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Differential reconstitution of zidovudine-induced inhibition of mitogenic responses by interleukin-2 in peripheral blood mononuclear cells from patients with human immunodeficiency virus infection

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Summary

Zidovudine (ZDV), an anti-human immunodeficiency virus (HIV) therapy, has been associated with reduction in mortality and improvement of patients with acquired immunodeficiency syndrome (AIDS). The ZDV recipients, however, experience a multitude of side effects of which bone marrow suppression is the most noteworthy, especially among patients with low CD4 cell counts. The effect of ZDV and interleukin-2 (IL-2) on phytohemagglutinin (PHA)-induced proliferative response of peripheral blood mononuclear cells (PBMs) from patients with HIV infection was investigated. ZDV 0.5 μg inhibited 40% of PHA-induced thymidine uptake in PBMs from healthy donors or patients with HIV, irrespective of their CD4 cell counts. However, IL-2 (10 U/ml) had differential effect on PHA-induced thymidine uptake that appeared to be dependent on absolute CD4 cell counts. While PBMs from patients with CD4 cell counts of 400/mm^3 or more did not respond to IL-2 (low responders), IL-2 enhanced the PHA-induced thymidine uptake in PBMs from patients with CD4 cell counts less than 400/mm^3 at an average of 60% (high responders). Moreover, IL-2 restored the ZDV-induced inhibition by almost 100% in the high responder group while it did not affect counts in the low responder group. The production of IL-2 in vitro, in response to PHA or recall antigens, was equivalently inhibited in both groups. These data suggest that

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ZDV and IL-2 could have an additive effect on immune parameters in certain groups of patients infected with HIV. The differential effect of IL-2 was independent of IL-2 receptor expression.

ZDV; IL-2; AIDS; HIV

Introduction

The acquired immunodeficiency syndrome (AIDS), a growing major health problem, is well recognized for its devastating and fatal complications. The severity of the disease is accounted for by the profound degree of immunosuppression observed which results in the development of opportunistic infections and malignancies. Human immunodeficiency virus (HIV), the causative agent of this syndrome, has a characteristic tropism for T cells with CD4 molecules on their surface leading to the continuous selective depletion of this T cell subpopulation (Gottlieb et al., 1981; Bowen et al., 1985). Recently azidothymidine (zidovudine, ZDV) has been shown to inhibit HIV replication effectively (Mitsuya et al., 1985) with encouraging improvement in the condition and a temporal increase of the helper/inducer CD4 cell counts of patients with advanced HIV infections (Fischl et al., 1987; Richman et al., 1987). In spite of the apparent efficacy of ZDV, it has been associated with a number of toxic side effects, the most serious of which is bone marrow suppression and its consequences (Richman et al., 1987; Gill et al., 1987).

Further studies will be required to search for methods to overcome ZDV-induced side effects. The introduction of new approaches utilizing combination therapy with ZDV in addition to other antivirals or biological response modifiers is currently being conducted. Also, systematic studies of ZDV interactions with other drugs that could interfere with its metabolism are underway (Richman et al., 1987). In this report peripheral blood mononuclear cell (PBM) responses to ZDV and/or interleukin-2 (IL-2) and IL–2 production by PBMs from healthy donors and patients with HIV infection were investigated.

Materials and Methods

Cell cultures and mononuclear cell blastogenesis assay

The PBMs were obtained from heparinized blood from normal blood donors or patients infected with HIV, which ranged from asymptomatic to meeting CDC requirements for AIDS by layering onto Ficoll-Hypaque gradients and separating them by density centrifugation. Mononuclear cells were washed three times and adjusted to a concentration 2×10^6 cells/ml in 20% heat-inactivated human serum known to be complement fixation antibody negative to CMV. PBMs (2×10^5 cells)

were incubated in a multiple of 6 wells for lymphokine detection or quadruplicates for blastogenic transformation assays in 96-well microtiter plates in 0.2 ml of RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 U-/ml) and streptomycin (100 μg/ml) in the presence or absence of optimum concentrations of antigens and mitogens. Control wells for PHA had RPMI media, while wells for CMV Ag control had antigen prepared from uninfected cell cultures treated the same way as CMV infected cultures. Supernatants of cultures incubated at 37°C in a 5% CO_2 atmosphere and treated with PHA 1:500 dilution of (Difco, Detroit, MI) or 1:40 dilution (Davis strain prepared as previously described, Pollard et al., 1978). CMV Ag were harvested at 72 h and 7 days, respectively. This dose of PHA or CMV Ag used gave an optimum response in PMNs from healthy controls at the indicated time from preliminary kinetic experiments. Samples were then stored at −70°C until assayed for IL-2. For blastogenic transformation assays, cultures were harvested at the same time as above. At 24 h before harvest tritiated thymidine was added to each well (0.5 μCi/ml, specific activity 6.7 Ci/mmol, ICN chemical and radioisotope Div., Irvine, CA). Cells were then collected onto glass filter paper discs and washed with a semi-automated microharvesting device (Skatron Inc., Sterling, VA). The discs were air dried and placed in a scintillation cocktail (Scintiverse, Fisher Scientific Co., Pittsburgh, PA) and [^{3}H]thymidine activity was determined by a Beckman LS9000 liquid scintillation counter.

IL-2 assay

IL-2 was determined by a human IL-2 ELISA test (Intertest 2, Genzyme Co., Boston, MA). Briefly, 100 μl of IL-2 standard or specimen was incubated in a 96-well polystyrene immunoplate coated with mouse monoclonal anti-IL-2 antibody specific for IL-2 for 6 h at 37°C. At the end of the incubation, samples were aspirated and the wells were washed 4 times with cold phosphate buffered saline (PBS)/Tween-20 washing buffer, then a second antibody (polyvalent rabbit anti-IL-2) was added for 1 h at 37°C in a humidified atmosphere. The plates were then washed 4 times with washing buffer and a 200 μl of a third antibody was added (goat anti-rabbit, alkaline-phosphatase-conjugated) and the plates were further incubated for another 1 h at 37°C. At the end of the incubation the plates were washed 4 times and a substrate reagent was added (para-nitrophenyl phosphate). A color reaction developed in 15–60 min at room temperature. The appropriate color change was measured by the absorbance of each well on an ELISA reader at 405 or 410 nm (alkaline-phosphatase setting). An IL-2 standard curve was constructed by plotting mean absorbance of the IL-2 standard dilutions versus the concentration of the IL-2 standards, and the IL-2 concentration in the test samples was determined by comparison with the curve.

CD4/CD8 ratios and IL-2 receptor expression

Absolute CD4 and CD8 cell numbers and IL-2 receptor (IL-2R) expression were determined by FACS analysis. For CD4 and CD8 cell determination whole blood

collected in EDTA was used. Blood samples (100 μl) were incubated with monoclonal antibodies to T4 and T8 (Coulter Immunology, Hialeah, FL) that were FITC-conjugated or with phosphate buffer saline (PBS) or mouse IgG-FITC conjugate as control. After a 45 min incubation, the cells were washed twice with PBS, the red blood cells were then lysed with Immuno-lyse (Coulter Immunology, Hialeah, FL) and fixed with 2% paraformaldehyde. The cells were washed 2 more times with PBS and resuspended in 1 ml of the same buffer. The cells were then analyzed on a Coulter EPICS clinical flow cytometer. The fluorescence intensity was measured at a wave length of 488 nM and the number of cells gated for lymphocytes was 3000 (Reinherz et al., 1982). For IL-2R, briefly, 1×10^6 PBM cells/ml were cultured in 1 ml in plastic tubes for 72 h in presence or absence of PHA or CMV Ag. At time of harvest the cells were washed and then stained with 20 μl FITC conjugate of the anti-IL-2R monoclonal antibody (Becton Dickinson, Mountainview, CA) for 30 min at 4°C. Stained cells (as well as unstained controls) were washed twice and resuspended in 0.5 ml of cold buffer to which 0.5 ml of 2% paraformaldehyde was added. The samples were then stored in the dark at 4°C (period not exceeding 5 days) until analyzed (Kelly et al., 1987).

Statistical analysis

Statistical analysis and levels of significance were determined by using the Student *t*-test.

Results

IL-2 response of PBMs from healthy donors and AIDS patients

The IL-2 response of PBMs from healthy CMV-seronegative and seropositive individuals to specific and nonspecific mitogens was determined. There was no significant difference in the IL-2 response to PHA between CMV-seropositive and seronegative individuals (Fig. 1A). The IL-2 response to CMV Ag between the groups differed significantly ($P<0.01$) with CMV-seropositive PBMs producing IL-2 while PBMs from seronegative individuals did not (Fig. 1B). These data suggest that the production of IL-2 was immune specific.

The PBMs from HIV-infected patients on the other hand failed to produce IL-2 to either specific (recall antigens) or nonspecific mitogens (Fig. 1C, D). This defect could be due to the fact that IL-2 is produced by CD4 cells which are partially depleted in these patients. Alternatively, the available cells could be functionally impaired or other IL-2 producing T cells could be dependent on a CD4 cell function. Since IL-2 is an important immune regulatory lymphokine that is required for proper humoral and cellular immune response to foreign antigens, the lack of its production could partially explain the vulnerability of AIDS patients to repeated infections.

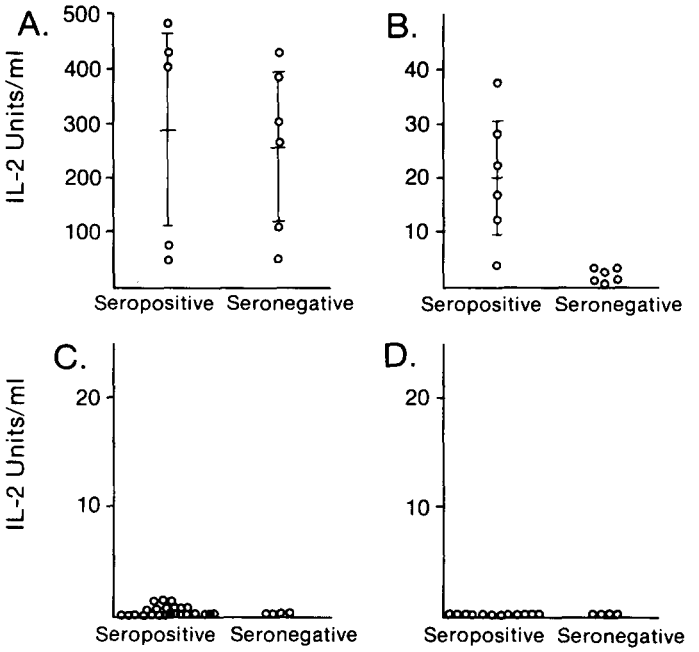

Fig. 1. IL-2 response to PHA (A,C) and CMV Ag (B,D) peripheral blood mononuclear cell cultures from CMV seropositive and seronegative healthy blood donors (A,B) and HIV-infected patients (C,D). PHA and CMV Ag responses were determined at 3 and 7 days respectively. The mean is shown by a bar ± SD.

Effect of IL-2 on PHA-induced mitogenic response in PBMs

The effect of IL-2 on PHA-induced proliferative response in PBMs from HIV-infected individuals was determined next. Patients were divided into two groups, a low responder and a high responder group (Tables 1 and 2 respectively) based on the ability of IL-2 (10 U/ml) to enhance PHA-induced ^{3}H-thymidine uptake by their PBMs. In group one, 3 out of 13 were diagnosed as AIDS (one was on ZDV) and the rest were HIV seropositive. In the high responder group 10 of 12 were AIDS (7 of those were on ZDV) and 2 HIV seropositive. The IL-2 enhanced the [^{3}H]thymidine uptake in the high responder and low responder group by an average of 63% and less than 1% respectively. The IL-2 also failed to enhance PHA-induced proliferative response in PBMs from healthy individuals (Table 1). When the absolute CD4 cell counts were determined in both groups, it appeared that the high responders all had less than 400 cells/mm^3, while the low responders had more than 400 cells/mm^3, except for one. These data suggest that there was a correlation between CD4 counts and IL-2 enhancing ability of the response to PHA.

It appears that the high responder group (majority were AIDS) produce insufficient amounts of endogenous IL-2 in response to PHA and adding IL-2 to their culture enhances their response, while the low responder group probably makes

TABLE 1

Effect of IL-2 on peripheral blood lymphocytes' proliferative response to PHA in HIV infected individuals (low responders)[a]

Patient No.	TI[b]	CD4/mm^3	PHA c.p.m.	PHA + IL-2[c] c.p.m.	Percent stimulation
1	65	799	27405	27260	−1
2	184	408	22761	19130	−16
3	93	580	19896	20575	3
4	70	490	21799	22487	3
5	57	570	21689	21609	0
6	52	2442	17320	15470	−11
7	76	720	22960	22059	−4
8	39	441	13220	13990	6
9	133	156	18749	21009	12
10	179	408	28752	26983	−6
11	40	440	5970	6992	17
12	32	ND	11235	12298	9
13	41	560	7748	7756	0
Mean	82	677	18423	18278	
	±	±	±	±	0.008
	14	163	1964	1745	
Healthy controls[d]					
Mean	118	824	18155	19415	
	±	±	±	±	7
	15	51	1754	1634	

[a]Low responders: patients whose PBLs' PHA-induced response was not enhanced by more than 20% in presence of IL-2.
[b]Transformation index (derived by dividing the cpm of PHA containing wells by the cpm of control wells).
[c]10 u/ml.
[d]Mean of 5 ± SE of the mean.

endogenous IL-2 sufficient to produce the PHA response but not sufficient to allow an excess to be detected in the supernatants. Moreover, cells from normal individuals produce enough endogenous IL-2 in response to PHA to drive the proliferative response and in excess so that it appears in the supernatant.

Effect of ZDV and IL-2 on PHA-induced mitogenic response in PBMs

ZDV has been shown to exert some toxic side effects in patients with AIDS. To determine if ZDV affects PBM cell responses to PHA, PBMs from normal healthy blood donors or patients with HIV infection were challenged with ZDV (0.5 μg/ml) and/or PHA. The dose of ZDV used corresponds to the mean peak plasma levels of ZDV after oral administration of therapeutic doses of the drug (Blum et al., 1988). The proliferative response, as determined by [^{3}H]thymidine uptake, was then measured at 72 h after culture initiation. ZDV inhibited thymidine uptake in PHA-

TABLE 2

Effect of IL-2 on peripheral blood lymphocytes' proliferative response to PHA in HIV-infected patients (high responders)[a]

Patient No.	TI[b]	CD4	PHA cpm	PHA+IL-2[c] cpm	Percent stimulation
1	243	ND	13370	21220	59
2	269	189	10758	12843	20
3	85	112	6719	10730	60
4	57	72	2635	5634	114
5	42	72	2042	3461	70
6	3	19	325	2611	703
7	46	87	4866	10313	112
8	8	285	4045	8714	116
9	107	81	12180	15435	27
10	204	374	18342	30816	68
11	90	ND	11680	20555	76
12	51	198	6018	8887	48
Mean	100	149	7748	12602	
	±	±	±	±	63
	25	47	1537	2320	

[a]High responders: patients whose PBMs' PHA-induced proliferative response was enhanced by more than 20% in presence of IL-2.
[b]Transformation index.
[c]10 U/ml.

treated lymphocytes from HIV infected (low responders and high responders) or healthy individuals by an average of 34, 44, and 31%, respectively (Tables 3 and 4). The effect of ZDV was not influenced by the absolute CD4 cell counts, CD4/CD8 ratios or the relative uptake of thymidine in the absence of ZDV. There was also no difference in the magnitude of the ZDV-induced inhibition between

TABLE 3

Effect of ZDV and IL-2 on PHA-induced proliferative response in PBMs from healthy donors

Sample	[^{3}H]Thymidine uptake[a]		
	cpm	Percent inhibition	Percent reversal of ZDV effect
PHA	18155 ± 754[b,c]		
PHA+ZDV	12512 ± 231[b,d]	31	
PHA+ZDV+IL-2	13566 ± 271[c,d]	25	19
PHA+IL-2	19415 ± 861	−7	

[a]Means from five healthy individuals. Determined at 72 h after culture initiation plus or minus SEM. The cpm for untreated controls (cells with RPMI) was 154, ZDV alone 130, ZDV and IL-2 951, IL-2 alone 1254.
[b]Statistically significant $P<0.01$.
[c]Statistically significant $P<0.01$.
[d]Statistically significant $P>0.05$.

TABLE 4

Effect of ZDV and IL-2 on PHA-induced proliferative response in PBMs from HIV-infected patients

Sample	[^{3}H]Thymidine uptake					
	Low responders[a]			High responders[b]		
	cpm	Percent inhibition	Percent reversal	cpm	Percent inhibition	Percent reversal
PHA	18353 ± 1860[c,d]			7748 ± 1537[e,f]		
PHA+ZDV	12161 ± 1042[c]	34		4328 ± 789[e]	44	
PHA+ZDV+IL-2	12013 ± 1230[d]	35	−1	8009 ± 1319[f]	−3	107
PHA+IL-2	18278 ± 1745	0		12602 ± 2320	−63	

[a]The cpm for untreated controls (cells with RPMI alone) was 243, ZDV alone 285, ZDV and IL-2 1057, IL-2 alone 1989.
[b]The cpm for untreated controls (cells with RPMI alone) was 96, ZDV alone 113, ZDV and IL-2 369, IL-2 alone 691.
[c]Statistically significant $P<0.01$.
[d]Statistically significant $P<0.01$.
[e]Statistically significant $P<0.01$.
[f]Statistically significant $P>0.05$.

healthy individuals and HIV patients. ZDV also diminished the IL-2 induced thymidine uptake. This is possibly due to its inhibitory effect of cellular DNA synthesis.

The effect of IL-2 on ZDV-induced inhibition of the proliferative responses in PBMs in both groups was then examined. When IL-2 was added to PHA-stimulated cultures in the presence of ZDV, the ZDV-induced inhibition of ^{3}H-thymidine uptake was reverted by 107% and −1% in high and low responder groups respectively (Table 4). Meanwhile, IL-2 reversed only 19% of the ZDV-induced inhibition of PBMs from healthy donors (Table 3), showing a pattern similar to that of the low responder group. Taken together these data show a correlation between absolute CD4 cells counts, the response to IL-2, and the ability of IL-2 to revert ZDV induced inhibitory effects.

IL-2 receptor expression on PBMs from HIV-infected patients

The relationship of IL-2R expression and CD4 cell counts in PBMs from HIV infected individuals was then investigated (Table 5). Although the percentage of cells with IL-2R in patients with CD4 counts more than 400/mm^3 was higher than from patients with CD4 counts less than 400/mm^3 by 24 h after PHA stimulation,

TABLE 5

IL-2R expression on PBMs from HIV-infected patients

Patient No.	Stimulation	CD4	% Cells positive		
			24 h	72 h	168 h
1	unstimulated	155	2.8	7.5	2.1
	PHA		4.7	37.6	–
	CMV Ag		–	–	61.5
2	unstimulated	216	2.9	7.9	0.6
	PHA		4.9	46.7	–
	CMV Ag		–	–	1.5
3	unstimulated	176	1.8	7.4	1.4
	PHA		6.7	36.6	–
	CMV Ag		–	–	28.3
4	unstimulated	470	3.1	13.5	2.63
	PHA		18.1	40.6	–
	CMV Ag		–	–	15.5
5	unstimulated	450	1.9	6.7	1.2
	PHA		15.3	40.3	–
	CMV Ag		–	–	33.1
6	unstimulated	75	6.0	11.3	4
	PHA		11.0	44.4	–
	CMV Ag		–	–	64.9
Normal[a] control	unstimulated	1124 ± 332	3.5 ± 2	3.5 ± 3	10.3 ± 2
	PHA		18.0 ± 5	43.2 ± 10	–
	CMV Ag		–	–	39.0 ± 22

[a]Mean ± SD of 5 healthy controls.

at 72 h there were no significant differences among the patients in both groups. These data apparently indicate that there was no correlation between CD4 cell counts and IL-2R expression. It also suggests that the failure of IL-2 to enhance PHA induced proliferative responses of PBMs from HIV patients with CD4 cell counts above 400/mm^3 was independent of IL-2R expression and was not due to possible downregulation of the receptor. In addition, it suggests that the enhancement observed among the high responder group was not due to positive upregulation of the receptor.

Discussion

Patients with AIDS have a multitude of immunologic abnormalities (Masur et al., 1981; Bowen et al., 1985; Lane and Fauci, 1985, Bonavida et al., 1986) that make them vulnerable to live-threatening opportunistic infections. The hallmark

of this syndrome is the gradual depletion of T cells with CD4 marker molecules on their surface (Gottlieb et al., 1981; Bowen et al., 1985). CD4 cells are central in regulating both the humoral and cell-mediated immune response through a cascade of events that are mediated by IL-2. Studies examining the production of IL-2 in response to mitogens in PBMs from AIDS have produced differing results. While some have shown no difference in IL-2 production between AIDS patients and uninfected controls (Reubin et al., 1985; Lane et al., 1985), others have shown impaired production of lymphokines in general (Murray et al., 1984) or of IL-2 in particular (Ciobanu et al., 1983; Kirkpatrick et al., 1985). The present observations support the latter although the extent and magnitude of this inhibition was less than reported by other investigators. These differences could be attributed to differences in patient populations, the time of harvest, and the IL-2 assay system utilized. Inhibition of IL-2 production is not surprising, since sera from patients with AIDS have been shown to suppress PHA-induced IL-2 production by normal blood mononuclear cells (Siegel et al., 1985). This suppression persisted and was not reversed by washing off the sera from the normal PBL cultures.

The proliferative responses to PHA in these patients were variable and apparently depended on absolute CD4 cell counts. Exogenously added recombinant IL-2 produced a differential enhancing effect on the PHA induced proliferative responses that was also dependent on CD4 cell counts. While IL-2 enhanced PHA responses from patients with CD4 cell counts of less than 400/mm^3 and completely reconstituted the ZDV induced inhibition of DNA synthesis, it failed to enhance the PHA-induced proliferative responses of PBMs from normal healthy donors or HIV-infected patients with CD4 cell counts above 400/mm^3. The lack of response in the latter group was independent of IL-2R expression. Although HIV-infected patients did not respond to specific antigens by making IL-2, the IL-2 receptor expression was not impaired. Others have also shown variable responses of PHA-induced PBMs from AIDS patients to IL-2 (Ciobanu et al., 1983). Their data failed to reflect any correlation between CD4/CD8 ratio and the response to IL-2. The CD4/CD8 ratios in patients in the present study were similar to theirs (data not shown) with no correlation between the ratio and the degree of response.

The inhibition of DNA synthesis in PHA-induced PBMs by ZDV suggests a possible explanation for the observed side effects associated with the in vivo administration of the drug. Among those are macrocytic anemia, neutropenia (Richman et al., 1987) and pancytopenia (Gill et al., 1987), all of which require reduction in the ZDV dose administered to the patients (Hirsch, 1988). It is possible that reduced amounts of ZDV in combination with IL-2 would produce an antiviral effect that is equivalent to that produced by high doses of ZDV administered alone, thus increasing the therapeutic benefits.

Cellular immune responses are important host defense mechanisms against opportunistic infections. Patients with AIDS have a deficient antiviral cytotoxic T cell response and natural killer cell activity (Rook et al., 1983, Quinan et al., 1985). These responses have often been shown to be augmented in lymphocytes from those patients by IL-2 (Rook et al., 1983). These observations, plus data from the present study, constitute a rationale for the potential usefulness of IL-2 as a therapy

for patients with AIDS. Early trials using IL-2 (Lane et al., 1984) for treatment of AIDS failed to show efficacy but produced information as to its tolerance and side effects. Therefore, its use in combination with ZDV might prove useful in modulating the immune response in AIDS and thereby alleviate some of the ZDV-induced toxicity by decreasing the dosages of ZDV required.

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