Clin. exp. Immunol. (1990) **80**, 241–246

Loss of natural killer activity as an indicator of relapse in acute leukaemia

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(Accepted for publication 24 November 1989)

SUMMARY

A role for naturally occurring cytotoxic cells in immunosurveillance against malignancy has been presumed in several studies. The natural killer activity (NKA) of peripheral blood mononuclear cells was therefore measured at regular intervals in patients with acute leukaemia and expressed specific cytotoxicity. Sixty controls had a median NKA of 33·6% (range 15·4–71). Seventy-three patients with acute lymphoblastic leukaemia (ALL) and acute non-lymphoblastic leukaemia (ANLL) with untreated or relapsed disease had a median activity of 2·4% (range 0–13·4) ($P < 0{\cdot}001$), while 57 patients who had achieved complete remission had a median activity of 22·7% (range 9·5–64·4). In 15 patients, reductions of NKA were seen prior to 16 episodes of relapse. In ten of these (nine with ANLL and one with ALL), 11 relapses were preceded within 10 weeks by drops in NKA to less than 30% of remission levels. The median NKA of the group prior to the drop in activity was 25·5% and the median first low value was 6·0%. Five patients who relapsed after allogeneic bone marrow transplantation had significant, sustained drops in NKA, 5–9 weeks earlier. The median NKA prior to the drop was 25·6% and the median first low value was 8·0%. We therefore conclude that there was a marked reduction in NKA in patients with active acute leukaemia when compared with healthy blood donors and that this activity substantially improved in complete remission. All patients who relapsed had significantly reduced NKA which in some, significantly preceded the time of relapse. These data suggest that the regular assessment of NKA in patients with acute leukaemia may be a useful diagnostic tool.

Keywords acute leukaemia natural killer relapse

INTRODUCTION

A role for perturbations of the immune system in the pathogenesis and management of acute leukaemia has been sought for many years (Herberman & Ortaldo, 1981; Ruco *et al.*, 1983; Komiyama *et al.*, 1984; Sorskaar, Lie & Forre, 1985; Dickinson *et al.*, 1985). To date, few studies have shown any impact on therapeutic results with the use of non-specific immunotherapy (Paton *et al.*, 1982). Based on multiple studies of the natural killer (NK) cell in many malignant processes, we sought to examine this cell, a specific component of the cellular immune system, for its putative role in acute leukaemia.

Changes in NK cell activity (NKA), assessed in peripheral blood lymphocytes, has been reported to be of significance in many malignant conditions (Tursz *et al.*, 1982; Balch *et al.*, 1983; Gastl *et al.*, 1984; Brenner, Friedman & Margolese, 1985), including leukaemia. NK cells are a heterogeneous population of lymphoid cells capable of spontaneous lysis of certain tumour and virus-infected cells (Trinchieri & Santoli, 1978; Ching & Lopez, 1979; Herberman & Ortaldo, 1981). They are characterized morphologically as large granular lymphocytes (LGL) and are present in blood and lymphoid tissues (Herberman & Holden, 1978; Herberman & Ortaldo, 1981). Their exact lineage is controversial since they have been reported to bear monocyte (Barada *et al.*, 1980), null lymphocyte (Bakacs *et al.*, 1977) and T lymphocyte surface antigens (Kay *et al.*, 1977), as well as some unique markers (Lanier *et al.*, 1983). Several investigators have reported decreased NKA in patients with untreated leukaemia (Herberman & Ortaldo, 1981; Balch *et al.*, 1983; Sorskaar *et al.*, 1985); however, both low (Matera & Giancotti, 1983) and high activities (Trentin *et al.*, 1989) have been reported in patients in remission. In order to address these discrepancies we examined NKA periodically in a large population of leukaemic patients, to determine any correlation between NK cytotoxicity, disease status and subsequent clinical events and the explanation for any abnormality.

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MATERIALS AND METHODS

Patients

All the subjects studied were adult patients of Alfred Hospital,

Melbourne, Australia. NKA was tested in 17 patients with active acute lymphoblastic leukaemia (ALL), 56 patients with active acute non-lymphoblastic leukaemia (ANLL), 11 patients with ALL and 46 patients with ANLL in chemotherapy-induced complete remission, as well as in 16 patients after allogeneic bone marrow transplantation (BMT) for acute leukaemia. The age range was 16–72 years and there was no selection based on sex (74 men, 72 women).

Patients with ANLL were treated on protocols of the Australian Leukaemia Study Group (generally with cytosine arabinoside and daunorubicin with or without etoposide) which included two courses of consolidation therapy after complete remission and then 2 years of maintenance at 8-week intervals, similar to protocols previously described (Yates *et al.*, 1982; Bishop *et al.*, 1986). Patients with ALL were treated with various programs based on vincristine, L-asparaginase, prednisolone, daunorubicin and etoposide. Patients undergoing allogeneic BMT were conditioned with cyclophosphamide 120 mg/kg and fractionated total body irradiation of 12–14 Gy. Post-grafting immunosuppression was with methotrexate and cyclosporin as described by Storb *et al.*, (1986).

A normal range of NK was assessed in our laboratory in 60 healthy blood donors (age range 17–74 years). Diagnosis of leukaemia and definition of remission status were made according to standard Cancer and Leukaemia Group B criteria (Yates *et al.*, 1982), as well as the absence of any cytogenetic marker of disease. Briefly, complete remission was defined by a normocellular bone marrow with blasts comprising less than 5% of nucleated cells in the absence of recognizable leukaemia and with a normal peripheral white blood cell differential and a platelet count greater than $100 \times 10^9/l$. All bone marrow smears were independently reviewed by a single haematopathologist to confirm the absence of leukaemic blasts in patients who subsequently relapsed.

Effector cells

Heparinized peripheral bood, diluted 1:2 with phosphate-buffered saline (PBS), was separated by density gradient centrifugation (Boyum, 1968) over lymphocyte separation medium. Peripheral blood mononuclear cells (PBMC) were washed and resuspended in RPMI 1640, containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI-FBS) (all from Flow Laboratories, Sydney, Australia).

Percoll density gradient centrifugation of effector cells

PBMC isolated as above were further fractionated by discontinuous Percoll density gradients (Timonen & Saksela, 1980). Briefly, density gradients were prepared with Percoll (Pharmacia, Sweden) in various concentrations in RPMI-FBS and carefully layered in 2·5% concentration increments from 50% Percoll at the bottom of the tube (fraction 6) to 37·5% at the top (fraction 1). PBMC were layered over fraction 1 and after centrifugation at 300 ***g*** for 45 min, all of the fractions were collected and individually assayed for morphology, surface markers and NKA. Fractions 2 and 3 were found to contain the majority of LGL with NK activity. In 15 patients, $CD16^+$ cells constituted 70–94% of the cells in these fractions (median 88%, mean 82·4% s.d. 9·9%). In all assays, cell numbers were adjusted so that the effective concentration of $CD16^+$ cells was 100%.

Target cells

The K562 cell line, derived from a patient with chronic myeloid leukaemia in blast crisis (Andersson, Nilsson & Gahmberg, 1979), served as the source of target cells. This cell line was maintained in RPMI-FBS at 37°C in a humidified air atmosphere with 5% CO_2.

Cytotoxicity assay

Cytotoxicity tests were performed by a standard ^{51}Cr release assay (Brunner, Engers & Cerottini, 1976) in a 96-well, U-shaped microplate (Lux, Flow Laboratories) in a total volume of 250 μl. One-million target cells were labelled with 100–200 μCi sodium chromate (CJS-4; Amersham, Australia) for 1 h at 37°C. Labelled target cells were washed and suspended in RPMI-FBS to a concentration of 5×10^4/ml. They were then mixed with different concentrations of an equal volume of effector cells to give final effector-to-target (E:T) ratios ranging from 50:1 to 3:1. Not all samples had a complete range of lower ratio assays performed, due to inadequate cell numbers available. After a 4-h incubation at 37°C, supernatant fluid was collected and cytotoxicity was estimated from released radioactivity according to the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Spontaneous ct/min}}{\text{Maximum} - \text{Spontaneous ct/min}} \times 100$$

where experimental was the median ct/min of the triplicate assay, spontaneous ct/min was spontaneous release by labelled target cells in microplate wells in the absence of effector cells and maximum ct/min was the total ^{51}Cr content of labelled cells and supernatant.

Assays were performed on patients at diagnosis then at approximately 4-week intervals, usually at the time of a diagnostic bone marrow aspirate and prior to commencing a further course of therapy. Patients with ANLL in complete remission were tested at 8-week intervals, prior to each course of maintenance therapy.

Subset analysis

One-million PBMC isolated as described above were suspended in PBS and 5% FBS (PBS-FBS) at 4°C and incubated for 30 min with monoclonal antibodies Leu7 (anti-HNK-1) or Leu11 (anti-CD16) (Becton Dickinson Monoclonal Center, Mountain View, CA). Cells were then washed twice in PBS-FBS and resuspended in 50 μl of an affinity-purified sheep anti-mouse fluorescein isothiocyanate (FITC) conjugate at a final dilution of 1:100 (DDAF; Silenus, Melbourne, Australia) for 30 min at 40°C. After washing with PBS-FBS, the cells were analysed in a fluorescence-activated cell sorter (FACS II, Becton-Dickinson, Sunnyvale, CA). Positively staining cells were those with a greater fluorescence intensity than cells labelled with second layer antibody alone and were expressed as a percentage of total viable cells. Approximately 10^4 randomly accumulated viable cells were analysed.

Statistical analysis

The Wilcoxon rank sum test was used to compare the NKA of the patient groups. A simple regression analysis was used to correlate NKA with cellular subsets.

RESULTS

Reproducibility of assays

Results were expressed as percentage specific cytotoxicity at an E:T ratio of 50:1. This always represented the maximal release for tested samples over the range of E:T ratios and is the most commonly cited value, although other modes of expression exist (Brunner *et al.*, 1976; Rusthoven, 1985; Lotzova Savary & Herberman, 1987). For 19 patients in complete remission, the ranges of NKA at the various ratios are shown in Table 1. As we wished to demonstrate any significance of a drop in NKA, the E:T ratio 50:1 was chosen as a point on the cytotoxicity curve at which any demonstrable drop would be significant (Yates *et al.*, 1982). Eight stable patients (in remission for 2 years or more) were tested at 1–3-month intervals, to assess the variability of results obtained in a single patient. The results of these experiments are shown in Table 2.

Activity of patient groups

The median NKA for 60 healthy volunteer controls was 33·6% (range 15·4–71%). The median NKA of 57 patients in complete remission (69 assays) was 22·7% (range 9·5–64·4) and of the 73 patients with untreated or relpsed disease (127 assays) 2·4% (range 0–13·4). The NKA of the active disease group was significantly less than that of the control group ($P<0·001$) and the remission group ($P<0·001$).

Correlation of NKA with cell subsets

Cells bearing the antigens HNK1 and CD16, recognized by monoclonal antibodies Leu7 and Leu11, were enumerated in 23 and 15 subects, respectively, from samples used to perform NK assays in patients with active disease or in remission. There was no correlation between numbers of HNK1 or CD16-bearing cells and NKA ($r=0·04$ and 0·55). After Percoll gradient centrifugation of PBMC from controls and patients with active disease, NK cells in fractions 2 and 3 from controls and patients were adjusted to the same concentration as described above. Depression of NKA in the NK cell-rich fractions was observed in patients (median NKA 1%, range 0–3%) and controls (median NKA 38·5% range 19–72%), while the numbers of NK cells (LGL) as determined by morphology and immunophenotyping were indistinguishable from controls.

Table 1. Cytotoxicity data on 19 patients in complete remission tested at various effector-to-target cell ratios

	Effector-to-target cell ratio				
	50:1	25:1	12:1	6:1	3:1
Median NKA (% cytotoxicity)	21·0	19·8	7·0	4·6	2·5
Mean NKA (% cytotoxicity)	23·4	14·5	9·6	4·0	3·8
s.d.	7·8	7·6	5·9	5·0	3·3

Table 2. Sequential testing of natural killer cytotoxicity in eight patients in stable complete remission

Patient	No. of tests	Median NKA (%)	Range
A	4	38·5	27–45
B	4	18·7	16–21
C	9	28·4	23·3–34·3
D	3	21·5	18·6–21·7
E	4	19·9	16–25·3
F	4	23·9	17·4–30·6
G	4	26·3	22–33
H	4	41·0	28·8–50·8

NKA, natural killer cell activity.

Prediction of leukaemic relapse

Patients in complete remission had assays performed at 4–8-week intervals as described above. If a change in activity was noted, the test was usually repeated within 2 weeks, where possible. In 16 studies of 15 patients in complete remission, there was a fall in NKA to 30% or less of baseline levels. This fall was maintained on at least two consecutive assays in the 14 episodes in which it was possible to perform repeated assays. All of these patients relapsed within 10 weeks of the date of the initially low level. At the time of the initial drop (always done on the day of a bone marrow examination) no patient had evidence of peripheral cytopenias, circulating blast cells, abnormal bone marrow examination or clonal cytogenetic abnormality. These results are summarized in Table 3. Sequential assays in eight patients in stable complete remission showed a consistently narrow range of NK activities (Table 2).

Of the 15 patients, five relapsed following allogeneic BMT. At the time of transplant, four patients had ALL in relapse and one had ANLL in first complete remission (unique patient numbers 5, 6, 8, 14 and 18). They relapsed a median of 129 days post-transplant (range 89–201 days). NKA on the assay prior to the first low level ranged from 15·5% to 38% (median 25·6%, mean 27·9% s.d. 9·5%). The value of the first low level ranged from 2·3% to 9% (median 8%, mean 6·6%, s.d. 3%). Ten patients (nine with ANLL and one with ALL) relapsed on 11 occasions, as one patient with ANLL had two relapses studied. The NKA before the first low level ranged from 14·2% to 39·7% (median 21·0%, mean 23·2%, s.d. 7·4%) and the value of the first low level ranged from 0·9% to 12·9% (median 5·7%, mean 6·6%, s.d. 3·9%). These patients are exclusive of the group described as active disease, many of whom had a reduction in NKA identified at the same time as morphological relapse.

Two patients out of 57 with sustained complete remissions of longer than 6 months maintained low NKA of between 9·5% and 15%. In addition, a 19-year-old man who underwent allogeneic BMT for ANLL in first remission, had NKA values of between 10 and 17·5% for over 1 year after the transplant.

DISCUSSION

It is now well established that freshly obtained mononuclear cells from peripheral blood are able to lyse target cells in the absence of prior sensitization. This function has been attributed to NK cells but despite intensive investigation, the significance of NK cell function *in vivo* remains to be established. NK cells have been postulated to be the significant mediators of immune surveillance (Baldwin, 1977) and to have importance in the control of malignant cell growth (Hanna & Fidler, 1981). Thus,

Table 3. Natural killer assays of patients in complete remission who subsequently relapsed

Patient no.	Disease	BMT	Remission Status	Consecutive NK assays 1	2	3	Interval to Relapse*
				(weeks from previous stay)			
1	ANLL	–	1st	20·7	5·2 (20)	5·1 (1)	5
2	ANLL	–	1st	27·6	12·9 (18)	7·6 (8)	6
3	ANLL	–	2nd	39·7	15·5 (8)	1·8 (22)	1
4	ANLL	–	2nd	27·5	6·2 (9)	1·1 (14)	8
5	ANLL	–	2nd	14·2	5·0 (5)	2·7 (21)	4
5	ANLL	–	1st	21·0	12·7 (17)	4·6 (9)	8
6	ANLL	–	2nd	16·0	8·6 (20)	1·0 (6)	5
7	ANLL	–	1st	21·8	4·5 (6)	2·3 (19)	3
8	ANLL	–	2nd	29·5	9·0 (9)	4·3 (5)	1
9	ANLL	–	1st	19·0	0·9 (27)	—	10
10	ALL	–	1st	17·7	5·7 (9)	1·8 (2)	2
11	ANLL	+	post-BMT	37·0	9·0 (10)	0 (5)	5
12	ALL	+	post-BMT	38·0	9·0 (3)	0 (2)	8
13	ALL	+	post-BMT	25·6	8·0 (7)	5·0 (1)	7
14	ALL	+	post-BMT	15·5	4·8 (9)	3·9 (4)	9
15	ALL	+	post-BMT	23·4	2·3 (4)	0 (5)	5

Results expressed as % cytotoxicity at an effector: target ratio of 50:1.

* Interval to relapse is expressed in weeks from the date of NK assay no. 2 in all patients except patient no. 3, NK assay no. 3.

Numbers in parentheses are the time interval in weeks between that assay and the assay reported in the previous column

Sustained, depressed NKA preceded cytological evidence of relapse by 1 to 10 weeks.

BMT, allogeneic bone marrow transplantation.

some investigators have proposed the development of leukaemia to be a direct consequence of reduced NKA (Matera & Giancotti, 1983).

In this study we have shown that the NKA of untreated patients or those with relapsed leukaemia was significantly lower than that found in control donors or in patients who had achieved complete remission (Stupp, Rosenkovitch & Izak, 1978; Matera & Giancotti, 1983; Dickinson *et al.*, 1985; Sorskaar *et al.*, 1985). Patients in remission exhibited NKA lower than those of the healthy controls but significantly higher than those of patients with active disease ($P<0{\cdot}001$). Normal ranges of NKA for normal donors and untreated patients in this study were virtually identical to those reported recently (Lotzova *et al.*, 1987).

It is possible that ongoing maintenance chemotherapy was responsible for the lower activity of most patients once complete remission had been achieved (Mantovani *et al.*, 1978); however, testing was almost always done at least 8 weeks after such therapy and a recent study suggested that there was no difference in NKA due to maintenance therapy (Trentin *et al.*, 1989). A large variation in the range of NKA between individuals in the healthy control group and the patients in complete remission was observed; however, individual subjects who did not subsequently relapse, showed little variability in sequential testing, thus confirming the reliability of the assay. The observations by one group of investigators that patients in complete remission had a markedly reduced level of NKA were not confirmed (Matera & Giancotti, 1983). It is possible that many of the 17 patients in that study were not truly in complete remission.

Various explanations for decreased cytotoxicity in leukaemia have been proposed. It has been suggested that depressed NK cell activity may be due to the presence of suppressor factors (Herberman & Ortaldo, 1981; Zeigler, Kay & Zarling, 1981; Zoller & Wigzell, 1982), defective function of NK cells (Kay & Zarling, 1984), or reduced numbers of circulating effector cells (Dickinson *et al.*, 1985; Hajto & Lanzrein, 1983). We were unable to correlate NKA with numbers of cells expressing antigens HNK1 and CD16. This finding was consistent with previous reports (Lanier *et al.*, 1983).

In many instances, samples of PBMC with high proportions of HNK1 and/or $CD16^+$ lymphocytes exhibited lower NKA than normal. Conversely, low proportions of these cells were found to display normal cytotoxicity. Thus, it is apparent that while NK cells may be encompassed with the HNK-1 and $CD16^+$ population, there is no direct correlation between the expression of these markers and NK function.

Depressed NKA was seen in Percoll-purified subsets of NK cells from patients with active disease while control donors retained normal NKA in comparable subsets. It is clear from these data that simple dilution of NK cells by a mass of leukaemic cells cannot be the sole explanation for the low or absent activity in patients with active disease. This conclusion is also supported by the observation that patients in otherwise stable complete remission dropped NKA prior to relapse without a change in the cell numbers or white cell differential.

This sudden, significant decrease in NK cell function often preceded leukaemic relapse and was observed in patients who had achieved complete remission by chemotherapy and in patients who had undergone allogeneic bone marrow transplantation for acute leukaemia. The findings in this latter group confirmed our earlier, preliminary observations (Tratkiewicz & Szer, 1987). A sustained fall in NKA was not seen other than in patients in whom the leukaemia subsequently relapsed.

It is therefore confirmed that there is a correlation between NKA and the status of disease in patients with ALL and ANLL. Additionally, the routine use of the NKA assay, in conjunction with morphologic analysis, may have a role in monitoring the remission status of these patients. Studies currently in progress are directed at elucidating the mechanism of reduced NKA patients with acute leukaemia.

ACKNOWLEDGMENTS

We wish to acknowledge the co-operation and assistance of the medical and nursing staff and the patients of the Clinical Haematology and Bone Marrow Transplantation unit. We thank Dr Brian G. Farragher for reviewing the bone marrow smears and peripheral blood of the patients and Dr Richard Boyd for helpful discussions.

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