*Eur J Cancer Clin Oncol*, Vol. 18, No. 11, pp. 1081–1088, 1982.
Printed in Great Britain.

0277-5379/82 111081-08$03.00/0


# Depressed Spontaneous Natural Killing and Interferon Augmentation in Patients with Malignant Lymphoma*

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**Abstract**—*A substantial proportion (44%) of peripheral blood lymphocyte samples from 41 patients with malignant lymphoma have been shown to have depressed or undetectable levels of natural cytotoxicity against the leukaemic cell line K562 in a 4-hr [$^{51}$Cr]-release assay. No correlation was found between low levels of natural killer (NK) cell activity and either the age of the patients, total or differential white blood counts, or the type or stage of disease. Furthermore, pre-treatment of lymphocytes with human lymphoblastoid (Namalva) interferon failed to enhance NK levels in 5/11 patients with Hodgkin's disease and 5/8 patients with non-Hodgkin's lymphoma, and was in contrast to the response of control peripheral blood lymphocytes assayed under the same test conditions. The lack of responsiveness to interferon of peripheral blood NK cells from lymphoma patients was not wholly associated with those patients shown to have low levels of spontaneous NK activity.*

## INTRODUCTION

NATURAL killer (NK) cell activity has been postulated as an important anti-tumour mechanism of possible *in vivo* relevance in controlling tumour growth [1–3]. Furthermore, virus-infected cells have been shown to be particularly susceptible to NK killing, and it has been suggested that NK effectors may operate as a first line of defence against viral infection [4, 5]. It has been shown that interferon [6, 7] and interferon inducers potentiate natural cell-mediated cytolysis *in vitro* and *in vivo* [8, 9], and may constitute a mechanism whereby interferon mediates its anti-cancer effect.

Reduced NK cell activity has been reported in tumour-bearing animals [10] and in several groups of patients with malignant disease [11–13]. However, not all cancer patients, especially those in the early stages of disease, have reduced NK cell reactivity [14, 15]. Most previous studies have been confined to patients with solid malignancies of specific organs, although two recent reports have shown a depressed NK cell reactivity in patients with chronic lymphatic leukaemia [15, 16].

In the present study peripheral blood lymphocytes isolated from patients with Hodgkin's and non-Hodgkin's lymphoma were assessed for natural killer function and for their response *in vitro* to interferon. Previous studies have shown lymphoma patients to have defective or altered immunocompetence [17–21], although to our knowledge NK cell function in these patients has not been reported.

## MATERIALS AND METHODS

### *Lymphoma patients and controls*

Ten-millilitre samples of heparinised peripheral blood were taken from a total of 41 patients with malignant lymphoma: 24 with Hodgkin's disease, 17 with non-Hodgkin's lymphoma. Thirty-five patients were assessed prior to any form of treatment and six were assessed in relapse. All patients were histologically typed using the Rye classification [22] for Hodgkin's disease and the Lukes and Collins classification [23] for non-

Accepted 10 June 1982.
10 June 1982.
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Hodgkin's lymphoma, and fully staged by the Ann Arbor criteria [24].

*Preparation of effector cells*

Lymphocyte-rich mononuclear cells (effector cells) were isolated by centrifugation of heparinised blood on Ficoll-Hypaque gradients [25]. These cells were washed three times, counted and re-suspended in RPMI 1640 supplemented with 10% calf serum (RPMI).

*Target cells*

The leukaemic cell line K562 [26] was kindly donated by Dr. M. Moore, Paterson Laboratories, Manchester. It was grown as a stationary suspension culture in RPMI.

*Interferon*

Human freeze-dried lymphoblastoid (Namalva) interferon (HIF) was kindly provided by Drs Fantes and Johnston, Wellcome Research Laboratories, and had a specific activity of approximately $6 \times 10^5$ HIF units/mg of protein. The HIF was aliquoted and stored at -80°C and diluted to 2000 units HIF/ml in RPMI prior to use in tests.

*Pre-treatment of effector cells with interferon*

Effector cells were re-suspended to cell concentrations of $2 \times 10^6$ cells/ml, 0.1 ml of cell suspension was then added to microtest plate wells (in triplicate) and 200 units of HIF in 0.1 ml RPMI were added. RPMI (0.1 ml) was added to control wells containing equivalent numbers of effector cells. The covered plates were incubated overnight (20 hr) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. Just prior to the addition of target cells, the plates were spun at 200 *g* for 2 min and 0.1 ml of the supernatant removed: the effector cells were gently re-suspended and $1 \times 10^4$ target cells in 0.1 ml of RPMI were added per well. The concentration of HIF used in these tests has previously been shown to be in excess of the amount of HIF to cause maximum increase in NK cytotoxicity.

*Four-hour [$^{51}$Cr]-release assay*

This assay was used to measure natural cytotoxicity. K562 target cells in 0.2 ml RPMI were labelled with $Na_2[^{51}Cr]O_4$ (100 $\mu$Ci) (Radiochemical Centre, Amersham, U.K.) for one hour at 37°C, after which time the cells were washed three times in RPMI and incubated for a further hour at 37°C. The cells were then washed three times, counted and re-suspended at a concentration of $1 \times 10^5$ cells/ml in RPMI. As described above, the effector cells (0.1-ml volumes) were added to the wells at effector to target (E:T) ratios of 40:1 and 20:1. [$^{51}$Cr]-labelled target cells (0.1-ml volumes) were added and the test incubated at 37°C in a 5% $CO_2$ atmosphere for 4 hr. The cells were sedimented at 200 *g* for 5 min and 0.1 ml of supernatant removed into separate wells. The plates were dried, sealed with Parafilm and the individual wells counted in a gamma spectrophotometer. The per cent [$^1$Cr]-release was determined using the following formula:

$$\%\ [^{51}Cr]\text{-release} = \frac{(\frac{1}{2}\ SN) \times 2}{(\frac{1}{2}\ SN) + (\text{cells} + \frac{1}{2}\ SN)} \times 100,$$

where SN = supernatant.

The results presented in this paper are expressed as % cytotoxicity, which was calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{(\text{test release}) - (\text{spontaneous release})}{100 - (\text{spontaneous release})} \times 100.$$

The spontaneous release (from target cells in presence of media alone in 4 hr) was usually in the range of 3–10%.

## RESULTS

Peripheral blood lymphocytes (PBL) isolated from patients with Hodgkin's and non-Hodgkin's lymphoma on Ficoll-Triosil were assayed for cytotoxicity against K562 target cells in a 4-hr [$^{51}$Cr]-release test and the cytotoxic values obtained compared with those of controls. Although the NK activity of controls showed a high degree of natural variation (Fig. 1), a significant number of lymphoma patients gave abnormally low levels of cytotoxicity. Twenty-seven per cent (11/41) of patients had cytotoxicity below 1 S.D. of the control value, whilst a further 15% (6/41) had cytotoxicity below 2 S.D. when lymphocyte reactivity was assessed at a 40:1 effector cell to target cell (E:T) ratio. A total of 9 patients with Hodgkin's disease and 6 patients with non-Hodgkin's lymphoma demonstrated cytotoxicity below any individual cytotoxicity shown by the controls. A similar decrease in NK cytotoxicity was evident when results were assessed at a 20:1 E:T ratio. Statistical analysis by Student's *t*-test of the grouped cytotoxicities showed that the NK reactivity of PBL from Hodgkin's disease patients was significantly different to that of the controls when compared at 40:1 ($P < 0.001$) and 20:1 ($P < 0.025$) ratios. However, the cytotoxicity of PBL from non-Hodgkin's lymphoma patients was not significantly different from control values. Regression analysis showed that neither the patient's age nor changes in absolute peripheral white blood cell count correlated with the cytotoxicity (Fig. 2); the

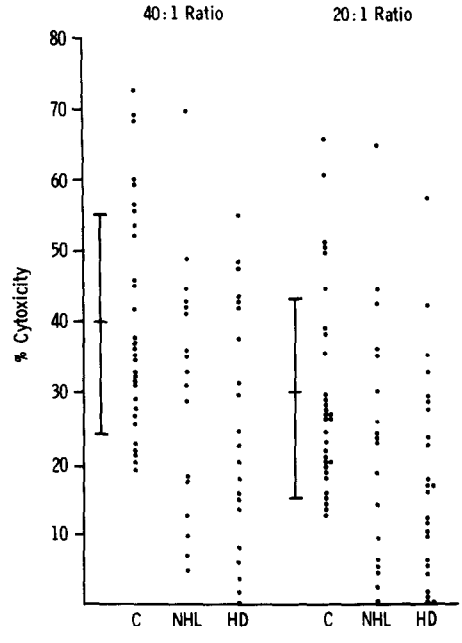


*Fig. 1. NK reactivity of peripheral blood lymphocytes from patients with Hodgkin's (HD) and non-Hodgkin's lymphoma (NHL) and control subjects (C) (bar lines indicate 1 S.D. of controls).*

correlation coefficients were calculated to be $r = -0.0159$ and $r = 0.1540$ for age and lymphocyte counts respectively. There was no correlation between the levels of NK activity and either the tumour histology or stage of disease, and these results are given in Tables 1 and 2. Furthermore, neutrophil and monocyte counts were performed on whole blood samples and the results subjected to regression analysis and compared with cytotoxicity values (Table 3). Total lymphocyte and monocyte counts failed to show any significant correlation with cytotoxicity in both groups of patients. A positive correlation was observed between neutrophil counts from non-Hodgkin's patients and the level of natural cytotoxicity ($P = <0.01$), although all the patients except one (JB) had neutrophil counts within the normal range. No such correlation was observed between cytotoxic values and neutrophil counts on blood obtained from patients with Hodgkin's disease.

*Impaired response to interferon stimulation*

PBL from patients and controls were pre-treated with interferon (IFN) overnight and assayed for cytotoxicity towards K562 cells; the cytotoxic values obtained following IFN exposure were compared to the spontaneous cytotoxic values by Student's *t*-test. The results are shown in Fig. 3 and are detailed in Table 4. All normal control PBL preparations (11/11) showed a significant enhancement ($P < 0.05 - P < 0.001$) in cytolytic activity towards K562 targets following treatment with IFN. In contrast, a high proportion of patients with Hodgkin's and non-Hodgkin's lymphoma failed to show increased cytotoxicity following pre-incubation with IFN. In 5/11 non-Hodgkin's lymphoma patients tests, a significant increase ($P < 0.05$) in cytotoxicity following HIF treatment could not be demonstrated. Analysis of the grouped data also showed that the percent increase in cytotoxicity of patients' PBL following exposure to human IFN was significantly less ($P = <0.001$) than the increase in cytotoxicity obtained with control PBL. The inability of patients' PBL to respond to IFN was not confined to those patients with low

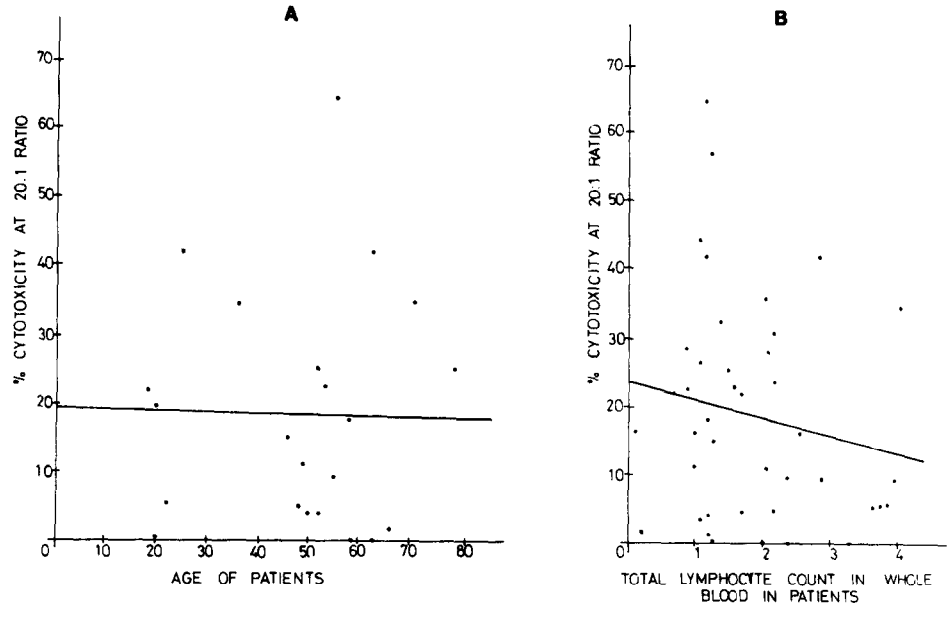


*Fig. 2. Regression analysis of NK cytotoxicity of peripheral blood lymphocytes from lymphoma patients against age (A) and total lymphocyte counts of patients (B).*

*Table 1. Clinical details of patients with Hodgkin's disease and details of spontaneous and interferon-induced natural killer cell activity*

| Patient | Sex | Age | Stage of disease* | Histology type† | Total blood lymphocyte count × $10^9$/l | NK activity at 40:1 or 20:1*‡§ | HIF stimulation‖ |
|---|---|---|---|---|---|---|---|
| PT | F | 42 | 1A | NS | 1.1 | + | NT |
| KF | M | 52 | 2A | MC | 2.1 | + | NT |
| IT | M | 22 | 3A | MC | 1.0 | + | NT |
| FT | F | 69 | 4B | LD | 0.6 | -§ | NT |
| MH | F | 63 | 1A | MC | 2.4 | -- | NT |
| JC | M | 27 | 3B | MC | 1.2 | - | NT |
| AT | M | 72 | 3B | MC | 2.4 | -§ | NT |
| GB | M | 25 | 3A | LP | 2.9 | + | NT |
| JL | F | 18 | 1A | NS | 0.7 | + | NT |
| KB | M | 48 | 2A | MC | 2.2 | -- | NT |
| WH | M | 49 | 1A | LP | 1.0 | + | NT |
| JC | F | 60 | 3B | NS | 1.6 | + | + |
| TB | M | 55 | 3B | NS | 2.9 | - | - |
| AJ | F | 39 | 1A | LP | 2.3 | - | - |
| MS | M | 8 | 1A | LP | 3.3 | -- | - |
| DP | M | 59 | Rec | MC | 1.4 | +§ | - |
| JC | F | 26 | 2A | NS | 0.9 | + | + |
| JN | M | 36 | Rec | MC | 4.1 | + | - |
| CB | F | 31 | 3B | MC | 1.1 | -- | - |
| FH | F | 22 | 4A | NS | 3.7 | - | NT |
| LB | F | 20 | 4B | MC | 1.3 | -- | - |
| JH | M | 37 | 2B | MC | 1.3 | +§ | + |
| GH | M | 46 | Rec | MC | 1.3 | + | NT |
| EG | M | 36 | Rec | NS | 2.1 | - | - |

*Staging by the Ann Arbor criteria (rec = recurrent disease).
†Histological type (Hodgkin's disease). LP, Lymphocyte predominance; NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depletion.
‡+ Normal; - below 1 S.D. of mean control value; -- below 2 S.D. of mean control value.
§Four-hour cytotoxicity assays.
‖+ Normal; - below minimum value of controls; NT, not tested.

*Table 2. Clinical details of patients with non-Hodgkin's lymphoma and details of spontaneous and interferon-induced natural killer cell activity*

| Patient | Sex | Age | Stage of disease* | Histology type† | Total blood lymphocyte count × $10^9$/l | NK activity at 40:1‡ | HIF stimulation§ |
|---|---|---|---|---|---|---|---|
| LC | F | 72 | 2A | 1 | 2.1 | + | NT |
| BP | F | 43 | Rec | 1 | 3.8 | - | NT |
| RP | M | 59 | 3A | 1 | 1.7 | + | NT |
| NT | F | 54 | 2A | 3 | 1.2 | + | NT |
| HC | M | 68 | 4A | 2 | 2.2 | + | NT |
| HS | F | 71 | 1A | 2 | | + | NT |
| GF | M | 59 | 4A | 4 | 2.0 | - | NT |
| JB | F | 79 | 1A | 3 | 1.5 | + | NT |
| JT | M | 50 | 2A | 1 | 1.2 | - | NT |
| WS | M | 56 | 2A | 3 | 4.0 | - | - |
| DP | F | 52 | 3A | 3 | 1.1 | + | - |
| RF | F | 53 | 1A | 1 | 0.9 | + | + |
| DB | F | 55 | 1A | 1 | 1.2 | + | - |
| JM | F | 68 | 4A | 2 | 1.7 | -- | - |
| MA | F | 66 | Rec | 3 | 0.2 | -- | - |
| JB | M | 63 | 4B | 3 | 1.2 | + | + |
| HS | M | 62 | 4B | 4 | 2.2 | + | + |

*Staging by the Ann Arbor criteria (rec = recurrent disease).
†Histological type (non-Hodgkin's lymphoma). 1, Small lymphocytic and small cleaved follicular centre cell FCC, follicular; 2, large cleaved FCC, follicular/diffuse; 3, large non-cleaved FCC, diffuse, and immunoblastic sarcoma; 4, others.
‡+ Normal; - below 1 S.D. of mean control value; -- below 2 S.D. of mean control value.
§+ Normal; - below minimum value of controls; NT, not tested.

*Table 3. Differential white blood cell counts: correlation with NK reactivity of PBL from patients with Hodgkin's disease and non-Hodgkin's lymphomas*

| Cell type | Hodgkin's disease patients ($n = 24$) | | Non-Hodgkin's lymphoma patients ($n = 17$) | |
|---|---|---|---|---|
| | Cell count/ml (±S.D.) ×10⁶ | Correlation coefficient* | Cell count/ml (±S.D.) × 10⁶ | Correlation coefficient* |
| Neutrophil | 5.81 ± 4.40 | 0.30 | 5.14 ± 3.00 | 0.62* |
| Lymphocyte | 1.90 ± 1.00 | 0.09 | 1.80 ± 0.94 | 0.40 |
| Monocyte | 0.33 ± 0.19 | 0.03 | 0.24 ± 0.17 | 0.09 |
| Total count | 19.23 ± 14.60 | 0.21 | 23.05 ± 17.8 | 0.24 |

*Regression analysis between cell counts and NK reactivity at 20:1 E:T ratio.
†$P = <0.01$.

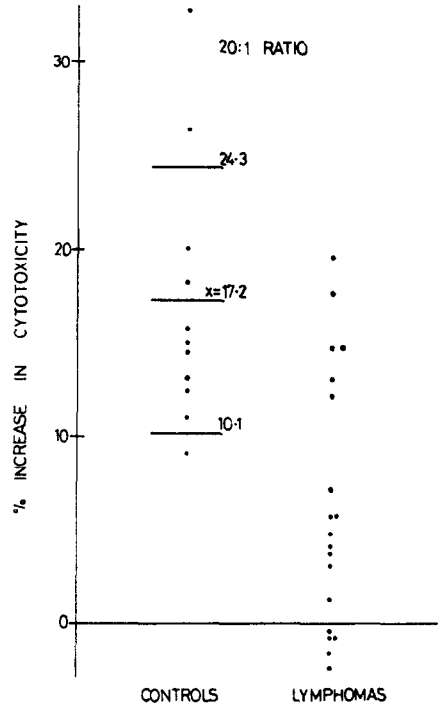


*Fig. 3. Percentage increase in cytotoxicity following treatment with α interferon (E:T ratio of 20:1) (bar lines indicate 1 S.D. of controls).*

NK levels (Tables 1 and 2), and both Hodgkin's and non-Hodgkin's lymphoma patients with seemingly normal NK reactivity failed to respond to HIF treatment (Table 4).

## DISCUSSION

In the present study cytotoxicity towards the NK-sensitive cell line K562 has been shown to be significantly impaired in a proportion of patients with malignant lymphoma, and this reduced activity was independent of the histological type of tumour or stage of disease. The former observation suggests a possible defect in number or cytolytic capacity of NK cells, and theoretically this could have occurred either before the onset of the disease or after the establishment of the lymphoma. Alternatively, suppressor cells might account for the impaired NK reactivity, either as a result of the presence of suppressor T cells or other cell types such as monocytes and/or their products [27, 28]. In peripheral blood, at least, no significant increase in the monocyte population was apparent in patients with low NK cell activity, and recent analysis of monocyte contamination of effector cells isolated from lymphoma patients' blood samples has shown them to represent less than one per cent of the population (Hawrylowicz and Rees, unpublished results). We have failed to observe a correlation between innate NK activity and monocyte content of effector cell preparations, but this is not to say that individual cells did not possess increased suppressor activity. In addition, Koren *et al.* [29] have reported that endogenous human natural killing is unaffected by the presence of monocytes from healthy individuals. In previous studies monocytes isolated from Hodgkin's disease patients were found to possess enhanced antibody-dependent cellular cytotoxicity [30] and an increased prostaglandin (PG) $E_2$ production, causing the suppression of T cell response to PHA [31]. Whether or not increased PG production is responsible for the observed decreased NK activity is not clear, although an increase in PG $E_1$ by monocytes has been shown to cause decreased natural killing [29].

In a recent study of natural cytotoxicity in patients with solid malignancies, NK activity was low or undetectable in 50% of patients with advanced disease [32]. Natural killing could be restored to within the normal range by pretreatment of PBL with interferon in only half the patients previously exhibiting low NK activity, despite the finding that interferon production by PBL upon stimulation with interferon-inducing agents appeared normal. The results obtained by these workers are similar to those shown here for patients with malignant lymphoma.

The augmentation of NK activity by interferon has been well documented [8, 9] and so the second finding reported here, namely that interferon-

*Table 4. Potentiation of NK reactivity by human interferon (E:T ratio of 20:1)*

| Experiment No. | Control PBL effectors -IFN | Control PBL effectors +IFN | Effectors from H.D. patients -IFN | Effectors from H.D. patients +IFN | Effectors from N.H.L. patients -IFN | Effectors from N.H.L. patients +IFN |
|---|---|---|---|---|---|---|
| 1 | 34.9 | 49.9* | 17.2 | 34.9** | — | — |
| | | | 29.9 | 28.2 | | |
| 2 | 28.9 | 42.1* | 27 | 32.8 | — | — |
| | | | 8.1 | 12.8* | | |
| 3 | 24 | 35.1* | 51.8 | 51.5 | — | — |
| 4 | 64.3 | 78.9* | 48.2 | 61.4* | 17.9 | 20.8 |
| | | | | | 68.1 | 68.9 |
| 5 | 68.4 | 80.5** | — | — | 48.7 | 54.5 |
| 6 | 54.1 | 80.6*** | — | — | 28.3 | 43.2*** |
| 7 | 30.2 | 39.3* | 42.5 | 49.6* | 37.1 | 49.3** |
| | | | | | 46 | 65.6*** |
| 8 | 54.9 | 73.1* | 12.9 | 16.8 | — | — |
| | | | 44.2 | 59* | | |
| 9 | 37.4 | 53.2* | — | — | 18.2 | 17.4 |
| 10 | 39.8 | 73*** | 11.9 | 13.1 | 13.2 | 10.7 |
| 11 | 41.9 | 54.4** | 23.1 | 26.8*** | — | — |

Significant boosting of NK reactivity by interferon.
*$P = <0.05$; **$P = <0.01$; ***$P = <0.001$.

mediated potentiation of NK activity is impaired in 5/11 Hodgkin's disease and 5/8 non-Hodgkin's lymphoma patients is more difficult to understand, particularly as prior knowledge of a patient's spontaneous level of NK cytotoxicity did not predict subsequent potentiation. Two possibilities exist: firstly, NK cells may already be in an activated state (possibly due to modulation of response by tumour products or circulating interferon), so that any attempt to enhance NK activity further is unsuccessful; and secondly, the defect in natural killer cell function may be such that the effector cells are unable to respond to the interferon. It has previously been found by us and other workers that exposure of the target cells to interferon can increase their resistance to NK lysis, and this could be argued as a possible explanation of our observations since target cells were incubated in the presence of interferon during the cytotoxicity assays. This is, however, unlikely, since significant enhancement of NK cytotoxicity was recorded for all control PBL assayed at the same time as PBL from Hodgkin's disease or non-Hodgkin's patients (see Table 4). In addition, pre-treatment of K562 target cells with interferon does not render them completely resistant to NK cytotoxicity in 4-hr assays (Hawrylowicz and Rees, unpublished results).

In addition to the above-mentioned study by Kadish and co-workers [32], Ziegler *et al.* [15] have recently studied patients with chronic B cell lymphocytic leukaemia (CLL), concluding that patients with advanced disease showed deficient NK cell activity and failed to response to *in vitro* pre-treatment with fibroblast ($\beta$) interferon. Platsoucas *et al.* [16] have also reported impaired NK cell activity and diminished responsiveness to leukocyte ($\alpha$) interferon in CLL patients. The results of these studies, together with those reported here, suggest that patients with lymphoid neoplasms, as well as patients with solid malignancies, have a defective natural resistance mechanism(s), and available evidence suggests that natural killer cell function is influenced by the lack of interferon production and the presence of inhibitory substances such as prostaglandins. Further understanding of the regulatory role played by interferon is of particular importance in view of the current interest in using interferon in the therapy of malignant disease.

**Acknowledgements**—We wish to thank Mrs. D. Edey and Mr. A. Clegg for their excellent technical assistance, the consultant medical staff of Weston Park Hospital for access to their patients, and Miss S. Francis and Mrs. C. Mullan for typing the manuscript.

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