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Abnormalities in the immune system of children with beta-thalassaemia major

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SUMMARY

We have studied both the humoral and cell mediated immune systems of 23 children with beta-thalassaemia major. In children who had not been splenectomized, a 3-fold expansion in the number of circulating B cells and a modest polyclonal gammopathy was present. Of these patients 70% had decreased numbers of circulating T4 cells; 83% were unresponsive to skin testing with *Candida albicans*, and the majority had decreased lymphocyte proliferative responses *in vitro*. In children who had been splenectomized, there was a 10-fold increase in the number of circulating B lymphocytes and a 2-fold increase in the number of T4 and T8 cells present in peripheral blood. Additionally, these patients as a group were more responsive to both skin testing and lymphocyte stimulation *in vitro* with *Candida albicans*. Seven patients had an inverted T4/T8 ratio. One child has positive serology to HIV by ELISA and Western Blot techniques with a normal T4/T8 ratio. Thus, while children with thalassaemia are at risk for exposure to HIV, the immunological abnormalities associated with the disease and/or its treatment necessitates cautious interpretation of any AIDS-related immunological changes.

Keywords thalassaemia B cells AIDS

INTRODUCTION

Patients with beta-thalassaemia major are exposed to large quantities of blood each year which places them at risk for development of the acquired immune deficiency syndrome (AIDS). We therefore screened 23 patients for immunological changes known to be associated with AIDS. In attempting to interpret the results, consideration had to be given to the immunological abnormalities previously described in patients with thalassaemia.

Most previous studies have examined patients with the non-tranfusion dependent 'intermedia' form of the disease (Musumeci *et al.*, 1979; Kapadia *et al.*, 1980; Munn *et al.*, 1981; Sinniah & Yadav, 1981; Tovo *et al.*, 1981; Rhalifa *et al.*, 1983). Immunological abnormalities reported in these studies have included: decreased opsonization and granulocyte phagocytosis (Rhalifa *et al.*, 1983), increased serum immunologlobulin levels (Tovo *et al.*, 1981; Sinniah *et al.*, 1981) and alterations in B and T cell numbers and function (Musumeci *et al.*, 1979; Kapadia *et al.*, 1980; Munn *et al.*, 1981). Contradictory data have been generated because of the clinical heterogeneity among patients with beta-thalassaemia in their medical care and in their exposure to potentially immunosuppressive viral agents with blood transfusions (Musumeci *et al.*, 1979). Factors such as splenectomy (Kapadia *et al.*, 1980), iron overload (Kapadia *et al.*, 1980), repeated exposure to foreign antigens at the time

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of blood transfusion (Gascon, Zdumbos & Young, 1984), and the use of the chelating agent, deferoxamine (DFO) (Lederman *et al.*, 1984) have profound effects on the immune system.

Our patients were children with transfusion dependent homozygous beta-thalassaemia. We have established baseline immunological data for this kind of multiply-transfused patient and examined then for changes associated with HIV infection.

METHODS

Subjects. Twenty-three patients of Greek or Italian ethnic background were studied. Twenty-one of 23 patients were greater than 7 years of age (mean age $\pm$ 1 s.d., 12·5 $\pm$ 4). All had been transfused since infancy with 15 ml/kg of washed packed red blood cells (PRBC) every 3–5 weeks to maintain a haemoglobin level greater than 10·0 g/dl (hypertransfusion) (Wolman, 1964). Fifteen of the 23 patients had been splenectomized after transfusion requirements exceeded 200 ml PRBC/kg/year. Twenty-one of the patients received chronic subcutaneous chelation therapy with DFO, 13 for established transfusional hemosiderosis. Fourteen of the 21 patients on the DFO regimen were 'compliant', defined as using 1–2 g at least 5 nights per week. Seven patients were less or non-compliant, using DFO only 0–3 nights per week. The two youngest patients had not begun chelation therapy.

None of our patients had experienced symptoms or signs associated with any phase of HIV infection. Seven of the 23 patients had required tonsillectomy because of severe hypertrophy and upper airway obstruction.

Enumeration of peripheral blood mononuclear cell subsets. Using standard methods for cell separation, the percentage and the absolute number of T cells expressing the antigens T3 (a pan T cell marker), T4 (helper/inducer cell marker) and T8 (suppressor/cytotoxic cell marker) were determined using appropriate monoclonal antibodies (Ortho Diagnostic, Canton, NJ) and a standardized indirect immunofluorescent technique. B cells were enumerated similarly using a monoclonal antisera directed against the B cell membrane determinant B1 (Coulter Immunology, Hialeah, FL). Comparisons were made between patient and adult control lymphocytes. Previous studies have shown that children attain an adult distribution of B lymphocytes by 3 years of age and T lymphocytes by age 7 (Falco, 1980; Nouri-Aria *et al.*, 1985).

Functional studies of T lymphocytes. T cells were stimulated by either PHA, *Candida albicans* or the antigens expressed on the surface of allogenic lymphocytes (MLR), using techniques previously reported from this laboratory (Dwyer, Johnson & Desaules, 1979). For the mitogen studies, three concentrations of PHA (Burroughs Wellcome, Research Triangle, NC) were used: 5 μg/ml (high), 1 μg/ml (medium) and 0·25 μg/ml (low) to allow for the construction of a dose response curve. In the antigen stimulation studies, cells were exposed to two concentrations of *Candida albicans* (a 1 : 2 and 1 : 20 dilution of a stock solution) (Hollister Stier, Elkhart, IN). MLR were established using 1×10^5 mitomycin treated stimulator cells (pooled mononuclear cells from four healthy adult donors).

The results throughout are reported as ct/min, i.e. the amount of uptake observed after stimulation minus the uptake noted in control wells (background count).

Spontaneous suppressor cell activity (SSCA). Immunoregulatory activity induced *in vitro* (SSCA) was assessed using an assay system previously reported from this laboratory (Dwyer & Johnston, 1982). Results are expressed for comparative purposes as the percentage reduction in the responsiveness to PHA seen when indicator cells are cultured in the presence of the suppressor cell population using the following formula:

$$\% \text{ suppression} = 1 - \frac{\text{ct/min with PHA and mitomycin C treated suppressor cells}}{\text{ct/min with PHA without suppressor cells}} \times 100$$

Delayed hypersensitivity. All patients were tested with 0·5 μg of PHA and 25 μg of *Candida albicans*. Erythema and induration were measured 24 (for PHA) and 48 h (for *Candida*) after intradermal injection. Comparisons were made with a group of historical controls in the same age ranges (Lawlor *et al.*, 1973).

Serological studies. IgM, IgG and IGA were measured by laser nephelometry. Children achieve

adult levels of IgG and IgA by approximately 7 years of age and IgM by 1 year of age (Buckley, Dees & O'Fallon, 1966). Immunoglobulin levels were compared against age-appropriate historical controls. Evidence for Epstein–Barr virus infection was determined by measuring antibodies to viral capsid antigen (VCA), Epstein–Barr nuclear antigen (EBNA) and early antigen (EA). IgM and IgG antibodies to cytomegalovirus were sought by complement fixation to HBsAg, anti-HBs, and anti-Hepatitis A antibodies were measured by routine methods (Francis, Hadler & Thompson, 1982).

Exposure to HIV was determined by ELISA and Western blot. ELISA testing was performed by EIA (Abbott Laboratories, N. Chicago, IL). Western blot assays were performed using an 11% polyacrylamide gel. Presence of antibody to the gP24 or gP41 glycoprotein band, when compared to a well-characterized standard, was considered to be antibody specific for HIV.

Statistical methods. The paired *t*-test (Colton, 1974) was used to compare patients to age-matched historical controls. When subgroups were compared, the two youngest patients (under age 7) were eliminated and the *t*-test for independent samples (Colton, 1974) was used. Ages for the subgroups did not differ statistically: mean age ± 1 s.d.: nonsplenectomized (11·7 ± 3·4), splenectomized (14 ± 4·3), low (13·6 ± 4·7) and high ferritin (13·2 ± 4·7). The chi-square test for association (Fleiss, 1972) was used to compare proportions of cases and controls positive for skin tests.

Multiple linear regression techniques (Draper & Smith, 1981) were used to assess effect of five independent variables (age, total units of blood received, years post splenectomy, serum ferritin level and deferoxamine use) on the immunologically dependent variables.

RESULTS

Hematological parameters

The absolute number of white blood cells, lymphocytes and neutrophils were increased in the thalassaemic patients ($P < 0·05$) in comparison to age appropriate historical controls (Albritton, 1952). In the nonsplenectomized group, the white blood cells, lymphocyte and neutrophil counts were decreased compared to control and splenectomized patients ($P < 0·05$) (Table 1).

Humoral immunity

The absolute number of B cells/μl of blood present in our patients are illustrated in Fig. 1. All groups had a significant increase in circulating B cells. The variation in B cell numbers could not be related to the patient's age, quantity of blood transfused, the amount of DFO taken or the ferritin levels of these patients. Splenectomy, however, had a marked effect on B cell levels. In the non-

Table 1. Haematological values (mean ± 1 s.d.)

Subjects	Age	Number	WBC* (cells/mm³)	ALC (cells/mm³)	ANC (cells/mm³)
Thalassaemia	12·5 ± 4·8	21	11,284† ± 5,373	4,731† ± 2,198	5,373† ± 2,736
NS	11·7 ± 3·4	6	5,660† ± 1,240	2,522† ± 1,073	2,844† ± 322
S	14·03 ± 4·3	15	13,293†‡ ± 3,423	5,519†‡ ± 1,947	6,276‡ ± 2,645

NS, Non-splenectomized patients; S, splenectomized patients; ALC, absolute lymphocyte count; ANC, absolute neutrophil count.

*All white blood cell counts corrected for nucleated red blood cells.

† Significant difference from historical controls in same age range ($P < 0·05$)

‡ Significant difference NS compared to S ($P < 0·05$)

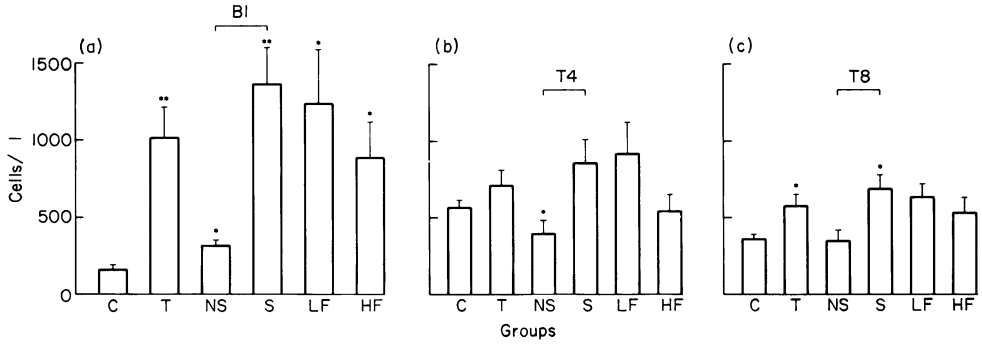

Fig. 1. (a) Circulating B lymphocytes in patients with thalassaemia. C, healthy adult controls; T, total thalassaemic population which is divided into NS, non-splenectomized patients, and S, splenectomized patients (all patients > 3 years of age); LF, patients with ferritin less than 3500 ng/ml; HF, ferritin levels equal to or greater than 3500 ng/ml. $*P<0{\cdot}05$; $**P<0{\cdot}001$ when compared to control subjects; bar, $P<0{\cdot}05$ between groups: NS versus S and LF versus HF. (b) (c) Circulating T4 and T8 cells in patients with thalassaemia. C, healthy adult controls; T, total thalassaemia population, which is divided into NS, non-splectomized, and S, splenectomized patients (all patients > 7 years of age); LF, patients with ferritin less than 3500 ng/ml; HF, ferritin levels equal to or greater than 3500 ng/ml. $*P<0{\cdot}05$; bar $P<0{\cdot}05$ between groups: NS versus S and LF versus HF.

Table 2. Quantitative immunoglobulin levels (mean ± 1 s.d.)

Subjects	Number	IgM (mg/dl)	IgG (mg/dl)	IgA (mg/dl)
Patients	21	116±51*	1377±412*	188±81*
NS	5	148±45*	1402±194*	140±32
S	14	107±52*	1427±467*	212±80*
LF	9	113±51*	1450±419*	209±60
HF	10	124±55*	1394±418*	179±89

NS, Non-splenectomized patients; S, splenectomized patients; LF, patients with ferritin levels less than 3500 ng/ml; HF, patients with ferritin levels equal to or greater than 3500 ng/ml.

*Significant difference from historical controls in same age range ($P<0{\cdot}05$).

splenectomized group, there was a 3-fold increase in the number of circulating B cells over normal ($P<0{\cdot}05$) whereas there was a 10-fold increase in the splenectomized group ($P<0{\cdot}001$).

The quantitative immunoglobulin levels are listed in Table 2. In comparison to age matched control values, IgG and IgM were elevated in all groups ($P<0{\cdot}05$). IgA was significantly elevated only in the splenectomized and total thalassaemic groups. The striking rise in circulating B cells was not matched by a proportional increase in serum immunoglobulins. There was a positive relationship between age and increases in IgG and IgA; ($R=0{\cdot}53$ and $R=0{\cdot}62$ respectively) and a negative relationship between DFO usage and IgA levels ($P<0{\cdot}05$) which was not effected by age.

Cell mediated immunity

Circulating T cells. The absolute numbers of circulating T4 and T8 cells are illustrated in Fig. 1. Decreased numbers of T4 cells compared to controls were found in the non-splenectomized group ($P<0{\cdot}05$). T8 cells were significantly increased in the splenectomized and total thalassemic groups ($P<0{\cdot}05$). Within subgroups, the splenectomized patients had a 2-fold increase in the circulating

Table 3. Circulating T cells in patients and 1 year follow-up with an inverted T4/T8 ratio

Patients	Splenectomy	T4 (cells/μl)		T8 (cells/μl)		T4/T8 ratio	
		1983	1984	1983	1984	1983	1984
Control†		567* ±252	623* ±191	358* ±171	418* ±118	1·6* ±0·36	1·5* ±0·38
1	NS	315	461	405	742	0·8	0·6
2	NS	382	1445	448	1308	0·9	1·1
3	NS	140	398	228	289	0·6	1·4
4	S	464	1320	546	1221	0·9	1·1
5	S	514	1890	1307	786	0·4	2·4
6	S	289	777	341	1020	0·9	0·8
7	S	459	2741	1165	3427	0·4	0·8

NS, patients who had not had a splenectomy; S, patients who had been splenectomized. Splenectomy state did not change from 1983 to 1984. All patients greater than 7 years of age.

* Data presented as mean ± s.d.

† Control number for 1983 ($n=23$) and 1984 ($n=26$).

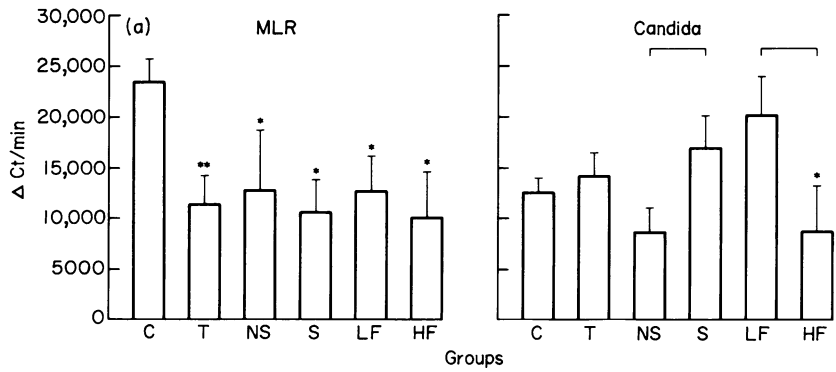

Fig. 2. Proliferative responses (Δ ct/min) of lymphocytes from control subjects and patients with thalassaemia stimulated by allogenic lymphocytes or *Candida albicans*. C, Healthy adult controls; T, total thalassaemic population, which is divided into NS, non-splenectomized patients, and S, splenectomized patients (all patients > 3 years of age); LF, patients with ferritin less than 3500 ng/ml; HF, ferritin levels equal or greater than 3500 ng/ml. *$P<0·05$; **$P<0·001$ when compared to control subjects; bar $P<0·05$ between groups: NS versus S and LF versus HF.

number of T4 and T8 cells in comparison to the non-splenectomized patients ($P<0·05$). The changes in the total number of circulating T3 cells paralleled those of the T8 subsets described above with T3 cells being slightly decreased in the non-splenectomized patients and markedly increased in the splenectomized patients (data not shown). Deferoxamine usage had a negative relationship with circulating T8 cells ($R=0·46$).

Seven of the 23 patients had T4/T8 ratios less than 1 (mean, 0·7). Of these, three who had not been splenectomized had a mean T4/T8 ratio of 0·8, while four who had been splenectomized had a mean T4/T8 ratio of 0·60. In the non-splenectomized patients, inverted T4/T8 ratios were secondary to a decrease in T4 cells in two of three patients. However, in the splenectomized patients, the decrease was secondary to an increase of the T8 cell in three of four patients.

One year follow-up studies were done on all patients with inverted T4/T8 ratios and/or positive

HIV serology. Four of the seven patients with a previously inverted T4/T8 had normal ratios when retested but persistent abnormalities in absolute numbers of T4 and T8 cells remained (Table 3). The one patient with positive HIV serology continued to have a normal ratio and number of T cells one year later.

T cell function. Proliferation following stimulation with the mitogen PHA was depressed in patients with thalassaemia. The mean ct/min ± 1 s.d. after PHA stimulation of control lymphocytes was 49,490 ± 11,974. This was reduced to 33,593 ± 15,536 and 30,157 ± 12,570 for patients who had not been splenectomized and those that had been splenectomized respectively ($P < 0{\cdot}05$ for both groups).

Similarly, the ability of lymphocytes to proliferate following stimulation with the antigens from *Candida albicans* and those expressed on the surface of allogeneic lymphocytes (MLR) was depressed. The MLR response was significantly decreased for all groups ($P < 0{\cdot}05$). The response to *Candida albicans* was decreased in comparing nonsplenectomized with splenectomized and low with high ferritin ($P < 0{\cdot}05$). There was a negative relationship between ferritin level and *Candida albicans* proliferation *in vitro* ($R = 0{\cdot}56$, $P < 0{\cdot}05$) (Fig. 2).

Spontaneous suppressor cell activity. The degree of spontaneous (i.e. *in vivo* generated) suppressor cell activity was increased on group analysis of our patients with thalassaemia. The mean ± 1 s.d. percentage decrease in the proliferative response to PHA of cells in our indicator system using suppressor cells from control subjects was 56 ± 13%. In patients with thalassaemia the figure was 69 ± 18%; a significant increase ($P < 0{\cdot}05$). Suppressor cell activity of splenectomized patients was less (64 ± 15%) but not significantly so.

Delayed hypersensitivity. The results of PHA and *Candida albicans* skin tests are shown in Table 4. Twenty-five percent of the patients did not respond to PHA compared to 95% of normals ($P < 0{\cdot}05$). Splenectomy and ferritin levels had no significant effect on responsiveness to PHA.

Fifty percent of the total patients and 83% of the non-splenectomized patients failed to respond to *Candida albicans* ($P < 0{\cdot}05$). In the non-splenectomized group, of those who responded to PHA, four of those five failed to respond to *Candida albicans*.

Virological studies

Serological screening for viral infection suggested that the population of patients had not experienced an unusual number of viral infections. Only one patient had antibody in his serum to HBsAg surface antigen. This patient had been transfused for most of his life in Greece and was

Table 4. The ability of patients with thalassaemia to produce delayed hypersensitivity reactions

	PHA*		*Candida**	
Subjects	Number positive	% positive	Number positive	% positive
Control	60/63†	95	37/43†	86
Thalassaemia	15/20	75‡	10/20	50‡
NS	5/6	83	1/6	17‡§
S	10/14	71‡	9/14	64
LF	6/9	67‡	5/9	56‡
HF	9/11	82‡	5/11	45‡

NS, Not splenectomized; S, splenectomized; LF, ferritin levels < 3500 ng/ml; HF, ferritin levels ≤ 3500 ng/ml.

* Patients were challenged with intradermal injections of PHA and *Candida albicans*; induration > 5 mm at 24 h (PHA) or 48 h (*Candida*) were regarded as positive.

† Age similar historical controls.

‡ Compared to historical control values in same age group ($P < 0{\cdot}05$).

§ NS compared to S ($P < 0{\cdot}05$).

positive when he first came to this country. No patient had evidence of hepatitis. Three patients had CMV titres greater than 1:14 as measured by complement fixation. One patient had an IgG anti-VCA titre of 1:320 but no antibody to EA in his serum. EB virus was not found in throat washings of 18 patients. Only one patient had evidence of exposure to HIV with a repeatedly positive ELISA and a positive western blot reactivity to HIV gP24 in serum drawn from 1983 to 1985.

DISCUSSION

In this study we have determined that patients with β-thalassaemia major have an immunological abnormality by a lymphocytosis and alterations in lymphocyte function. Factors that might lead to the development of these abnormalities include iron overload (Kapadia *et al.*, 1980), splenectomy (Kapadia *et al.*, 1980), the use of DFO (Lederman *et al.*, 1984), repeated exposure to allogeneic antigens in blood (Gascon, Zdumbos & Young, 1984), and exposure to immunosuppressive viruses and liver damage following hepatitis. In our study, the only significant factors contributing to immunological abnormalities were iron overload, deferoxamine use, and splenectomy.

Iron overload has been implicated as a major cause of immunological disturbances in thalassaemia. The mechanisms suggested include toxic effects of high iron levels on lymphocyte function (Matzner *et al.*, 1979; Buffe & Rimbaut, 1975; Bryan & Leech, 1981) and redistribution of B lymphocytes from the spleen and lymph nodes to the circulating pool (DeSousa, Smithyman & Tan, 1978). In our patients both mechanisms received support. High ferritin levels appeared associated with a decrease in lymphocyte responsiveness to *Candida albicans*, while the marked increase in circulating B cells was consistent with a redistribution effect. Increased B cell numbers were also present in children who had not been treated with DFO or who had just begun transfusion therapy which may suggest a co-existing intrinsic immuno-regulatory defect.

DFO is a potent inhibitor of T-cell responsiveness to mitogens (Lederman *et al.*, 1984) at levels of 20 mmol/l of the drug *in vitro*. In our patients, we found a significant negative relationship between DFO and IgA levels and T8 cells numbers.

A major finding in this study was the immunological differences between non-splenectomized and splenectomized patient groups. In the non-splenectomized group we found a 3-fold increase in circulating B cells and a modest polyclonal gammopathy. The majority of these patients were unresponsive to skin tests to *Candida albicans*. Additionally, they had decreased numbers of T4 cells and decreased lymphocyte proliferative responses. In the splenectomized group, there was a 10-fold increase in B lymphocytes and a 2-fold increase in T4 and T8 cells. As a group, these patients were more responsive to skin testing and stimulation *in vitro* with *Candida albicans*. We speculate that the differences between these two groups are related to splenectomy rather than the treatment or the age of the patient because these variables were similar in the two groups. In addition, preliminary results obtained on two patients studied pre- and post-splenectomy demonstrated the same kinds of changes described above (Wood *et al.*, 1985). A recent study on another group of beta-thalassaemia patients found similar changes related to splenectomy (Grady *et al.*, 1985).

Only a few studies have been published which utilized monoclonal antibodies directed against T cell subsets in the analysis of immunological dysfunction in thalassaemia (Vierucci, 1984; Guglielmo *et al.*, 1984; Grady *et al.*, 1985). In our initial study, seven patients had inverted T4/T8 ratios that could not be attributed to exposure to CMV, EBV, HB, or HIV when studied. One year later, four of these patients had a normal T4/T8 ratio. However, abnormal numbers of T4 and T8 cells persist. Although there may be major fluctuations in the lymphocyte subsets of these patients, they have persistently demonstrable abnormalities. The one patient with positive serology for HTLV-III did not have an inverted T4/T8 ratio or any other T cell abnormalities when studied 1 year later.

Patients with beta-thalassaemia major appear to have an intact immune system with only moderate abnormalities of T and B lymphocyte numbers and function. Our one child with positive serology for HIV may have a latent infection and may yet develop AIDS-associated immunological changes. A pattern of positive HIV serology to gP24 alone has been reported in other patients in the early stages of AIDS (Petricciani *et al.*, 1985).

Further study of the immunological abnormalities and the incidence and effects of HIV infection in these children are warranted. There have been more than 134 cases of transfusion-associated AIDS (Update: AIDS, 1985) and, to date, three individuals with beta-thalassaemia major have been reported with HIV infection. Two of these patients had a transient lymphadenopathy syndrome and one developed CDC-defined AIDS (Ivelez-Garcia, Robles-Cardona & Fradera, 1985; Giardina *et al.*, 1985). Despite the immunological abnormalities described in this study, all of the children we studied are well. Thus the challenge to understand the basic immunological changes associated with beta thalassaemia may be of greater importance than the clinical consequences.

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