysed. Cytotoxic T lymphocyte (CTL) clones were found to lyse cells expressing HIV-1 envelope or reverse transcriptase. In contrast to the cytotoxic response detected with PBMC, the cloned CTLs were major histocompatibility complex (MHC) class I restricted. Our finding that a cloned CTL line lysed cells expressing highly divergent HIV envelopes strongly suggested that a conserved epitope was recognized. Identification of these shared epitopes may assist in designing a vaccine for HIV-1 that could stimulate MHC-restricted cytotoxic responses.**

Individuals infected with human immunodeficiency virus (HIV) may develop several types of immunological responses, which may potentially limit the spread of infection. These responses usually include antibodies to a number of viral components as well as neutralizing activity against HIV, yet there is no convincing evidence that the presence of such neutralizing activity can protect infected individuals from developing the acquired immunodeficiency syndrome (AIDS) (1–3). It is believed that the generation of cytotoxic cells may be required to confer protective immunity against some viruses (4, 5). A number of different cell types with cytotoxic function may participate in surveillance against infection. Major histocompatibility complex (MHC)-restricted cytotoxic cells of both the $CD8^+$ and $CD4^+$ cell lineages have been detected in individuals after infection with a number of DNA and RNA viruses (6–15). In one well-characterized retroviral animal model, MHC-restricted cytotoxic T lymphocytes (CTL) appeared to be required to confer protection against the leukemic complications of murine leukemia virus (16). Cells with so called "natural killer" (NK) cell activity, implying an ability to execute cytolysis in the absence of MHC restriction, may be important for protection against HIV as well. Multiple cellular phenotypes and lineages, including those that express CD16, Leu 19, or CD3, with either an α/β or γ/δ T-cell antigen receptor rearrangement may mediate such NK cell activity (17–20). Cells which bear receptors for immunoglobulin may execute their cytolytic activity through either an antibody-independent or an antibody-dependent mechanism.

The scope of the real and potential cytotoxic effector responses against HIV-infected target cells has not been fully delineated. In this regard, there have been some recent reports describing cytolytic responses against target cells expressing HIV-specific proteins in cells obtained from HIV-seropositive individuals. One group has detected cytolytic activity in peripheral blood mononuclear cells (PBMC) against MHC class I or class II matched target cells expressing HIV-1 envelope proteins (21). Also, they have found MHC class I-restricted responses against targets expressing the *pol* gene product, reverse transcriptase (22). Others have detected cytolytic activity against envelope proteins mediated by MHC-unrestricted $CD16^+$ cells (23). In addition, it has been reported that $CD8^+$ cells derived from the broncho-alveolar lavages of HIV-seropositive individuals with interstitial pneumonitis are cytolytic for autologous macrophages infected with HIV and are class I restricted (24). These studies suggest that different cell types may mediate cytotoxic responses against HIV *in vivo*. In these studies, cytotoxic responses detected in the majority of patients were directed at HIV envelope proteins derived from a single isolate. Since there is marked diversity among isolates in envelope protein sequences, it is probable that these cytotoxic effector mechanisms were recognizing conserved amino acid sequences within the envelope region. This contrasts with observations that antibody- and cell-mediated proliferative responses generated in animals immunized with envelope proteins of HIV are directed against a portion of the envelope contained within a hypervariable region and are specific for the HIV isolate from which they were derived (25–27).

In an effort to characterize the nature of the cell-mediated cytotoxic response to the HIV envelope protein, we have examined freshly separated unfractionated and cloned cell lines from a healthy HIV-1-seropositive individual for cytotoxic responses to variant HIV envelope proteins. We have found that freshly isolated PBMC recognize in an MHC-nonrestricted manner divergent envelope proteins of HIV-1 but not a more distantly related envelope protein derived

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte(s); PBMC, peripheral blood mononuclear cells; LCL, lymphoblastoid cell line(s); E:T ratio, effector-to-target cell ratio.

†To whom reprint requests should be addressed at: National Institutes of Health, Building 10, Room 11B-13, Bethesda, MD 20892.
§Permanent address: Istituto Pathologia Medica, Bari, Italy.
¶Present address: Eniricerche, Milan, Italy.

from the simian immunodeficiency virus (SIV). In addition, we have demonstrated this same phenomenon at the clonal level. However, in contrast to the cytotoxic response detected with PBMC, the cloned CTL were MHC class I restricted. These observations suggest that patients infected with HIV may develop a group-specific CTL response, and identifying these shared epitopes may assist in designing a vaccine for HIV-1 that could stimulate MHC-restricted cytotoxic responses.

MATERIALS AND METHODS

Cell Populations. Unfractionated PBMC, obtained from a healthy HIV-1-seropositive homosexual man, were separated over Hypaque/Ficoll, resuspended in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD) with 10% fetal calf serum, and assayed on the day of separation. HIV-specific cytotoxic cell lines were cloned by the method of Moretta (28) by limiting dilution from PBMC of the HIV-1-seropositive patient, using 50,000 irradiated [4000 roentgens (R); 1 R = 0.258 mC/kg] feeder cells derived from a HIV-seronegative patient, phytohemagglutinin (0.25 μg/ml; Burroughs–Wellcome, Research Triangle, NC), and purified interleukin 2 (Electro-Nucleonics, Rockville, MD). Individual clones were screened for cytotoxicity against autologous cells infected with recombinant vaccinia virus expressing control or HIV-1 proteins. Clones with cytotoxic activity against target cell lines expressing one of the HIV-1 proteins, but not against the targets expressing the control protein, were expanded, subcloned, and retested against infected target cell lines expressing HIV-1 envelope protein or reverse transcriptase. Lymphoblastoid cell lines (LCL) were generated from the PBMC of the patient (autologous) and from MHC HLA class I- and II-matched or mismatched donors by transformation with culture supernatant derived from marmoset cells infected with the B95-8 strain of Epstein–Barr virus and were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. SUPT1, a cell line expressing high levels of CD4, was cocultured with LCL target cells infected with HIV-1 envelope-expressing recombinant vaccinia virus to monitor envelope fusion activity (syncitium formation).

Cytotoxicity Microassay. A 4-hr ^{51}Cr release cytotoxicity microassay was performed as described (21). LCL cells 2×10^6 were infected with recombinant vaccinia virus (10 plaque-forming units per cell) for 10–14 hr, labeled with 250 μCi (1 Ci = 37 GBq) of sodium [^{51}Cr]chromate (Amersham), washed twice, and then resuspended to the appropriate concentration for use as target cells. Multiple effector-to-target cell (E:T) ratios were examined in triplicate or quadruplicate. Percent specific cell lysis was determined by the formula [(mean experimental release − mean spontaneous release)/(mean total release − mean spontaneous release)] × 100. Experiments in which the mean spontaneous release exceeded 25% were discarded.

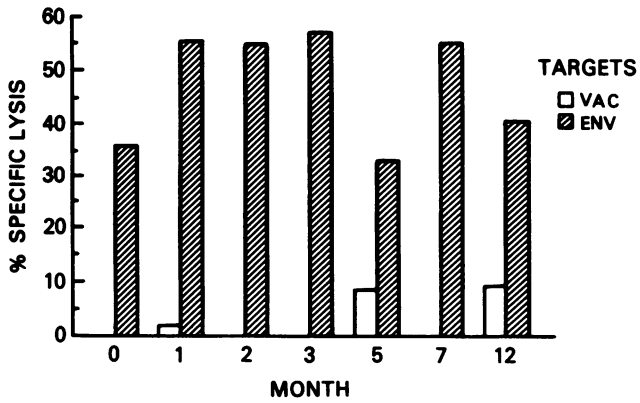

FIG. 1. Cytotoxicity directed against target cells expressing HIV-1_{IIIB} envelope proteins (ENV) by fresh unfractionated PBMC from a HIV-1-seropositive healthy homosexual man over a 1-year period. VAC, control target cells infected with vaccinia virus VSC-8. E:T ratios of 100:1, 25:1 (not shown), and 6:1 (not shown) were tested for each target cell line in quadruplicate during the month indicated (abscissa) after initial testing.

Recombinant Vaccinia Viruses. Viruses used for infecting the target cells included the following: (*i*) the control VSC-8, expressing the *Escherichia coli lacZ* gene product (21); (*ii*) VPE-7, expressing the envelope open reading frame of HIV-1_{IIIB}; (*iii*) VSC-40, expressing the *gag* gene product precursor p55 (21); (*iv*) RF, expressing the envelope open reading frames of HIV-1_{RF}; (*v*) WR-194, expressing the open reading frames of the envelope region of SIV isolate 251, derived from a macaque monkey (SIV_{mac}); (*vi*) VCF-21, expressing the p65 protein, reverse transcriptase, from the *pol* gene of HIV-1_{IIIB} (22). In each construct, the HIV gene was regulated by the vaccinia virus p7.5K promoter and was inserted into the thymidine kinase gene of vaccinia virus strain WR.

RESULTS

Blood samples were obtained from a healthy 34-year-old homosexual man, first noted to be seropositive for HIV-1 by ELISA and Western blotting in January 1985. He has been followed since that time as a participant in a prospective study of healthy HIV-seropositive individuals. Laboratory evaluation has shown a stable level of $CD4^+$ cells in his

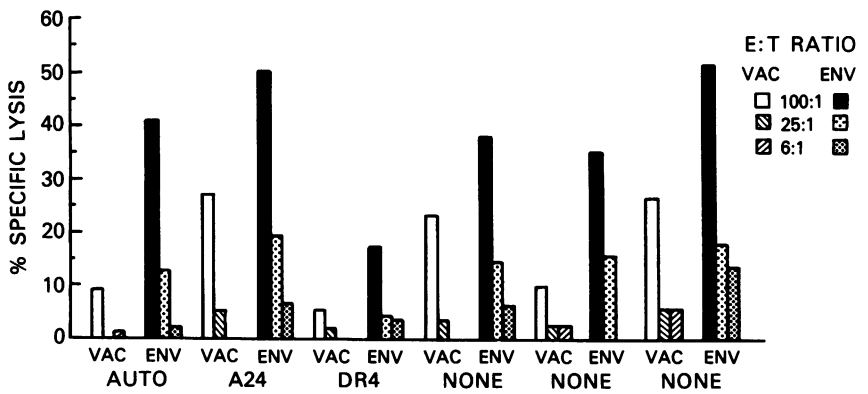

FIG. 2. Lysis of MHC-mismatched target cells expressing HIV-1 envelope proteins by fresh unfractionated PBMC. LCL cells generated from the PBMC of donors typed for HLA class I (A, B, C) and class II (Dr, DQ) were infected with either VSC-8 (VAC control) or VPE-7 (ENV). HLA antigens common to the target and effector cells are indicated on the abscissa. AUTO designates the autologous LCL generated from the patient. NONE indicates that no HLA antigens were common to target and effector cells.

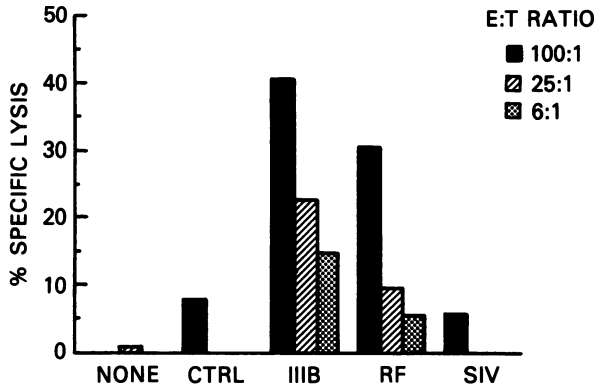

FIG. 3. Cytotoxicity of autologous LCL target cells expressing different envelope proteins by fresh unfractionated PBMC. Target cells were infected with recombinant vaccinia virus expressing the envelope proteins from the IIIB isolate (VPE-7), the RF isolate, or SIV_{mac}. Target cells infected with VSC-8 were used as a control. Expression of envelope protein by target cells was monitored by a syncytium assay as described in *Materials and Methods*, except uninfected H-9 cells were cocultured with LCL expressing the envelope from SIV_{mac}.

peripheral blood (392–443 cells per mm^3). PBMC examined for HIV-specific cytotoxicity against $HIV\text{-}1_{IIIB}$ envelope protein has demonstrated persistently significant and stable activity over a period of observation of 1 year (Fig. 1). Comparable assay of cytotoxic activity against gag proteins has shown intermittent and lower responsiveness (data not shown).

To further characterize this cytotoxic response, freshly separated PBMC were tested against target cells which expressed MHC proteins that were matched and mismatched at class I and II (Fig. 2). Significant cytotoxic responses could be detected even in the absence of MHC restriction. The lytic activity against the nonautologous lines expressing envelope proteins was always greater than that seen with the controls, although the extent of lysis of control cells from different individuals varied considerably. This high activity against envelope protein-expressing targets was not the result of differences in the spontaneous release of chromium from the envelope-expressing target cells, which appeared to be comparable to the control vaccinia-expressing target cells. Cytolytic activity against envelope-expressing targets was totally eliminated by treatment of PBMC with antibody recognizing CD3 and complement. Partial to complete abrogation of the envelope-specific cytotoxic response also was seen after treatment of the cells with anti-CD8 plus complement, but no diminution of the cytotoxic response was noted when anti-CD4 was used.

Next, we tested the PBMC for cytotoxic activity against other envelope proteins derived from $HIV\text{-}1_{RF}$ and SIV_{mac} to determine if the cytotoxic cells could recognize more than one form of viral envelope. As shown in Fig. 3, comparable cytotoxic activity could be detected against $HIV\text{-}1_{IIIB}$ and $HIV\text{-}1_{RF}$ envelope proteins, which share about 79% identity in amino acid sequences (29). However, no activity was seen against target cells expressing the envelope protein of SIV_{mac}, which is distantly related to HIV-1, sharing only 34% identity with the envelope sequence of $HIV\text{-}1_{IIIB}$ (29). Examination of PBMC from six other individuals demonstrated similar activity against $HIV\text{-}1_{IIIB}$ and $HIV\text{-}1_{RF}$ envelope proteins, while only one of six showed cytotoxic activity against the envelope of SIV_{mac} (data not shown). While these observations demonstrate that cytotoxicity of populations of PBMC against HIV-1 may be group specific, the possibility exists that individual cells or different cell types might mediate cytotoxicity against the two different envelope proteins independently, and thus give the appearance of a group-specific response. This response might occur particularly if individuals are infected with multiple strains of HIV-1.

To resolve the issue of group antigenic specificity, we cloned CTL from this patient by the technique of limiting dilution as previously described (28). The precursor frequency of cytotoxic T cells directed against HIV-1 proteins was 1/1000 in the PBMC. Four of five cytotoxic cell lines generated manifested specificity for the $HIV\text{-}1_{IIIB}$ envelope-expressing targets, while the other was specific for the *pol* gene product, reverse transcriptase (Fig. 4). Immunohistochemical staining revealed that these lines were $CD8^+$ $CD4^-$ (data not shown). To further analyze their specificity, we examined the ability of one of these clones, RR-1B10, to lyse targets expressing variant envelope proteins. Consistent with the unfractionated PBMC, RR-1B10 could lyse targets expressing envelope protein derived from either IIIB or RF strains but not the one expressing SIV_{mac} envelope (Fig. 5).

When clone RR-1B10 was evaluated for MHC restriction, it was capable of lysing target cells expressing the MHC class I B14 allelic product but not other class I, class II, or mismatched targets (Fig. 6). Thus, this MHC restriction contrasted with the MHC-unrestricted activity seen in the uncloned population.

DISCUSSION

These observations offer an approach to identifying conserved epitopes of HIV that may be critical in developing a vaccine that could stimulate a CTL response to protect

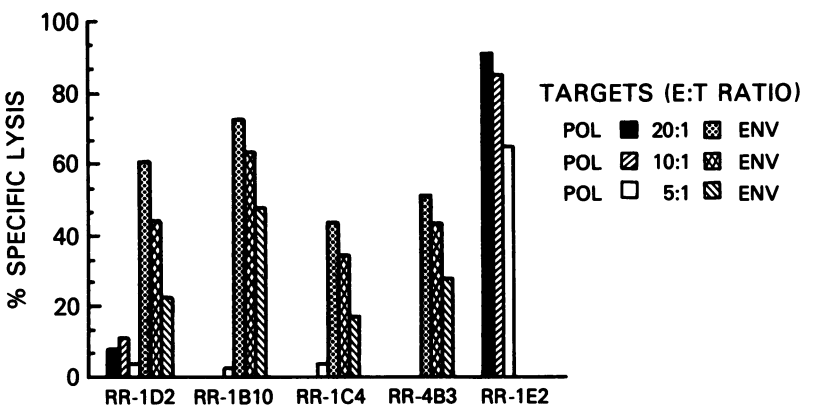

FIG. 4. Cytotoxicity of target cells expressing envelope protein (ENV) or reverse transcriptase (POL) by cloned cytotoxic cell lines. Clones were tested against the infected target cell lines at the indicated E:T ratios in triplicate. Results from five clones with consistent cytotoxic activity against target cells expressing HIV-1 proteins are shown.

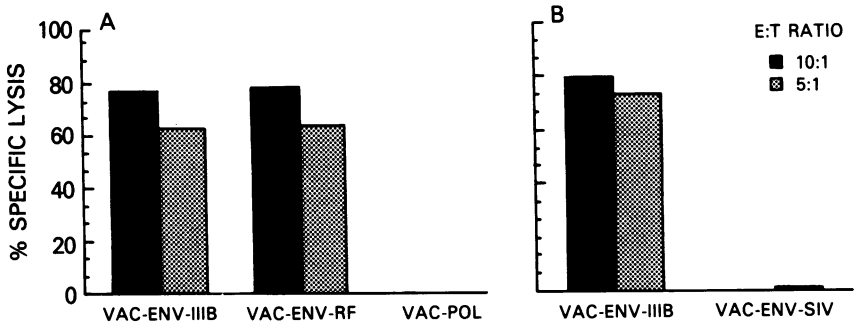

FIG. 5. Cytotoxicity of target cells expressing different envelope proteins by a cloned cell line. Cytotoxicity of clone RR-1B10 was tested against autologous target cells expressing IIIB or RF envelope or IIIB reversing transcriptase (POL) proteins (*A*) or the envelope protein from IIIB or SIV_{mac} (*B*) in triplicate.

against the range of HIV isolates to which a seronegative individual in a high risk population may be exposed. Recent animal studies indicated that MHC class I-restricted cytolytic responses in mice were generated against an amino acid sequence in the second hypervariable envelope domain and that cross-reactivity against the IIIB and RF isolates could not be achieved (27). While the bulk population of murine lymphocytes generated in those animals was type specific, a minor population which was not detectable may have been capable of responding to other more conserved epitopes. Alternatively, the immunogenic and immunodominant domains recognized by mice or other animals may not coincide with the epitopes recognized by humans. Ranki *et al.* (30) have reported that cells from seropositive patients proliferate in response to different peptides generated from the envelope sequences than do cells from animals immunized with HIV-1. It would seem critical, therefore, to map the epitopes stimulating cytotoxic responses by using cells derived from human subjects who have been naturally infected with HIV as well as individuals immunized with candidate vaccines. Since the envelope-specific cell-mediated response against naturally occurring infections appears to be at least in part MHC unrestricted (Fig. 2) (23), and in this patient, T-cell mediated, it will be necessary to address these studies at the clonal level. We were unable to evaluate our other three envelope-specific clones from this patient for MHC restriction due to their limited ability to propagate *in vitro*, but we suspect that in addition to the MHC-restricted clone shown (Fig. 6), other T cell clones generated from this patient may be MHC unrestricted.

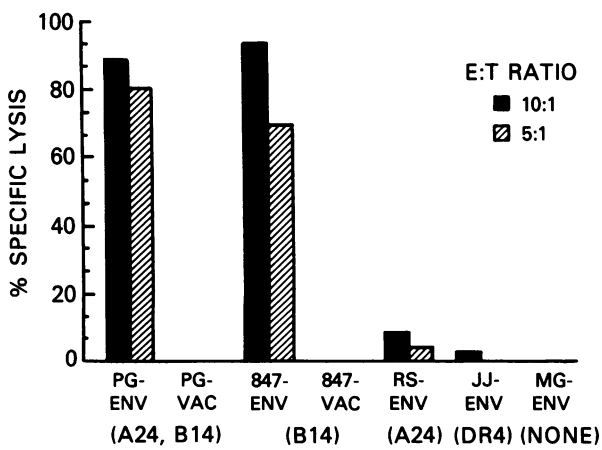

FIG. 6. MHC restriction of an envelope-specific cytotoxic clone. Cytotoxicity of clone RR-1B10 for LCL target cell lines from different donors matched with the HLA type of the effector cells and infected with the recombinant vaccinia virus expressing the IIIB envelope (ENV) or the control *lacZ* gene product (VAC) was measured. Target cells matched only at A24, or DR4, or mismatched (NONE) were three of the LCL used for the experiments in Fig. 2.

While the technical approach used here favors the selection of group-specific CTL by virtue of the initial screening for cytotoxic clones against one envelope protein derived from a single isolate, to which the patient very likely was not exposed, other approaches may be needed to assess type-specific immune responses. The group specificity shown here for clone RR-1B10 appears to be limited to an envelope epitope common to HIV_{IIIB} and $HIV\text{-}1_{RF}$ but not SIV_{mac}, although other epitopes may be present that are common to all three strains and could stimulate CTL responses. Since it appears that the frequency of group-specific clonable cells is relatively high (1 in 1000) in this and other patients (data not shown), the method outlined should be sufficient to characterize regions of HIV envelope and probably other proteins that could stimulate HIV-specific CTL responses. The ability to respond to a particular epitope is dependent on the MHC protein with which it associates, and therefore it will be necessary to analyze the MHC-restricted HIV-specific cytotoxic responses of subjects displaying a broad range of HLA alleles. We have been successful in cloning envelope-specific CTL from several other individuals representative of HIV-seropositive patients of different clinical stages and HLA types. The next step is to subclone and characterize them with regard to MHC restriction and group specificity.

We are grateful to C. Flexner and P. Johnson for providing their recombinant vaccinia viruses VCF-21 and WR-194, respectively, and M. Rust for editorial assistance. S.M. was supported through the generosity of Eniricerche, Milan, Italy.

1. Robert-Guroff, M., Brown, M. & Gallo, R. C. (1986) *Nature (London)* **316**, 72–74.
2. Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, A. G., Carne, C. A., Weller, I. V. D. & Tedder, R. S. (1985) *Nature (London)* **316**, 69–72.
3. Weiss, R. A., Clapham, P. R., Weber, J. N., Dalgleish, A. G., Lasky, L. A. & Berman, P. W. (1986) *Nature (London)* **324**, 572–575.
4. Zinkernagel, R. M., Welsh, R. M. & Caron, M. G. (1976) *J. Immunol.* **117**, 1495–1502.
5. Zinkernagel, R. M. & Doherty, P. C. (1979) *Adv. Immunol.* **27**, 51–177.
6. Starr, S. E. & Allison, A. C. (1977) *Infect. Immun.* **17**, 458–462.
7. Oakes, J. E. (1975) *Infect. Immun.* **12**, 166–172.
8. Lukacher, A. D., Braciale, V. L. & Braciale, T. J. (1984) *J. Exp. Med.* **160**, 814–826.
9. Byrne, J. A. & Oldstone, M. B. A. (1984) *J. Virol.* **51**, 682–686.
10. Rook, A. H., Quinnan, G. V., Jr., Frederick, W. J. R., Manischewitz, J. F., Kirmani, N., Dantzler, T., Lee, B. B. & Curier, C. B. (1984) *Am. J. Med.* **76**, 385–392.
11. Chesebro, B. & Wehrly, K. (1976) *J. Exp. Med.* **143**, 85–99.
12. Blank, K. J. & Lilly, F. (1977) *Nature (London)* **269**, 808–810.
13. Jacobson, S & Biddeson, W. E. (1986) *Concepts in Viral*

Pathogenesis (Springer, New York), Vol. 2, pp. 187–194.
14. Mitsuya, H., Matis, L. A., Megson, M., Bunn, P. A., Muray, C., Mann, D. L., Gallo, R. C. & Broder, S. (1983) *J. Exp. Med.* **158,** 994–999.
15. Kannagi, M., Sugamura, K., Kinoshita, K.-I., Uchino, H. & Hinuma, Y. (1984) *J. Immunol.* **133,** 1037–1041.
16. Earl, P., Moss, B., Morrison, R. P., Wehrly, K., Nishio, J. & Chesebro, B. (1986) *Science* **234,** 728–731.
17. Borst, J., van de Griend, R. J., van Oostveen, J. W., Ang, S.-L., Melief, C. J., Seidman, J. G. & Bolhuis, L. H. (1987) *Nature (London)* **325,** 683–688.
18. Ferrini, S., Bottino, C., Biassoni, R., Poggi, A., Sekaly, R. P., Moretta, L. & Moretta, A. (1987) *J. Exp. Med.* **166,** 277–282.
19. Hoffman, R. W., Bluestone, J. A., Leo, O. & Shaw, S. (1985) *J. Immunol.* **135,** 5–8.
20. Siliciano, R. F., Pratt, J. C., Schmidt, R. E., Ritz, J. & Reinherz, E. L. (1985) *Nature (London)* **317,** 428–430.
21. Walker, B. D., Chakrabarti, S., Moss, B., Paradis, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S. & Schooley, R. T. (1987) *Nature (London)* **328,** 345–348.
22. Walker, B. D., Flexner, C., Paradis, T. J., Fuller, T. C., Hirsch, M. S., Schooley, R. T. & Moss, B. (1988) *Science* **240,** 64–65.
23. Weinhold, K. J., Lyerly, H. K., Matthews, T. J., Tyler, D. S., Ahearne, P. M., Stine, K. C., Langlois, A. J., Durack, D. T. & Bolognesi, D. P. (1988) *Lancet* **i,** 902–905.
24. Plata, F., Autran, B., Martins, L. P., Wain-Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J.-M. & Debre, P. (1987) *Nature (London)* **328,** 348–351.
25. Palker, T. J., Clark, M. E., Langlois, A. J., Matthews, T. J., Weinhold, K. J., Randall, R. R., Bolognesi, D. P. & Haynes, B. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1932–1936.
26. Rusche, J. R., Javaherian, K., McDanal, C., Petro, J., Lynn, D. L., Grimaila, R., Langlois, A., Gallo, R. C., Arthur, L. O., Fischinger, P. J., Bolognesi, D. P., Putney, S. D. & Matthews, T. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3198–3202.
27. Takahashi, H., Cohen, J., Hosmalin, A., Cease, K. B., Houghten, R., Cornette, J. L., DeLishi, C., Moss, B., Germain, R. N. & Berzofsky, J. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3105–3109.
28. Moretta, A. (1985) *Eur. J. Immunol.* **15,** 148–155.
29. Myers, G., Rabson, A. B., Josephs, S. F., Smith, T. F. & Wong-Staal, F. (1988) *Human Retroviruses and AIDS* (Los Alamos Natl. Lab., Los Alamos, NM).
30. Ranki, A., Mattinen, S., Anpnen, J., Ashon, P. & Krohn, K., in *Fourth International Conference on AIDS* (Stockholm Intl. Fairs, Stockholm), Book 1, p. 191 (abstr.).