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Modulation of the defective natural killer activity seen in thalassaemia major with desferrioxamine and α-interferon

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SUMMARY

We previously observed that natural killer (NK) activity toward K562 cells is markedly depressed in patients with β-thalassaemia major. Here we report that these patients also exhibit significantly decreased ($P < 0.005$) NK cytotoxicity against human fibroblasts infected with herpes simplex virus-type 1 (HSV-1) and that the amount of α-interferon (α-IFN) generated during the latter assays is significantly less than normal ($P < 0.005$). This decreased production of α-IFN may account in part for the decreased NK activity seen in the thalassaemia patients. On the other hand, the cytotoxicity of their mononuclear cells (MNC) toward both K562 cells and HSV-1-infected fibroblasts could be augmented to the same extent as that of normal MNC by preincubation with α-IFN suggesting that thalassaemia MNC are capable of responding to this lymphokine despite their reduced ability to produce it. Moreover, preincubation of thalassaemia MNC with desferrioxamine (DFO), an iron-chelating agent, consistently increased the lysis of K562 cells indicating that the transfusion-induced iron overload which these patients experience may also contribute to the defective NK function seen in this disease. We have now found that preincubation of such MNC with DFO has no effect upon production of α-IFN when the MNC are cocultured with either HSV-1-infected fibroblasts or K562 cells. Combining DFO and α-IFN resulted in an increase in the NK activity of both normal and thalassaemia MNC against the two targets which was greater than that with α-IFN alone. In fact, preincubation of thalassaemia cells with this combination increased their NK activity toward K562 targets to that of untreated normal cells. This was true when either unfractionated MNC or NK-enriched fractions were used as effector cells. These results suggest that DFO and α-IFN enhance NK activity by different mechanisms, both of which appear to be reversibly impaired in thalassaemia patients.

Keywords thalassaemia α-interferon desferrioxamine natural killer activity herpes simplex virus-type 1

INTRODUCTION

Patients with β-thalassaemia major must receive repeated blood transfusions to prevent the life-threatening anaemia which otherwise arises as a consequence of their disease. As chronic transfusion therapy leads to severe iron overload, a therapeutic splenectomy is usually performed

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early in the second decade to reduce the rate of iron loading (Modell, 1977; Graziano *et al.*, 1981). For the past several years we have been investigating the effects of blood transfusion, splenectomy and iron overload on the immune function of patients with thalassaemia. Those with β-thalassaemia intermedia, a clinically milder form of this disease, have elevated serum levels of IgG and IgA and a high percentage of circulating cells which bear surface immunoglobulins, the increases being related to both serum iron levels and splenectomy (Kapadia *et al.*, 1980). In patients with thalassaemia major, significant correlations have been reported between the number of transfusions received and increases in the percentages of both T suppressor cells (Grady *et al.*, 1985) and circulating cells containing cytoplasmic immunoglobulin (Akbar *et al.*, 1985). Furthermore, a significant correlation has been observed between the amount of blood transfused and decreased natural killer (NK) activity against K562 target cells (Gascon, Zoumbos & Young, 1984; Kaplan *et al.*, 1984; Akbar *et al.*, 1986). In one report an association between decreased NK activity and transfusion-induced iron overload has been suggested (Akbar *et al.*, 1986). Clearly, thalassaemia patients experience marked alterations in immune function as a consequence of their transfusion therapy. In this study we have further investigated the mechanisms affecting NK function in patients with thalassaemia major. Their mononuclear cells (MNC) exhibit significantly less NK activity against human fibroblasts infected with herpes simplex virus-type 1 (HSV-1) than normal MNC. Desferrioxamine (DFO), an iron-chelating agent, partially restores this NK activity. The thalassaemia MNC also produce significantly less α-interferon (α-IFN) when incubated with HSV-1-infected targets but show normal augmentation of NK activity in the presence of exogenous α-IFN. Thus, there appears to be a defect in the production of this lymphokine by thalassaemia patients. Finally, combining α-IFN with DFO had an additive effect upon the NK activity of MNC from both normal individuals and thalassaemia patients. The fact that DFO did not increase the production of α-IFN by thalassaemia MNC implies that DFO and α-IFN enhance NK activity by different mechanisms, both of which appear to be reversibly impaired in patients with thalassaemia major.

MATERIALS AND METHODS

Patient and control groups. The thalassaemia major patients studied regularly attended the blood transfusion clinic at New York Hospital–Cornell Medical Center. All were chronically transfused with deglycerolized (frozen thawed) packed red cells, most being splenectomized in order to reduce their transfusion requirement. The control group consisted of healthy laboratory personnel.

Isolation of mononuclear cells (effector cells). Mononuclear cells were isolated from heparinized peripheral blood. Following centrifugation on a density gradient of sodium metrizoate/Ficoll (Lymphoprep; Accurate Chemical Co., Westbury, NY), mononuclear cells were harvested from the interface, washed twice with Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) and once with RPMI-1640 (Gibco) containing penicillin (100 U/ml; Gibco), streptomycin (100 mg/ml; Gibco) and glutamine (20 μM; Gibco). The cellular composition of such mononuclear cell preparations has been previously reported (Grady *et al.*, 1985). The cells were finally resuspended in the modified RPMI-1640 described above further supplemented with 10% fetal bovine serum (FBS, Gibco). After appropriate adjustment of their concentration, these cells were used as effector cells in the cytotoxicity (NK) assay. In some experiments the effector cell preparations were enriched for NK cells on Percoll gradients as previously described (Akbar *et al.*, 1986).

Target cells. K562 cells, a human erythroleukaemia cell line, were grown in FBS-supplemented RPMI-1640 (complete medium). The cells were in log phase when used. The cells were radiolabelled by incubating them for 1 h at 37°C with $Na_2\,^{51}CrO_4$ (NEZ-030S; NEN Research Products, Boston, MA). They were then washed twice with RPMI-1640, resuspended in complete medium and counted.

Human fibroblasts were cultured and infected with herpes simplex virus-type 1 as described previously (Fitzgerald *et al.*, 1983). Briefly, monolayers of low passage human foreskin fibroblasts were infected with HSV-1 at a multiplicity of infection of 5:1 for 2 h. They were then trypsinized,

washed with RPMI-1640, resuspended in complete medium and labelled as described above. After labelling the cells were washed twice with RPMI-1640, resuspended in complete medium and counted.

Cytotoxicity assay. This assay is described in detail elsewhere (Fitzgerald *et al.*, 1983). Briefly, 5×10^3 ^{51}Cr-labelled K562 cells or HSV-1-infected fibroblasts in 0·1 ml of complete medium were dispensed into the wells of flat-bottomed microtitre plates (Costar, Cambridge, MD) after which effector cells were added in 0·1 ml of complete medium. Each assay was performed in triplicate at effector:target (E:T) ratios ranging from 50:1 to 3:1. The cultures were incubated for either 5–6 h (K562 cells) or 14–16 h (HSV-1-infected fibroblasts) at 37°C in a humidified atmosphere containing 5% CO_2, after which aliquots (100 μl) were removed from each well and their radioactivity determined in a gamma counter (Ultrogamma, model 1280; LKB Instruments Inc., Rockville, MD). The percentage cytotoxicity was calculated according to the following equation.

$$\% \text{ cytotoxicity} = \frac{\text{ct/min (exp)} - \text{ct/min (spon)}}{\text{ct/min (tot)} - \text{ct/min (spon)}} \times 100$$

where spontaneous release was that from target cells incubated in complete medium alone and total release was that from target cells incubated in complete medium containing 2·5% Triton X-100 (Sigma Chemical Company, St Louis, MO).

Modulators of NK activity. Desferrioxamine (DFO) was obtained from the Ciba Pharmaceutical Company (Summit, NJ). A fresh (daily) stock solution of DFO (11 mM) was prepared in RPMI-1640. Stepwise 10-fold dilutions of this stock solution were used in the assays.

Recombinant human α-interferon (α-IFN) was obtained from Hoffmann-LaRoche, Inc. (Nutley, NJ). A stock solution of this material (2×10^4 IU/ml) was prepared in RPMI-1640 and stored at 4°C.

Incubation of effector cells with α-IFN and/or DFO. The effect of α-IFN on NK function was tested in two ways. First, α-IFN (10 μl) was added in triplicate directly to cell cultures at the time of initiation, the final volume being 210 μl. The cultures used for determination of spontaneous and total release were treated similarly, α-IFN having no significant effect on these parameters. RPMI-1640 was added to control cultures. Supernatants were harvested and counted as previously described.

In a second set of experiments, either DFO (20 μl), α-IFN (10 μl) or a combination of the two agents was added in triplicate to 100 μl of effector cells and the cultures incubated for 5–6 h at 37°C in 5% CO_2. After this preincubation, target cells (100 μl) were added to the appropriate wells to give an E:T ratio of 50:1 and the cultures then incubated for an additional 5–6 (K562 cells) or 14–16 h (HSV-1-infected fibroblasts). The supernatants were harvested and counted as before. In this set of experiments, the stimulating agents were not removed after the preincubation period. Their concentration was approximately halved, however, on addition of the target cells.

Determination of supernatant α-IFN. Aliquots of supernatant were collected from the microtitre plates upon termination of those NK assays using effector cells at an E:T ratio of 50:1. Their interferon content was determined using a cytopathic effect inhibition assay as described previously (Fitzgerald, von Wussow & Lopez, 1982). The interferon produced during such assays was shown to be α-IFN (Fitzgerald *et al.*, 1982). The amount of interferon was measured (IU) with an International Human Leukocyte IFN Standard (G-023-901-527) obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases (NIAID).

Statistics. Student's *t*-test was used to determine the significance of the results.

RESULTS

NK activity against HSV-1-infected fibroblasts. We determined the NK activity of unfractionated mononuclear cells from 12 normal individuals and 15 patients with thalassaemia major against both HSV-1-infected and uninfected human fibroblasts. As shown in Fig. 1(a), there was no difference in the cytotoxicity of either effector population toward uninfected targets. However, when the cytotoxic response of the two MNC populations was determined against HSV-1-infected

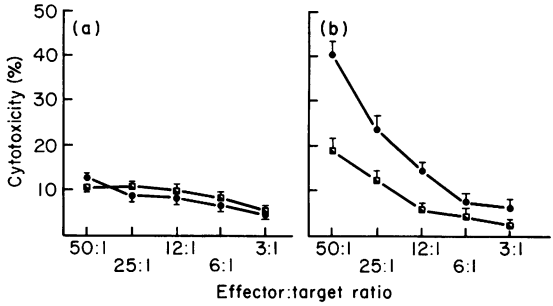

Fig. 1. A comparison of the NK activity of mononuclear cells from 12 normal individuals (●) and 15 thalassaemia patients (◘) against (a) uninfected and (b) HSV-1-infected fibroblasts over a range of effector:target ratios. The effector and target cells were incubated together for 14–16 h before the supernatants were harvested and NK activity determined. Mean values are plotted, the error bars representing the s.e.m.

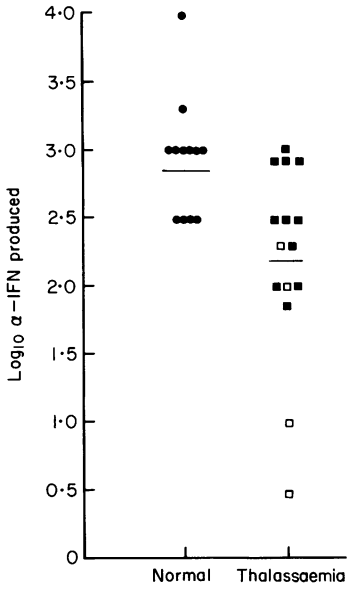

Fig. 2. α-Interferon production following co-culture of mononuclear cells from 12 normals (●), 11 splenectomized thalassaemics (■) and four non-splenectomized thalassaemics (□) with HSV-1-infected fibroblasts for 14–16 h at an E:T ratio of 50:1. Mean (±s.e.m.): normal, 2·94±0·13; thalassaemia, 2·19±0·18; $P<0·005$.

targets, the diminished ability of thalassaemia cells to kill the virally infected targets became manifest (Fig. 1b), the cells from both splenectomized and nonsplenectomized patients behaving similarly. The difference in cytotoxicity between the two populations was significant ($P<0·05$) at E:T ratios of 50:1, 25:1 and 12:1.

α-Interferon production during NK assays. α-Interferon production during NK assays involving MNC from 12 controls and 15 patients with thalassaemia major were compared, both uninfected and HSV-1-infected fibroblasts as well as K562 cells being employed as targets. There was no production of α-IFN in response to either K562 cells or uninfected fibroblasts. However, as shown

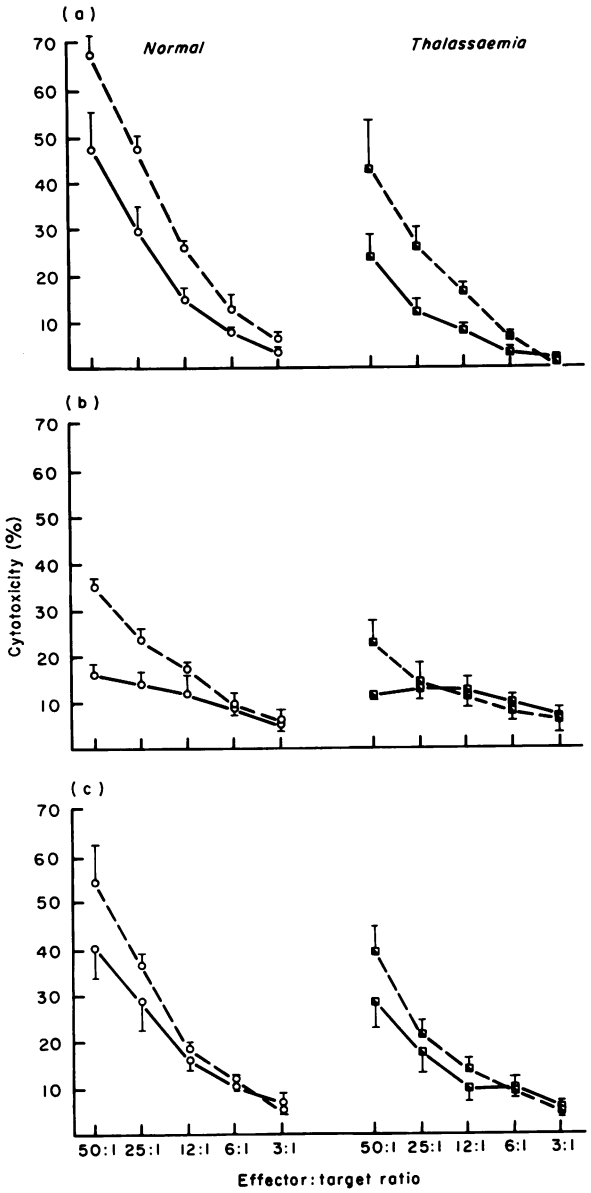

Fig. 3. A comparison of the NK activity of MNC from four normal individuals (○) and five patients with thalassaemia major (■) against (a) K562 cells, (b) uninfected fibroblasts and (c) HSV-1-infected fibroblasts in the presence (dotted line) and absence (solid line) of exogenous α-IFN, the latter being added at the beginning of the NK assays. Mean values are plotted, the error bars representing the s.e.m.

in Fig. 2, when HSV-1-infected fibroblasts were used as targets at an E:T ratio of 50:1, the amount of α-IFN produced by thalassaemic MNC (geometric mean, 155 IU; range of s.d., 30–776 IU) was significantly less ($P<0{\cdot}005$) than that produced by normal MNC (871 IU; 316–2399 IU), irrespective of splenectomy. Serum samples from the individuals studied did not contain detectable amounts of α-IFN (data not shown).

NK activity in response to exogenous α-IFN. As MNC from thalassaemia patients produced less α-IFN in response to HSV-1-infected targets, we evaluated the ability of exogenous α-IFN to augment

Table 1. The effect of exogenous α-IFN (+/− DFO) on the cytotoxicity of normal and thalassaemia MNC against both K562 cells and HSV-1-infected fibroblasts*

		Control	α-IFN†		α-IFN+DFO†‡	
Effector	Target	Killing (%)	Killing (%)	Change (%)	Killing (%)	Change (%)
Normal ($n=3$)	K562 cells	40·7±7·1	67·5±8·0	65·8	72·3±6·0	77·6
Thalassaemia ($n=6$)	K562 cells	23·5±2·5	49·5±4·8	110·0	63·3±3·9	169·4
Normal ($n=3$)	HSV-1-infected fibroblasts	48·2±7·0	74·1±2·3	53·7	84·1±0·4	74·5
Thalassaemia ($n=5$)	HSV-1-infected fibroblasts	11·0±2·8	22·1±4·0	100·9	29·3±5·4	166·4

* Cytotoxicity (%) of MNC preincubated alone or with α-IFN (+/− DFO) for 5–6 h before addition of K562 targets. ^{51}Cr release was measured 5–6 h later.

† MNC preincubated with α-interferon (α-IFN; 2×10^3 IU/ml) for 5–6 h.

‡ MNC preincubated with desferrioxamine (DFO; 0·1 mM) for 5–6 h.

Table 2. The effect of desferrioxamine (DFO) on the production of α-interferon by HSV-1-infected fibroblasts

		α-Interferon (IU)	
Effector cell	No.	−DFO	+DFO
Normal	1	1000	1000
	2	300	3000
	3	300	3000
	4	1000	3000
	5	2000	2000
Thalassaemia	1	1000	1000
	2	100	100
	3	3	10
	4	200	300
	5	300	700
	6	700	700
	7	700	700

Mononuclear cells were preincubated at 37°C for 6 h with and without desferrioxamine (0·1 mM) before addition of the HSV-1-infected fibroblasts, α-interferon production being determined 14–16 h later.

their cytotoxicity. In these experiments α-IFN was added at the initiation of NK assays which lasted for 5–6 h in the case of K562 targets and 14–16 hours in that of infected and uninfected fibroblasts. α-IFN enhanced the NK activity (K562 cells) of MNC from each of the four normals and five patients studied at all E:T ratios, however the mean increases were not significant (Fig. 3a). With either infected (Fig. 3b) or uninfected (Fig. 3c) fibroblasts as targets, exogenous α-IFN had its

maximal enhancing effect on NK activity at an E:T ratio of 50:1. Again however, none of the increases were significant when compared with the activity of cells cultured in the absence of added α-IFN.

NK activity following preincubation with α-IFN and/or desferrioxamine (DFO). In a previous study, we showed that preincubation with DFO had little or no effect on the NK activity of normal MNC toward K562 cells while increasing the activity of thalassaemic MNC in every instance (Akbar *et al.*, 1986). Using HSV-1-infected fibroblasts as targets, we have found that both normal and thalassaemia MNC are consistently more cytotoxic following preincubation with DFO although here too the mean increases were not significant (data not shown). Given these results together with those of adding exogenous α-IFN, we studied the effect of combining these agents on the NK activity of the two effector populations against both K562 cells and HSV-1-infected fibroblasts (Table 1). In these experiments, the MNC were preincubated with α-IFN or DFO plus α-IFN for 5–6 h before the target cells were added. Spontaneous release of radioactivity from the targets was not affected by preincubation with either DFO or α-IFN. Preincubation with α-IFN significantly increased the NK activity of normal and thalassaemia MNC toward K562 targets ($P < 0{\cdot}05$ and 0·001, respectively) and HSV-1-infected fibroblasts ($P < 0{\cdot}025$ and 0·05, respectively), the percentage increase in activity toward both targets being greater in the case of thalassaemia MNC (Table 1). When the effector cells were preincubated with both DFO and α-IFN in every instance the resulting cytotoxicity against the two targets was greater than that with α-IFN alone, although the mean increases were not statistically significant. Once again the increases were most pronounced with MNC from thalassaemia patients. Similar results were obtained with MNC fractions enriched for NK cells on Percoll gradients (data not shown). Finally, in experiments carried out with uninfected fibroblasts as targets, DFO caused little or no increase in cytotoxicity while α-IFN had a moderate enhancing effect (data not shown).

α-IFN production in the presence of DFO. To determine if the increase in NK activity caused by preincubation with DFO could be due to increased production of α-IFN we analysed the supernatants from NK cultures involving HSV-1-infected fibroblasts which had been preincubated for 14–16 h in the presence or absence of DFO. Preincubation with DFO had little or no effect on α-IFN production by the MNC from seven thalassaemia patients (Table 2). However, in the case of two of the five normal individuals studied, preincubation with DFO resulted in a 10-fold increase in the amount of α-IFN produced.

DISCUSSION

In this study we have further investigated the mechanisms involved in the decreased NK activity observed in patients with β-thalassaemia major. It was previously shown that blood transfusion leads to a decrease in NK activity (Gascon *et al.*, 1984; Kaplan *et al.*, 1984; Akbar *et al.*, 1986). In thalassaemia patients who are chronically transfused, we showed that there is a significant correlation between the decrease in NK activity toward K562 targets and the number of transfusions received and that the effect may be due in part to iron overload (Akbar *et al.*, 1986). The fact that NK cells are heterogeneous with respect to target specificity (Fitzgerald *et al.*, 1983) and that NK activity toward HSV-1-infected fibroblasts correlates with resistance to this virus (Lopez *et al.*, 1983; Messina *et al.*, 1986) prompted us to investigate the NK activity of our patients toward the latter target. We now report that NK activity against HSV-1-infected fibroblasts is also significantly decreased in these patients. This was not due to a decrease in the proportion of Leu 11b-staining cells as thalassaemia patients had normal proportions of these cells (Akbar *et al.*, 1986). Furthermore, the decreased activity did not arise as a consequence of splenectomy as peripheral blood MNC from splenectomized and nonsplenectomized patients behaved similarly (data not shown).

A number of studies have shown that co-culture of MNC populations with NK-sensitive target cells induces the production of interferon (Trinchieri *et al.*, 1978; Timonen *et al.*, 1980; Ronnblom, Ramstedt & Ulm, 1983), α-IFN being elaborated when HSV-1-infected fibroblasts are used as targets (Fitzgerald *et al.*, 1982). We found that the MNC from thalassaemia patients produced

significantly less ($P < 0{\cdot}005$) α-IFN when co-cultured with HSV-1-infected fibroblasts than those from normals. As generation of α-IFN appears to be essential for cytotoxicity (Steinhauer, Doyle & Kadish, 1985), a decrease in its production could be one reason for the decreased NK activity in thalassaemia patients. The cell responsible for producing α-IFN in these cultures has been identified as a large granular lymphocyte that does not stain with Leu 11b and lacks other NK markers (P.A. Fitzgerald-Bocarsly *et al.*, unpublished observations). It is not known at present if the decreased production of α-IFN by thalassaemic MNC is due to a reduction in the number of these cells or if there is a defect in production/secretion of this lymphokine. It has been shown however, that the amount of α-IFN generated in assays involving HSV-1-infected fibroblasts cannot completely account for the increased lysis of infected versus uninfected cells (Fitzgerald *et al.*, 1982). It is clear from our studies that while MNC from thalassaemia patients produce less α-IFN than normal, their activity toward both K562 cells and HSV-1-infected fibroblasts can be markedly increased by preincubation with exogenous α-IFN, an apparent normalization of cytotoxicity being observed toward K562 cells.

We previously found that preincubation of thalassaemia but not normal MNC with desferrioxamine can augment NK activity against K562 targets (Akbar *et al.*, 1986). Here we report that similar treatment augments the cytotoxicity of MNC from both normals and thalassaemia patients toward HSV-1-infected fibroblasts. When either population of MNC was preincubated with both DFO and α-IFN an increased enhancement of killing was observed toward the two targets, the effect being greatest in the case of thalassaemia MNC. The fact that DFO had no effect on α-IFN production suggests that these compounds increase NK activity by two different mechanisms both of which appear to be reversible.

Patients with thalassaemia develop severe iron overload as a consequence of their transfusion therapy. *In vitro,* iron-containing compounds have been shown to inhibit the stimulation of lymphocytes by both T cell mitogens (Matzner *et al.*, 1979; Bryan & Leech, 1983) and alloantigens (Bryan *et al.*, 1981). In addition, ferric citrate (Nishiya & Horowitz, 1982) and iron-saturated transferrin (Baines, Lafluer & Holbein, 1983) have been shown to inhibit the killing of K562 cells. Our results with DFO suggest that iron overload *in vivo* may contribute in part to the decreased NK activity which thalassaemia patients express. Decreased α-IFN production could also be a factor. It is not yet clear whether decreased production of this lymphokine is the result of iron overload or some other phenomenon. Thus, we have not ruled out the possibility that repeated antigenic challenges stemming from transfusion therapy may contribute to the decreased NK function seen in this disease.

Apart from a transfusion-related decrease in NK activity (Akbar *et al.*, 1986), thalassaemia patients display a transfusion-related increase in circulating cells containing cytoplasmic immunoglobulin (Akbar *et al.*, 1985) and increased levels of serum immunoglobulins (Wasi, Wasi & Thongchareon, 1971; Tovo *et al.*, 1981). This, together with an increase in the proportion of circulating B cells which bear activation markers (unpublished observations), implies that these patients have increased B cell differentiation *in vivo*. As NK cells can regulate B cell function (Abruzzo & Rowley, 1983; Arai *et al.*, 1983; Brieva, Targan & Stevens, 1984), one explanation for the increased B cell differentiation in thalassaemia is decreased NK regulation of B cell function. Related findings support this hypothesis. For instance, it has recently been shown that α-IFN inhibits *in vitro* antibody synthesis by normal human lymphocytes (Pelton & Denman, 1985). The transfusion-related increase in T suppressor cells which has been found in thalassaemia patients (Grady *et al.*, 1985) may be a response to the increased B cell differentiation seen in this disease.

In conclusion, thalassaemia patients express a significant decrease in NK activity toward K562 cells and HSV-1-infected fibroblasts, reasons for which may include decreased production of αIFN and/or iron overload. We are presently investigating these and other factors which may contribute to decreased NK activity and the mechanism(s) whereby this may relate to the markedly altered immune function observed in these patients.

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