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Immunological abnormalities in thalassaemia major. I. A transfusion-related increase in circulating cytoplasmic immunoglobulin-positive cells

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SUMMARY

Multiply transfused patients with β-thalassaemia major have elevated serum immunoglobulin levels and a transfusion-related increase in ($T8^+$) T-suppressor cells. We report here that these patients also have a significantly increased ($P < 0{\cdot}001$) proportion of B-cells that contain cytoplasmic immunoglobulin when visualized immediately upon isolation. On the other hand, the same cell populations do not exhibit an increase in the proportion of immunoglobulin-secreting cells as measured by a reverse haemolytic plaque assay. These results, together with those of ongoing studies, suggest that the cells containing the cytoplasmic immunoglobulin are likely to be terminally differentiated B-cells which persist in the circulation. While the reason for this phenomenon is not yet known, we have found that the increase in these cells is transfusion-related.

Keywords thalassaemia transfusion serum immunoglobulins cytoplasmic immunoglobulins circulating cytoplasmic immunoglobulin-positive cells

INTRODUCTION

Patients with β-thalassaemia major have an inherited dysfunction of their β-globin genes and must receive repeated blood transfusions to prevent the development of life-threatening anemia. The transfusions, however, lead to severe iron overload. A therapeutic splenectomy is generally performed early in the second decade to reduce the rate of iron loading (Modell, 1977; Graziano *et al.*, 1981). We have previously shown that the percentage of T-suppressor ($T8^+$) cells increases linearly with the number of units of blood transfused in both splenectomized and non-splenectomized patients (Grady *et al.*, 1985). Similar findings have been observed in patients with sickle cell disease who require transfusion therapy (Kaplan *et al.*, 1984). On the other hand, natural killer (NK) activity is decreased in patients receiving multiple transfusions (Gascon, Zoumbos & Young, 1984; Kaplan *et al.*, 1984).

To obtain a better understanding of the immunological changes which occur in multiply transfused patients, we have been further investigating the abnormalities observed in patients with thalassaemia major. Serum immunoglobulin levels are markedly elevated in these patients (Wasi, Wasi & Thongcharoen, 1971; Tovo *et al.*, 1981; Munn *et al.*, 1984). We now report that there is a transfusion-related increase in the proportion of their circulating mononuclear cells which contain cytoplasmic immunoglobulin (CIg) and that these cells are possibly terminally differentiated B-cells which persist in the circulation.

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MATERIALS AND METHODS

Patient and control groups. The thalassaemia major patients were drawn from those who regularly attend the blood transfusion clinic at New York Hospital-Cornell Medical Center. All were chronically transfused, deglycerolized (frozen thawed) packed red cells being employed since 1968. Most of the patients had been splenectomized in order to reduce their transfusion requirement. Measurements of serum chemistries were made every 3 months and of serum iron and transferrin saturation every 6 months. In comparing these parameters with those of our immunological studies, we used the values determined closest to the time of the immunological studies. The control group consisted of healthy laboratory personnel.

Isolation of mononuclear cells (MNC). Mononuclear cells were isolated from heparinized peripheral blood within 1 h of venipuncture. Following centrifugation on a sodium metrizoate/ficoll gradient (Lymphoprep; Accurate Chemical Co., Westbury, New York, USA), the isolated cells were resuspended in RPMI-1640 (GIBCO, Grand Island, New York, USA) 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).

Preparation of T-enriched and T-depleted MNC fractions. T-enriched and T-depleted populations of MNC were prepared by rosetting with neuraminidase-treated sheep red blood cells as described previously (Akbar, Jones & Wright, 1984). The purity of the T-enriched and T-depleted fractions after E-rosetting of normal and thalassaemic MNC is shown in Table 1.

Preparation of monocyte-depleted MNC fractions. Mononuclear cell suspensions (5×10^6 cells/ml) were incubated for 1 h at 37°C in plastic Petri dishes (60 × 15 mm; Becton Dickinson, Oxnard, California, USA). The non-adherent cells were collected, washed twice, and used as a monocyte-depleted population.

Enumeration of cells bearing surface immunoglobulin. B-lymphocytes were identified by direct immunofluorescent staining of membrane immunoglobulins (Ig). Briefly, aliquots of MNC suspensions were incubated on ice for 30 min with fluorescein isothiocyanate-conjugated (FITC-conjugated) goat anti-human polyvalent immunoglobulins (Meloy, Springfield, Virginia, USA). After washing three times in cold phosphate buffered saline (PBS; GIBCO), the cells were pelleted by centrifugation, resuspended in cold glycerol, mounted on slides and examined under ultraviolet illumination using a Leitz fluorescent microscope (Optilux II). The number of fluorescent lymphocytes was expressed as a percentage of the total number of lymphocytes counted (Table 1). A minimum of 100 cells were counted per slide, duplicate slides being prepared for each sample.

Enumeration of monocytes. Monocytes (macrophages) were identified in two ways. First, cytocentrifuge preparations of mononuclear cell populations were stained for acid α-naphthyl acetate esterase activity as previously described (Koski, Poplack & Blaese, 1976), the results being

Table 1. Composition of mononuclear cell subpopulations after E-rosetting

		E-rosetting cells (%)	SIg^+ cells* (%)	NSE^+ cells† (%)
Normal	T-enriched	87·0 ± 5·6‡	3·0 ± 1·8	1·4 ± 0·4
	T-depleted	1·0 ± 0·2	39·9 ± 8·2	22·7 ± 5·0
Thalassaemia	T-enriched	86·3 ± 6·7	5·7 ± 4·4	1·0 ± 0·2
	T-depleted	1·0 ± 0·2	48·3 ± 2·4	12·3 ± 4·4

* Cells positive for surface Ig.
† Cells positive for non-specific esterase.
‡ Mean ± s.e.m. of at least four experiments.

expressed as the percentage of esterase-positive cells (Table 1). Secondly, the proportion of monocytes was determined by indirect immunofluorescent staining with a mouse monoclonal antibody (M1; Ortho Pharmaceutical Co., Raritan, New Jersey, USA). Briefly, the monoclonal antibody was added to aliquots of mononuclear cell suspensions (2×10^6 cells/ml) and the mixtures incubated on ice for 30 min. After being washed twice with Hanks' balanced salt solution (HBSS, GIBCO), the cell pellets were resuspended in RPMI-1640 and incubated with FITC-conjugated goat anti-mouse polyvalent immunoglobulins (Meloy). The cells were then washed and counted as described above.

Enumeration of cells staining for cytoplasmic immunoglobulin (CIg). Cytocentrifuge preparations were made within 2 h of isolating the mononuclear cells. The cells were fixed overnight in dry acetone at −20°C and then stained with FITC-conjugated goat anti-human antisera (Meloy) for 30 min at room temperature. The slides were then washed four times with PBS, mounted in 90% glycerol and examined under ultraviolet illumination as described above. The number of cells bearing cytoplasmic immunoglobulin was expressed as a percentage of the total number of mononuclear cells counted.

Enumeration of phagocytic cells. Aliquots (0·5 ml) of freshly isolated MNC (3×10^5 cells/ml) were incubated for 45 min at 37°C with 0·5 ml of a 1:50 dilution of packed latex particles (diameter, 1·101 μm; Dow Chemical Co., Indianapolis, Indiana, USA) in RPMI-1640 containing 20% fetal bovine serum (GIBCO). The cells were then washed three times in HBSS and cytocentrifuge preparations made as described above. Cells that contained more than three latex particles were considered to be phagocytic. The results were expressed as the percentage of MNC that were phagocytic.

Trypsinization of MNC to remove surface protein. Freshly isolated MNC were treated with trypsin to remove surface proteins, specifically surface Ig. Briefly, trypsin (500 μg/ml; Type XI, Sigma Chemical Co., St Louis, Missouri, USA) and cycloheximide (10 μg/ml; Sigma) were added to MNC and the suspension incubated for 30 min at 37°C. The cycloheximide was used to prevent re-expression of surface proteins after their removal. The cells were then washed four times in HBSS after which cytocentrifuge preparations were made, fixed and stained with FITC-conjugated goat anti-human polyvalent Ig as previously described.

Statistics. Student's *t*-test and linear regression analysis were used to analyse the results.

RESULTS

Determination of cytoplasmic Ig in thalassaemic MNC when visualized immediately upon isolation

As shown in Fig. 1, unfractionated MNC preparations from 21 thalassaemic patients exhibited a significantly ($P < 0{\cdot}001$) greater proportion of cells bearing small amounts of cytoplasmic Ig (mean 15·0%) than those of the controls (mean 1·5%). This cytoplasmic Ig was visualized upon fixing and staining the cells immediately after isolation and was clearly distinct from the bright cytoplasmic fluorescence seen in both normal and thalassaemic cells cultured for 7 days in the presence of pokeweed mitogen (Fig. 2). The fact that both splenectomized and non-splenectomized patients displayed markedly elevated proportions of circulating CIg-positive cells suggests that the phenomenon did not arise as a consequence of splenectomy. Moreover, we found that freshly isolated MNC from 10 thalassaemia patients failed to secrete significant amounts of Ig as shown by the results of a reverse haemolytic plaque assay. Finally, the increase in spontaneous cytoplasmic Ig could not be attributed to a particular Ig isotype as the proportions of cells bearing cytoplasmic IgG, IgA and IgM were all increased (Table 2).

Evidence that the spontaneous cytoplasmic Ig is present within B-cells

As thalassaemia patients have elevated levels of serum Ig, one possible explanation for the elevated proportion of MNC bearing cytoplasmic Ig could be non-specific internalization of Ig by monocytes. To investigate this possibility, MNC from five patients were incubated with latex particles and then stained for cytoplasmic Ig to see if CIg^+ cells were also phagocytic as evidenced by internalization of latex particles (Table 3). Cells from the five individuals examined showed

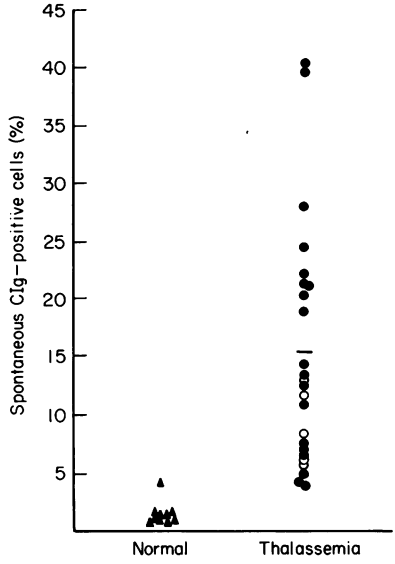

Fig. 1. A comparison of the percentages of circulating CIg$^+$ cells seen in normals (n = 10) and patients with thalassaemia major (n = 25). The open circles represent patients who were not splenectomized.

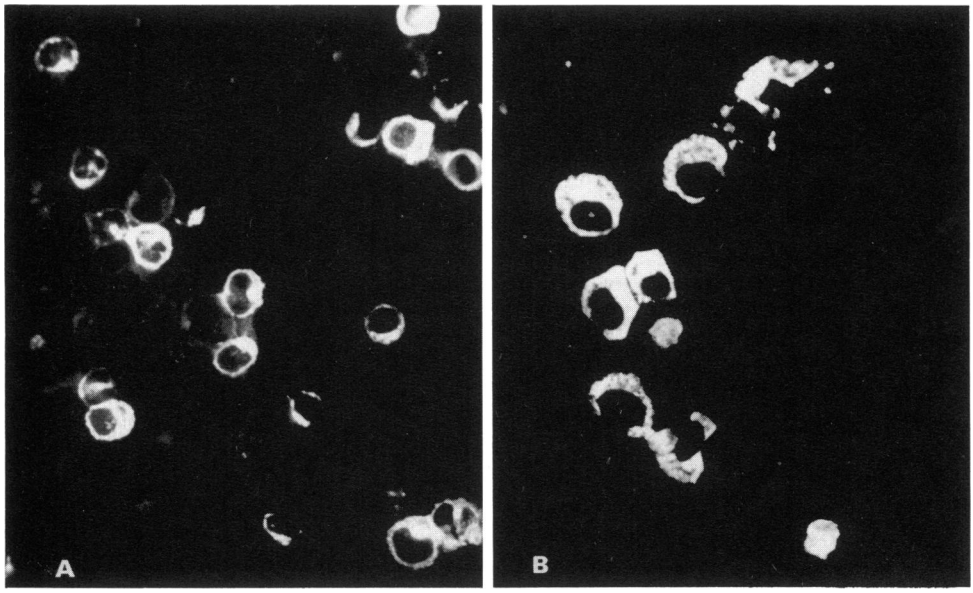

Fig. 2. (A) Circulating CIg$^+$ cells from a patient with thalassaemia major. The cells were fixed with FITC-conjugated goat anti-human polyvalent Ig immediately upon isolation. (B) Cytoplasmic Ig in cells from a normal individual cultured for 7 days in the presence of pokeweed mitogen.

variable proportions of cytoplasmic Ig but similar proportions of phagocytic cells. No cells were observed which contained both cytoplasmic Ig and phagocytized latex particles. Furthermore, in three separate experiments removal of adherent cells (monocytes) reduced the mean proportion of M1$^+$ cells from 14·9 to 4·3% while that of CIg$^+$ cells showed little change (8·0 versus 7·2%; Table 4). Thus, it seems unlikely that the increased proportion of circulating cells exhibiting cytoplasmic Ig in thalassaemia is due to internalization of Ig by monocytes.

Table 2. Percentages of freshly isolated thalassaemia cells bearing cytoplasmic IgG, IgA and IgM*

	Spontaneous CIg$^+$ cells (%)†			
Experiment	Total Ig	IgG	IgA	IgM
1	20·5	9·9	5·0	6·3
2	4·0	0·8	2·7	1·9
3	6·0	1·4	2·0	2·9
4	3·3	2·0	0·5	0·5

* Four different individuals tested by immunofluorescence.

† Cytocentrifuge preparations of unfractionated MNC were made and stained simultaneously for total Ig and each of the three isotypes. Results expressed as the mean percentage of cells containing cytoplasmic Ig relative to the total number of cells counted on duplicate slides.

Table 3. Phagocytic capacity of thalassaemic cells bearing spontaneous cytoplasmic Ig*

Experiment	CIg$^+$ cells (%)	Phagocytic cells (%)	Cells containing CIg and latex (%)
1	5·5	3·6	0·0
2	8·6	6·6	0·0
3	21·4	5·3	0·0
4	19·2	4·2	0·0
5	40·6	4·6	0·0

* Aliquots (0·5 ml) of freshly isolated MNC (3×10^5/ml) were incubated with latex particles as described in materials and methods. After washing, cytocentrifuge preparations were made, fixed and stained for polyvalent cytoplasmic Ig. Results expressed as mean percentage of cells containing cytoplasmic Ig and/or latex paticles relative to the total number of cells counted on duplicate slides.

The small amounts of spontaneous cytoplasmic Ig could also be due to staining of serum immunoglobulins bound to Fc receptors expressed on the surface of the cells. T-cells, B-cells and monocytes all bear these receptors. Staining of monocytes via Fc receptor-bound Ig can be ruled out as phagocytic cells (monocytes) did not contain cytoplasmic Ig (Table 3). When freshly isolated MNC from two patients were fractionated into T-enriched and T-depleted populations prior to staining for cytoplasmic Ig, it was found that the T-enriched fractions were markedly depleted of CIg$^+$ cells compared to the unfractionated populations (1·5 and 1·6% versus 6·2 and 13·0%). Conversely, the T-depleted fractions were greatly enriched for CIg$^+$ cells (22·7 and 34·7% versus 13·0 and 6·2%). A cell-marker analysis of T-enriched and T-depleted fractions from both normals and thalassaemia patients is presented in Table 1. Thus, it is unlikely that visualization of Ig bound to Fc receptors on T-cells or monocytes can account for the elevated proportions of circulating CIg$^+$ cells in thalassaemia.

Finally, in previous studies (Kapadia *et al.*, 1980; Grady *et al.*, 1985), we showed that

Table 4. Effect of monocyte depletion on the proportion of CIg-positive cells*

Experiment	Monocyte depletion	$M1^+$ cells (%)	CIg^+ cells (%)
1	–	12·5	6·0
	+	4·3	5·4
2	–	15·8	9·0
	+	5·8	7·8
3	–	16·5	8·9
	+	2·8	8·3

* Aliquots of mononuclear cells from three patients were stained separately for monocytes (M1) and cytoplasmic Ig (CIg) before and after adherence to plastic. The results are expressed as the mean percentage of positive cells on duplicate slides.

unfractionated MNC from thalassaemia patients exhibit highly significant increases in the proportion of cells bearing surface Ig (B-lymphocytes). We therefore investigated the possibility that the small amount of cytoplasmic Ig observed in the freshly isolated MNC of thalassaemia patients is due to staining of surface Ig on B-cells. MNC from two patients were trypsinized, thereby reducing the proportion of cells bearing surface Ig from 11·8 and 22·2% to 1·5 and 6·4%, respectively. However, little change was noted in the proportions of CIg^+ cells (4·1 and 5·0% versus 5·7 and 5·8%, respectively). Thus, visualization of surface Ig on B-cells can also be ruled out as the source of CIg-positive cells.

The effect of transfusion on the proportion of circulating CIg^+ cells in thalassaemia major

We determined the number of CIg^+ cells in 25 patients who had received up to 800 transfusions of blood. As shown in Fig. 3, there was a significant correlation between the proportion of CIg^+ cells and the number of units received ($P < 0{\cdot}001$).

DISCUSSION

It is widely recognized that both splenectomized and non-splenectomized patients with homozygous β-thalassaemia have elevated levels of serum Ig (Wasi *et al.*, 1971; Constantoulakis *et al.*, 1978; Tovo *et al.*, 1981). Neither transfusions *per se* nor liver damage can account for this as elevated serum Ig levels are found in even young patients who have received few transfusions and in whom serum liver enzyme levels are normal (Tovo *et al.*, 1981). Our experience is in agreement with these findings. Moreover, the patients that we have investigated do not have an increased incidence of infections to account for the elevated levels of serum Ig.

Recently, iron and transferrin have received considerable attention as potent modulators of immune function (Baines, Lafluer & Holbein, 1983; Brieva & Stevens, 1984; Neckers & Cossman, 1983). While patients with thalassaemia major have severe iron overload, we have not found any correlation between elevated serum Ig levels and serum iron or transferrin saturation. However, in an earlier study of patients with thalassaemia intermedia (Kapadia *et al.*, 1980), a clinically milder form of the disease requiring few if any transfusions, patients with high serum iron levels were shown to have higher circulating levels of IgG and IgA than patients with a low serum iron. Thus, a plateauing of serum iron levels may explain the lack of correlation in those who were heavily transfused. It is possible that tissue iron load would correlate more closely with serum Ig than serum

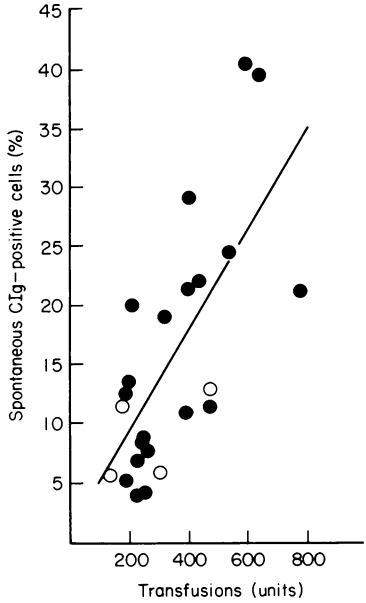

Fig. 3. Linear regression analysis of the percentage of circulating CIg^+ cells as a function of the number of transfusions that the thalassaemia patients received ($r = 0{\cdot}641$, $P < 0{\cdot}001$). The open circles represent patients who were not splenectomized.

iron. On the other hand, the length of exposure to excess iron may be of greater importance in modulating Ig production than its actual circulating level.

We have been investigating B-cell function in these patients to determine if the high levels of serum Ig seen *in vivo* are reflected by the reactivity of peripheral blood MNC *in vitro*. The normal circulating B cell pool in humans is thought to contain cells at various stages of activation ranging from the resting B cell to fully differentiated B cells capable of secreting immunoglobulins spontaneously (Muraguchi *et al.*, 1984; Hirano, Teranishi & Onoue, 1984). In diseases such as AIDS (Lane *et al.*, 1983), systemic lupus erythematosus (Kallenberg *et al.*, 1983) and rheumatoid arthritis (Pardo & Levinson, 1983) where B cell activation is present, there are elevated numbers of fully differentiated B cells which secrete Ig spontaneously. Upon stimulation with PWM, however, mononuclear cells from these patients secrete less Ig than normal (Lane *et al.*, 1983; Kallenberg *et al.*, 1983). In patients with thalassaemia, the presence of elevated levels of serum Ig together with increased numbers of CIg-positive cells and peripheral blood mononuclear cells which respond poorly to stimulation with both PWM and formalin-fixed *Staphylococcus aureus* (unpublished observations) indicates that B-cell activation also occurs in this disease. Unlike the situation which exists in the other diseases cited, the lack of cells that secrete Ig spontaneously in thalassaemia may indicate that the circulating CIg^+ cells are at a late (postsecretory) stage of B-cell differentiation. We have ruled out the possibility that the spontaneous CIg^+ cells found in thalassaemia are B cells with surface/Fc receptor-bound Ig. It is unclear at the present time why these differentiated cells persist in the circulation. The finding that elevated levels of serum Ig and increased proportions of cells bearing spontaneous cytoplasmic Ig are found in both splenectomized and non-splenectomized patients indicates that splenectomy cannot account for the results.

We previously demonstrated a transfusion-related increase in the number of T-suppressor cells in the peripheral blood of patients with thalassaemia major (Grady *et al.*, 1985). We have now found a transfusion-related increase in the proportion of spontaneous CIg^+ cells in these patients. It is possible that the increase in T-suppressor cells may be directed against activated B cells. The fact that the increase in suppressor cells does not maintain the proportion of spontaneous CIg^+ cells at normal levels suggests that an additional regulatory mechanism for activated B cells may be

deficient in these patients. Natural killer (NK) cells have been shown to regulate the terminal stages of humoral responses (Brieva, Targan & Stevens, 1984). We have found that NK activity is decreased in these patients compared to that of normals (unpublished observations). At present we are investigating the possibility that the observed increase in spontaneous CIg^+ cells in thalassaemia is due to diminished NK regulation of terminally differentiated B-cells.

In conclusion, we have shown that patients with thalassaemia major have elevated serum Ig and a transfusion-related increase in spontaneous CIg^+ cells. It is of importance to determine if the immunological abnormalities reported here are also found in patients who have been multiply transfused for other reasons.

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