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Immunomodulation of Natural Killer Activity in Children with Acute Lymphoblastic Leukaemia

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Abstract—*Modulation of NK activity of PBLs from ALL patients was studied following exposure to IFN-α, staphylococcal protein A and interleukin-2. Only 52% of ALL patients responded to IFN-α stimulation, where the majority of controls showed positive enhancement of NK activity. Protein A failed to cause a significant stimulation of ALL patient PBLs whereas all controls showed a positive response. The majority of ALL patient and child control PBLs were however able to produce significant levels of IFN-γ (protein A stimulation) and IFN-α (Sendai virus stimulation), although significantly more of both types of interferon could be induced in adult PBL samples. The ability of IL-2 to activate NK activity of ALL PBL samples showed a similar trend to IFN-α stimulation; thus, not all ALL patients showed positive augmentation of NK activity upon IL-2 stimulation. It is clear from these results that interferons and IL-2 may not necessarily lead to activated NK cytolytic activity, and in the present study approx. 50% of ALL patients failed to respond to lymphokine stimulation.*

INTRODUCTION

IN THE accompanying paper we have shown that natural killer (NK) cell activity in children undergoing maintenance chemotherapy for acute lymphoblastic leukaemia (ALL) is severely depressed [1]. The present communication reports on the ability of biological response modifiers to influence, *in vitro*, the spontaneous levels of NK activity exhibited by those patients.

NK cell cytotoxicity can be augmented *in vitro* [2–4] and *in vivo* [5, 6] by interferon (IFN) and IFN-inducers [7]. Recently it has also been shown that interleukin-2 (IL-2) potentiates the lytic ability of NK cells [8–10]. Since NK cells are known to secrete a variety of lymphokines including IFN and IL-2 [11], a mechanism for self regulation exists.

It has previously been shown that leukocytes from ALL children produce normal levels of IFN-alpha (IFN-α) upon stimulation with viral antigen [12] and it is therefore possible that the response of leukocytes to lymphokines is abnormal in ALL children rather than lymphokine production *per se*. To test this hypothesis we have investigated the ability of exogenously supplied IFN-α staphylococcal protein A (an IFN-γ inducer [13]) or IL-2 to augment endogenous NK activity in ALL patients compared to normal controls. Evidence presented suggests that activation of NK cells by IFN-α and IL-2 share a common pathway (at least, in part) that differs from IFN-γ augmentation.

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MATERIALS AND METHODS

Patients

Details of ALL patients and controls used in this study are given in the accompanying paper [1]. Unselected children aged 3–15 years were included in the study, and 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. Heparinized 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 2½–12 years) attending either orthopaedic outpatients at the Children's Hospital, Sheffield or undergoing routine tests prior to orthopaedic surgery, or from healthy adult volunteers.

Cytotoxicity assay

The NK activity of peripheral blood lymphocytes (PBLs) was measured in a 4 h ^{51}Cr release assay

vs. K562 target cells, as described previously [1]. All blood samples from ALL patients were free of circulating leukaemic cells, that might act as cold competitors in NK assays.

Pre-treatment with IFN-α, staphylococcal protein A or IL-2

Human lymphoblastoid (Namalwa) interferon IFN-α (specific activity 7×10^7 IU/mg protein) was kindly supplied by Dr. K. Fantes (Wellcome Research Laboratories, Beckenham). IFN-α was diluted in RPMI supplemented with 10% new born calf serum (RPMI-NBCS) to a concentration of 10,000 IU/ml and stored at −70°C prior to use. In tests IFN-α was used at a final concentration of 100 UI/ml in RPMI-NBCS. PBLs (2.5×10^6/ml) were prepared as described previously and incubated overnight in the presence or absence of IFN-α at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Following overnight incubation, the cells were washed (× 2) in RPMI-NBCS and resuspended to 1.2×10^6 cells/ml prior to use in the cytotoxicity assay.

Staphylococcal protein A (Sigma) was used for pre-treatment of PBLs at a final concentration of 50 μg/ml, in a protocol similar to that described for IFN-α above.

Clarified supernatant (100,000 ***g***) from a gibbon lymphosarcoma line (MLA-144) was used as a source of IL-2. This source of IL-2 is lectin free and contains no detectable levels of any other lymphokines. The MLA-144 cell line was kindly provided by Dr. H. Rubin (National Cancer Institute, Frederick, U.S.A.). MLA-144 supernatant was used at 50% v/v in the same manner as for IFN-α and protein A in tests to investigate NK activation by IL-2.

IFN-α production by PBLs

IFN-α was induced by infecting 1×10^6 PBLs with Sendai virus (3 plaque-forming units/cells) for 24 h at 37°C. Supernatants were harvested, clarified, adjusted to pH 2, and stored at +4°C prior to assay.

IFN-γ production by PBLs

Following incubation of PBLs with protein A for 18 h at 37°C, the supernatants were harvested, clarified and stored at −20°C prior to assay.

Interferon assay

Interferon was assayed by the ability of samples to protect V_3 cells against the cytopathic effect of Semliki Forest Virus [14]. Results are reported in international units (IU)/ml measured against the MRC research standard B for human leukocyte (α) interferon, 69/19 and against a N.I.H. reference standard for IFN-γ.

Statistics

Lytic units (LU) of NK activity were calculated from cytotoxicity values by Von Krogh analysis [15]. Statistical analysis was by the Mann–Whitney rank test [16]. Paired data were analysed using the paired Student's *t*-test.

RESULTS

Immunomodulation of ALL NK cell activity by IFN-α, protein A and IL-2

The ability of IFN-α to increase endogenous NK activity against K562 was assessed in 59 ALL samples and 12 child controls. As is evident from Fig. 1a, baseline NK cell activity of the ALL patients was significantly lower than that of controls ($P < 0.0004$). Median NK activity in patients was 3.3 LU/10^7 PBL (range 0–225.8) vs. 69.3 LU/10^7/PBL (range 5.3–328.1) in the controls. After pre-treatment with IFN-α the median NK cell activity in the ALL children was 41.0 LU (range 0–327.6) compared with 175.6 LU (range 60.8–1176.3) in child controls ($P < 0.0001$). According to paired *t*-test data IFN-α significantly enhanced endogenous NK activity of PBLs from ALL patients ($P < 0.001$) as well as controls ($P < 0.02$). However, when individual patients or controls were assessed, only 31/59 (52%) of ALL children exhibited a positive response to IFN-α, whereas the NK activity of 11/12 (91%) of control samples was augmented. Parallel studies on PBLs from adult controls showed that the NK activity of 13 (84%) samples was augmented by exposure to IFN-α.

Similar studies to those described above were carried out using 50 μg/ml protein A on 36 ALL children and eight controls (Fig. 1b). Pre-treatment with protein A did not increase NK cytotoxicity of PBLs from ALL patients ($P > 0.11$). Median values were 0 LU (range 0–401.4) for endogenous NK activity and 0.2 LU (range 0–279.2) following pre-treatment with protein A. Assessment of paired data also failed to show a positive effect for protein A ($P > 0.1$). By contrast, the NK activity of PBLs from child controls was significantly enhanced by protein A pre-treatment whether analysed as a group ($P < 0.0014$) or as paired data ($P < 0.02$). Spontaneous NK activity (median 71.4 LU; range 42.6–165.9) was increased to 248.7 LU (range 123.3–694.0) following overnight incubation of control PBLs with protein A. Of the 36 ALL samples tested only 11 (30%) showed significant enhancement of spontaneous NK activity, whereas the endogenous NK activity of 8/8 (100%) child controls was augmented. Again, PBLs from adult controls showed a positive response to pre-treatment with protein A (data not shown).

The enhancing effect of IL-2 on spontaneous NK activity was investigated in 42 ALL samples and

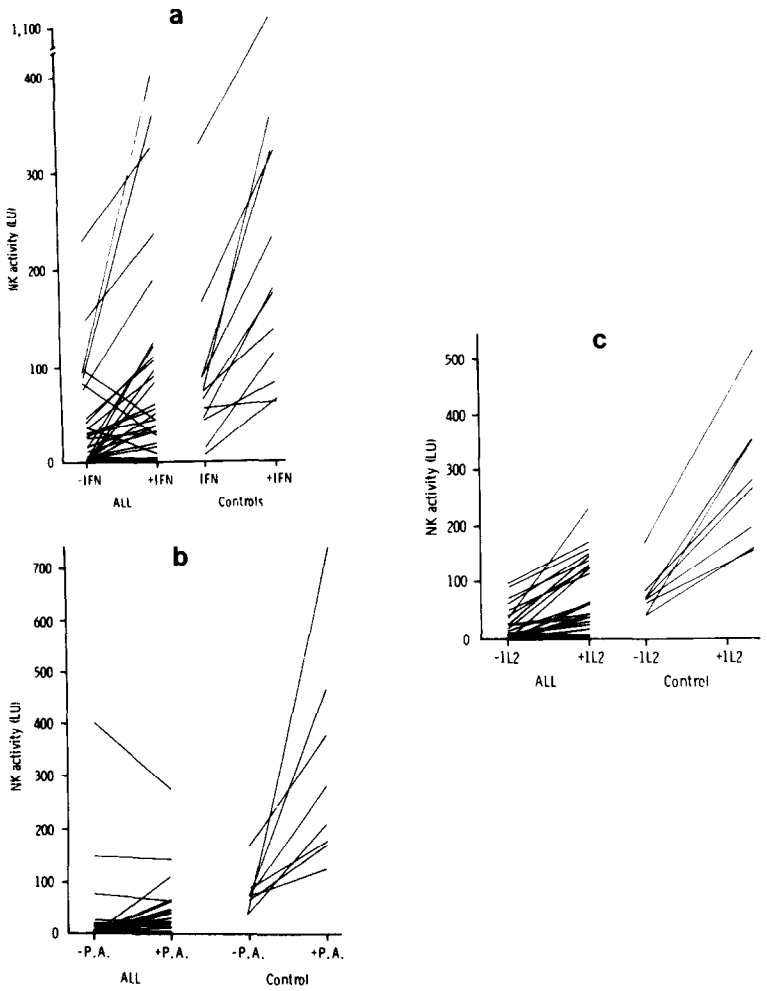

Fig. 1.a. Endogenous and IFN-α activated NK activity of PBLs from ALL patients (n = 59) *and child controls* (n = 12). *b. Endogenous and protein A activated NK activity of ALL patients* (n = 36) *and child controls* (n = 8). *c. Endogenous and IL-2 activated NK activity of ALL patients* (n = 42) *and child controls* (n = 8).

eight child controls (Fig. 1c). A similar response to that shown for IFN-α pre-treatment was demonstrated. Endogenous NK activity against K562 targets (median 2.2 LU; range 0–100.3) was increased (median 25.5 LU; range 0–237.6) in PBLs from ALL patients following IL-2 pre-treatment ($P < 0.02$; paired data $P < 0.001$). A similar trend was observed in control samples ($P < 0.01$; paired data $P < 0.001$). Spontaneous NK activity (median 71.4 LU; range 42.5–165.9) was augmented (median 288.8 LU; range 165.7–498.0) after incubation with IL-2. Whereas the NK activity of 8/8 (100%) of child control PBLs was augmented by IL-2 pre-treatment, only 24/42 (57%) of PBL samples from ALL children showed positive modulation by IL-2. Although IL-2 enhanced the endogenous NK activty of PBLs from ALL patients ($P < 0.02$) and controls ($P < 0.001$), it did not restore the lytic ability of PBLs from ALL children to the level observed in controls ($P < 0.0001$).

Effect of IFN-α and IL-2 pre-treatment on the NK activity of PBLs from the same patient

The effect of IFN-α and IL-2 pre-treatment on the NK activity of PBLs from the same donor was studied in 25 ALL samples and seven controls. All control PBL samples demonstrated a positive response to modulation by both IFN-α and IL-2 (Table 1). By contrast, the NK activity of only 11/25 PBL samples from ALL patients was observed to be augmented by both IFN-α and IL-2 (Table 1). There was no qualitative difference in the response of PBLs from ALL patients to IFN-α or IL-2; that is the NK activity of PBLs was augmented by both IFN-α and IL-2, or there was no enhancement by either lymphokine. There were quantitative differences, however. In some PBL samples enhancement by IFN-α was greater than for IL-2 (e.g. patients 14 and 23, Table 1) and in other samples the reverse was true (e.g. patients 3 and 15).

Spontaneous NK activity in PBL samples responding to lymphokine augmentation compared with non-responders

The endogenous NK activity of PBL samples responding to IFN-α, protein A or IL-2 pre-treatment was compared with the spontaneous activity of PBLs which failed to respond to lymphokine

Table 1. Modulation of human NK activity of PBLs from ALL patients or controls by IFN-α and IL-2

Sample No.	NK activity (LU) for: ALL patients Spontaneous	IFN-α	IL-2	Controls Spontaneous	IFN-α	IL-2
1	0.6	49.7*	48.4*	66.3	173.0*	166.0*
2	0	0.2	6.4	74.2	357.3*	269.9*
3	34.7	57.1*	143.5*	165.9	317.2*	498.0*
4	12.4	32.3*	60.6*	42.6	79.2*	165.7*
5	0	0	0	72.4	135.2*	360.0*
6	37.6	91.6*	103.0*	88.6	317.1*	284.9*
7	0	0	0	43.5	178.9*	366.3*
8	0	0	0			
9	0	0	0			
10	0	0	0			
11	100.3	151.4*	165.4*			
12	0	0	0			
13	0	0	0			
14	0	79.2*	16.5*			
15	2.9	9.9*	119.5*			
16	0	0	0			
17	2.4	76.6*	51.6*			
18	10.3	49.8*	23.6*			
19	73.4	214.7*	135.0*			
20	0	0	0			
21	0	0	0.9			
22	1.6	0	0			
23	11.1	57.7*	27.5*			
24	2.0	2.1	2.0			
25	0	2.4	2.4			

*Significantly greater than spontaneous activity ($P < 0.05$).

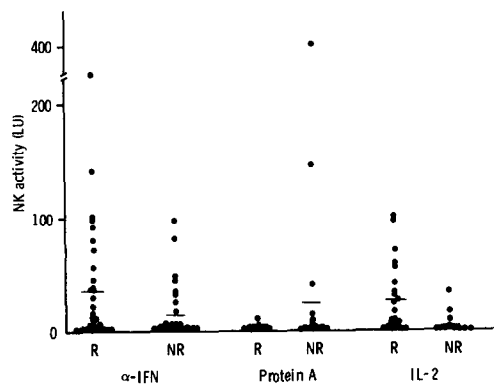

Fig. 2. Endogenous NK activity of PBL samples from patients responding to IFN-γ, protein A or IL-2 activation (R) or non-responders (NR).

stimulation (Fig. 2). The results demonstrated that the endogenous NK activity of PBL populations failing to respond to IFN-α or IL-2 was significantly lower ($P < 0.0725$; and $P < 0.0001$ respectively) than PBLs from responder populations. The median endogenous NK activity was 13.9 LU (range 0–96.7) in non-responders and 36.6 LU (range 0–225.8) in PBL samples enhanced by IFN-α. Spontaneous NK activity was 27.6 LU (range 0–100.3) in ALL patients responding to IL-2 pre-treatment and 2.7 LU (range 0–34.3) in those in which IL-2 had no augmenting effect. Also, there was no significant difference between the spontaneous NK activity exhibited by PBL samples responding or not responding to protein A pretreatment ($P > 0.94$). Median endogenous NK actvity was 25.6 LU (range 0–401.4) in PBL populations responding to protein A and 2.1 LU (range 0–10.4) in those which did not.

IFN production in ALL patients and controls

IFN-α levels induced by Sendai virus were measured in ALL patients ($n = 35$), child ($n = 11$) and adult controls ($n = 9$) (Fig. 3a). IFN-α produced by PBLs from ALL children (median 575 UI/ml; range 16–3715) was not significantly different from that produced by child controls (median 1513 IU/ml, range 371–2630) ($P > 0.6$). However, IFN-α levels induced in PBL samples from adult controls (median 2630 IU/ml, range 1549–15,135) were significantly higher than in either ALL patients ($P < 002$) or child controls ($P < 004$).

IFN-γ produced following incubation of PBLs with protein A (50 μg/ml) was assesssed in ALL patients ($n = 23$), child ($n = 6$) and adult controls ($n = 8$) (Fig. 3b). IFN-γ produced by PBLs from

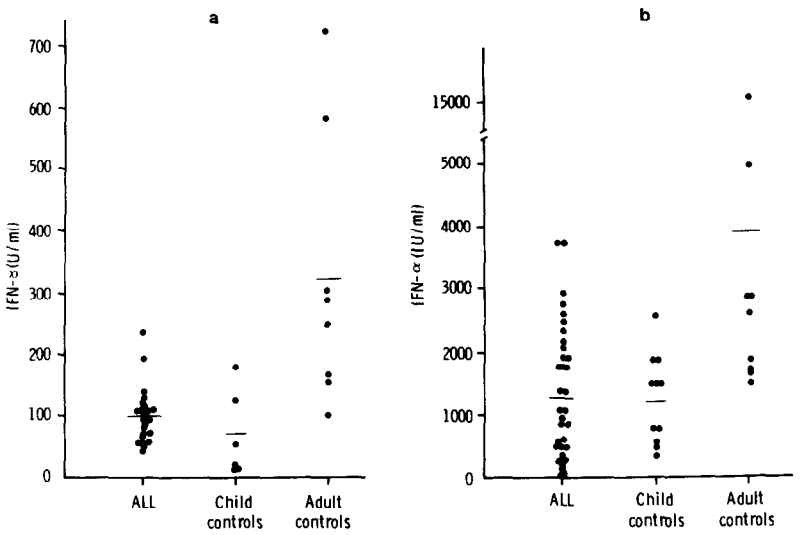

Fig. 3.a. IFN-α levels (IU/ml) *obtained following stimulation by Sendai virus of PBLs from ALL patients, child controls or adult controls. b. IFN-γ* (IU/ml) *obtained following stimulation with protein A of PBLs obtained from ALL patients, child controls or adult controls.*

ALL children (median 100 IU/ml; range 9.3–239.9) was not significantly different from that produced by child controls (median 53.7; range 19.5–177.8) ($P > 0.19$). PBLs from adult controls, on the other hand, produced significantly more IFN-γ (median 295 IU/ml; range 100–724.4) than either the ALL patients ($P < 0.0007$) or child controls ($P < 0.01$).

DISCUSSION

Endogenous and lymphokine-augmented NK activities have been studied in a number of diseases where there is immunological perturbation such as in cancer [17], immunodeficiency [18] and autoimmune syndromes [19]. Here we have demonstrated that the ability of INF-α, staphylococcal protein A and interleukin-2 to augment spontaneous NK activity in ALL patients undergoing maintenance chemotherapy is impaired.

In contrast to the study of Dickinson *et al.* [20] we have demonstrated that IFN-α pre-treatment enhanced endogeneous NK activity in ALL children undergoing maintenance chemotherapy when the results were analysed as a group. However, the induction of IFN-γ by protein A was unable to augment spontaneous NK activity to the level observed in controls. These data support previous findings in systemic lupus erythematosis (SLE) [21] and multiple sclerosis (MS) [19], although another recent report shows that the NK-cell activity defect in MS can be corrected *in vitro* by treatment with recombinant IFN-α [22]. In rheumatoid arthritis, another disease in which immunosuppressive therapy is commonly employed, IFN-α modulation of NK activity appears to be normal [19]. Therefore, while immunosuppressive therapy may contribute to the impaired response to IFN-α modulation of NK cytotoxicity observed in ALL children it is unlikely to be the sole cause of this anergy.

Although group data suggest that the endogenous NK activity of ALL children can be augmented by pre-treatment with IFN-α, analysis of individual patients has shown that the NK activity of only 52% of patient samples can be augmented by this lymphokine. These findings support previous studies in cancer patients [17] which have shown that NK activity is not affected by IFN treatment. In addition, studies in our own laboratory into the NK status of patients with Hodgkin's lymphoma have also shown that only a proportion of individuals are capable of responding to IFN-α modulation [23, 24].

The augmentation of NK cytolytic capacity by IFN is due in part to the recruitment of non-cytotoxic NK precursors [25–27] and to an increase in the rate of lysis and recycling of individual cytotoxic effector cells [28]. The finding that the spontaneous NK activity of PBLs responding to IFN-α was significantly greater than that of non-responding populations suggests that in ALL children IFN-α acts to enhance existing 'active' NK cells rather than on non-cytolytic precursors. It was, however, not possible to address this question directly through the use of single cell assays in the present study. Some PBL samples exhibiting baseline endogenous NK activity were augmented by IFN-α and conversely other samples did not respond to IFN-α, suggesting perhaps, that their NK activity was fully activated *in vivo*.

The ability of staphylococcal protein A to augment the spontaneous NK activity of ALL children was also studied. Evidence suggests that protein A enhances NK activity indirectly via the induction of IFN-γ [13], although a recent report infers that

the augmenting effect may be IFN-γ independent [29]. The data presented here shows that protein A is unable to augment the endogenous level of NK activity in ALL children, even though IFN-γ levels similar to those from child controls are induced by this treatment. We have previously shown that these levels of IFN-γ are capable of augmenting NK activity in normal PBLs although it is possible that the exposure time of PBLs to IFN-γ may have been inadequate to fully activate NK effectors (R.C. Rees, unpublished observations). It is of interest that IFN-α induction by Sendai virus is unimpaired in ALL patients.

Recently, IL-2 has been demonstrated to augment the NK activity of normal donors [9, 10] and it is largely held that the enhancing effect is independent of IFN-γ induction [30, 31]. The finding that IL-2 can augment the endogenous NK activity of ALL children, whereas protein A cannot, supports this view. Again, as for IFN-α, IL-2 is not capable of enhancing the NK activity of all PBL populations isolated from ALL children. Indeed, in experiments where the enhancing effects of IFN-α and IL-2 were tested on the same patient, no qualitative difference in the response to the two lymphokines was detected. This indicates that IFN-α and IL-2 could share, at least in part, a common activation pathway and that is likely to differ from the mechanism of NK augmentation by IFN-γ.

The results presented in this paper indicate that the inability of NK cells from some ALL patients to respond to positive lymphokine regulation may contribute to the vulnerability ALL children show to virus infections. In addition, the observation that the lytic activity of NK cells isolated from ALL patients may be refractory to IFN-α and IL-2 modulation is of importance when considering the possible therapeutic use of lymphokines in ALL patients.

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