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A distinct pattern of cytokine production from blood mononuclear cells in multitransfused patients with β-thalassaemia

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SUMMARY

The unstimulated and induced production of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-3, IL-6, stem cell factor (SCF), IL-1β, tumour necrosis factor-alpha (TNF-α), TNF-β, interferon-gamma (IFN-γ) and transforming growth factor-beta (TGF-β) was determined after culture of blood mononuclear cells from 22 patients with severe β-thalassaemia in a regular transfusion programme, five non-regularly transfused patients with β-thalassaemia intermedia and nine normal persons. A distinct pattern of cytokine production in thalassaemic patients was detected, namely a low unstimulated production of all cytokines and a significant increase in the stimulated production of IFN-γ, TNF-α and IL-1β; these abnormalities were more pronounced in the more heavily transfused older patients. The increased production of the above cytokines, which usually characterize the acute response to infectious agents and have a negative effect on erythropoiesis, may explain the deterioration of anaemia found in thalassaemic patients during acute infections.

Keywords β-thalassaemia cytokines transfusions

INTRODUCTION

β-thalassaemia is a hereditary disease, characterized by decreased or absent production of β-globin chains, resulting in ineffective erythropoiesis and severe anaemia. Recent work has shown that the ineffective erythropoiesis present in β-thalassaemia is the result of an increased rate of apoptosis of the marrow erythroid cells [1]. Transfusions lead to iron overload and also to immune derangements, both of which exert a negative effect on the functional integrity of the immune system in multitransfused patients with thalassaemia. There are already a large number of reports describing immune abnormalities in thalassaemia, namely defective function of polymorphonuclear neutrophils and monocytes [2,3], decrease of $CD4^+$ cells and increase of $CD8^+$ cells [4–6], diminished mitogen responses [5] and low natural killer (NK) cell activity [7,8]. These and probably other undetected abnormalities may explain the tendency for severe or unusual infections. The severity of infections in thalassaemic patients is manifested by a deterioration of the degree of anaemia and by an increased incidence of sepsis. It is well established that the production of cytokines from cells of the immune system (IL-1, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), etc.) plays a major role in the generation of cardinal manifestations of acute infections, like fever and anaemia. In a recent study increased levels of TNF-α and undetectable or normal values for IL-2 and IL-6 were found in serum of patients with β-thalassaemia [9].

In the present study we investigated the production of cytokines, both unstimulated and induced, from blood mononuclear cells from patients with β-thalassaemia with different transfusion history and correlated the results with those obtained from non-transfused patients with β-thalassaemia intermedia and from normal persons.

PATIENTS AND METHODS

The study was approved by the ethical committee of the hospital and informed consent was obtained from all individuals included in the investigation. Multiple transfused patients were divided in three subgroups (C,D,E), according to age. Two other groups were the thalassaemia intermedia patients (B) with a history of occasional transfusions, and healthy controls (A). Table 1 shows the study groups and the corresponding mean age, mean pretransfusion haemoglobin value, mean number of transfusions and mean serum ferritin value. Splenectomy status, anti-hepatitis C virus (HCV) positivity and treament with IFN-α are also shown.

Cells were isolated from heparinized peripheral blood by Ficoll–Hypaque density gradient centrifugation. Cells at the interphase, namely lymphocytes, monocytes and NK cells, were collected, washed twice with RPMI, resuspended in RPMI + 10% fetal calf serum (FCS) at a concentration of 2×10^6 cells/ml and

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Table 1. Patient characteristics

		n	Age, mean (years) ± s.e.m.	Hb, mean (gm/dl) ± s.e.m.	Transfusion, mean ± s.e.m.	Ferritin, mean (mg/ml) ± s.e.m.	Splenectomized	Anti-HCV-positives	IFN-α-treated
A	Normals	9	28·6 ± 2·9	13·8 ± 1·3	0	78 ± 26	0/5	0/5	0/5
B	Thalassaemia intermedia	5	34 ± 4	9·5 ± 0·4	6 ± 3·7	2168 ± 560	4/5	0/5	0/5
C	β-thalassaemia major 5–10 years	4	7·8 ± 1·3	10 ± 0·3	160 ± 28·7	3309 ± 650	0/4	2/4	2/4
D	β-thalassaemia major 11–20 years	10	16·2 ± 0·8	10·3 ± 0·1	327 ± 26·8	3035 ± 355	2/10	5/10	4/10
E	β-thalassaemia major >20 years	8	26·3 ± 1·3	10·2 ± 0·1	621 ± 48·6	4347 ± 1295	4/8	5/8	4/8

incubated at 37°C, 5% CO_2. Cultures were performed in triplicate wells with either medium alone, lipopolysaccharide (LPS; 50 ng/ml) or phytohaemagglutinin (PHA; 3 μg/ml). Supernatants were collected after 3 days and kept at −70°C. Cytokine concentrations were measured by ELISA.

The following cytokines (minimum detectable levels in pg/ml shown in parentheses) were determined: granulocyte-macrophage colony-stimulating factor (GM-CSF) (1·5), granulocyte colony-stimulating factor (G-CSF) (7·2), IL-6 (0·7), IL-3 (7·4), IL-1β (0·3), TNF-α (4·4), transforming growth factor-beta (TGF-β) (7·0) (all from R&D Systems, Abingdon, UK), and IFN-γ (100) from Endogen Inc. (Cambridge, MA).

RESULTS

Characteristics of the patients

As shown in Table 1, there was a positive correlation between mean total number of transfusions and age in multiple transfused thalassaemic patients (groups C–E); the correlation between mean number of transfusions (or age) and ferritin levels, on the other hand, was poor, reflecting both the inability of ferritin to serve as an ideal marker of iron load and the different intensity of deferioxamine therapy among patients. Statistical analysis showed that history of splenectomy, anti-HCV positivity and IFN-α treatment were not important discriminating factors.

Unstimulated cytokine production

Figure 1 shows unstimulated cytokine production, as determined in supernatants from unstimulated liquid cultures of blood mononuclear cells. Decreased unstimulated production of GM-CSF, G-CSF, IL-3, IL-6, TNF-α and IL-1β was found in older multitransfused patients with β-thalassaemia (group E), compared with both normal persons (A) and non-transfused patients with thalassaemia intermedia (B).

Younger thalassaemic patients (groups C and D) showed cytokine production values that were not statistically significantly different from the two control groups (A and B), although a trend

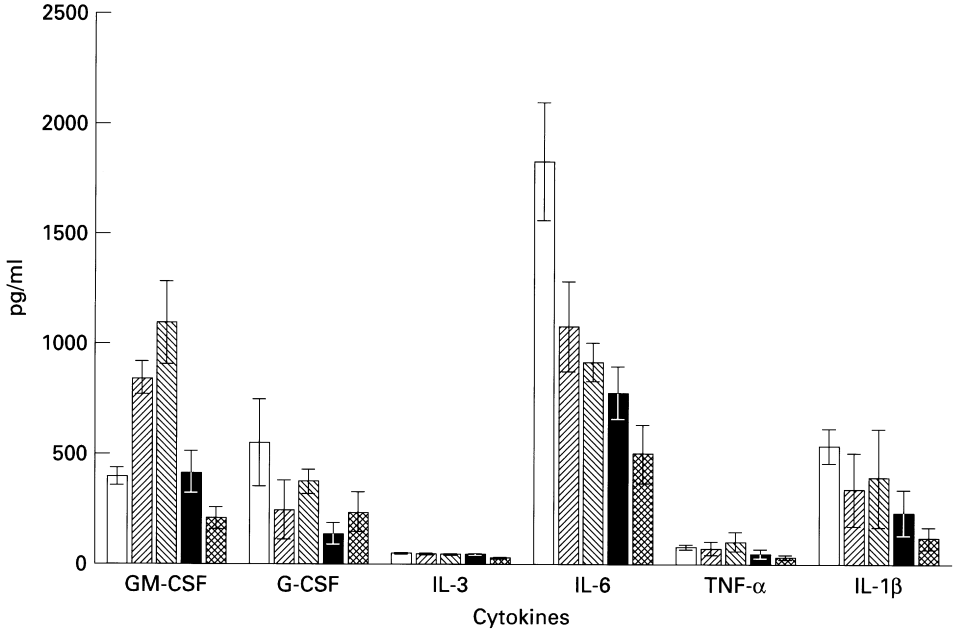

Fig. 1. Unstimulated production of cytokines from blood mononuclear cells after incubation of 2×10^6 cells/ml at 37°C, 5% CO_2 for 3 days. Results are shown as pg/ml (× ± s.e.m.). □, Normals; ▨, thalassaemia intermedia; ▧, β-thalassaemia-positive 5–10 years; ■, β-thalassaemia-positive 11–20 years; ▩, β-thalassaemia-positive > 20 years.

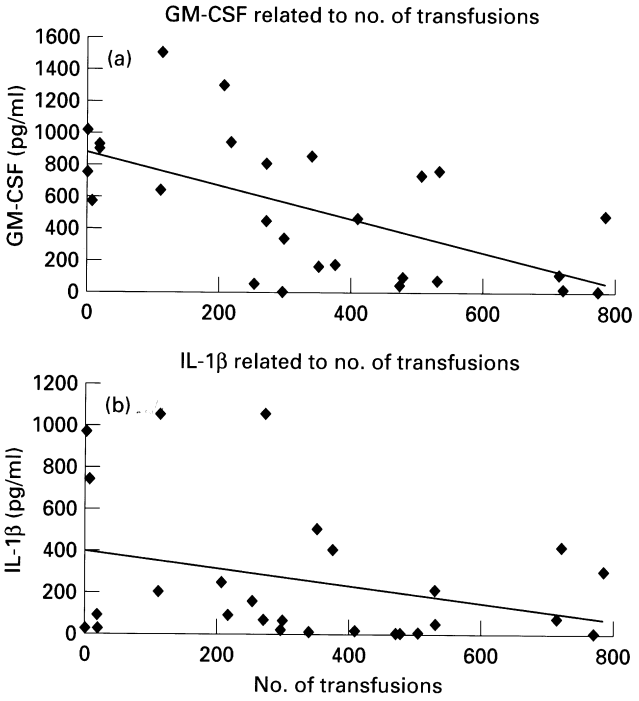

Fig. 2. Comparison between unstimulated production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1β from blood mononuclear cells from patients with thalassaemia and number of transfusions. For GM-CSF the correlation coefficient (r) was $-0{\cdot}522$ ($P = 0{\cdot}0025$) and for IL-1β r was $-0{\cdot}287$ ($P = 0{\cdot}0726$).

towards lower unstimulated cytokine production was evident mainly in group D.

When a comparison was made between the number of transfusions and unstimulated production of GM-CSF and IL-1β (Fig. 2) an inverse correlation was found, in that less transfused patients had a higher production of both cytokines.

Induced production of cytokines

Figure 3 shows the production of cytokines from cultures of mononuclear cells performed in the presence of LPS or PHA. Data are presented as percentage increase after stimulation, in comparison with unstimulated (medium alone) cultures. It is evident that older multitransfused patients (group E) manifested a tremendous increase in the production of certain cytokines after stimulation, namely of TNF-α (2036%), IFN-γ (2846%) and IL-1β (500%), and much less so for GM-CSF (211%), G-CSF (40%), IL-3 (324%) and IL-6 (75%). The relative increase of stimulated production for non-transfused patients of similar age with thalassaemia intermedia was 205% for TNF-α, 23% for IFN-γ and 377% for IL-1β. This increase of stimulated cytokine production was also observed in the other groups of younger transfused thalassaemic patients (groups C and in particular D) and, as is also evident from the figure, this significant increase of induced cytokine production concerned only cytokines with a negative action on haematopoiesis (IFN-γ, TNF-α, IL-1β) and not the positive regulators (GM-CSF, IL-3, IL-6).

Statistical analysis showed that the above differences were related to the number of transfusions, but not to ferritin levels, previous splenectomy, the presence of chronic HCV infection or treatment with IFN-α (data not shown).

DISCUSSION

Decreased unstimulated production of several cytokines and a significant increase of induced production of cytokines with a negative action on haematopoiesis were detected in multitransfused patients with β-thalassaemia.

These findings were unrelated to ferritin levels, previous splenectomy, the presence of chronic HCV infection or treatment with IFN-α, and were strongly correlated with age and the number of transfusions. Age and transfusion numbers were in complete accordance in thalassaemic patients, but the absence of abnormalities in the production of cytokines in older patients with β-thalassemia intermedia, which are not regularly transfused, suggests that transfusion history is the major determining factor.

The absence of a correlation between ferritin levels and cytokine production does not contradict the above findings. Serum ferritin determination is a good but not ideal marker of iron overload [10], because it is influenced by several unrelated factors. On the other hand, we can speculate that cytokine

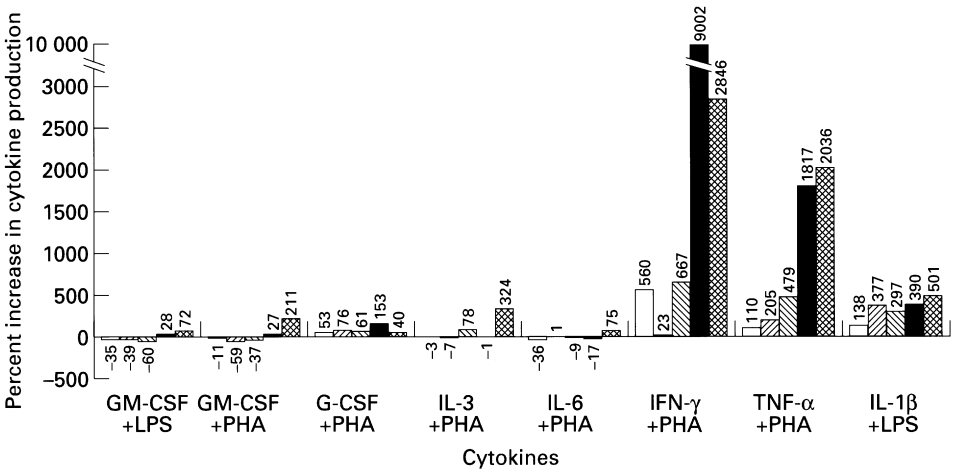

Fig. 3. Percent increase of production of cytokines from 2×10^6 blood mononuclear cells/ml after incubation with phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) at 37°C, 5% CO_2 for 3 days. Results are shown as the mean percentage increase. □, Normals; ▨, thalassaemia intermedia; ▧, β-thalassaemia major 5–10 years; ■, β-thalassaemia major 11–20 years; ▩, β-thalassaemia major > 20 years.

production is related more to the event of transfusion *per se* (frequency and chronicity of antigenic stimulation) than to its iron overload-related sequelae.

The finding of 'overproduction' of certain cytokines, such as TNF-α, IFN-γ and IL-1, after stimulation of mononuclear cells in multiple transfused patients may be relevant to some of the observed consequences of infections in these patients. Acute infections in thalassaemic patients are accompanied very often by a deterioration of anaemia and increased transfusion needs. The overproduction of cytokines with a negative effect on haematopoiesis, such as TNF-α and IFN-γ [11,12], could explain the above complication. The same holds true concerning the propensity of thalassaemic patients to develop sepsis after an acute, initially localized, microbial infection, which could be explained by the overproduction of TNF-α and IL-1.

In conclusion, we believe that clinical studies in thalassaemic multiple transfused patients, with serial measurements of serum cytokines during steady-state and acute infections, are needed in order to validate the above *in vitro* data.

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