Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8198–8202, December 1985
Medical Sciences

Influence of the human T-lymphotropic virus/lymphadenopathy-associated virus on functions of human lymphocytes: Evidence for immunosuppressive effects and polyclonal B-cell activation by banded viral preparations

(retroviruses/B and T lymphocytes/immunomodulation/acquired immunodeficiency syndrome)

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Contributed by Robert A. Good, July 3, 1985

ABSTRACT **The etiologic agent for the acquired immunodeficiency syndrome (AIDS) is now firmly established as the retrovirus termed the human T-lymphotropic virus type III (HTLV-III) or the lymphadenopathy-associated virus, LAV. The disease is characterized by profound and progressive loss of immunity, but molecular evidence indicates that only a few cells in peripheral blood are being productively infected with this virus. In the present study we have investigated a disrupted HTLV-III viral preparation for biologic effects on normal lymphoid cells. Relatively dilute concentrations of this preparation were found to stimulate immunoglobulin secretion by peripheral blood lymphocytes; at the same dosages, the preparation was inhibitory for the B-cell differentiation responses that are induced with other known polyclonal B-cell activators, pokeweed mitogen, *Staphylococcus aureus*, and Epstein–Barr virus. This preparation was also inhibitory at high concentrations for T-lymphocyte proliferative responses to phytomitogens and antigens and resulted in a reduced expression of Tac antigen on phytohemagglutinin-activated lymphocytes. Paradoxically, incubation of lymphocytes of certain healthy donors with the HTLV-III preparation alone resulted in increased expression of Tac and Leu-12 antigens. These findings show that a disrupted preparation of HTLV-III virus can mimic many of the immunologic abnormalities present in patients with HTLV-III infection. Nonviable viral proteins may be responsible for some of the immunologic perturbations that occur in HTLV-III-infected states.**

Acquired immunodeficiency syndrome (AIDS) is a disease that has appeared in epidemic proportions among certain populations in the United States and throughout the world (1–3). This immunodeficiency disease has recently been shown to be associated with infection with a lymphotropic retrovirus by both French and American scientists that has been dubbed human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV virus) (4–6). Because the virus infection is destructive or damaging to infected lymphocytes, difficulty was experienced at first in maintaining and producing virus for study. More recently, however, a cell line, H9, has been discovered that withstands infection well and permits production of large amounts of this virus (5). Study of the virus and its components has thus become possible. Patients infected with HTLV-III/LAV virus often exhibit progressive losses of the T lymphocytes from the blood, progressive decreases of numbers of the T4 lymphocyte subpopulations, and diminishing T-cell function. Evidence that the virus can infect cells other than the cells of T4 lineage has recently appeared; B lymphocytes (7) and even cells of the brain (8) have been found to be infected with this agent. However, a paradox has been that the best evidence available to date seems to indicate that only a few among many lymphoid cells in the peripheral blood or lymphoid organs can be shown by the most sensitive methods available to be productively infected with the virus (9). Thus, this agent, which produces profound and progressive loss of lymphoid functions (10) in many infected persons rendering them immunodeficient and susceptible to devastating opportunistic infections (3), does not seem to be infecting a high proportion of their lymphoid cells.

One possible resolution to this apparent paradox could be that the virus itself or components of the virus might be inhibitory or injurious to T lymphocytes and thus, by virtue of these toxic actions, lead to progressive inhibition of the functions of the lymphoid cells. Infection with HTLV-III/LAV virus appears in many persons to produce a polyclonal B-cell activation (11, 12), leading to accumulation of B cells and plasma cells in the lymphoid tissues, increases in circulating levels of each immunoglobulin isotype, and elevated serum levels of antibodies and autoantibodies (11). At the same time, patients with AIDS are unable to respond normally with antibody production to otherwise effective antigenic stimulation either *in vivo* or *in vitro* (11–14). This influence too could be attributable to direct influence of components of the virus.

To test this hypothesis, we carried out a series of experiments to ascertain whether or not the HTLV-III virus or any of its nonviable components can interfere with the functions of human lymphocytes *in vitro*. We have found that crude preparations of the banded virus exert rather dramatic inhibitory effects on a number of functional responses of both T and B lymphocytes. The virus preparation inhibits responsive T-cell proliferation to phytomitogens, to common antigens, and to alloantigens. Indeed, in line with clinical findings, preparations of the virus can even induce polyclonal B-cell activation at relatively low dilution and, at the same concentration, can inhibit responses of B cells to other known polyclonal B-cell activators. Thus, in these preliminary experiments, we show that nonviable preparations of the killed HTLV-III/LAV virus, and thus nonreplicating virus, appear to mimic influences on lymphocyte functions

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Abbreviations: ISC, immunoglobulin-secreting cells; ARC, AIDS-related complex; AIDS, acquired immunodeficiency syndrome; HTLV-III/LAV, human T-lymphotropic virus type III/lymphadenopathy-associated virus; PBL, peripheral blood lymphocytes; Con A, concanavalin A; PWM, pokeweed mitogen; PHA, phytohemagglutinin; NK, natural killer; EBV, Epstein–Barr virus; SAC, *Staphylococcus aureus* of Cowan strain.

encountered with the AIDS-associated HTLV-III/LAV virus infection in humans.

MATERIALS AND METHODS

HTLV-III Virus and Control Preparations. The virus was purified from supernatants of H9 cell cultures supporting the continuous production of HTLV-III (5). Concentrated culture fluids were purified through sucrose density gradients of 20–60% by weight in TNE buffer (10 mM Tris·HCl, pH 7.4/0.1 M NaCl/1 mM EDTA, pH 7.2). Virus bands were collected, treated with 0.5% Triton X-100 in 0.6 M NaCl, and homogenized. After centrifugation for 1 hr at 100,000 × g, the particulate-free material was collected and adjusted to a concentration of 1 mg of protein per ml. Supernatants of uninfected H9 cell cultures were similarly treated; the protein derived was used as "control" material in the same dilutions as the viral material.

Analyses of Lymphocyte Functions and Membrane Antigens. Peripheral blood lymphocytes (PBL) were isolated from venous samples of healthy volunteers on sodium metrizoate gradients (Lymphoprep; Nyegaard, Oslo). For all functional assays described below, various dilutions of the HTLV-III and control preparation were added directly to the cultures at the time of initiation of each cell culture.

Proliferative responses of lymphocytes to optimal concentrations of phytomitogens [phytohemagglutinin (PHA; 20 μg/ml) concanavalin A (Con A; 50 μg/ml), and pokeweed mitogen (PWM; 1:125 dilution)], to common antigens [tetanus (1:1000 dilution of stock with 630 limit flocculation units/ml) and candida (1:500 dilution of stock with 1025 limit flocculation units/ml)], and to alloantigens (mixed lymphocyte culture, MLC) were tested as described by Cunningham-Rundles *et al.* (14). B-lymphocyte differentiation into immunoglobulin-secreting cells (ISC) in response to stimulation with PWM (1:100 dilution), *Staphylococcus aureus* of Cowan Strain (SAC, 0.015%), and Epstein–Barr virus (EBV, 1:5 dilution of a supernatant of a B-95-8 marmoset infected cell line) was tested as described before (16, 17). Natural killer (NK) cell responses of lymphocytes for ^{51}Cr-labeled K562 cells were tested at effector/target ratios of 50:1 in 14-hr assays as described (18).

For membrane antigen analysis of lymphocytes, cells were stained with fluorescein isothiocyanate-conjugated monoclonal antibodies OKT4, OKT8, OKT11 (Ortho Diagnostic Systems), anti-IL-2 receptor, and anti-Leu-12 (Beckton Dickinson), and the percentage of positive cells was determined by flow cytometry (19) with an Epics C cell sorter (Coulter Electronics).

RESULTS

Viability Testing. The viral preparation was first evaluated to determine whether it or the control preparation was toxic to cells. It can be seen in Table 1 that after an incubation period of 24 hr, during which PBL were in direct contact with viral or control preparations, no toxicity was noted at dilutions of 1:30 or more. Cell cultures contained 98–100% viable cells after test periods up to 4 days at dilutions of 1:100 or greater of virus preparation. Experiments reported herein were performed with viral preparations at dilutions of 1:100 or greater.

Proliferative Responses of Lymphocytes to Phytomitogens, Antigens, or Allogeneic Cells. Summarized on Table 2 are observations that indicate that the preparation of the HTLV-III virus inhibited the proliferative responses of PBL to the phytomitogens PHA, Con A, and PWM. It will be seen in the table that the preparation of the virus used was not by itself mitogenic for human lymphocytes but inhibited proliferative responses to phytomitogens at dilutions of 1:100 and, to some extent, at a dilution of 1:300. Also in Table 2 are recorded data that pertain to influences of the virus preparations on proliferative responses to alloantigens and to the common tetanus and candida antigens, to which lymphocytes of normal persons regularly respond because of prior sensitization. The proliferative responses to these antigens were inhibited more effectively by the viral antigen preparation than the control material. No inhibition of these lymphoid functions was evident at dilutions of 1:500 of the viral preparation.

Table 1. Viability of cells incubated with HTLV-III or control preparation

Incubation period, hr	Preparation added	% viable cells with dilution of preparations*		
		1:30	1:100	1:300
24	HTLV-III	98	98	99
	Control	96	99	99
48	HTLV-III	86	98	99
	Control	77	98	99
96	HTLV-III	66	99	100
	Control	58	99	99

*Representative of four experiments.

Influence on B-Cell Differentiation into ISC and Antibody-Secreting Cells. In Fig. 1 *Upper* the influence of the preparation of HTLV-III on B-cell differentiation into ISC is shown. In these experiments addition of the virus preparations to cultures of PBL induced an impressive proportion of the lymphocytes to become ISC. This influence on the development of ISC was demonstrable even at dilutions of the virus preparations as high as 1:15,000. This activity of the virus preparations to induce ISC responses, although lower than PWM, was at least as impressive as the capacity of EBV, an accepted polyclonal B-cell activator at inducing immunoglobulin secretion by B lymphocytes.

In Fig. 1 *Lower* are recorded observations of experiments designed to assess the influence of the virus preparations on the ISC response induced by known polyclonal B-cell activators PWM, SAC, and EBV. The figure shows that the virus preparation inhibited the influence of all three of the polyclonal B-cell activators to induce ISC among PBL. The inhibition was rather pronounced at the dilution 1:1,500, which was the most effective concentration that stimulated immunoglobulin secretion when the viral preparation was

Table 2. Influence of HTLV-III or control preparation on lymphocyte proliferation

Stimulus	Preparation added	[^{14}C]Thymidine uptake with dilution of preparations,* cpm			
		0	1:100	1:300	1:500
Mitogens					
PHA	HTLV-III	22,583	7,215	16,607	22,501
	Control		16,258	22,953	24,542
Con A	HTLV-III	9,670	4,776	7,490	9,884
	Control		8,036	9,519	10,249
PWM	HTLV-III	2,829	516	987	2,749
	Control		2,287	2,981	2,869
Antigens					
Candida	HTLV-III	9,933	1,100	6,644	10,127
	Control		4,500	7,427	9,635
Tetanus	HTLV-III	9,936	688	5,272	9,557
	Control		3,965	6,642	9,199
Allogeneic cells	HTLV-III	16,996	2,322	12,536	16,231
	Control		5,362	13,310	15,920
No addition	HTLV-III	202	79	64	72
	Control		57	62	102

*Representative of eight experiments.

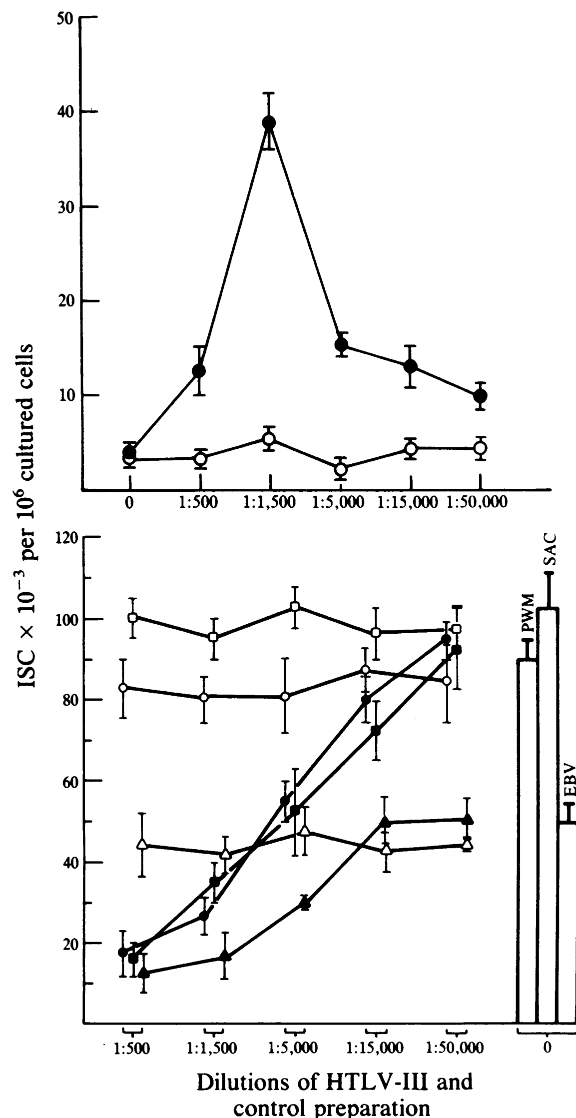

FIG. 1. (*Upper*) HTLV-III preparation-driven induction of ISC in PBL cultures of healthy volunteers. Results represent means ± SD of responses of lymphocytes from five donors cultured with HTLV-III (●) or control (○) preparation. (*Lower*) Inhibition of B-cell differentiation with the HTLV-III preparation. Results represent means ± SD responses of five donors whose cells were cultured with PWM (●, ○), EBV (▲, △), or SAC (■, □) in the presence of the HTLV-III preparation (●, ▲, ■) or control preparation (○, △, □).

used by itself. The inhibitory activity could be diluted out only at concentrations of viral material as low as that present in a 1:50,000 dilution.

Effect on NK Cell Activity. At very high concentrations of virus preparation, NK activity was inhibited (Table 3). The control preparation was also inhibitory of NK activity, and the difference between the effects of the two preparations was not of sufficient magnitude to be significant.

Influence on Antigenic Expression on the Surface of PBL. The possibility that a component or components of the virus might activate the T lymphocytes from normal donors was next tested. In Table 4 are presented results relating to the expression of the Tac antigen, which has been found to be a receptor for interleukin 2. This antigen was induced on the surface of PBL after 24 hr of exposure to the HTLV-III virus preparation. Further, an increase in expression of the surface antigen Leu-12, which identifies B lymphocytes, was also noted in lymphocytes exposed to these viral preparations for 24 hr. By contrast, the proportion of cells bearing demonstrable T4, T8, and T11 antigens, which identify peripheral blood T lymphocytes, was slightly reduced in PBL samples after exposure to the viral products.

Table 3. Effect of HTLV-III and control preparations (prep) on NK cell activity

Treatment*	% kill†	
	HTLV-III prep	Control prep
Dilution of added prep		
1:30	16.8	15.4
1:100	26.2	30.7
1:200	30.4	34.1
1:300	34.7	37.6
1:400	39.3	41.3
1:500	47.0	52.0
No addition	57.2	
Spontaneous release	22.5	

*Mixtures of effector and target cells were incubated for 14 hr in the absence (no addition) and presence of various dilutions of HTLV-III and control preparation. Spontaneous release represents target cells incubated without effector cells.
†Results are representative of five experiments.

Consideration of the findings reported in Tables 4 and 5 reveals a provocative paradox with respect to the Tac antigen. Whereas the viral preparation by itself appeared to induce cells to express Tac antigen, a putative marker of T-cell activation, the virus preparation at the same time was inhibitory for expression of Tac antigen that normally appears on T cells after exposure to PHA. Control preparations had no effect on membrane antigen expression in PBL. Influences of viral antigen preparations described above were also variable from donor to donor with respect to the magnitude of effects on the expression of membrane antigens in lymphocytes.

DISCUSSION

AIDS, the most threatening epidemic disease of our time, is caused by a retrovirus infection that sometimes produces a profound, progressive, highly lethal immunodeficiency disease. The immunodeficiency that occurs in patients infected by the HTLV-III virus has been considered to be the consequence of invasion of the T4 helper subpopulation of lymphocytes that destroys these cells and leads to an immunodeficiency (10) that culminates in life-threatening opportunistic infections or may lead to the increased occurrence of a peculiar constellation of cancers. However, a provocative paradox has emerged, which relates to the fact that only a few of many lymphocytes, perhaps as few as 1 in 1000 or even 1 in 100,000 cells, appear to be infected by the virus in amounts that can be readily demonstrated even by highly sensitive molecular biological analyses (9). If so few lymphocytes or even so few T4 cells are productively infected by

Table 4. Influence of HTLV-III preparation at 1:300 dilution on lymphocyte membrane antigens

Membrane antigen	% positive cells in PBL culture	
	Without HTLV-III	With HTLV-III
Leu-12	13.5	26.2
T11	74.8	63.5
Tac (IL-2 receptor)	2.7	8.4
T4	38.5	30.6
T8	30.1	21.0

Lymphocytes were incubated for 24 hr with and without a 1:300 dilution of the HTLV-III preparation. Results are representative of five experiments.

Table 5. Influence of the HTLV-III preparation at 1:300 dilution on the expression of Tac antigen

Additions	% Tac-positive cells		
	Exp. 1	Exp. 2	Exp. 3
None	3.0	6.0	9.1
PHA	35.1	35.7	41.5
PHA/HTLV-III	25.6	20.0	31.5

Lymphocytes incubated for 48 hr with PHA (20 μg/ml) in the presence and absence of a 1:300 dilution of the HTLV-III preparation.

the virus, the question must be raised as to what other basis might exist for the profound, progressive deficiency of immunologic functions and the progressive loss of the T4 subpopulation of lymphocytes. In addition, patients with AIDS and with manifestations associated with virus infections including AIDS-related complex (ARC) or the lymphadenopathy syndrome show not only defects of T-cell functions but evidences of excessive proliferation and differentiation of B lymphocytes and elevations of serum immunoglobulin (11–13). The latter changes have been attributed to polyclonal activation of the B lymphocytes, but the mechanism of this activation too has remained obscure up to the present.

In the present report we show that a noninfective component(s) of the HTLV-III virus exerts influences on both T and B lymphocytes *in vitro* that may explain, in part at least, changes of lymphocyte numbers and functions that have been observed in patients. We show that exposure of lymphoid cells to high concentrations of noninfectious virus inhibits their proliferative responses to phytomitogens, to two widely distributed antigens, and to allogeneic cells. In much lower concentrations, the same preparation of the virus was shown to be capable of inducing polyclonal activation of B lymphocytes that leads to the synthesis and secretion of immunoglobulin by the B lymphocytes. In addition, preparations of viral components over a wide range of doses inhibited induction of immunoglobulin secretion by known polyclonal B-cell activators. Inhibition of B-cell activation was demonstrated with B-cell activators that are T-cell dependent (PWM), T-cell independent (EBV), or partially T-cell dependent (SAC).

An additional influence of the noninfectious preparations of virus studied herein was to change the pattern of expression of certain surface antigens on PBL. The most dramatic of the changes attributable to exposure to the virus components was to increase the percentage of cells expressing Leu-12, an antigen that is known to be present on all B lymphocytes from the peripheral blood. In addition, the expression of Tac antigen, a receptor for interleukin 2, was increased by these viral components. Paradoxically, with both Tac expression and B-cell activation, the preparations that induced these cellular events also appeared to inhibit induction of Tac and to inhibit polyclonal B-cell activation by other activators. These findings are in agreement with the observations reported in patients with AIDS or ARC (11, 12, 20).

At the present time it is not clear how all these influences of killed virus are to be explained, but influences of different concentrations or different components of the virus and differing responsive cell populations could help to explain the results obtained.

These influences *in vitro* of noninfectious components of the HTLV-III virus could help resolve at least some of the paradoxes pointed out above and could contribute toward an understanding of the pathogenesis of several important immunopathologic concomitants of HTLV-III infection, ARC, and/or AIDS. The polyclonal B-cell activation, defective proliferative and functional responses of the T lymphocytes, and even susceptibility to infection and susceptibility to certain cancers might then be attributable not to a direct cytotoxic action of virus infection of the T4 cells, but rather to indirect influences of virus components at differing concentrations. Could it be that the production of virus or its antigens is more localized or that virus is preferentially produced by different cells than is now considered likely and that much of the immunodeficiency of AIDS and also the polyclonal B-cell activation seen in this disease will be attributable to virus or virus components delivered to the affected cells from a more remote site? If viral components can exert an influence to inhibit clonal proliferation of T4 cells, then the progressive decline in numbers of these cells in patients with frank AIDS might be better explained. Experiments to test this postulate seem urgent.

The dose requirement for the putative viral antigens in the preparations used appears to be quite large for some of the influences reported herein but this requirement too could be consonant with the temporal relationships and/or possible dose–response relationships of the individual viral influences observed. Thus, they could be related to alterations of lymphoid cells and functions seen at different times during the course of disease in patients who exhibit progressive AIDS. For example, one of the earliest if not first influence of HTLV-III/LAV virus infection is to induce polyclonal activation of B lymphocytes and to produce increased levels of circulating immunoglobulins. Similarly, the polyclonal B-cell activation in the present *in vitro* studies showed activity of the virus preparation at the lowest concentration exhibiting biological effects.

What is needed more than anything else in the light of the present observations are studies to identify in molecular terms which of the virus components is responsible for each of the actions we have observed and report herein.

With other retroviral infections, immunodeficiencies occur in high frequency (21), and already some of these, such as those produced by the Feline Leukemia Virus (FeLV), have been attributable to influences of noninfectious viral components (22, 23). Even particular antigens, like the p-15 component of the feline leukemia virus (23), have already been tentatively implicated as the cause of immunodeficiency in the feline leukemia virus infection. Identification and preparation of large amounts of individual noninfectious viral components could yield a most important immunomodulatory agent or set of agents. Such analyses might permit development of new immunostimulants, immunodepressants, and/or immunomodulators of value to transplantation surgery, transplantation biology, control or management of autoimmunities, anticancer therapy, and even control of reproduction. The biologic tricks used by the HTLV-III/LAVvirus might then become a blessing in disguise. From this perspective, identification of the site or sites of production of virus and its components may turn out to be a most critical issue. Already it is known that infection with the virus may involve not only T cells but B cells (7), macrophages, and even the central nervous system (8). Where else?

Perhaps from the standpoint of AIDS itself, identification of the molecules and possibly also of the genetic control of production of these molecules could become a focus of incisive investigation that could lead directly to inhibition of their action—e.g., by immunologic means—and thus to correction of the highly lethal immunodeficiency associated with the HTLV-III infection.

We are grateful to Dr. M. G. Sarngadharan for advice and encouragement, to Maria Marecki and Corine Leombruno for expert technical assistance, and to Kay Suwczinsky for typing the manuscript.

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