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Iron-related disturbances of cell-mediated immunity in multitransfused children with thalassemia major

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SUMMARY

Immunological abnormalities have been observed in many haemophiliacs receiving clotting factor concentrates. To determine whether similar changes also occur after repeated blood transfusions we estimated T cell subsets and cutaneous delayed hypersensitivity (CDH) in 50 multitransfused children with β-thalassemia major (β-TM). All patients were also tested for anti-HTLV-III/LAV antibodies. A diminished percentage of T lymphocytes (E-rosettes, $T3^+$), and $T4^+$ cells and a low T4/T8 ratio was found in patients as compared to age and sex matched controls ($P < 0{\cdot}001$). Negative CDH tests to specific antigens (Multi-test) were also found in a significantly larger proportion of β-TM children ($P < 0{\cdot}01$). Antibodies against HTLV-III/LAV were negative in all patients. Decreased T4/T8 ratio in β-TM children was primarily due to a reduction of $T4^+$ cells and was inversely correlated to the patients' age, number of units of transfused blood ($P < 0{\cdot}05$) and especially to ferritin serum levels and annual iron balance ($P < 0{\cdot}001$). These findings indicate that immunological abnormalities in β-TM patients appear to be acquired, transfusion-associated and related to iron load which depends on the appropriate chelation therapy.

Keywords iron-load ferritin β-thalassemia major multitransfused subjects

INTRODUCTION

Epidemiological studies have shown that HTLV-III/LAV virus is transmissible through blood or blood products and so subjects who need repeated blood transfusions are at high risk for the development of the acquired immunodeficiency syndrome (AIDS) (Curran *et al.*, 1984; Landesman, Ginzburg & Weiss, 1985; Feorino *et al.*, 1985). Sporadic reports of AIDS in haemophiliacs treated with factor VIII concentrates have attracted research on this group of patients while investigation of multitransfused subjects with congenital haemolytic anaemias has not aroused the same degree of interest. Thus, relevant data for patients with thalassemia major or sickle-cell disease is very limited (Gascon, Zoumbos & Young, 1984; Kaplan *et al.*, 1984; Neri *et al.*, 1984). This point, together with the fact that β-thalassemia is a major health problem in Greece, led us to investigate if immunological abnormalities similar to those seen in haemophiliacs also occur in thalassemic children receiving multiple blood transfusions.

The aim of the study was to determine firstly, if there are abnormalities in T cell subpopulations which may influence the natural history of HTLV-III/LAV infection in these children and secondly to define the responsible specific risk factors for these abnormalities.

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SUBJECTS AND METHODS

Patients. The study group consisted of 50 children with β-thalassemia major (β-TM), 25 females and 25 males, aged 2 to 18 years (mean 7·3 ± 3·7) who were regularly transfused with packed red cells from Greek donors every 2–4 weeks at the Blood Transfusion Centre for β-Thalassemia of our Hospital. Additional criteria for the patients to be included in the study were that they were free of acute infection or other chronic disease, that they did not take any drugs and that they had not been vaccinated within a period of 2 months.

Thirteen of the patients had been receiving transfusions for 1–3 years (mean 1·9 ± 0·6) and 37 for 4–18 years (mean 6·9 ± 3·7). Four patients were splenectomized and 15 used filters for leukapheresis during transfusions. Forty-one of the 50 patients were receiving systematic subcutaneous chelation (using a special pump) 2–5 times per week over a period of 6 months to 11 years. In 9/50 children no treatment of haemosiderosis had been tried. Twenty-six children had serological markers of previous exposure to hepatitis B virus (HBV) and the remaining 24 had been vaccinated against HBV with a good antibody response.

Controls. The control group comprised 50 children, 24 males and 26 females, aged 3 to 18 years (mean 7·7 ± 3·1) who were selected from the Surgery Department and the Outpatient Clinics where they had come for treatment of minor trauma, surgery of hernias, recurrent abdominal pain or headaches. An additional criterion for the selection of controls was that they should not belong to any of the groups at high risk for the development of AIDS. Special emphasis was placed on reporting if they had ever been transfused with blood or blood products. To compare the results of delayed hypersensitivity skin reaction in our patients we used as controls another group of 80 healthy children aged 3 to 16 years who had served as the control group in another study of ours.

Collection of blood samples. Blood samples were collected from all patients at 0830 h just before regular blood transfusion and at least 15 days after the previous one. Routine blood test and T cell measurements were immediately performed, and a serum sample was stored at −70°C for the other serological tests. The same procedure was followed for the controls. Careful clinical examination and detailed history preceded the bleeding of patients and controls. In all cases informed consent was obtained from the parents.

Specimen preparation. Mononuclear cells were obtained from heparinized venous blood by Ficoll-Hypaque separation within 1 h of venipuncture.

T lymphocyte determination. T lymphocytes were measured as E-rosette forming cells (E-RFC) with neuraminidase-treated sheep red blood cells, following conventional techniques. The T-lymphocyte subpopulations were quantified by direct immunofluorescence using fluorescein-conjugated monoclonal antibodies OKT_3 (CD3) OKT_4 (CD4) and OKT_8 (CD8) (Ortho Pharmaceutical Corporation) and a Zeiss microscope. Tests were done according to the recommended procedures of manufacturers (Ortho Monograph, 1984).

Delayed type hypersensitivity skin tests. Cutaneous delayed type reactivity to specific antigens (tetanus and diphtheria toxoids, streptococcus, tuberculin, proteus, trichophyton and candida) and to dinitrochlorobenzene (DNCB) was tested by the multipuncture technique (Multi test, Merieux) and by contact sensitization as previously described (Cassimos *et al.*, 1980).

Anti-HTLV-III/LAV antibodies. Antibodies against HTLV-III/LAV were detected by ELISA and confirmed by Western blot assay and immunofluorescence (the antigen was kindly provided by Professor Montagnier, Institute Pasteur, Paris).

Other assays. Immunoglobulin and serum transferrin concentrations were measured in Nor-Partigen immunoplates (Hoechst) by radial immunodiffusion. Serum ferritin levels were measured by the double antibody ^{125}I radioimmunoassay (Diagnostic Products Corporation, Los Angeles). Serum alanine aminotransferase (ALT) was measured by the UV spectrophotometric method (Sclavo Diagnostics).

Estimation of iron stores. For estimation of total iron stores we calculated the ratio of iron excreted in the urine to iron introduced with transfusion in the previous year. This annual iron balance is calculated according to the formula (Kattamis, 1985):

Annual iron balance = Fe received through transfusions (mg) − (number of desferrioxamine infusions × mean daily urinary iron × 1·5).

Statistical analysis. Comparisons between patient and control groups were made using the Mann-Whitney test for quantitative measurements and the chi-square test with Yate's correction for qualitative measurements. Comparisons of the mean values of certain parameters (number of transfused blood units, serum ferritin levels, annual iron balance, etc.) between patients with low (< 1 s.d. of the control mean) and with normal T4/T8 ratio were made using Student's t-test. The relationships of T4/T8 ratio with other variables were examined using single and multiple regression analysis.

RESULTS

Haemoglobin, ferritin, transferrin. Haemoglobin and serum transferrin levels were significantly lower in patients than in control subjects. On the contrary, serum ferritin levels were significantly higher in patients than in controls ($P < 0{\cdot}001$, Table 1).

Immunoglobulins. Serum immunoglobulin levels did not differ significantly between patient and control groups (Table 1). In two of four splenectomized β-TM children the IgM level was at the lowest normal limit for their age.

T lymphocytes. The immunological phenotype of circulating lymphocytes is summarized in Table 1. Total leukocyte and lymphocyte counts did not differ significantly between patients and controls but the percentage of lymphocytes was higher in β-TM children ($P < 0{\cdot}05$). Also the percentage of pan-T cells (E-rosettes, $T3^+$) and $T4^+$ cells and the ratio of T4/T8 were significantly lower in β-TM children than in the control group ($P < 0{\cdot}001$). The percentage of $T8^+$ cell count was higher in patients ($P < 0{\cdot}05$).

Distribution of the T cell subset values is illustrated in Figs. 1 and 2. Thirty β-TM children (60%) presented with a T4/T8 cell ratio lower than one standard deviation of the control mean value and nine ß-TM children (18%) with a ratio lower than two standard deviations. The range of T4/T8 values in these nine patients was from 0·41 to 1·09 (Fig. 2).

Table 1. Comparison of laboratory parameters between patients and controls

Variable	Patients		Controls		Mann-Whitney	
	Median	Range	Median	Range	ζ	P
Haemoglobin	8·7	5·9–12·8	12·5	10·3–15	8·00	$<0{\cdot}0001$
Ferritin	2002	49–4900	33·7	1–196	8·53	$<0{\cdot}0001$
Transferrin	1·65	0·98–3·99	3·09	2·14–4·30	8·15	$<0{\cdot}0001$
IgG	13·1	5·08–31·5	14·3	8·02–22·6	0·59	0·55
IgA	1·71	0·42–6·03	1·44	0·42–3·80	1·72	0·085
IgM	1·91	0·32–5·15	2·26	0·83–3·92	0·54	0·59
Leucocytes	7953	2500–15000	8000	4500–15500	0·20	0·84
Lymphocytes (absolute number)	2963	1175–7200	2495	1250–7285	1·23	0·22
Lymphocytes %	42	21–79	34	14–57	2·22	0·026
E-rosettes (absolute number)	1756	341–4550	1632	731–5027	0·57	0·57
E-rosettes %	57	41–70	64	51–75	3·99	0·0002
$T3^+$ (absolute number)	1703	331–4095	1540	805–5544	0·29	0·77
$T3^+$%	55·3	34·8–68·9	63·0	48·5–75	4·52	$<0{\cdot}0001$
$T4^+$ (absolute number)	1044	291–3182	1118	541–3533	0·41	0·68
$T4^+$ %	36·8	13·7–50	43·9	31·9–50·2	4·47	$<0{\cdot}0001$
$T8^+$ (absolute number)	770	141–1987	600	297–1894	2·03	0·042
$T8^+$ %	26·1	13·1–53·8	25·2	16–33	1·24	0·22
T4/T8	1·34	0·42–2·09	1·69	1·4–2·56	4·58	$<0{\cdot}0001$

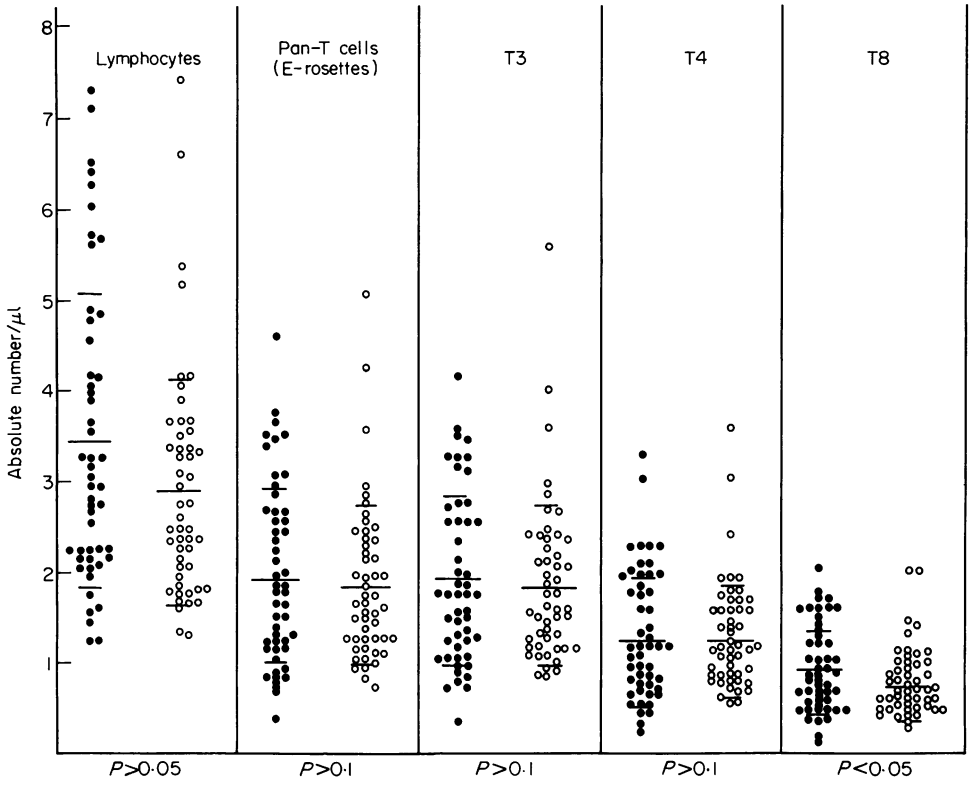

Fig. 1. Absolute number of lymphocyte populations (distribution and mean values ± s.d.) in (●) patient and (○) control groups.

Cutaneous delayed hypersensitivity. Table 2 shows that the prevalence of negative skin tests to specific antigens and to DNCB was higher in patients than in the control group but statistical analysis showed that the difference was significant only for specific antigens ($P < 0{\cdot}01$).

Anti-HTLV-III/LAV antibodies. Antibodies against HTLV-III/LAV were found in 2/50 patients with the ELISA test but none was confirmed either by Western blot or immunofluorescence techniques.

Correlation of T4/T8 ratio values with clinical and laboratory parameters in ß-TM children. The mean T4/T8 ratio values did not differ significantly ($P > 0{\cdot}1$) between females and males, splenectomized and non-splenectomized, infected and non-infected with HBV, those using and not-using filters for leukapheresis and those with positive and negative skin tests. In addition, comparison between patients with low (0·41–1·45) and normal (> 1·45) T4/T8 ratios showed that the two groups did not differ significantly in the mean age of onset of blood transfusions, time interval between previous transfusion and bleeding, age of onset or duration of chelation and in the mean Hb, transferrin, ALT and immunoglobulin levels ($P > 0{\cdot}1$). On the contrary, it was found that patients with low ratios of T4/T8 had significantly higher values for age (8·25 years versus 5·95 years $P < 0{\cdot}05$), number of units of transfused blood (136·6 versus 40·5, $P < 0{\cdot}05$), annual iron balance (1904·4 mg versus 309·4 mg, $P < 0{\cdot}001$) and serum ferritin levels (2756 ng/ml versus 1525 mg/ml $P < 0{\cdot}001$) than those with normal ratios. An inverse relationship between T4/T8 ratio values and the above mentioned parameters was confirmed by single regression analysis (Fig. 3). Multiple regression analysis also showed that the best single predictors were ferritin (F_1, $48 = 9{\cdot}15$, $P < 0{\cdot}01$), iron balance (F_1, $36 = 10{\cdot}44$, $P < 0{\cdot}01$) and number of transfusions (F_1, $48 = 6{\cdot}27$, $P < 0{\cdot}02$).

Comparison of T cell subsets between patients with low and normal T4/T8 ratios showed that

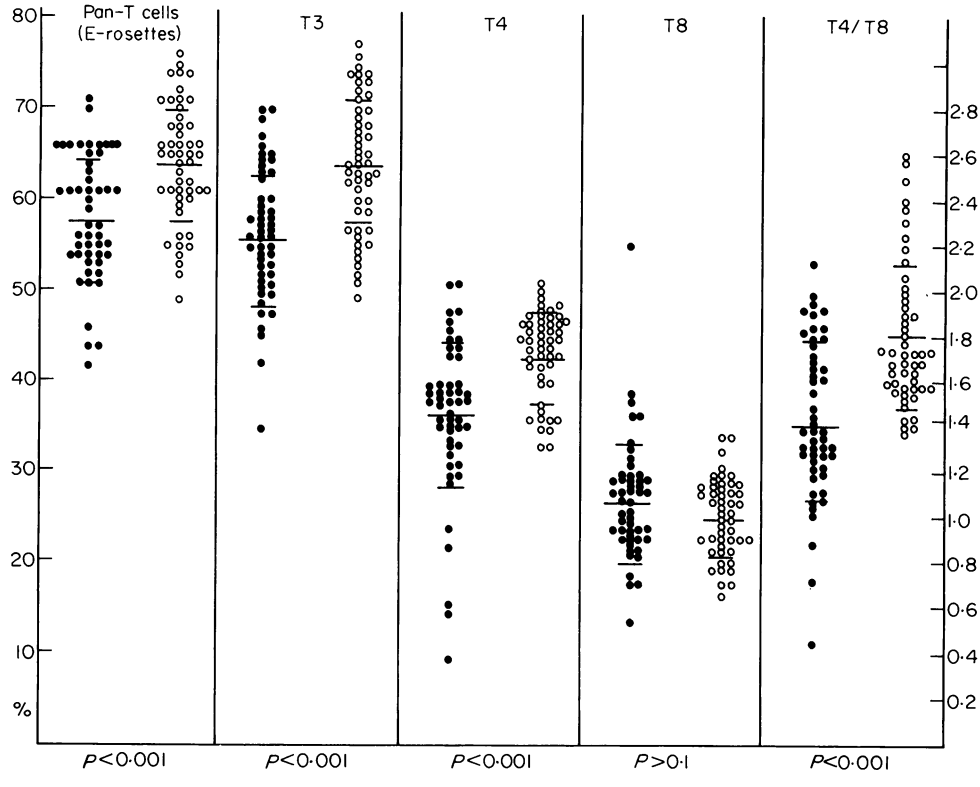

Fig. 2. Percentage of lymphocyte populations (distribution and mean values ± s.d.) in patient and control groups.

Table 2. Results of cutaneous reactivity to recall antigens (Multi-test) and to dinitrochorobenzene (DNCB) in patients and controls

Group study	Number of subjects	DNCB		Number of subjects	Multi-test	
		+ (%)	− (%)		+ (%)*	− (%)
Patients	45	41 (91·1)	4 (8·9)	46	36 (78·3)	10 (21·7)
Controls	80	78 (97·5)	2 (2·5)	80	77 (96·2)	3 (3·8)
Statistical analysis		$\chi^2 = 1{\cdot}36$ $P = 0{\cdot}11$		$\chi^2 = 8{\cdot}36$ $P = 0{\cdot}004$		

* Positive reaction in one or more antigens.

the former had a lower absolute count (1058 versus 1474, $P < 0{\cdot}05$) and percentage (32·4% versus 40·9%, $P < 0{\cdot}001$) of $T4^+$ cells and a higher percentage (28·2% versus 23·3%, $P < 0{\cdot}001$) of $T8^+$ cells than the latter. These findings were confirmed by regressing the T4/T8 ratio on the above mentioned parameters (%$T4^+$: F1, 48 = 22·9, $P < 0{\cdot}001$. Absolute number of $T4^+$: F1, 48 = 7·7, $P < 0{\cdot}02$. %T8+: F1, 48 = 19·4, $P < 0{\cdot}001$).

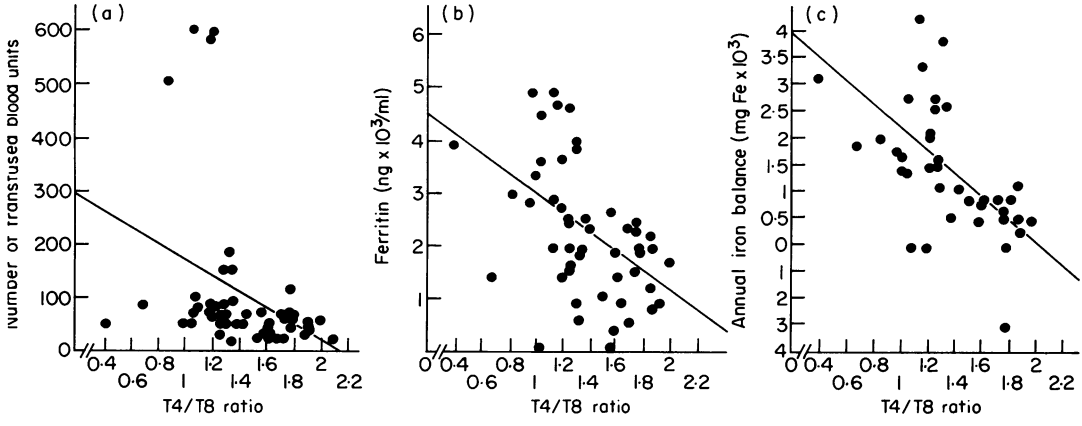

Fig. 3. Correlation between patient T4/T8 ratio and (a) blood units transfused, (b) ferritin level and (c) annual iron balance. (a) $r=0{\cdot}34$, $y=-140x+196$, $t=2{\cdot}5$, $P<0{\cdot}05$. (b) $r=0{\cdot}46$, $y=-1648{\cdot}5x+4572$, $t=3{\cdot}60$, $P<0{\cdot}001$. (c) $r=-0{\cdot}54$, $y=-1970+3996$, $t=3{\cdot}86$, $P<0{\cdot}001$.

DISCUSSION

This study indicates that children with homozygous β-TM who receive multiple blood transfusions show abnormalities in the distribution of T cell subsets and in the cutaneous delayed hypersensitivity reaction to recall antigens. The analysis of circulating T lymphocytes showed that 60% of the patients appear to have an imbalance in T cell subsets and 18% have a significantly low ratio of T4/T8 as compared to that of matched controls (< 2 S.D., range 0·41–1). This decrease in T4/T8 ratio is primarily due to a reduction of T helper/inducer cells ($T4^+$) and partly to an increase of T suppressor/cytotoxic ($T8^+$) cells. Lymphocytosis found in our patients explains the absence of a significant difference in the mean absolute count of these T subsets between patient and control groups. Similar findings as far as T cell subpopulations are concerned have also been reported by others in β-TM children (Neri *et al.*, 1984) and in multitransfused patients with βTM and sickle-cell anaemia (Kessler *et al.*, 1983; Kaplan *et al.*, 1984).

An increased frequency of negative skin tests to specific antigens was also observed in patients (21·7%) in comparison to the control group (3·8%). These findings agree with results reported previously in another series of β-TM children (Kanakoudi-Tsakalidis *et al.*, 1977). No significant changes in the serum immunoglobulin levels were found in the patients' group except for two splenectomized children with low IgM level which can be due to splenectomy as previously described (Koren *et al.*, 1984).

Abnormalities in cell-mediated immune response of our patients is difficult to explain. Kaplan *et al.* (1984), who also found a similar pattern of T subset alterations in transfused subjects with sickle cell anaemia but not in non-transfused patients, postulated that these immunological defects appear to be transfusion-associated and not specific to sickle cell disease or ß-TM. Several other studies have demonstrated the association between blood transfusions and immunosuppression (Opelz & Terasaki, 1974; Opelz & Persijn, 1981; Gupta & Good, 1981; Dupont *et al.*, 1983; Kessler *et al.*, 1983; Gascon *et al.*, 1984; Neri *et al.*, 1984). In our study it was found that the diminished T4/T8 ratio was inversely correlated to the number of blood units transfused, to the serum ferritin levels and the annual iron balance. These data indicate that immunoregulatory disturbances in our patients appear to be acquired, transfusion-associated and in significant correlation with the annual iron balance which depends on the appropriate chelation therapy.

Our findings favour the view that iron, iron-binding proteins and products of red cell breakdown influence the immune response of multitransfused subjects, especially the traffic and distribution of lymphoid cells (Dupont *et al.*, 1983; De Sousa, 1983). The real mechanism of this influence is not yet clearly understood (De Sousa, 1983; Editorial, 1984; Brock & De Sousa, 1986).

Several hypotheses have been proposed based on the findings of studies *in vitro* and *in vivo* (Nishiya *et al.*, 1980; Munn *et al.*, 1981; Gupta & Good, 1981; Bryan & Leech, 1983; Dupont *et al.*, 1983; Brock & De Sousa, 1986). The role of ferritin and iron overload in T cell abnormalities found in our patients is evident. We cannot exclude the effect of other factors proposed to be associated with immunosuppression in multitransfused subjects, as repeated exposure to alloantigens, development of antiidiotypic antibodies, hyperimmunization etc. (Fischer *et al.*, 1980; Smith *et al.*, 1981; Suciu-Foca *et al.*, 1982; Shearer, 1983; Woodruff & VanRood, 1983; Terasaki, 1984). However we can exclude the effect of HBV because there was no difference in the mean T4/T8 ratio between infected and noninfected β-TM children and also the effect of HTLV-III/LAV as no antibodies against this virus were found in our patients.

Serum transferrin level in our patients was found to be significantly lower and ferritin level significantly higher than in controls, which means that saturation of transferrin with iron was very high. Transferrin seems to be important in promoting lymphocyte proliferation only when it is partly saturated with iron, there being less proliferation if the saturation is more than 80%. A possible explanation for this phenomenon is that lymphocytes in the latter case acquire more iron than they can process (Brock & De Sousa, 1986). From this point of view iron overload in our patients could also influence the T lymphocyte function of DTH.

There is another explanation for the association between repeated blood transfusions and immunosuppression. The latter may be a protective mechanism as a part of the normal immune response to chronic alloantigenic stimulation (Kaplan *et al.*, 1984). By decreasing T helper/inducer function or increasing T suppressor activity or by combination of both accelerated acute phase response may be avoided (Woodruff & Van Rood, 1983; Terasaki, 1984). However, prolonged depression of T cell immune response may lead to undesirable immunosuppressive status which may be a major factor in the development of malignancies, opportunistic infections or in the pathogenesis of AIDS (Shearer, 1983; Woodruff & Van Rood, 1983; Kaplan *et al.*, 1984). In this context β-TM children who receive multiple blood transfusions and who express a decreased $T4^+$ cell activity should be considered as a high risk population for the development of clinical infection with HTLV-III/LAV knowing that this retrovirus has a selective tropism and cytopathic effect on $T4^+$ lymphocytes (Klatzmann *et al.*, 1984). The evident association between iron overload and T cell abnormalities observed in our patients suggests that correct and continuous regulation of annual iron balance in β-TM children is an important factor in minimizing this immunological disturbance.

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