

**Prospective study of cytotoxic T lymphocyte responses to influenza and antibodies to human T lymphotropic virus-III in homosexual men. Selective loss of an influenza-specific, human leukocyte antigen-restricted cytotoxic T lymphocyte response in human T lymphotropic virus-III positive individuals with symptoms of acquired immunodeficiency syndrome and in a patient with acquired immunodeficiency syndrome.**

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*J Clin Invest.* 1985;76(4):1699-1704. <https://doi.org/10.1172/JCI112158>.

**Research Article**

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## Prospective Study of Cytotoxic T Lymphocyte Responses to Influenza and Antibodies to Human T Lymphotropic Virus-III in Homosexual Men

### Selective Loss of an Influenza-specific, Human Leukocyte Antigen-restricted Cytotoxic T Lymphocyte Response in Human T Lymphotropic Virus-III Positive Individuals with Symptoms of Acquired Immunodeficiency Syndrome and in a Patient with Acquired Immunodeficiency Syndrome

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#### Abstract

Peripheral blood leukocytes (PBL) from 18 homosexual men who did not have acquired immunodeficiency syndrome (AIDS) and from 9 heterosexual men were repetitively tested for their ability to generate HLA self-restricted cytotoxic T lymphocyte responses to influenza virus (flu-self) over a 2-yr period. The sera of the same donors were tested for antibodies to human T lymphotropic virus-III (HTLV-III). Six of the homosexual and none of the heterosexual donors consistently generated weak cytotoxic T lymphocyte responses to flu-self. Seven of the homosexual and none of the heterosexual donors were seropositive for antibodies to HTLV-III. No obvious correlation was detected between weak flu-self cytotoxic T lymphocyte responses and antibodies to HTLV-III. However, one homosexual donor generated no detectable cytotoxic T lymphocyte activity to flu-self, although he was a strong responder to HLA-alloantigens. This donor had an OKT4:OKT8 ratio of 0.4 and was seropositive for HTLV-III antigens; HTLV-III virus was identified in his PBL; and he developed AIDS during the course of this study. A second donor with lymphadenopathy and who was seropositive for HTLV-III antigens exhibited marginal cytotoxic T lymphocyte activity to flu-self which he subsequently lost. PBL from two patients, one with Kaposi's sarcoma and one with generalized lymphadenopathy, were also tested for cytotoxic T lymphocyte responses to flu-self and to alloantigens. Both donors failed to generate cytotoxic T lymphocyte to flu-self, but generated strong cytotoxic T lymphocyte responses to alloantigens. The selective loss of an HLA-restricted cytotoxic T lymphocyte response without loss of HLA alloantigenic cytotoxic T lymphocyte activity may be an important functional immunologic characteristic in the development of AIDS.

#### Introduction

The immunologic disturbances that are associated with acquired immunodeficiency syndrome (AIDS)<sup>1</sup> are complex and varied,

*Received for publication 15 May 1985 and in revised form 8 July 1985.*

1. *Abbreviations used in this paper:* AIDS, acquired immunodeficiency syndrome; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked im-

and not all components of the immune system are similarly affected. For example, the syndrome is characterized by T cell deficiencies (1-6) as well as B cell hyperplasia (6, 7). Although the helper:suppressor T cell ratio is reversed, this reversal in AIDS resides mainly in a real reduction in the helper cell compartment (8) and within a particular helper cell subpopulation (9). To better understand the immunologic deficiencies that lead to and result from AIDS, it is important to address functional aspects of the AIDS syndrome not only in patients, but also in prospective studies of donors who are at risk for developing AIDS.

In an earlier study (10), we reported that 25-30% of homosexual men from the Washington, DC area who did not have AIDS exhibited reduced cytotoxic T lymphocyte (CTL) responses to influenza virus recognized in association with self HLA antigens (flu-self). Since the initiation of this prospective study, the likely etiologic agent for AIDS has been identified as a retrovirus of the human T lymphotropic virus (HTLV) family (11-15). Furthermore, tests have been developed for detecting antibodies to HTLV-III (14, 15), and HTLV-III can be isolated by culture from donors' lymphocytes (12, 13). As a follow-up of a previous report (10), we present a more intensive study of repetitive anti-flu-self CTL tests performed on several donors over a 2-yr period; we have investigated whether there is a correlation of decreased anti-flu-self CTL with the presence of antibody to HTLV-III. One donor developed AIDS 10 mo. after joining the study. He was seropositive for antibodies to HTLV-III, and HTLV-III virus was detected by electron microscopy. This donor as well as two donors with lymphadenopathy and one patient with AIDS presented an unusual pattern of potent CTL responsiveness to HLA alloantigens and of unresponsiveness to flu-self which may represent a T cell functional pattern that could be characteristic for onset of the syndrome.

#### Methods

*Generation of and assay for in vitro CTL responses.* Heparinized peripheral blood was separated on Ficoll-Hypaque and established in tissue culture for detection of retrovirus or for immunological tests. CTL responses to influenza A/Bangkok/RX73 (H3N2) and HLA alloantigens were generated during 7-d culture in 24-well plates as described elsewhere

munosorbent assay; flu-self, influenza virus associated with self HLA antigens; HTLV, human T lymphotropic virus; PHA, phytohemagglutinin.

(10, 16). Influenza-immune CTL and alloimmune CTL were assayed on phytohemagglutinin (PHA)-stimulated autologous-infected or allogeneic targets, respectively, at four effector:target cell ratios as described previously (10, 16). Repetitive CTL assays were performed for each donor over a period of up to 2 yr and the mean values of percent lysis for all assays were calculated for each effector:target cell ratio. The CTL activity was then converted to lytic units by determining the reciprocal of the number of effectors per  $10^7$  cells required to give 20% lysis.

**Analysis of OKT antigen expression.** Peripheral blood leukocytes (PBL) were analyzed for surface expression of OKT3, OKT4, and OKT8 (generously provided by Dr. G. Goldstein, Ortho Pharmaceuticals, Raritan, NJ) by indirect immunofluorescence using flow microfluorometry as previously described (17).

**Isolation of HTLV-III from patient lymphocytes.** Fresh PBL were incubated in growth media (RPMI 1640, 20% fetal bovine serum, 0.29  $\mu$ g/ml glutamine) supplemented with 5  $\mu$ g/ml PHA-P for 48 h at 37°C. These cells were then grown in media containing 10% T cell growth factor. Cell cultures were monitored for release of retrovirus by testing for reverse transcriptase activity in supernatant fluid, by electron microscopic study, and by transmission of virus to fresh umbilical cord blood or to adult peripheral blood or bone marrow leukocytes (12, 13).

**Detection of antibody to HTLV-III in sera and plasma.** Sera were tested for the presence of antibody to HTLV-III structural proteins by ELISA and Western blot procedures as previously described (14, 15).

## Results

18 homosexual and 9 heterosexual donors, who were included in an earlier study (10), were repetitively tested for CTL responses to flu-self during a 2-yr period. The lytic unit values of CTL activity obtained from the means of these repetitive tests are summarized in Table I as well as the serological data for anti-

**Table I. Summary of Cytotoxic T Lymphocyte Responses to Influenza-infected Autologous Cells and Serum Antibody to HTLV-III in Non-AIDS Homosexual and Heterosexual Donors**

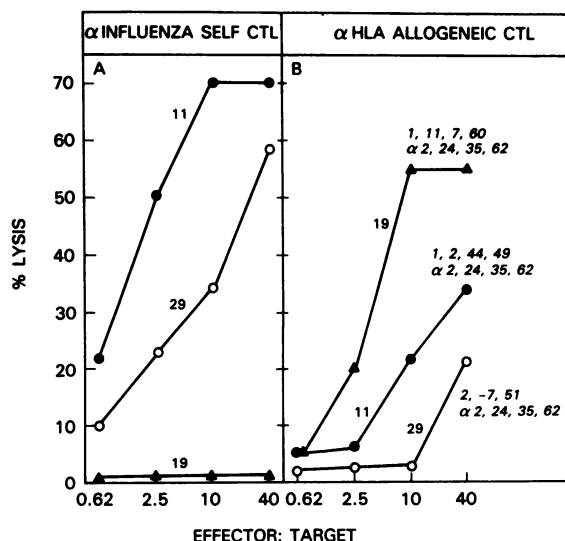
Homosexual donors			Heterosexual donors		
Donor number	Lytic units*	Antibody to HTLV-III†	Donor number	Lytic units*	Antibody to HTLV-III†
8	1,400	—	25	670	—
5	1,400	—	28	330	—
4	670	—	30	330	—
15	400	—	34	300	—
16	330	—	26	250	—
11	330	+	27	200	—
7	200	—	41	100	—
18	111	+	22	50	—
12	76	+	29	50	—
14	67	—			
21	67	+			
17	50	—			
49	25	—			
1	10	+			
10	8	—			
2	8	—			
50	<1	+			
19	0	+			

\* Cytotoxic T lymphocyte activity is expressed in terms of lytic units per  $10^7$  effector cells for 20% lysis (see Methods).

† Serum antibodies to HTLV-III was determined by ELISA and Western blot techniques (14, 15).

bodies to HTLV-III. 6 of the homosexual donors exhibited anti-flu CTL activity that was 25 lytic units or more below that of the lowest heterosexual controls. 7 of the 18 homosexual donors were seropositive for antibodies to HTLV-III, whereas none of the heterosexual donors were antibody-positive. Three of the six weakest homosexual CTL responders and four of the intermediate-to-strong homosexual CTL responders were seropositive. It may be relevant that the two lowest homosexual anti-flu-self CTL responders, donors 19 and 50, who were also seropositive, exhibited the lowest OKT4:T8 ratios of any donors in this study (10), i.e., 0.4 for both donors, and both had a history of lymphadenopathy.

As a part of this investigation, one homosexual couple (donors 11 and 19 shown in Table I) that had maintained a nearly monogamous sexual relationship for 7 yr was studied in detail for a 12-mo. period. PBL from donor 11 consistently generated strong anti-flu-self CTL responses, whereas donor 19 was the only donor in this prospective study who failed to generate any CTL activity specific for flu-self (see Table I). However, donor 19 was seropositive for antibodies to influenza virus (data not shown), indicating that this donor had been exposed to this virus and that he was not a genetic nonresponder to influenza. In a comparative experiment, PBL from donors 11 and 19 and from donor 29 (a male heterosexual control) were sensitized in vitro to flu-self as well as to HLA alloantigens. The effector cells generated were assayed on the appropriate target cells 7 d later. As shown in Fig. 1 A, PBL from donor 19 did not generate any detectable CTL activity to flu-self, whereas PBL from both donors 11 and 29 generated strong anti-flu-self CTL activity. However, PBL from all three donors were able to generate a CTL response to HLA alloantigens (Fig. 1 B). Thus, in contrast to the absence of CTL activity to flu-self, donor 19 generated a potent CTL response to HLA alloantigens, which was, in fact, stronger than that of either donors 11 or 29. Similar results were obtained from a second test performed 2 mo. later (data not shown). However, subsequent bleeds and tests for CTL function against HLA alloantigens at 6 and 9 mo. in the study of this



**Figure 1.** Cytotoxic T lymphocyte responses of two homosexual donors (nos. 11 and 19) and one heterosexual donor (no. 29) to (A) influenza A virus-infected autologous leukocytes and (B) HLA alloantigens. The HLA-A and -B antigens expressed by the responder (above) and stimulator (below) cells are shown for the allogeneic response.

couple indicated a decline in CTL activity by PBL from donor 19 (Fig. 2). The allogeneic CTL responses of donor 11 and heterosexual control donors retained normal levels (data not shown).

At intervals during the study, PBL from donors 11 and 19 (as well as from heterosexual controls) were analyzed for the proportion of OKT3<sup>+</sup>, OKT4<sup>+</sup>, and OKT8<sup>+</sup> cells (Table II). PBL from donor 19 consistently exhibited a reduced number of OKT3<sup>+</sup> and OKT4<sup>+</sup> cells as well as a reduced T4:T8 ratio compared with heterosexual or other homosexual donors in this study (10). PBL from donor 11 exhibited OKT3<sup>+</sup>, OKT4<sup>+</sup>, and OKT8<sup>+</sup> cells in the normal range (10) throughout the period of this study. A further reduction in the T4:T8 ratio of donor 19 was observed at the tenth month of the study, at which time this donor was diagnosed with *Pneumocystis carinii* and AIDS.

A second donor in our prospective study who had a 3-yr history of unexplained lymphadenopathy also generated very weak CTL responses to flu-self (Table I, donor 50) and had a T4:T8 ratio of 0.4. A more detailed study of his CTL responses to flu-self and HLA alloantigens is summarized as a function of time (with controls) in Fig. 3. At the time he entered the study, PBL from this donor generated a weak response to flu-self and a normal response to HLA alloantigens. The normal range for CTL responses to alloantigens among male heterosexuals is from 10 to 40% at an effector:target ratio of 40:1 (10). At the sixth month of the study, donor 50 generated an allogeneic response comparable with the earlier test. (His anti-flu-self response was not tested at this time.) However, by the twelfth month, PBL from donor 50 generated almost no CTL activity to flu-self, but an elevated response to HLA alloantigens. PBL from this donor also generated strong CTL responses to PBL from at least four other allogeneic donors (data not shown).

The selective loss of a self + X CTL response in donors 19 and 50 raised the possibility that such a pattern of T lymphocyte function might be a functional characteristic of AIDS development. Therefore, we tested the ability of PBL from two patients to generate CTL to flu-self and to HLA alloantigens using PBL from HLA-mismatched donors. Donor 81 was a homosexual male diagnosed with AIDS who presented with Kaposi's sarcoma, a T4:T8 ratio of 0.38, and an absolute number of T4<sup>+</sup>

Table II. Analysis of T Cell Subsets of Donors 19 and 11 as a Function of Time Using OKT Monoclonal Reagents

Time of test (mo.)	Donor number tested	Percent of PBL that expressed OKT markers			T4:T8
		OKT3	OKT4	OKT8	
0	19	48	14	36	0.4
	11	72	41	32	1.3
2	19	43	12	29	0.4
	11	71	38	26	1.5
3	19	36	10	28	0.4
	11	65	36	23	1.6
10	19	63	11	61	0.18
12	19	73	1	71	0.01

Donor PBL were analyzed for surface expression of OKT3, OKT4, and OKT8 (Ortho Pharmaceuticals, Raritan, NJ) by indirect immunofluorescence using flow microfluorometry as previously described (17).

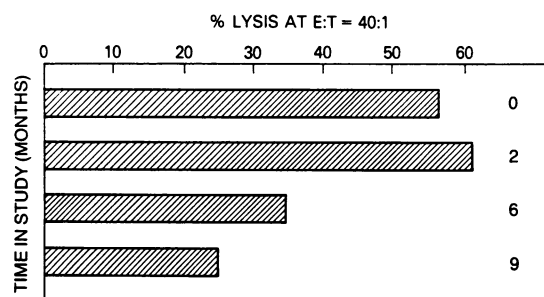


Figure 2. Decline of HLA allogeneic cytotoxic T lymphocyte potential of donor 19 as a function of time. Percent lysis is shown at effector:target cell ratio of 40:1 for tests performed at months 0, 2, 6, and 9 in the study. HLA-A, -B type of donor 19 was 1, 11, 7, 60; and of the allogeneic stimulators were (top to bottom): 2, 24, 35, 62; 2, 24, 44, -; 2, 24, 35, 57; 2, 24, 35, 62. Stimulator and target cells from the same donor were used in the experiments of months 0 and 9.

cells of 331/mm<sup>3</sup>. Donor 82 was a female with von Willebrand's disease; she was diagnosed with a generalized lymphadenopathy, and had a T4:T8 of 0.83 and an absolute number of T4<sup>+</sup> cells of 710/mm<sup>3</sup>. Donor 82 had received blood transfusions as well as Factor VIII concentrates. Although this donor was a patient, she did not meet Centers for Disease Control criteria for diagnosis of AIDS. Both donors were antibody-positive for HTLV-III. The CTL responses to flu-self and to HLA alloantigens are shown in Fig. 4 for these two patients as well as the CTL responses of

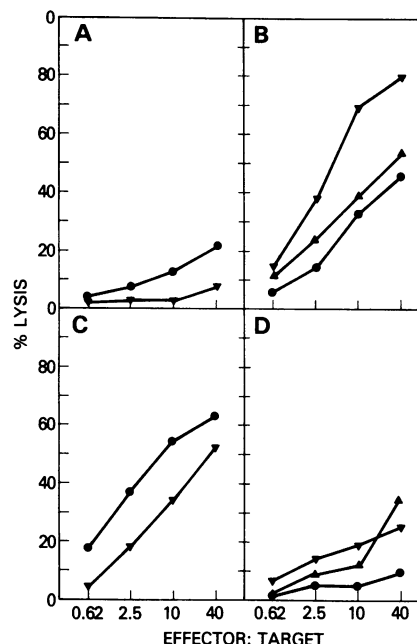


Figure 3. Cytotoxic T lymphocyte responses of homosexual donor 50 as a function of time in the prospective study. (●) indicates month 0; (▲) indicates month 6; (▼) indicates month 12 of the study. Anti-flu-self and allogeneic CTL responses for donor 50 are shown in (A) and (B), respectively. Control anti-flu-self and allogeneic CTL responses are shown in (C) and (D), respectively. The target cells used for anti-flu-self CTL were influenza-infected and uninfected autologous (control) T cell blasts. The target cells used for allogeneic CTL were T cell blasts from the allogeneic donors used for stimulation and autologous T cell blasts (control). In both cases the uninfected autologous targets were not lysed by the CTL (data not shown).

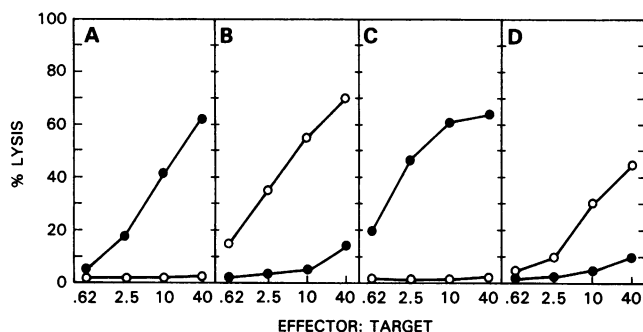


Figure 4. Cytotoxic T lymphocyte responses of donor 81 (a patient with Kaposi's sarcoma) (A, B) and of donor 82 (a patient with generalized lymphadenopathy) (C, D) to flu-self (A, C) and to HLA alloantigens (B, D). Donor 81 and 82 are indicated by (○); control donors 29 (A, B) and 87 (C, D) are indicated by (●). The same types of target were used as those described in Fig. 3.

sex-matched non-AIDS controls run in the same experiments. The data of Fig. 4 A illustrate that the AIDS patient donor 81 generated no CTL activity to flu-self, whereas control donor 29 generated a strong anti-flu-self CTL. In contrast, the results of Fig. 4 B indicate that donor 81 generated a strong allogeneic CTL response, even stronger than the control. Similarly, lymphadenopathy patient donor 82 generated no CTL response to flu-self, whereas the control donor generated a strong anti-flu-self CTL (Fig. 4 C). This AIDS patient also generated a stronger allogeneic CTL response than did the non-AIDS control (Fig. 4 D). As noted above, the weak allogeneic CTL responses of the controls are within the range of CTL activity to HLA alloantigens (Tung, K. S., F. Koster, D. C. Bernstein, P. Kriebel, S. M. Payne, and G. M. Shearer, manuscript submitted for publication).

During the course of this study serum samples from donors 11, 19, and 50 were collected and tested for antibody to HTLV-III. On the fourth and ninth months of the study, sera from both donors 11 and 19 were positive for antibody to HTLV-III by ELISA and Western blot assays (14, 15), whereas sera from more than 100 randomly selected donors were negative. Furthermore, donor 11 converted from seronegative to seropositive during the first 4 mo. of the study, whereas the serum from donor 19 was positive for HTLV-III antibody from the start of the study. Donor 50 was seropositive for HTLV-III at the time that he entered the study. At the sixth month of the study of this couple, PBL from donors 11, 19, and 29 were established in culture and monitored for the release of virus by reverse transcriptase (12, 13). As shown in Fig. 5 A, retrovirus (that was subsequently characterized as HTLV-III by electron microscopy) was produced by lymphocytes from donor 19, but not from donors 11 or 29. This virus was also demonstrated to be transmissible to fresh PHA-stimulated and cord lymphocytes (Fig. 5 B). Similarly, cultures of PBL from donor 50 generated elevated levels of reverse transcriptase activity (data not shown).

## Discussion

The results of the present study demonstrate the following: (a) The homosexual donors who were weak or strong CTL responders to flu-self retained their respective CTL profiles for up to 2 yr. (b) There is no apparent correlation between weak anti-flu-self CTL response status and the detection of antibody to HTLV-III in the sera of the donors. (c) Of the homosexual donors

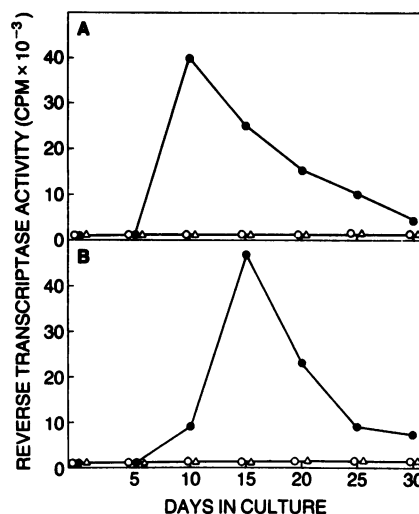


Figure 5. Magnesium-dependent reverse transcriptase activity in supernatant fluids from T lymphocytes grown in vitro from donors 11, 19, and 29. PBL were stimulated with PHA and grown in the presence of T cell growth factor. (A) Primary cultures of donor 11 (○), donor 19 (●), and donor 29 (Δ). (B) Cord blood lymphocytes treated with conditioned medium collected 12 d postincubation of cell cultures from donor 11 (○), donor 19 (●), and donor 29 (Δ).

studied, only one who later developed AIDS exhibited no CTL response to flu-self. (d) Despite his lack of CTL activity to flu-self, this donor exhibited an abnormally strong CTL response to HLA alloantigens. (e) One homosexual donor with a history of lymphadenopathy lost his flu-self CTL response, but retained strong allogeneic CTL activity. (f) One patient with AIDS and another patient with generalized lymphadenopathy also exhibited a total absence of CTL responsiveness to flu-self, but had strong CTL activity to HLA alloantigens.

The range of CTL responses to flu-self and to HLA alloantigens is wide in both heterosexual and homosexual donors. From our experience, the range for anti-flu-self CTL in heterosexual donors is 50–1,000 lytic units/ $10^7$  cells, and is <1–100 lytic units/ $10^7$  for allogeneic responses (Tung, K. S., F. Koster, D. C. Bernstein, P. Kriebel, S. M. Payne, and G. M. Shearer, manuscript submitted for publication). For HTLV-III-negative homosexual males, the range for anti-flu-self CTL is 10–1,400 lytic units/ $10^7$  cells, and is <1–200 lytic units/ $10^7$  cells for allogeneic CTL (Tung, K. S., F. Koster, D. C. Bernstein, P. Kriebel, S. M. Payne, and G. M. Shearer, manuscript submitted for publication). Except for donors 19 and 50, the ranges for HTLV-III-positive homosexual donors was indistinguishable from those of HTLV-III-negative homosexuals. The significant points of our study are that PBL from HTLV-III antibody-positive donors with other symptoms of AIDS generate no CTL activity to flu-self, but often generate elevated CTL responses to HLA alloantigens.

Of the six homosexual donors of Table I whose anti-flu-self CTL activity was at least 25 lytic units below the responses of the weakest heterosexual donors, three were seropositive and three were seronegative for antibody to HTLV-III. Furthermore, four of the seropositive donors generated intermediate-to-strong anti-flu-self CTL responses. The reduced but positive anti-flu-self CTL responses of some antibody-negative homosexual donors may be independent of AIDS, and may be a characteristic

of the immune potential of homosexual men that is not directly associated with HTLV-III infection. It is also possible that anti-flu-self CTL responses of the antibody-positive donors who currently exhibit normal or reduced but positive responses to flu-self will decline with time, and that these donors will develop other symptoms of AIDS.

The complete loss of anti-flu-self CTL activity observed in donors 11 and 50 (in contrast to the other donors shown in Table I) may indicate a CTL functional defect before AIDS diagnosis or in early AIDS cases. In contrast with the other donors of Table I, both donors 11 and 50 had T4:T8 of 0.4 or less, presented a history of persistent, generalized lymphadenopathy, and had detectable retrovirus activity in cultures of their PBL. Donor 11 developed AIDS, whereas donor 50 has continued to present with lymphadenopathy. Thus, the complete loss of, but not necessarily a reduction in, anti-flu-self CTL activity may be associated with AIDS development. This interpretation is supported by the data of two patients, donor 81 (Kaposi's sarcoma) and donor 82 (generalized lymphadenopathy), both of whom failed to generate anti-flu-self CTL, but generated strong allogeneic CTL.

The selective loss of a self + X CTL response observed thus far in one donor who developed AIDS in our prospective study, in another virus-positive donor with lymphadenopathy, and in two patients raises the possibility that the functional loss of CTL activity in the development of AIDS will first affect the CTL responses to antigens that are recognized in association with self HLA antigens (i.e., self + X responses). This appears to be paralleled by an increase in allogeneic CTL activity which may be reduced in the later stages of AIDS. The early selective loss of the flu-self CTL response without any detectable loss of HLA allogeneic CTL activity cannot be attributed to the CTL response to influenza virus being weaker than that to HLA alloantigens. In fact, in PBL obtained from healthy heterosexual donors, the anti-flu-self CTL is the more potent CTL response (W. E. Bidison and G. M. Shearer, unpublished observations) (see controls in Figs. 3 and 4).

The selective early loss of anti-flu-self CTL activity in the development of AIDS may have implications for the importance of human self + X responses in surveillance against certain types of infections and malignancies, as well as for possible differences in the repertoires of human self + X and allogeneic T lymphocyte populations. Since the OKT4<sup>+</sup> T cell population (18), and possibly the functional T4<sup>+</sup> subset of T4<sup>+</sup>, Leu 8<sup>+</sup> cells, appears to be the primary cytopathic target of HTLV-III (9), these findings raise the possibility that the self + X human CTL response is more dependent on this subset of helper cells than is the HLA allogeneic CTL response. It is of interest that Singer et al. (19, 20) have recently observed in a murine CTL model that self + X responses strictly require L3T4<sup>+</sup> helper cells (considered to be the murine equivalent of OKT4<sup>+</sup> cells), whereas allogeneic CTL responses can utilize either L3T4<sup>+</sup> helpers, or alternatively, Lyt2<sup>+</sup> helper cells (considered to be the murine equivalent of OKT8<sup>+</sup> cells). Similarly, it has been recently demonstrated that class II major histocompatibility-restricted graft-vs.-host induced immunodeficiency in mice abrogates self + X CTL responses without affecting allogeneic responses (21). These murine models that distinguish different pathways of helper T cell function for self + X vs. allogeneic CTL responses are consistent with the results of the present study. The absence of a flu-self CTL response reflects an HTLV-III-induced selective loss of a subpopulation of T helper cells that is involved in self + X CTL, and

suggests that the near-normal allogeneic CTL response of donor 19 and the elevated allogeneic responses of donors 50, 81, and 82 were mediated via different helper cell populations. Based on the murine model (19, 20), one might expect that these two human helper subsets would be of the T4<sup>+</sup> and T8<sup>+</sup> phenotypes, respectively. Recent reports suggest that the markers of the inducer T cell subset initially lost in AIDS (9) and the helper T cell for influenza virus-specific CTL (22) may be more complex than simply a T4<sup>+</sup> cell.

The observation that allogeneic T cell responses can remain intact or are elevated even after self + X T cell responses have been lost raises the possibility that allogeneic helper cell populations could be utilized in therapeutic protocols at early stages of AIDS to provide T cell help via alloantigen-induced interleukin-2 production for self + X restricted T cell and for B cell responses that may be important in surveillance against opportunistic infections associated with AIDS. However, there is another side to the AIDS paradox involving attempts to reconstitute immune functions. For example, it is possible that elevated T cell function will result in enhanced immune destruction of HTLV-III-infected lymphocytes, and thus would lead to the further decline of the immune system (23). Therefore, an elevated T cell response to alloantigens may indicate that helper factors are being generated that could promote immune-mediated destruction of virus-infected lymphocytes in HTLV-III-positive individuals.

## Acknowledgments

The authors express their gratitude to the doctors and nurses of the Clinical Oncology Program of the National Cancer Institute for providing blood and clinical information on donors 81 and 82; Dr. R. B. Wigton, Pulmonary Associates, Alexandria, VA, for providing clinical information on donor 19; Dr. H. Golding and Dr. A. Singer for discussion of the data; Dr. A. Singer and Dr. R. Yarchoan for reviewing the manuscript, and Ms. J. Kress for preparation of the manuscript.

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