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# Spontaneous Natural Killer Cell Activity in Childhood Acute Lymphoblastic Leukaemia

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**Abstract**—*Endogenous NK activity was measured in ALL patients and compared with child and adult control values. ALL children undergoing maintenance chemotherapy showed significantly lower NK activity than control groups; however, patients off treatment and in remission expressed cytotoxicity within the normal range of the control groups. The expression of the HNK-1 marker in the ALL children was not significantly different from child controls, although ALL patients failed to show the same correlation between cytotoxic activity and HNK-1 expression. The target binding capacity of PBLs from ALL children was significantly greater compared with controls but did not correlate with NK activity. In addition plasma from ALL patients was not inhibitory for NK cytolytic activity, suggesting an innate defect in cytotoxicity mediated by NK cells rather than a plasma inhibitory factor. Patients failing to display NK activity against K562 target cells appeared to mediate killing of measles virus infected (Raji) targets.*

## INTRODUCTION

THE TREATMENT of childhood acute lymphoblastic leukaemia (ALL) is characterized by the induction of a remission state maintained by long-term chemotherapy. Such drug regimens have been shown to cause a severe depletion in circulating T- and B-lymphocyte populations [1, 2] with consequent abnormalities in immunoglobulin production [3, 4] and response to mitogen stimulation [2, 5, 6]. This phenonomenon of drug-induced immunosuppression has been used as an explanation for the increased incidence of infection observed in ALL patients undergoing chemotherapy as compared to that in normal individuals [7, 8].

Recently, the importance of non-specific innate resistance mediated, for example, by interferon and natural killer (NK) cells, in combating viral infections has been emphasized [9]. In children with ALL undergoing maintenance chemotherapy the status of natural resistance may be especially relevant since it has previously been reported that circulating NK activity is depressed in ALL children [10–12].

In the present study, we report further on the apparent functional abnormality of NK cells in childhood ALL, using conventional NK-susceptible target cells and measles virus-infected target cells.

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## MATERIALS AND METHODS

*Patients*

Forty-two unselected children (25 boys and 17 girls) aged 3–15 years were included in the study. All had been receiving remission maintenance therapy on the MRC UKALL VIII treatment schedule for a period of up to 3 years. Therapy consisted of continuous 6-mercaptopurine and methotrexate pulsed with prednisolone and/or vincristine. Dosage schedules were adjusted on a sliding scale in the face of neutropenia or thrombocytopenia. Heparinzed blood samples (5 ml) were obtained immediately prior to the intravenous administration of drugs.

Control blood samples were obtained from 21 children (eight boys and 13 girls; age $2\frac{1}{2}$–12 years) attending either orthopaedic outpatients at the Children's Hospital, Sheffield or undergoing routine tests prior to orthopaedic surgery. Additional control blood samples were obtained from healthy adult volunteers.

*Isolation of peripheral blood lymphocytes (PBLs)*

Mononuclear cells were separated from heparinized blood [13], harvested and washed (× 2) in RPMI-1640 (Flow Laboratories Ltd.), supplemented with 10% v/v new born calf serum (Gibco Ltd. Paisley, U.K.), 0.225% w/v sodium bicarbonate (BDH Ltd., Atherstone, U.K.), 20 mM Hepes buffer (BDH), penicillin (100 U/ml) and streptomycin (100 μg/ml) (RPMI-NBCS). The PBLs were

counted and diluted in RPMI-NBCS to a concentration of $2.5 \times 10^6$ in 1 m volumes in plastic bijoux. The cells were then incubated overnight at 37°C in a 5% $CO_2$ in air atmosphere.

*Target cells*

The human leukaemic cell line K562 and the Raji cell line were used in NK cytotoxicity assays, and were maintained as stationary suspension cultures in RPMI-NBCS.

Raji cells infected with measles virus at a multiplicity of infection (MOI) of 0.50 for 48 h were used to measure natural cytotoxicity towards virally infected targets.

*Cytotoxicity assay*

Cytotoxicity was measured in a 4 h $^{51}Cr$ release assay. Target cells were labelled for 1 h with 100 μCi Na $^{51}Cr_2O_4$ (Radiochemicals Centre, Amersham, U.K.) in a 0.1 ml volume at 37°C. The cells were washed (× 2) in RPMI/NBCS and incubated for a further hour. The targets were then washed (× 3), counted and diluted to $1 \times 10^5$ ml.

Following overnight incubation effector cells were washed (× 2) in RPMI-NBCS, counted and diluted to $1.2 \times 10^6$/ml. Serial dilutions were performed in the wells of flexible round-bottomed microtitre plates (Becton-Dickinson) and target cells added (final volume of 0.2 ml/well) to give effector : target (E : T) ratios of 12 : 1, 6 : 1 and 3 : 1, in 0.2 ml triplicate volumes. The plates were incubated for 4 h at 37°C in a humidified 5% $CO_2$ in air atmosphere. At the end of this period the cells were pelleted (5 min at 200 ***g***) and half the supernatant from each well removed into separate wells. The plates were dried with parafilm and individual wells counted in a Packard gamma-spectrophotometer. The test release (%) was calculated and the specific $^{51}Cr$ release (cytotoxicity) calculated using the following formula:

$$\frac{\text{Sample \% release} - \text{spontaneous \% release}}{100 - \text{spontaneous \% release}} \times 100.$$

*Conjugate assay*

$10^5$ K562 and $10^5$ PBLs were admixed in a volume of 0.2 ml in a 10 ml conical test tube and left at room temperature (RT) for 10 min, centrifuged for 5 min at 100 ***g***, and the pellets allowed to stand on ice for a futher 5 min. The cells were then gently resuspended and kept on ice until they were counted. The number of PBLs binding to K562 target cells was enumerated by microscopic observation in a haemocytometer; at least 200 PBLs were counted in each sample.

*Staining of PBLs by indirect immunofluorescence*

$5 \times 10^5$ PBLs were centrifuged for 5 min at 500 ***g*** and the supernatant removed. One hundred μl of anti-Leu-7 monoclonal antibody (Becton-Dickinson Ltd.) was added, the cells resuspended and kept at RT for 20 min, and then washed in PBS (× 2). One hundred μl of fluorescein conjugated goat anti-mouse anti-serum (Tago) was added, the cells resuspended and allowed to stand at RT for a further 20 min, prior to washing (× 3) in PBS and resuspending in the residual PBS. The percentage staining of PBLs was enumerated by flow cytometry using a FACS 420 (Becton-Dickinson). Stock solutions of antisera were titrated against normal PBLs and diluted accordingly.

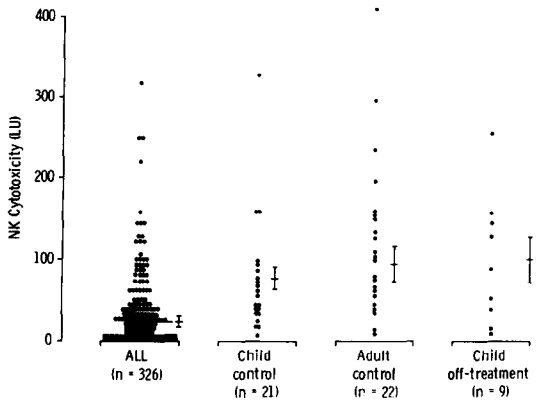


*Fig. 1. Spontaneous levels of NK cytotoxicity in ALL children and controls. Bar lines indicate mean ± SE. LU/$10^7$ lymphocytes were calculated at 20% killing.*

*Statistics*

Lytic units (LU) of NK activity were calculated from cytotoxic values by Von Krogh analysis [14]. Statistical analysis was by Student's *t* test and regression analysis.

## RESULTS

*Spontaneous levels of NK cytotoxicity in ALL children—correlation with clinical parameters*

Endogenous NK activity was measured in ALL patients, and in child and adult controls. NK activity of ALL children undergoing maintenance chemotherapy (LU = 20.8 ± 2.1) was significantly depressed with respect to that of the child (LU = 75.3 ± 15.6, $P < 0.001$) and adult (LU = 125.0 ± 20.7, $P < 0.001$) control groups (Fig. 1). Only 27/352 (7.0%) of PBL samples from ALL children tested had lytic activity above the mean for the child control group, which were comparable by age to ALL patients. There was no significant difference between the lytic activity of the control groups. Thirty-seven of the 42 ALL children tested had either no activity or only a baseline level of activity on at least one occasion during the study.

*Table 1. Natural cytotoxicity of ALL PBLs against measles-infected and K562 target cells*

| PBLs from | | Target cells—cytotoxicity in LU* Raji | MV-Raji | K562 |
|---|---|---|---|---|
| Patients | 1 | 0.1 | 44.4 | 20.1 |
| | 2 | 0.1 | 81.3 | 82.9 |
| | 3 | 0.1 | 55.6 | 70.9 |
| | 4 | 0.1 | 21.7 | 0.1 |
| | 5 | 0.1 | 110.7 | 0.1 |
| | 6 | 0.1 | 183.6 | 139.3 |
| | 7 | 0.1 | 54.8 | 64.7 |
| Controls | 1 | 0.6 | 75 | 31.3 |
| | 2 | 0.1 | 67 | 12.0 |
| | 3 | 0.1 | 16 | 24.3 |
| | 4 | 6.3 | 72.6 | 41.0 |
| | 5 | 8.7 | 95.9 | 165.3 |

*PBLs isolated from (a) ALL patients and (b) adult controls were assayed simultaneously for their ability to lyse measles virus-infected (MV-Raji) and non-infected Raji cells and K562 targets. Cytotoxicity is expressed as LU/$10^7$ at 20% killing.

In addition, we studied the NK activity of PBLs isolated from seven patients with ALL who were off treatment and in remission (Fig. 1). No significant difference between the lytic ability of PBLs from normal children and those ALL patients off chemotherapy was demonstrated ($P < 0.30$), although these cytotoxicities were significantly elevated above those of children still undergoing chemotherapy ($P < 0.001$).

Regression analysis showed no correlation between age ($r = 0.22$, $n = 27$) or duration of treatment ($r = 0.04$, $n = 27$) and the expression of NK function in ALL patients. There was no significant difference with respect to sex in either patient or control groups, and total white cell, lymphocyte or neutrophil counts at the time of sampling also showed no correlation with NK status. Neither erythrocyte 6-mercaptopurine levels at the time of sampling nor the dose of 6-mercaptopurine correlated with the NK function of PBLs from ALL patients. During the course of the study several patients relapsed, although no significant difference was observed between the NK activity in patients prior to relapse, and those children who had not relapsed.

PBLs from seven ALL patients were tested against K562, Raji and measles virus-infected Raji (MV-Raji) target cells in a 6 h chomium-release assay (Jermy, Jennings and Rees, paper in preparation). The results in Table 1 show that whilst all the PBL populations tested (7/7) demonstrated significant cytotoxicity against MV-Raji and not uninfected targets, only 4/7 PBL samples were able to 'kill' K562 targets, contrasting with the activity of PBLs from normal individuals. Although in some experiments, low level cytotoxicity is expressed against Raji cells alone, a greater susceptibility to 'killing' is exhibited by MV-Raji. However, PBLs from normal adults were capable of killing MV-Raji and K562 targets equally well.

*Studies on the mechanism of depressed NK cytotoxicity in ALL*

The percentage of PBLs expressing the HNK-1 marker in ALL children (4.6 ± 0.8 SE) was lower than in normal adults (8.8 ± 1.4 SE, $P < 0.02$) but was not significantly different from child controls (5.0 ± 0.9, $P < 0.5$). Whereas both adult and child controls exhibited a positive correlation between HNK-1 expression and NK activity ($P < 0.001$ and $P < 0.05$, respectively), no such relationship was demonstrated for PBLs from ALL patients ($n = 46$, $r = 13$) (Fig. 2).

The percentage of K562 target binding PBLs from ALL children (15.7 ± 1.3) was significantly enhanced compared with controls (7.1 ± 0.9, $P < 0.001$) although there was no correlation between NK activity and percentage target-binding PBLs for either group (Fig. 3).

In order to test whether serum factors were contributing to NK suppression, ALL plasma samples were incubated with PBLs from normal individuals and their effect on NK activity studied. In three separate experiments (Table 2), there was no consistent effect on the NK activity when plasma

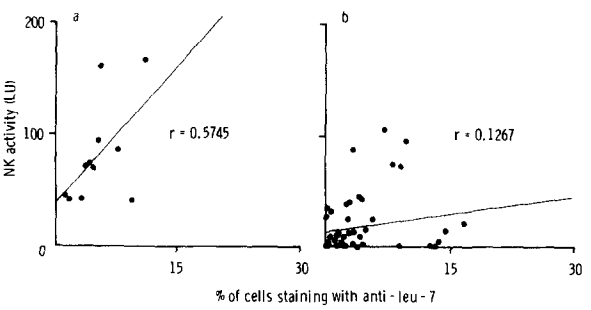


*Fig. 2. Regression analyis of NK activity (LU) against percentage HNK-1$^+$ cells for (a) child controls and (b) ALL patients.*

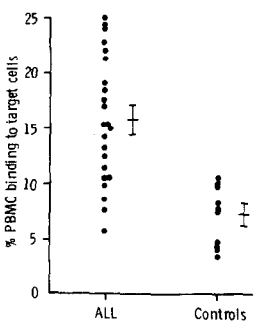


*Fig. 3. Percentage of K562 binding PBLs for ALL and child controls (bar lines indicate mean ± SE).*

*Table 2. The effect of plasma from ALL patients on normal NK activity*

| Plasma* from | LU (percentage value of media control) Exp. 1 | Exp. 2 | Exp. 3 |
|---|---|---|---|
| ALL patients | 54.9 (165.8) | 89.9 (138.1) | 225.5 (92.0) |
| | 22.4 (67.6) | 74.5 (114.4) | 132.9 (54.0) |
| | 38.8 (117.2) | 78.8 (121.0) | 217.4 (88.1) |
| | 33.4 (100.9) | 95.6 (146.8) | 239.8 (98) |
| | 39.7 (119.9) | 100.7 (154.7) | 176.8 (72) |
| | 34.0 (102.7) | | |
| | 27.1 (81.9) | | |
| Controls | 23.9 (72.2) | 57.3 (88) | 199.1 (81) |
| | 23.6 (71.2) | 87.0 (133.6) | 184.5 (75) |
| | 38.6 (116.6) | 123.5 (189.7) | 88.4 (36.8) |
| | 23.0 (69.4)* | 88.2 (135.4) | 174.4 (71.5) |
| None | 33.1 | 65.1 | 243.9 |

*2.5 × $10^6$ PBLs from normal individuals were incubated in 20% v/v patient or control plasma in RPMI medium for 18 h at 37°C. The cells were extensively washed and used in 4 h-cytotoxicity assays against K562 targets. Cytotoxicity values are expressed as LU/$10^7$ lymphocytes at 20% killing for three separate experiments. Figures in brackets indicate percentage value of the media control.

from ALL patients or controls was preincubated with normal PBLs *in vitro*. In addition, no effect on NK activity was observed when plasma was present throughout the cytotoxicity assay (data not shown).

## DISCUSSION

The results presented here confirm previous studies [9, 10] that demonstrated the NK activity of children undergoing maintenance chemotherapy for ALL to be severely depressed. In common with other workers [15, 16], it was found that following cessation of chemotherapy, NK activity in ALL children returned to normal levels. Abo *et al.* [17] have reported a correlation between age and NK activity in normal individuals. There was no such correlation in the present study. However, the expression of HNK-1 on PBLs from ALL children, adult and child controls was found to be consistent with that reported by these authors.

No correlation between age, duration of treatment, sex, white blood cell count, lymphocyte count or neutrophil count at the time of sampling and NK function was established. Interestingly, neither dosage of 6-mercaptopurine nor levels of 6-thioguanine nucleotide, the active metabolite of 6-mercaptopurine, was related to NK activity in ALL children (results not shown).

The depressed NK activity observed in ALL can be attributed to either reduced NK cell numbers or abnormal function of existing NK cell populations. The results of this study support the latter of these two hypotheses since no correlation could be established between HNK-1 expression and ALL cytotoxicity. These results support recent findings in patients with ALL [12] and malignant lymphoma [18] but contradict those in carcinoma patients [19] and renal transplant patients receiving azathioprine [20]. The depressed NK function in ALL cannot be explained by a reduction in the number of PBLs with 'receptors' for K562 since target-binding lymphocytes are significantly increased in ALL children compared with controls. Further analysis is required to evaluate whether the target-binding lymphocytes are functionally immature or functionally inactive LGLs, as has been reported in SLE [21] and CLL [22], or defective in their lytic mechanism. It is unlikely that all target-binding lymphocytes from ALL children are NK cells since 15.7% of PBLs bind to K562 targets but only 4.6% of PBLs express the surface determinant, HNK-1, and the possibility that PBLs from ALL children non-specifically block NK target structures on K562 cells cannot be ruled out.

Soluble factors, particularly prostaglandins, have been shown to suppress NK activity in normal individuals [23]. Preincubation for 18 h in vitro of normal PBLs with ALL plasma has shown no conclusive suppressive effect in this study; although this appears to negate a direct role for a soluble suppressor factor in ALL plasma, the presence of a molecular species mediating its effect indirectly by initiating the production of a suppressor factor from other cell types, for example adherent monocytes, should not be discounted. Suppressor cells of human natural killer cell activity have been described in both normal individuals and cancer patients [24] but it has not been possible to address this question in the present study.

Since ALL patients are highly susceptible to viral infections it was considered pertinent to investigate

the ability of PBLs from ALL children to 'kill' virus-infected targets as well as the classically NK sensitive target K562. The preliminary data reported indicate that PBLs from ALL children can lyse measles virus-infected cells, and not their uninfected counterparts irrespective of their capacity to 'kill' K562 targets. This would suggest that while ALL children have demonstrable depressed NK activity towards K562 cells, PBLs from these patients appear to maintain a natural cytotoxicity against measles virus-infected target cells.

In summary, we have shown that in ALL children the depressed NK activity observed using K562 targets is not due to depletion of NK cells *per se* but appears to be a functional abnormality. The evidence (presented in the accompanying paper) that endogenous NK activity is largely responsive to immunomodulation by interferon and interleukin-2 supports this view. In addition, preliminary data suggests a more prudent selection of appropriate target cells for use in cytotoxicity assays should be made for assessing NK activity in cancer patients.

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