*Eur J Cancer Clin Oncol*, Vol. 24, No. 3, pp. 369–376, 1988.
Printed in Great Britain

0277-5379/88$3.00+0.00


# Increased Number of CD4 Cells Able to Bind to Natural Killer Cell Targets in the Peripheral Blood of AIDS Related Complex Patients

VINCENZO BARNABA,* GUIDO VALESINI,* GIANNI GATTAMELATA,* ROSALBA BENVENUTO,* ANDREA VELARDI† and FRANCESCO BALSANO*

**Istituto I Clinica Medica, Università di Roma 'La Sapienza', Roma, Italy and †Istituto di Clinica Medica I, Università di Perugia, Perugia, Italy*

**Abstract**—*CD4 cells forming conjugates with natural killer target cells (K562 cells) were measured in the peripheral blood of anti-HIV antibody positive AIDS related complex (ARC) patients and in three control groups (asymptomatic individuals at risk, normal healthy people and patients with acute hepatitis B). These CD4 cells, which are unable to kill K562 cells, were significantly increased in ARC patients as compared to the control groups. Our data indicate that classical CD4 cells are partially replaced, in ARC patients, by a population of natural killer-target binding granular CD4 lymphocytes, and suggest that the functional abnormalities of helper T cells in these patients may be in part a consequence of the relative predominance of these non IL-2 and BCGF producing cells within the circulating CD4 population.*

## INTRODUCTION

THE acquired immunodeficiency syndrome (AIDS) is a severe disease of the immune system caused by a horizontally transmitted retrovirus. This virus, which has been referred to variously as human T-cell lymphotrophic virus type III (HTLV-III), lymphadenopathy associated virus (LAV), AIDS-associated retrovirus (ARV), and human immunodeficiency virus (HIV), will be called HIV in this paper [1].

The AIDS syndrome is the most severe clinical manifestation of HIV infection, but there appears to be a wide range of milder conditions, including persistent generalized lymphadenopathy and AIDS-related complex (ARC), a constellation of signs and symptoms manifested by persons belonging to high-risk groups [2].

HIV preferentially infects and destroys lymphocytes of the T helper subset [3], leaving the host unable to cope with a variety of infectious and neoplastic diseases.

It has been found that the CD4 antigen is the viral receptor for this virus, and that the infection can be blocked by monoclonal antibody to CD4 antigen [4, 5].

Accepted 7 September 1987.
Correspondence to: Guido Valesini, Clinica Medica I, Policlinico Umberto I, 00161 Roma, Italy.

Recently a subpopulation of cells bearing the T helper phenotypic marker (CD4) but sharing cytochemical and ultrastructural characteristics with natural killer (NK) cells, and accounting for 20% of the total adult blood CD4 cell subset, has been identified within the circulating pool of human lymphocytes [6]. These cells form conjugates *in vitro* with the NK target cell lines K562 and MOLT-4 without killing them, and contain cytoplasmic granules similar in ultrastructure and cytochemistry to those previously defined as large granular lymphocytes (LGL) with NK function [7, 8].

In patients with B-cell lymphoproliferative disorders and various solid tumors, NK target binding granular CD4 cells are increased (up to 80% of the whole CD4 population) and co-express NK-related surface antigens such as Leu7 and CD11 which are virtually absent on normal circulating CD4 cells [9]. A cell with a similar phenotype, CD4-Leu7, is selectively located within the germinal centers of normal and lymphoma lymphoid tissues [10]. Functional analysis of these populations [9] using clonal techniques [11] indicates that, in contrast to typical CD4 cells, CD4 cells with NK features are not capable of producing interleukine 2 (IL2) and B cell growth factor (BCGF).

The aim of the present study was to give further information about the changes induced by HIV in ARC patients by evaluating the NK-target binding

*Table 1. Clinical and laboratory features of patients with AIDS-related complex as compared with controls*

| | Total T cells | CD4/CD8 ratio | CD4 binding cells/mm | (%) | IgG (mg/dl) | IgA (mg/dl) | IgM (mg/dl) |
|---|---|---|---|---|---|---|---|
| *ARC patients' clinical features* | | | | | | | |
| 1. Hom. Fever, lap, wl, MCC | 831 | 0.6 | 181 | (56) | 2225 | 270 | 307 |
| 2. Hom. Fever, lap, MCC, d., w a | 991 | 0.6 | 186 | (51) | 1277 | 225 | 110 |
| | 1268 | 0.6 | 143 | (40) | 2202 | 144 | 177 |
| 3. Hom. Fever, lap | 552 | 0.9 | 181 | (55) | — | — | — |
| 4. Hom. Fever, lap, wl | 910 | 1.0 | 140 | (35) | 2162 | 115 | 110 |
| 5. Hom. Fever, lap | 1248 | 0.6 | 197 | (37) | — | — | — |
| 6. Hom. Fever, lap, d. | 1547 | 1.7 | 258 | (32) | 1399 | 189 | 243 |
| 7. Hom. Fever, lap | 1100 | 0.4 | 160 | (42) | 2102 | 146 | 151 |
| 8. Hom. Fever, lap | | | | | | | |
| 9. d.a. Fever, lap, wl, MCC, t.r. | 1382 | 0.5 | 239 | (53) | 2162 | 238 | 267 |
| 10. d.a. Fever, lap, d. | 813 | 0.8 | 169 | (41) | 2198 | 170 | 172 |
| 11. d.a. lap, MCC, CMV | 854 | 0.9 | 128 | (34) | 2354 | 99 | 632 |
| 12. d.a. Fever,lap, MCC | 1395 | 2.0 | 278 | (29) | 3193 | 217 | 255 |
| 13. d.a. Fever, lap, CMV | 873 | 0.8 | 170 | (37) | — | — | — |
| 14. d.a. Fever, lap | 1698 | 1.4 | 258 | (23) | 2220 | 245 | 268 |
| 15. d.a. Fever, lap, MCC, CMV | 2375 | 0.9 | 343 | (30) | — | — | — |
| Mean | 1189 | 0.9* | 202† | (39)‡ | 2082§ | 183 | 226 |
| (S.D.) | (454) | (0.4) | (60) | (10) | (475) | (54) | (140) |
| Asymptomatic at risk (22) | | | | | | | |
| Mean | 1290 | 1.3 | 166 | (22) | 1412 | 251 | 329‖ |
| (S.D.) | (311) | (0.4) | (82) | (8) | 204 | (70) | (167) |
| Hepatitis controls (6) | | | | | | | |
| Mean | 1426 | 1.3 | 160 | (22) | 2120 | 220 | 180 |
| (S.D.) | (320) | (0.3) | (30) | (1.5) | (205) | (32) | (15) |
| Healthy controls (22) | | | | | | | |
| Mean | 1290 | 1.7 | 169 | (20) | 1230 | 210 | 200 |
| (S.D.) | (422) | (0.2) | (41) | (5) | 120 | (30) | (33) |

*$t$ 3.9, $P < 0.0005$.
†$t$ 1.8, $P < 0.05$.
‡$t$ 7.1, $P < 0.0005$.
§$t$ 4.6, $P < 0.0005$.
‖$t$ 2.6, $P < 0.0005$.
Hom.: Homosexual men; d.a.: drug abusers; lap: lymphoadenopathy; wl: weight loss; d.: diarrhea; wa.: warts; t.r.: toxo-retinitis; MCC: mucocutaneous candidiasis; CMV: cytomegalovirus viremia.The $P$ values refer to the comparison with the healthy control group.

capability of their CD4 cell population.

The results indicate that the proportion of CD4 cells capable of binding to NK targets is increased in ARC patients as compared with either normal or high risk controls.

## MATERIALS AND METHODS

### *Patients*

Fifteen ARC patients (eight homosexual males and seven drug abusers), followed by the Istituto Clinica Medica I, Università di Roma 'La Sapienza', were studied. The criteria for the diagnosis were three or more of the following: lymphoadenopathy of > 1 cm at two or more noncontiguous, extra-inguinal sites for at least 3 months; intermittent or continuous fever of > 38°C for more than 1 month; loss of ≥ 10% of body weight; unexplained mucocutaneous candidiasis; and persistent diarrhea (Table 1).

The control groups consisted of 22 asymptomatic subjects (11 homosexual males and 11 drug abusers) matched for age and life style with the patient group, 22 healthy heterosexual males, and six patients with acute hepatitis B virus infection (HBsAg positive). The last two control groups had no history of drug abuse.

The clinical and laboratory features of all four groups are shown in Table 1.

### *Anti-HIV antibodies*

Anti-HIV antibodies were sought in the sera of patients and controls using an ELISA test (Abbott HTLV-III EIA, Abbott Laboratories) with Western Blot confirmation.

### *Cell separation and T cell subsets*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood

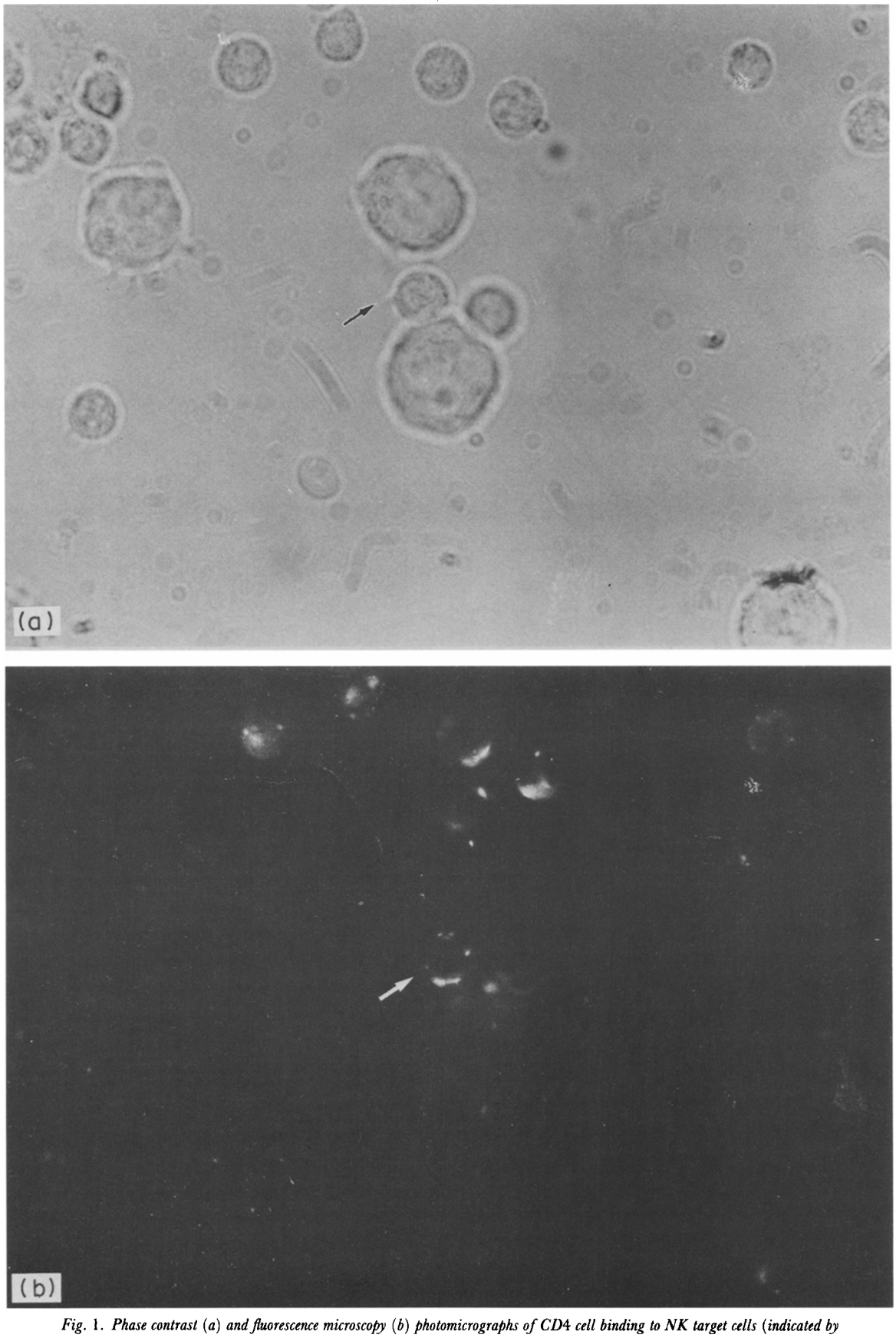


*Fig. 1. Phase contrast (a) and fluorescence microscopy (b) photomicrographs of CD4 cell binding to NK target cells (indicated by the arrow). The ability of K562 binding is not a property of all CD4 cells and not all the K562 binding cells are CD4.*

through Ficoll–Hypaque density gradient centrifugation and washed three times in RPMI 1640 (Eurobio, Paris, France) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, U.S.A.), 1% glutamine (Gibco), 25 mM hepes (Gibco), 100 U/ml penicillin and 10 μg/ml streptomycin (Eurobio) ('complete medium').

Monoclonal antibodies (MoAbs) OKT3, OKT4, OKT8 and OKIa (recognizing respectively a pan-T antigen; at cell subset consisting of helper-inducer T cells, a T cell subset consisting of suppressor-cytotoxic T cells; and monocytes, B and activated T-lymphocytes) were purchased by Ortho, Raritan, NJ, U.S.A. MLR4 and 5/9 MoAbs, recognizing respectively activated T lymphocytes and a minor subset of T lymphocytes (20 ± 5%) responsible for maximal helper activity for B cell differentiation, were kindly provided by G. Corte [12, 13].

One hundred microliters of the PBMC suspension adjusted to $10 \times 10^6$ cells/ml was incubated with 5 μl of MoAb for 30 min at 4°C in phosphate buffered saline (PBS). After washing in complete medium, the cells were incubated with 100 μl of FITC-conjugated goat anti-mouse IgG serum for 30 min at 4°C. After three additional washings the cells were resuspended in 50 μl of PBS. The antibody labelled cellular suspensions were then mounted wet and examined using a Leitz Orthoplan microscope.

*Enumeration of CD4 cells binding to NK target (K562 cells)*

PBMC ($0.5 \times 10^6$) were stained with OKT4 MoAb and FITC-conjugated goat antimouse serum (Ortho) and mixed with $0.5 \times 10^6$ K562 cells (human erythroblastoid cell line) in 6 × 50 mm test tubes.

In some experiments, $0.5 \times 10^6$ PBMC were stained using directly labeled F(ab')2 fragments from the OKT4 antibody (prepared by pepsin digestion following Hudson and Hay [14]) to exclude the possibility that the labeling of the CD4 cells with an IgG2a (OKT4) antibody and fluorescent anti-Ig prior to binding exposes the Fc fragment of the antibody, which might interfere with an Fc receptor positive cell, such as K562.

In our experiments, as in the previous report of Velardi *et al.* [6], the binding of CD4 positive cells to K562 cells proved unaffected by the presence of OKT4 antibody on the surface.

The lymphocyte-target cell mixtures were centrifugated at 100 ***g*** for 7 min and the pellet was incubated in a 37°C water bath for 10 min. After incubation all but one drop of the supernatant was discarded and the cell sediment was gently resuspended and examined under a fluorescence microscope. CD4 cell binding to K562 cells was estimated by comparing the count of fluorescent cells forming conjugates to the total number of fluorescent cells (Fig. 1a,b). At least 200 CD4 cells were scored in each experiment.

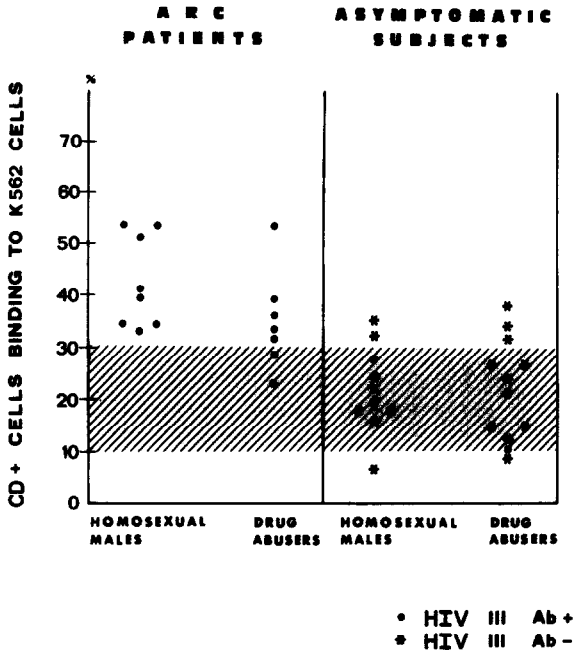


*Fig. 2. Percentage of CD4 cells binding K562 in ARC patients and asymptomatic at risk subjects. The shaded area represents the normal range.*

*Statistical analysis*

Statistical comparisons were performed using Student's *t*-test [15].

## RESULTS

*Anti-HIV antibody positivity*

Anti-HIV antibodies were detectable in all the ARC patients and in three of the 22 asymptomatic 'at risk' controls (two homosexual and one drug abuser); all healthy controls and hepatitis patients were seronegative (Fig. 2).

*Phenotypic analysis of lymphocytes*

There was no difference among groups in the number of CD3 cells per $mm^3$ (Table 2), but the CD4/CD8 ratio was significantly decreased in ARC patients (0.9) over asymptomatic 'at risk' individuals (1.3), hepatitis patients (1.3) and healthy controls (1.7) (Table 1). The absolute number of cells with a CD4 phenotype was significantly less in ARC patients (mean 577 ± 308) than in normal (mean 835 ± 258) or 'at risk' controls (mean 800 ± 230) (Table 2). CD8 cells were significantly increased in ARC patients (mean 690 ± 172; $P < 0.01$) over normals (468 ± 159; $P < 0.01$), while 'at risk' individuals had a non-significant increase (mean 540 ± 208) (Table 2). Thus, the decreased CD4/CD8 ratio was determined both by

*Table 2. Peripheral blood lymphocyte subsets (cells/mm³)*

| | Groups | *n* | Mean | S.D. | *t* | *P* |
|---|---|---|---|---|---|---|
| CD4 | ARC | 15 | 202 | 60 | 1.8 | < 0.05 |
| b.K562 | 'at risk' controls | 22 | 166 | 82 | — | ns |
| | healthy controls | 22 | 169 | 41 | | |
| CD3 | ARC | 18 | 1207 | 480 | — | ns |
| | 'at risk' controls | 19 | 1297 | 510 | — | ns |
| | healthy controls | 22 | 1290 | 422 | | |
| CD4 | ARC | 18 | 577 | 308 | 2.4 | <0.01 |
| | 'at risk' controls | 19 | 800 | 230 | — | ns |
| | healthy controls | 22 | 835 | 258 | | |
| CD8 | ARC | 18 | 690 | 272 | 2.6 | <0.01 |
| | 'at risk' controls | 19 | 540 | 208 | — | ns |
| | healthy controls | 22 | 468 | 159 | | |
| Ia | ARC | 18 | 226 | 110 | — | ns |
| | 'at risk' controls | 19 | 194 | 100 | | |
| | healthy controls | 22 | 198 | 96 | | |
| 5/9 | ARC | 18 | 173 | 84 | 2.7 | <0.005 |
| | 'at risk' controls | 19 | 182 | 94 | 2.4 | <0.01 |
| | healthy controls | 22 | 281 | 142 | | |
| MLR4 | ARC patients | 18 | 122 | 69 | 1.9 | <0.025 |
| | 'at risk' controls | 19 | 148 | 89 | 2.5 | <0.005 |
| | healthy controls | 22 | 68 | 92 | | |

The *P* values refer to the comparison with the healthy control group.

a decrease in CD4 cells and an increase in CD8 cells.

A decrease of 5/9 cells was detected in the ARC group and, to a lesser extent, in asymptomatic at risk subjects, while MLR4 cells were increased in both groups (Table 2).

*NK-target binding capability of CD4 cells*

The percentage of CD4 cells binding to K562 cells was abnormally high in 13 of 15 ARC patients and in five out of 22 asymptomatic 'at risk' individuals (Fig. 2); the absolute number of K562-binding cells was also increased over all three control groups, though to a lesser degree (Table 1). There was no difference in K562 cell binding between homosexual ARC patients and those who were drug addicts.

Labelling of CD4 cells with IgG2a (OKT4) antibody and fluorescent anti-Ig or with labelled F(ab')2 fragment from OKT4 antibody produced no difference in the percentage of binding to K562 cells, excluding the possibility of an artifact.

## DISCUSSION

The results of this study demonstrate not only a decrease of CD4 cells in ARC patients but an even more significant decrease in 5/9 cells, a category which comprises the entire helper/inducer subset [12]. This may account for the severe impairment of helper function in HIV infection that eventually can lead to opportunistic infections and malignancies. Moreover, our results demonstrate that in ARC patients an increased proportion of CD4 cells are capable of binding to NK targets.

Recently, it has been shown that 20% of normal adult blood CD4 cells exhibit granular lymphocyte morphology and the NK-related property of binding to NK-target cells (K562 or MOLT-4), though without killing them [6]. In the circulation of patients with lymphoproliferative disorders and various solid tumors, and within the germinal center of normal and lymphoma lymphoid tissues, such cells also express Leu7 and CD11 NK-related surface antigens [9]. Cloned CD4 cells co-expressing NK-related surface antigens, in contrast to typical CD4 cells, are not capable of producing IL-2 and BCGF and, thus, of providing helper function [11]. Our present data indicate that CD4 cells in HIV antibody positive ARC patients are partially replaced by a population of NK-target binding granular CD4 lymphocytes.

Several *in vitro* T-cell functional abnormalities have been identified in AIDS and ARC patients, including deficiencies in blast transformation, lymphokine production, IL-2 receptor expression, alloreactivity and ability to provide help to B cells [16]. Such abnormalities are a consequence of the infection of CD4 cells by the retrovirus HIV, which is cytotropic and cytopathic for this T cell subset. Our present data suggest that the helper T cell functional abnormalities in ARC patients may, at least in part, be a consequence of the relative predominance of a subgroup of NK target-binding

but non IL-2 and BCGF producing granular cells within the circulating CD4 population.

Studies in patients with the Chediak–Higashi syndrome have shown that granular CD4 lymphocytes share more characteristics with NK cells than with T-lineage cells [6]. NK cell function in these patients is impaired, and the Leu7 NK population displays characteristic lysosomal inclusions [17]. NK-target binding CD4 cells from Chediak–Higashi patients exhibit lysosomal abnormalities and share a series of defects with bone marrow derived cells such as granulocytes, monocytes, platelets and B cells, whereas non-binding CD4 cells contain normal Gall bodies without evidence of lysosomal fusions and have normal T cell function [18]. Moreover, in normal individuals, a three-fold lower level of NK-target binding CD4 cells are found in cord blood as compared with adult blood, a development pattern typical of NK-cells [6]. These data suggest that NK-target binding CD4 cells may share a developmental origin with NK cells.

The relative predominance of NK-target binding CD4 cells in ARC patients could result from selective viral destruction of typical, non-NK-target binding CD4 cells by HIV, and thus be another reflection of the developmental divergence of these two subsets. Our findings of a greater divergence between patients and controls in the proportion of NK-target binding cells rather than in their absolute number lends support to this hypothesis. HIV infection could then represent an experimental model for further studies of the characteristics and functional properties of CD4 granular lymphocytes.

Lane *et al.* [16] have demonstrated that the CD4 cells in AIDS patients were quantitatively and qualitatively abnormal. The quantitative defect is easily explained with the cytopathic effect of HIV infection [3], while the qualitative abnormality could be related to the increased proportion of non-functional CD4 granular lymphocytes within the residual population of circulating CD4 cells.

**Acknowledgements**—Supported in part by a grant (86.01553.52) from Consiglio Nazionale delle Ricerche (CNR) and by the Fondazione Andrea Cesalpino (Roma).

## REFERENCES

1. Gallo RC, Salahuddin SZ, Popovic M *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLVIII) from patients with AIDS and at risk for AIDS. *Science* 1984, **224**, 500–503.
2. Fauci AS, Masur M, Gelman EP, Markham PD, Hahn BH, Lane HC. The acquired immunodeficiency synrome: an update. *Ann Intern Med* 1985, **102**, 800–813.
3. Klatzmann D, Barre-Sinoussi F, Nugeyre MT *et al.* Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. *Science* 1984, **225**, 59–63.
4. Dalgleish AG, Beverly PCL, Clapman PR, Crawford DH, Greaves MF, Weiss RA. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 1985, **312**, 763–766.
5. McDouglas JS, Mawle A, Cort SP *et al.* Cellular tropism of the human retrovirus HTLVIII/LAV. *J Immunol* 1985, **135**, 3151–3162.
6. Velardi A, Grossi CE, Cooper MD. A large subpopulation of lymphocytes with T helper phenotype (Leu3/T4+) exhibits the property of binding to NK targets and granular lymphocyte morphology. *J Immunol* 1985, **134**, 58–64.
7. Grossi CE, Webb SR, Zicca A *et al.* Morphological and histochemical analysis of two human T cll subpopulation bearing receptors for IgM or IgG. *J Exp Med* 1978, **147**, 1405–1417.
8. Timonen T, Ortaldo JR, Herberman RB. Characterization of human large granular lymphocytes and relationship to natural killer and K cells. *J Exp Med* 1981, **153**, 569–582.
9. Velardi A, Clement L, Grossi CE. Qualitative and functional analysis of a human lymphocyte subset with T helper (Leu3/T4) phenotype and NK cell characteristics in patients with malignancies. *J Clin Immunol* 1985, **5**, 329–334.
10. Velardi A, Tilden AB, Millo R, Grossi CE. Isolation and characterization of Leu7+ germinal center cells with T-helper cell phenotypic and granular lymphocyte morphology. *J Clin Immunol* 1986, **6**, 205–215.
11. Velardi A, Mingari MC, Moretta L, Grossi CE. Germinal center cells coexpressing T4 (helper) and Leu7 (NK) antigens display T-lineage functional characteristics. *Fed Proc* 1985, **44**, 1291.
12. Corte G, Mingari MC, Moretta A, Damiani G, Bargellesi A, Moretta L. Human T cell subpopulations defined by monoclonal antibodies: 1. A small subset is responsible for proliferation to allogenic cells or to soluble antigens and for helper activity for B cells differentiation. *J Immunol* 1982, **128**, 16–19.
13. Corte G, Moretta L, Daminai G, Mingari MC, Bargellesi A. Surface antigens specifically expressed by activated T cells in humans. *J Immunol* 1981, **11**, 162–164.
14. Hudson L, Hay FC. *Practical Immunology*. Oxford, Blackwell, 1980.
15. Swinscow TDV. *Statistics at Square One*. London, British Medical Association, 1977.
16. Lane HC, Depper JM, Greene WC, Whalen G, Waldmann TA, Fauci AS. Qualitiative

analysis of immune function in patients with the acquired immunodeficiency syndrome. *N Engl J Med* 1984, **313**, 79–84.
17. Haliotis T, Roder J, Klein M, Ortaldo J, Fauci AS, Herberman RB. Chediak–Higashi gene in humans. I. Impairment of natural killer function. *J Exp Med* 1980, **151**, 1039–1048.
18. Grossi CE, Crist WM, Abo T, Velardi A, Cooper MD. Expression of the Chediak–Higashi lysosomal abnormality in human peripheral blood lymphocytes subpopulations. *Blood* 1985, **65**, 837–844.