Clin. exp. Immunol. (1985) **62,** 128–135.

Natural killer cells in intravenous drug abusers with lymphadenopathy syndrome

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(Accepted for publication 29 May 1985)

SUMMARY

We have investigated 25 intravenous drug abusers with the clinical and laboratory features of lymphadenopathy syndrome (LAS) and 10 AIDS patients for the expression of NK activity. LAS and AIDS patients had low NK cytotoxicity compared to normal donors. The defective NK cytotoxicity was analysed in the eight LAS subjects with most marked depression. NK effectors were identified by morphology (large granular lymphocytes, LGL) and monoclonal antibody-defined surface markers (B73.1, N901, HNK1). LAS patients had normal percentages of LGL and B73.1$^+$ and N901$^+$ cells, with the exception of two subjects with very low frequency of B73.1$^+$ and N901$^+$ cells. The percentage of HNK1$^+$ cells was increased in LAS, probably because of the reactivity of this reagent with a subset of conventional OKT8$^+$ cells, relatively augmented in LAS subjects. Depletion of monocytes did not enhance NK activity consistently. LAS patients had a normal frequency of cells capable of binding K562. In-vitro exposure to interferon beta (natural) or gamma (recombinant) augmented the defective NK activity of LAS subjects. Thus, patients with LAS have defective NK activity that cannot be accounted for by a low frequency of the relevant effector cells or by monocytic suppressors. These observations suggest a functional defect of NK cells at one or more of the post-binding steps required for the completion of killing.

Keywords AIDS lymphadenopathy syndrome intravenous drug abusers Natural killer cells

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) and AIDS-related disorders (lymphadenopathy syndrome, LAS, or pre-AIDS) are relatively new diseases emerging in well defined groups at risk (Centers for Disease Control Task Force, 1982 a,b,c,d).

Retroviruses have been implicated in the etiology of these disorders (Broder & Gallo, 1984; Laurence, Gottlieb & Kunkel, 1984). Alterations of the number and function of T cells probably play a major role in the pathogenesis of immunodeficiency and have been the object of extensive studies particularly in AIDS (Seligmann *et al.*, 1984; Pinching, 1984; NIH Conference, 1984). On the other hand, whether and to what extent alterations of mechanisms of natural resistance contribute to immunodeficiency has been the object of less attention, and most available information relates to Natural Killer (NK) function in AIDS patients (Siegal *et al.*, 1981; Gerstoft *et*

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al., 1982; Poon *et al.*, 1983; Rook *et al.*, 1983; Aiuti, Sirianni & Seminara, 1983; Metroka *et al.*, 1983; Lopez, Fitzgerald & Siegal, 1983; Laurence *et al.*, 1983; Pitchenik *et al.*, 1983; Lazzarin *et al.*, 1984; Lifson *et al.*, 1984; Reddy, Chinoy & Grieco, 1984). Natural killer cells are credited with an important role as a first line of resistance against foreign cells, infectious agents and possibly cancer cells (Herbermann & Ortaldo, 1981; Trinchieri & Perussia, 1984).

Moreover NK cells may be involved in regulation of haemopoietic elements (Trinchieri & Perussia, 1984) and of other immune populations through the release of lymphokines (Timonen *et al.*, 1980; Kashara *et al.*, 1983; Scala *et al.*, 1984).

Patients with AIDS have been shown to have defective NK activity that can be stimulated *in vitro* by interleukin 2 (IL-2) and, less frequently, by interferon (IFN) (Rook *et al.*, 1983; Metroka *et al.*, 1983; Lifson *et al.*, 1984; Reddy *et al.*, 1984). Decreased NK cell function has also been reported in studies in AIDS-related syndromes, but the pathogenesis of this defect has not been investigated (Siegal *et al.*, 1981; Gerstoft *et al.*, 1982; Laurence, Gottlieb & Kunkel, 1983; Metroka *et al.*, 1983; Lopez, *et al.*, 1983; Pitchenik *et al.*, 1983). We have recently described the occurrence of a LAS outbreak among intravenous drug abusers clustering in a suburban area of Milan, Italy (Lazzarin *et al.*, 1984). The purpose of this investigation was to analyse NK activity in patients with LAS. Results obtained indicate that LAS patients have defective NK activity that can be stimulated by in-vitro exposure to IFN. The defective NK cell function could not be accounted for by a low relative frequency of the relevant effector cells, identified morphologically and by monoclonal antibody defined surface markers, or by the presence of monocytic suppressor cells. The frequency of NK cells capable of binding K562 target was normal, thus suggesting a functional impairment of the effector population.

MATERIALS AND METHODS

Human subjects. Twenty-five intravenous drug abusers with LAS (22 males and three females) formed the case list of this study. The LAS patient population was identified according to the criteria of the Centers of Disease Control (Centers for Disease Control Task Force, 1982d) and the outbreak of this disorder in Milan has been previously described (Lazzarin *et al.*, 1984). Briefly, all subjects live in a restricted surburban area. Six of the males are homosexuals. The most prominent clinical findings were persistent unexplained lymphadenopathy, with typical follicular hyperplasia (Parravicini *et al.*, 1984), fever, weight loss, night sweats and malaise. Immunological abnormalities included lymphopenia, decreased $OKT4^+/OKT8^+$ ratio, anergy or hypoergy to recall skin antigens and elevated levels of IgG (Lazzarin *et al.*, 1984).

Ten symptom-free drug addicts (SFDA) from the same area and social groups, and 14 normal laboratory control donors were studied in parallel. None of them was homosexual. Anti-HTLV III antibody determinations were performed by indirect immunofluorescence on H-4 infected cells (Popovic *et al.*, 1984). Twenty of the 25 (80%) LAS patients and six of the 10 (60%) SFDA tested were positive for anti-HTLV III antibodies. None of the normal donors were positive for anti-HTLV III antibody determinations.

We have also studied 10 patients with AIDS, with no apparent relationship to the LAS population. Four of these were homosexual and six were drug abusers, with one subject falling in both risk groups; four had anamnestic history of travel and life in Africa and California. Diagnosis was made according to the criteria of the Centers for Disease Control (Centers for Disease Control Task Force, 1982b). All patients' were examined for anti-HTLV III antibodies and five were found positive: one of these became negative in a subsequent determination.

All AIDS patients had severe respiratory tract infections, and two had Kaposi's sarcoma. At the time of writing seven of the 10 AIDS patients have died.

Peripheral blood mononuclear cells (MNC). Heparinized peripheral venous blood was diluted 1:4 with PBS and mononuclear cells were separated by centrifugation at 400 ***g*** for 30 min on Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md., USA). Mononuclear cells were washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 10% FBS.

Mononuclear cell counts and the relative percentage of different cellular populations (monocytes, lymphocytes, large granular lymphocytes) were assessed by morphology in May

Grunwald-Giemsa stained cytospins of mononuclear cell suspensions. Non-adherent cells were obtained incubating mononuclear cell suspensions in tissue culture plastic Petri dishes for 45–60 min at 37°C, in a humidified atmosphere containing 5% CO_2. Non-adherent cells were collected, centrifuged at 400 *g* for 5 min, and finally resuspended in RPMI 1640 medium with 10% FBS.

Monoclonal antibody (moab) defined-surface markers. The moab HNK1, reactive with NK cells and some T lymphocytes (Abo & Balch, 1981) was obtained through the courtesy of Dr T. Abo (Birmingham, Alabama, USA). B73.1, recognizing the Fcγ receptor of NK cells and polymorphs (Perussia *et al.*, 1983) was a kind gift of Dr G. Trinchieri (Wistar Institute, Philadelphia, PA, USA). The moab N901 (gift of Dr J. Griffin, Dana Farber, Cancer Institute, Boston, MA, USA) reacts with NK cells and some immature myeloid cells (Griffin *et al.*, 1983). MNC were labelled by indirect membrane immunofluorescence and analyzed in a FACS IV apparatus, as previously described (Colotta *et al.*, 1984).

Cytotoxicity assay. ^{51}Cr-labelled K562 tumour cells (1×10^4) were cultured with MNC (MNC/K562 ratio employed routinely was 25:1) in 0·2 ml of RPMI 1640 medium with 10% FBS in round bottomed microplate wells (Sterilin, Teddington, Middlesex, UK).

The incubation time routinely employed was 4 h. Isotope release was calculated as $(A/B) \times 100$, where A is the isotope in the supernatant and B is the total incorporated radioactivity released by incubation with 1% sodium dodecyl sulfate.

Specific lysis was calculated by subtracting spontaneous isotope release of tumour cells alone. Spontaneous ^{51}Cr release from K562 cells was 0·5–1·5% for 1 h incubation.

Conjugate assay (Grimm & Bonavida, 1979). The conjugation capacity of MNC was measured by mixing equal numbers (1×10^5) of MNC and K562 target cells in 0·2 ml medium, followed by incubation for 5 min at 29°C and centrifugation at 120 *g* for 10 min.

The pellet was suspended by gentle aspirations with a Pasteur pipette, necessary to dissociate multicellular clusters, which hampered the analysis of conjugates at a single cell level. The frequency of conjugate-forming cells was determined by microscope inspection of at least 200 MNC.

IFNs. Human recombinant IFN (γ) was obtained from Bhoeringer (Wien, Austria). Partially purified (12×10^6 units/mg proteins) human IFN (β) was obtained by Serono (Rome, Italy) and Sclavo (Siena, Italy). Effector cells (MNC) were cultured for 12 h in the presence of 1000 U/ml IFN in RPMI 1640 with 10% FBS.

Statistical analysis. NK activity of the cells from various patient populations examined was analysed by Duncan's new multiple range test.

RESULTS

The NK activity of peripheral blood mononuclear cells of subjects with LAS is shown in Fig. 1.

As previously reported (Lazzarin *et al.*, 1984), and confirmed in this study, the LAS patients identified among drug addicts in Milan had lymphopenia (lymphocyte count = 1321 ± 448/mm^3 compared to 2433 ± 355 for controls, $P < 0{\cdot}01$) with a decrease of the OKT4$^+$/OKT8$^+$ ratio ($0{\cdot}7 \pm 0{\cdot}3$ compared to $1{\cdot}7 \pm 0{\cdot}4$ for controls, $P < 0{\cdot}01$), hypergammaglobulinemia (2950 ± 756 mg/dl compared to 1244 ± 306 mg/dl for controls, $P < 0{\cdot}01$) and defective responsiveness to recall skin antigens (50% of the subjects did not respond to seven recall antigens).

NK cytotoxicity was titrated at effector to target ratios ranging from 50:1 to 6:1, but only results at 25:1 are presented, with comparable differences being found at the other ratios tested. LAS subjects had significantly ($P < 0{\cdot}01$) less cytotoxicity than normal donors tested in parallel with a mean value of $28{\cdot}2 \pm 10$% and $10 \pm 9{\cdot}3$% for the control and LAS group respectively. MNC from SFDA had intermediate values. These findings confirm our previous observations in the same LAS population (Lazzarin *et al.*, 1984). In particular, 15 of the 25 LAS patients had very low cytotoxicity values ($\leqslant 10$% at 25:1, Fig. 1), and these were used for subsequent analysis. As illustrated by the examples given in Table 1, defective NK function of LAS patients was a consistent finding upon repeated examinations. When NK activity values were considered in relation to anti-HTLV III reactivity, no difference was apparent with $10{\cdot}0 \pm 8{\cdot}0$% specific lysis for positive LAS subjects ($n = 20$) and $11{\cdot}4 \pm 13{\cdot}7$% for the negative ones ($n = 5$). Similarly no difference was observed when

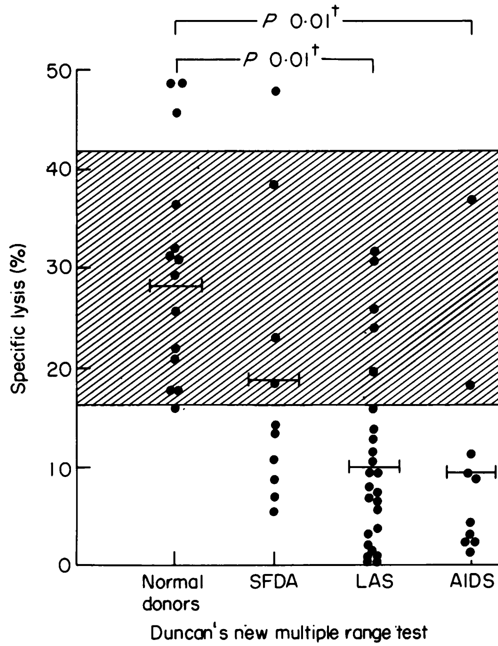

Fig. 1. NK activity of subjects with LAS. Results presented refer to an E:T ratio of 25:1.

homosexual LAS patients were compared to the heterosexual ones. Also shown in Fig. 1 are results with cells from 10 patients with AIDS, seven of whom also had low levels of reactivity.

In an effort to elucidate the mechanism of defective NK cytotoxicity in LAS patients, we examined the frequency of the relevant effector cells using either morphological criteria (LGL morphology) or three moab-defined surface markers.

Table 1. Defective NK activity of LAS subjects on repeated assessment

Patient *n*°	Month (1984)	MNC	Specific lysis (%)
1	March	Normal donor	45·6
		Patient	13·9
	May	Normal donor	24·8
		Patient	4·5
	July	Normal donor	52·2
		Patient	5·9
2	April	Normal donor	32·1
		Patient	11·2
	May	Normal donor	24·8
		Patient	7·8
3	May	Normal donor	24·8
		Patient	2·8
	July	Normal donor	52·2
		Patient	0·0
4	May	Normal donor	24·8
		Patient	10·8
	July	Normal donor	52·2
		Patient	10·5

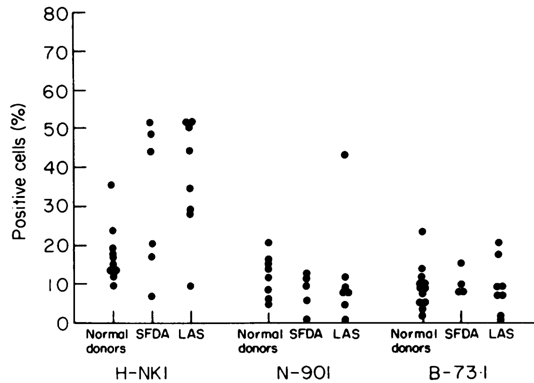

Fig. 2. Expression of moab-defined NK cell markers by MNC from LAS subjects with defective cytotoxicity.

As shown in Fig. 2 most LAS subjects with marked defects of NK function had an appreciable proportion of NK effectors, as judged by expression of moab-defined surface markers. Only two patients had very low levels of B73.1$^+$ and one of N901$^+$ cells. In four out of eight LAS subjects the frequency of HNK1$^+$ cells exceeded control values.

Similarly, when coded cytocentrifuge preparations were examined the frequency of cells with LGL morphology was similar in control ($16{\cdot}6 \pm 6{\cdot}3\%$ mean ± s.d.) and LAS ($19{\cdot}6 \pm 10{\cdot}6\%$) group. These results suggest that in most LAS patients with defects of NK cytotoxicity the reduced activity cannot be accounted for by a low frequency of relevant effector populations.

The capacity of cells of the monocyte-macrophage lineage to down-regulate NK function under various conditions is well established (e.g. Bordignon *et al.*, 1982). Thus we examined the NK cytotoxic potential of LAS effector cells after monocyte depletion. As shown in Fig. 3, monocyte depletion by adherence caused a modest augmentation of NK activity of LAS MNC only in two out of six experiments performed. As expected, the NK cytotoxicity of control donors was also not consistently modified by the same procedure.

The NK lytic reaction requires, as a first step, recognition and binding of target cells. The K562 binding capacity of LAS MNC with defective NK function was therefore assessed. The frequency of binders was similar in LAS patients ($6{\cdot}3 \pm 3{\cdot}1\%$, $n = 5$) and controls ($6{\cdot}2 \pm 0{\cdot}8\%$, $n = 3$).

A variety of agents have been shown to augment the cytotoxicity of human NK cells and the capacity of LAS NK cells to respond to positive regulatory signals was assessed.

Sufficient numbers of MNC to test this point were available from 3 patients. As shown in Table 2 exposure to IFN (natural β or recombinant γ) augmented the NK activity of LAS MNC to an extent comparable to that of controls in this limited series of assays.

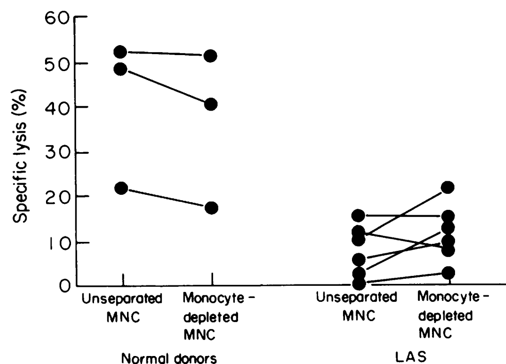

Fig. 3. Effect of monocyte depletion on the defective NK activity of subjects with LAS.

Table 2. Effects of IFN on NK activity of LAS patients

		Specific lysis (%) after incubation with:		
		medium	IFN-β	IFN-γ
Normal donors	1)	$14{\cdot}2 \pm 4{\cdot}8$	$21{\cdot}6 \pm 3{\cdot}6$*	$24{\cdot}2 \pm 3{\cdot}6$*
	2)	$15{\cdot}2 \pm 1{\cdot}6$	$35{\cdot}4 \pm 8{\cdot}6$†	$24{\cdot}6 \pm 5{\cdot}4$†
	3)	$13{\cdot}4 \pm 3{\cdot}0$	$26{\cdot}0 \pm 4{\cdot}2$†	$9{\cdot}2 \pm 2{\cdot}6$
SFDA	1)	$44{\cdot}0 \pm 5{\cdot}0$	$59{\cdot}8 \pm 2{\cdot}2$†	$51{\cdot}6 \pm 6{\cdot}6$*
	2)	$14{\cdot}0 \pm 2{\cdot}8$	$27{\cdot}8 \pm 3{\cdot}6$†	$19{\cdot}0 \pm 3{\cdot}4$*
	3)	$12{\cdot}6 \pm 2{\cdot}6$	$15{\cdot}0 \pm 2{\cdot}4$	$11{\cdot}6 \pm 3{\cdot}8$
LAS	1)	$0{\cdot}0 \pm 1{\cdot}0$	$6{\cdot}4 \pm 3{\cdot}6$*	$12{\cdot}0 \pm 1{\cdot}4$*
	2)	$8{\cdot}8 \pm 1{\cdot}6$	$12{\cdot}6 \pm 4{\cdot}8$	$17{\cdot}8 \pm 2{\cdot}0$*
	3)	$10{\cdot}8 \pm 5{\cdot}2$	$19{\cdot}6 \pm 4{\cdot}8$	$18{\cdot}0 \pm 3{\cdot}6$

* = $P < 0{\cdot}05$
† = $P < 0{\cdot}01$

DISCUSSION

The present study was designed to investigate NK activity in a group of intravenous drug abusers with LAS, recently identified in a Milanese suburban area. The outbreak of LAS in these subjects, their immunocompetence and lymphnode pathology have been described elsewhere (Lazzarin *et al.*, 1984; Parravicini *et al.*, 1984).

Peripheral blood MNC from LAS patients had defective NK activity compared with those from normal controls. A group of SFDA from the same area had NK levels intermediate between normal donors and LAS patients. It must be noted that at the time of writing one of the SFDA has developed LAS and that 6/10 SFDA tested are positive for anti-HTLV III antibodies, thus questioning the adequacy of these subjects as an appropriate control group (see Human subjects, Materials and Methods).

In an effort to elucidate the mechanism responsible for defective NK function in LAS patients, the frequency of NK effectors was evaluated morphologically and using moab-defined surface markers, focussing on those subjects who were functionally most impaired. LAS patients had normal levels of morphologically defined LGL and of N901$^+$ or B73.1$^+$ cells, with the possible exception of two subjects. Thus, inasmuch as morphology and monoclonal antibodies accurately identify NK cells, these results suggest that defective NK activity in LAS cannot be accounted for by a low frequency of the relevant effectors. Interestingly, four out of eight LAS patients tested had a considerably higher frequency than normal of HNK1-positive cells. HNK1 moab has been shown to react with NK cells and with a subset of conventional OKT8$^+$ lymphocytes (Lanier *et al.*, 1983). Therefore it can be speculated that the higher frequency of HNK1$^+$ cells in LAS is in fact the expression of the elevation of the relative proportion of OKT8-positive cells, previously described in these subjects (Lazzarin *et al.*, 1984). Double marker studies will be required to verify this likely possibility.

The expression of NK activity is subjected to positive and negative regulatory influences. Macrophage mediated suppression is the most thoroughly documented mechanism of cell-mediated down regulation of NK function, that plays an important role under physiological or pathological conditions, or after the administration of pharmacological agents (Bordignon *et al.*, 1982; Herberman & Ortaldo, 1981; Trinchieri & Perussia, 1984). Removal of monocytes from LAS MNC did not consistently augment NK activity. Thus, monocyte-mediated suppression is not the only or major determinant of reduced NK cytotoxicity in patients with LAS.

While NK cell cytotoxic function was reduced in LAS patients, NK effectors retained the ability to respond to stimulation with IFN (natural β or recombinant γ). This finding apparently contrasts

with the reported lack of responsiveness to IFN of AIDS NK cells (Rook *et al.*, 1983; Reddy *et al.*, 1984).

Although minor experimental differences between the protocols used in the two studies (e.g. time of incubation with IFN) caution against 'mechanical' comparisons, it is of interest that these investigators found an appreciable response to IFN-β in only one of six AIDS patients examined (Rook *et al.*, 1983). Hence one could speculate that progression to AIDS is associated with the development of unresponsiveness to IFN of NK cells.

The results reported here show that patients with LAS have defective NK activity, that cannot be accounted for by a low frequency of the relevant effector cells or by monocytic suppression. These observations suggest a functional defect at one or more of the post-binding steps required for NK cytotoxicity.

This work was supported by finalized project Oncology and by the Lombardy Region. The generous contribution of Italian Association for Cancer Research, Milan, Italy, is gratefully acknowledged.

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