

Cytokine production in transient hypogammaglobulinemia and isolated IgA deficiency

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Background: Transient hypogammaglobulinemia of infancy and isolated IgA deficiency are characterized by normal numbers of circulating B lymphocytes. It is likely that no single abnormality, but rather different factors, may be relevant for the delayed onset of IgG synthesis in transient hypogammaglobulinemia or for the differentiation defect of B cells in IgA deficiency. These factors may include defective production of cytokines or an abnormal response of B cells to various mediators. Alternatively, some cytokines may act as inhibitory factors of B-cell function.

Methods: The ability of peripheral blood mononuclear cells from children with proved or probable transient hypogammaglobulinemia (30 patients) and IgA deficiency (15 patients) to secrete several cytokines on stimulation with phytohemagglutinin *in vitro* was analyzed.

Results: An enhanced production of tumor necrosis factor (TNF)- α , TNF- β , and IL-10 was observed in transient hypogammaglobulinemia; whereas secretion of IL-1, IL-4, and IL-6 was essentially similar in the control and patient groups. Increased frequency of mononuclear cells secreting TNF- α was seen in the patient groups. Apart from elevated production of TNF- α , no other abnormalities in cytokine synthesis in selective IgA deficiency were observed. *In vitro* observations showed that exogenously added TNF- α and TNF- β inhibited IgG and IgA secretion by pokeweed mitogen-stimulated mononuclear cells. During follow-up of 10 children, normalization of serum IgG level was associated with a decrease in previously elevated TNF- α and TNF- β production, but IL-10 production remained unchanged.

Conclusion: These results suggest that TNF may be involved in the regulation of IgG and IgA production and can be associated with an arrest of IgG and IgA switch of B cells in hypogammaglobulinemia. The balance between TNF and IL-10 may be important for the normal development of IgG-secreting B cells. (*J Allergy Clin Immunol* 1997;100:556-62.)

Key words: Transient hypogammaglobulinemia, isolated IgA deficiency, tumor necrosis factor, cytokines

Abbreviations used

mAb:	Monoclonal antibody
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PHA:	Phytohemagglutinin
PWM:	Pokeweed mitogen
THI:	Transient hypogammaglobulinemia of infancy
TNF:	Tumor necrosis factor

Several cellular and molecular defects have been identified in various forms of human immunodeficiency. In particular, *btk* gene mutations have been described in X-linked hypogammaglobulinemia.^{1,2} However, the mechanism or mechanisms responsible for humoral deficiency in hypogammaglobulinemia with the presence of circulating B cells (e.g., common variable immunodeficiency, transient hypogammaglobulinemia, isolated IgA deficiency, antibody deficiency) has not been established.

Transient hypogammaglobulinemia of infancy (THI) is characterized by an abnormal delay in onset of IgG synthesis.³ It is a self-limiting condition in which IgG production becomes normal at 3 to 5 years of age.⁴ The incidence and classification of THI is a subject of controversy,^{5,6} but recent evidence indicates that it is not uncommon.^{4,7} Some affected children have a high incidence of infections (mostly of the respiratory tract), asthma, or both and food allergy or intolerance.^{7,8} Although deficiency of T helper cells⁸ and delayed functional maturation of B lymphocytes⁹ have been postulated as contributing factors to the pathogenesis of THI, the actual cause remains unknown.

It is known that various cytokines act as growth factors for B cells and molecules responsible for their maturation and final differentiation.¹⁰ However, little is known about defects or abnormal expression of cytokine genes in hypogammaglobulinemia. Recent studies¹¹ indicate that in common variable immunodeficiency in adults, an elevated production of interferon- γ by peripheral blood mononuclear cells (PBMCs) is seen. In contrast, phorbol myristate acetate-induced synthesis of other cytokines, including tumor necrosis factor (TNF)- α , is unaltered. However, it is unclear whether this has any bearing on depressed immunoglobulin production or whether it is associated with abnormalities of cellular immunity known to occur in common variable immunodeficiency.

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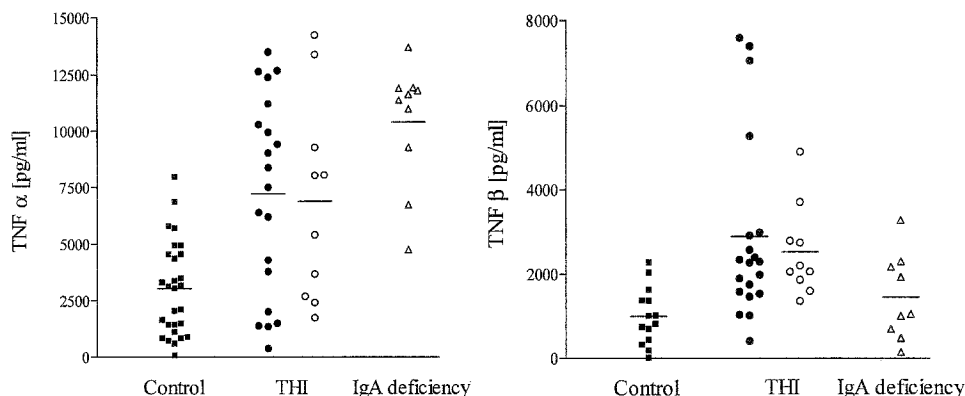


FIG. 1. Production of TNF- α and TNF- β by PHA-stimulated PBMCs of children with probable THI (filled circles), proved THI (open circles), and isolated IgA deficiency (open triangles). Short horizontal lines show the mean level of cytokines. Differences between control and patient groups are significant at $p < 0.05$.

However, no aberrant cytokine gene expression has been described in THI to date. In an isolated IgA deficiency, measurable serum levels of IL-4 and IL-6 were more frequently seen, whereas transforming growth factor- β was decreased.¹²

Our preliminary data indicated that synthesis of bioactive TNF by PBMCs of children with humoral immunodeficiency is increased.¹³ This study extends these observations and shows that both TNF- α and TNF- β production of phytohemagglutinin (PHA)-stimulated PBMCs was increased in children with THI, whereas in isolated IgA deficiency only TNF- α production was elevated. Interestingly, IL-10 production was also elevated in THI but not in isolated IgA deficiency. These observations indicate that aberrant cytokine production may occur in humoral immunity disorders with normal B-cell numbers.

METHODS

Patients

The patients studied consisted of children with recurrent respiratory tract infections attending the outpatient clinic for immunodeficiency from 1989 to 1996. Thirty patients with abnormally low serum IgG levels (>2 standard deviations below the mean for age, according to our laboratory values) and 15 children with isolated IgA deficiency were selected from approximately 980 referrals. The mean IgG level in children with hypogammaglobulinemia was 3.14 ± 1.00 gm/L, the mean IgA level was 0.29 ± 0.11 gm/L, and the mean IgM level was 1.23 ± 0.61 gm/L. Ten patients reached normal values during longitudinal follow-up (proved THI),⁷ and 20 patients had a single low IgG measurement or IgG remained depressed during the observation period (probable THI). The mean age for the patient group was 15 ± 7 months (mean $\pm$ SD), the median age was 13 months, and the male-to-female ratio was 2.3:1. The control group ($n = 40$) was formed from the examined children with recurrent infections in whom immunodeficiency was ruled out. The mean age for the control group was 16 ± 6 months (mean $\pm$ SD), the median age was 14 months, and the male-to-female ratio was 1.9:1. The mean serum level of IgG in this group was 9.40 ± 3.11 gm/L, the mean serum level of IgA was 0.91 ± 0.60 gm/L, and the mean serum level of IgM was

1.70 ± 0.77 gm/L. Isolated IgA deficiency was defined as an IgA serum level below 0.05 g/L, with IgM and IgG levels within the normal range. The mean age for patients with isolated IgA deficiency was 5 years. An appropriate control group consisting of seven children (mean age, 5.4 ± 1.1 years) was formed, and the cytokine studies were performed. Because the profiles of TNF- α and TNF- β were similar to those of the control group consisting of younger children, only the latter is shown in the results. Samples of blood were taken during routine diagnostic procedures. Informed consent was obtained from the parents. The control and patient samples were run in parallel. The level of circulating CD3, CD4, CD8, CD19, and CD22 lymphocytes, assessed by cytofluorimetric analysis after staining with appropriate antibodies, was within the normal range in both the patient and control groups. Also, no abnormalities in the lymphocyte response to mitogens were observed.

Cell separation and cultures

PBMCs were isolated by standard Isopaque/Ficoll (Pharmacia, Uppsala, Sweden) gradient and suspended in RPMI-1640 medium (Biochrom, Berlin, Germany). Monocytes were separated from PBMCs by counterflow centrifugal elutriation with a JE-5.0 elutriation system (Beckman, Palo Alto, Calif.) equipped with a 5 ml Sanderson separation chamber as previously described.¹⁴ T cells were isolated from elutriated lymphocytes by rosetting with neuraminidase-treated sheep erythrocytes followed by the Isopaque/Ficoll gradient,¹⁵ and the remaining lymphocytes were used as the B-cell-enriched population. Monocytes were approximately 88% to 96% pure as determined by staining with anti-CD14 monoclonal antibody (mAb) (Leu-M3; Becton-Dickinson, Mountain View, Calif.) and flow cytometry analysis. T cells were approximately 97% CD3⁺, and the B-cell-enriched population contained approximately 75% CD19⁺ cells.

For cytokine production, PBMCs (1×10^6 /ml) suspended in RPMI-1640 medium with 10% fetal calf serum (Biochrom) were cultured in microtiter plates (Nunc, Roskilde, Denmark) for 48 hours at 37°C in 5% CO₂ in the presence or absence of 2.5 μ g/ml PHA (Murex Diagnostics, Dartford, U.K.). Supernatants were harvested and maintained at -70°C until they were tested for cytokine production.

For in vitro studies of immunoglobulin synthesis, the B-cell-enriched population isolated from healthy donors was cultured with mitomycin C-treated (Sigma, St. Louis, Mo.) T lympho-

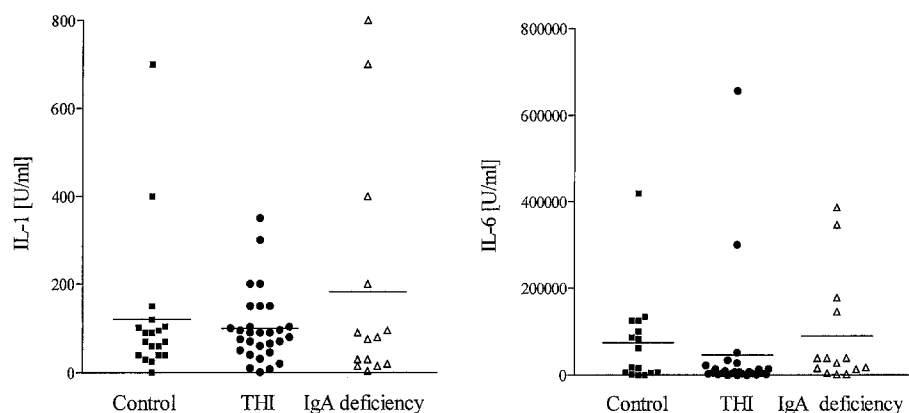


FIG. 2. Production of bioactive IL-1 and IL-6 by PHA-stimulated PBMCs.

cytes at a ratio of 1:1 and 5% monocytes. The cells (1×10^6 ml) were stimulated with pokeweed mitogen (PWM) (Gibco, Paisley, U.K.) at a final dilution of 1:1000 and cultured in triplicate in microtiter plates for 7 days. In some experiments various doses (0.1 to 100 U/ml) of human recombinant TNF- α (specific activity, 4×10^7 U/mg; the kind gift of Professor J. W. Stec, Polish Academy of Science, Łódź, Poland) or recombinant TNF- β (R&D Systems, Abingdon, Oxon, U.K.) were added to the cultures.

Measurements of cytokines

The levels of TNF- α , TNF- β , IL-1, IL-4, IL-6, and IL-10 in the culture supernatants were determined by ELISA (EASIA, Medgenix, Fleurus, Belgium).

Bioactive IL-1 was measured by the thymocyte comitogenic proliferation assay as previously described.¹⁶ Recombinant IL-1 (NIBSC, London, U.K.) was used as a standard. For IL-6 determination serially diluted supernatants were added to IL-6-dependent B9 line, and cell proliferation was assessed 3 days later by use of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye reduction test.¹⁷ As a standard, recombinant IL-6 (kindly provided by Professor T. Kishimoto, Osaka University, Japan) was used. The results were expressed in units per milliliter. The same supernatants were also tested by ELISA.

Determination of immunoglobulin levels in the culture supernatants

IgG and IgA in the supernatants were quantified by ELISA. Microtiter plates (Nunc-Immuno Plate MaxiSorp) were coated with 100 μ l of goat anti-human IgG or anti-human IgA (1 μ g/ml; Jackson ImmunoResearch Lab., Westgrove, Pa.). After overnight incubation at 4°C, the plates were washed, and 100 μ l/well of serial dilutions of the culture supernatants were added in triplicate. Plates were then incubated overnight at 4°C and washed. To detect bound IgG or IgA alkaline phosphatase-conjugated goat anti-human serum, IgG or IgA antibody (Jackson) was added, followed by 100 μ l/well of p-nitrophenyl phosphate. The plates were read with an ELISA reader (Lab-systems Multiskan Plus) at 492 nm. Serial dilutions of N Protein Standard SY (Behring, Marburg, Germany) were used to obtain the concentration curve.

TNF- α ELISPOT assay

Nitrocellulose filters (0.5 cm; Millipore GmbH, Wien, Austria) were placed on 96-well Maxisorp plates (Nunc) and, after

washing, coated for 4 hours at room temperature with 50 μ l of purified mouse anti-human TNF- α mAb (2 μ g/ml; Pharmingen, San Diego, Calif.). The wells were washed with phosphate-buffered saline (PBS) and blocked for 18 hours at 4°C with 3% bovine serum albumin (Sigma) in PBS. The plates were washed, and various numbers of PBMCs in RPMI-1640 medium containing 10% human AB serum (2×10^5 cells/ml) were added to the wells in duplicate. Plates were then incubated overnight at 37°C in 5% CO₂. After washing with 0.05% Tween in PBS, 50 μ l of biotin-conjugated mouse anti-human TNF- α mAb (1 μ g/ml; Pharmingen) was added to the wells for 18 hours at 4°C. The plates were then washed thoroughly with Tween-PBS, treated with 50 μ l of 1:1000 dilution of avidin-conjugated alkaline phosphatase (Jackson) for 3 hours, and washed again. Single cells secreting cytokine (spots) were visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Bio-Rad, Richmond, Calif.). The reaction was stopped after 10 minutes by washing with rotation, and spots were enumerated under $\times 20$ magnification.

Statistical analysis

The two-sample rank test of Mann-Whitney was used as a two-tailed test to compare the values obtained from the patient and control groups. The difference was regarded as significant when the *p* value was less than 0.05.

RESULTS

Production of TNF- α and TNF- β by PBMCs of children with THI and isolated IgA deficiency

Our previous results indicated an increased production of bioactive TNF in children with humoral immunodeficiency.¹³ This study continued these observations in THI and isolated IgA deficiency and addressed the issue of the type of TNF involved. Production of both TNF- α and TNF- β by PHA-stimulated PBMCs was significantly ($p < 0.05$) elevated in THI (both probable and proved) in comparison with control (Fig. 1). The spontaneous release of TNF- α and TNF- β by unstimulated PBMCs was not enhanced (data not shown). Surprisingly, significantly ($p < 0.001$) enhanced production of TNF- α , but not TNF- β , was observed in isolated IgA deficiency (Fig. 1). The level of TNF- α in the sera of children with THI was also determined and was found to be similar (19 ± 17 pg/ml) to that of the control group

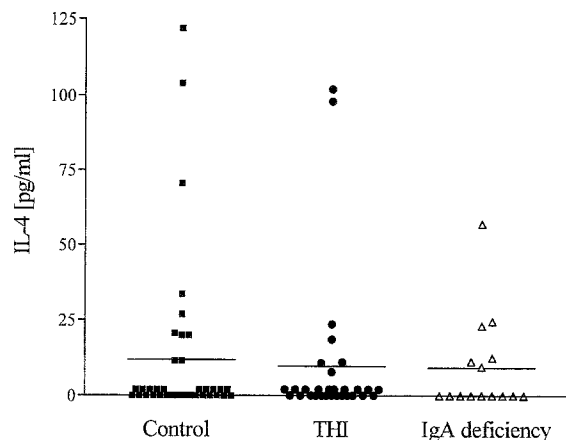


FIG. 3. Production of IL-4 by stimulated PBMCs as determined by ELISA.

(31 ± 40 pg/ml). TNF- β was not detectable in the sera of the patients or control subjects. Analysis of the expression of TNF receptor types I and II (flow cytometry with htr-9 and utr-1 mAbs¹⁷) on unstimulated and PHA-stimulated PBMCs showed no difference between THI and control groups (TNF receptor II: 9.1 ± 5.2 ; 8.7 ± 4.3 for stimulated cells, respectively).

The number of TNF- α -secreting cells in transient hypogammaglobulinemia

The increased level of cytokines in the culture supernatants may be due to an increased secretion by the same number of cells, an increased number of secreting cells, or both. To determine the actual number of TNF- α -secreting PBMCs we have used an ELISPOT assay, which enables the assessment of cytokine-producing cells at the single-cell level. The number of unstimulated PBMCs releasing TNF- α was similar in the control and hypogammaglobulinemia groups. However, after stimulation with PHA this number was significantly higher in the patients with THI (not shown). Also, spots formed by PBMCs of the patients were larger, implicating enhanced production by a single cell.

Production of IL-1, IL-6, IL-4, and IL-10

The production of other cytokines was also assessed. We have chosen IL-1, IL-4, IL-6, and IL-10 because they are involved, at different stages, in the regulation of B-cell function. Fig. 2 shows that the release of bioactive IL-1 and IL-6 by PBMCs was comparable in both the patient and control groups. The same supernatants were tested for IL-1 and IL-6 content by ELISA. Similarly, no differences were found (data not shown). Production of IL-4 as determined by ELISA was also similar in all groups (Fig. 3). IL-10 production was somewhat elevated in the patient groups (Fig. 4). However, this increase reached statistical significance in proved THI ($p < 0.05$) but not in probable THI ($p < 0.1$) or IgA deficiency (not significant).

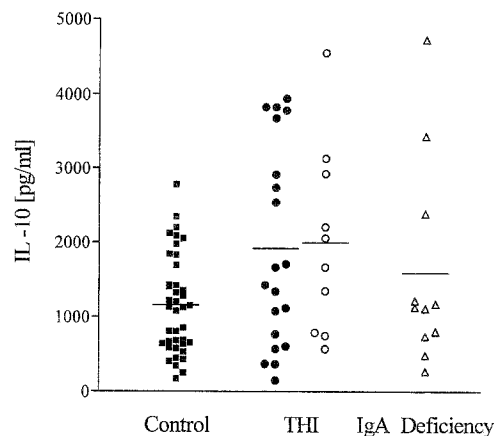


FIG. 4. Production of IL-10 in probable THI (filled circles), proved THI (open circles), and isolated IgA deficiency (open triangles).

Inhibition of IgG and IgA production by TNF in PWM-stimulated cultures

Finding an enhanced production of TNF by PBMCs in the patient groups prompted *in vitro* observations to establish whether TNF is able to inhibit immunoglobulin secretion by normal B cells. The cells (B-cell-enriched lymphocytes, mitomycin C-treated T cells, and monocytes) from normal donors were stimulated with PWM and cultured in the presence of TNF- α or TNF- β . Fig. 5 shows that IgG and IgA synthesis were markedly inhibited by both TNF- α and TNF- β .

Longitudinal testing

Some patients with THI were followed up, and the serum level of IgG and production of TNF- α and TNF- β by their PBMCs were determined 6 to 12 months after the first investigation. It has been noted that when the serum level of IgG increased (without substitution therapy with immunoglobulins), production of TNF- α and TNF- β by PBMCs decreased (Fig. 6). However, no changes in IL-10 levels were observed. These results suggest that increased production of TNF may be associated with delay in the normal development of IgG production.

DISCUSSION

The main findings of this study were increased production of TNF- α , TNF- β , and IL-10 by PBMCs of children with probable and proved THI and a lack of major changes in the release of other cytokines, including IL-6 and IL-4. In an isolated IgA deficiency the only abnormality found was an elevated TNF- α release.

The reason for the delay, sometimes up to 40 months, in the production of IgG in THI is unknown.¹⁸ The level of circulating B cells is normal. It is known that neonatal lymphocytes are deficient in their ability to produce immunoglobulin, and several cytokines (IL-2, IL-4, IL-6, IL-10) are required for induction of immunoglobulin secretion.¹⁹ Whether limited availability of cytokines may be linked to the delayed IgG secretion in some children is unclear. Current studies do not support such a notion as

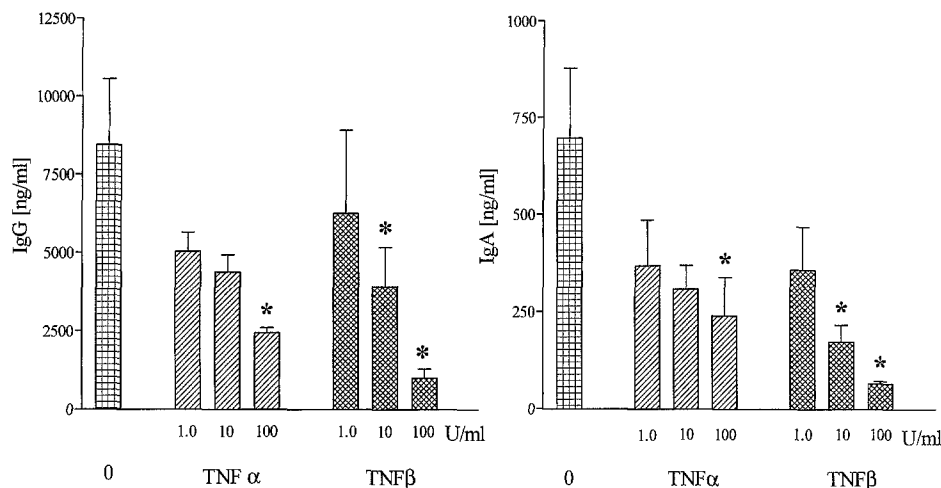


FIG. 5. Inhibitory effect of exogenous TNF- α and TNF- β on PWM-induced IgG or IgA synthesis. *Significant inhibition ($p < 0.05$) decreased in comparison with immunoglobulin synthesis by TNF-untreated PWM-stimulated PBMCs.

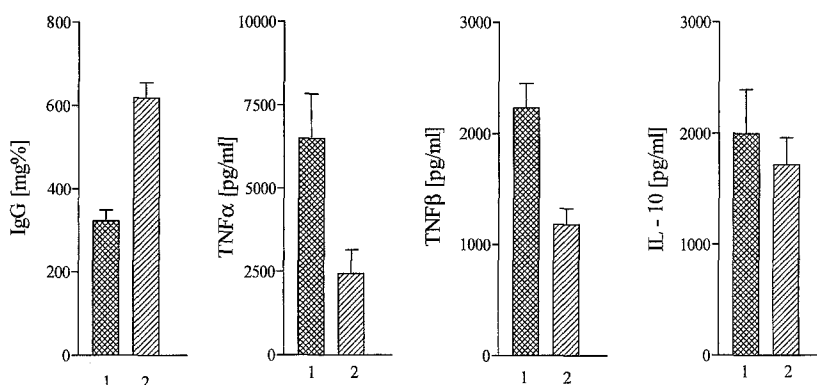


FIG. 6. Comparison of IgG serum level and production of TNF- α , TNF- β , and IL-10 in children with proved THI. Hatched columns indicate first investigation. Striped columns indicate second investigation (6 to 12 months later).

production of IL-6 and IL-4 was comparable to that in the control group of children with normal levels of IgG, whereas IL-10 was increased. Recently, Matheson et al.²⁰ have shown that production of IL-4 by PBMCs of patients with hypogammaglobulinemia (age 4 to 57 years) is not depressed. The major abnormality seen in THI was an enhanced production of both types of TNF, but no changes in the expression of TNF receptors were observed. TNF- α and TNF- β can be linked to abnormal B-cell function because in vitro observations showed that recombinant TNF- α and TNF- β significantly inhibited PWM-induced IgG production by normal B cells. This is partly in keeping with findings of Kashiwa et al.,²¹ indicating that TNF- α inhibits PWM-stimulated immunoglobulin synthesis without affecting cell proliferation. However, we are not aware of the data on the suppressive effect of TNF- β on immunoglobulin secretion. The data from the ELISPOT assay indicated that in patients with THI the number of cells secreting TNF- α spontaneously was similar to that seen in control subjects. In contrast,

frequency of cells secreting TNF- α after PHA stimulation in the THI group was increased. This seems to indicate that enhanced expression of the TNF- α gene occurs only after stimulation. Overall, these results indicate that increased TNF- α production in the patients in this study was probably due to both enhanced release and an increased number of circulating secreting cells.

The finding of an enhanced production of IL-10 in some patients with THI was unexpected. IL-10 is a potent growth and differentiation factor for B lymphocytes.²² TNF- α acts as a regulator of IL-10 expression by PBMCs in vitro²³ and mediates the induction of IL-10 release in endotoxemia in human beings and chimpanzees.²⁴ This may suggest the existence of an autoregulatory feedback mechanism. Alternatively, production of IL-10 may be a part of the protective mechanism leading to inhibition of TNF production as observed in experimental sepsis.²⁴ On the other hand, IL-10 is an IgG switch factor,²⁵ and its enhanced synthesis may represent the response designed to induce differentiation of B

cells. This reasoning may be supported by the lack of elevated IL-10 in patients with IgA deficiency (see below). The question of whether the balance between TNF production (suppressing IgG synthesis) and IL-10 production (inducing IgG switch) is critical for regulation of IgG synthesis during immune system development arises.

Although in this study the identification of cells responsible for an enhanced production of cytokines was not undertaken for logistic reasons (i.e., the small volume of blood samples), it is probable that monocytes, as a major source of TNF- α and IL-10, were involved. This reasoning is in keeping with our other observations indicating that TNF- α predominantly affects monocytes.²⁶ The target cell for the action of TNF- β has not been established, but it may be the T helper cell.²¹ However, our preliminary experiments indicate that TNF- α and TNF- β may act directly on B cells.

The follow-up observations in some children with THI provided further evidence for the role of TNF in the regulation of IgG production. Normalization of IgG serum level during follow-up was accompanied by decreased production of TNF- α and TNF- β , whereas IL-10 did not show similar changes.

Current evidence indicates that the underlying mechanism of selective IgA deficiency is due to abnormal immunoregulation, probably genetically determined, which results in the defective maturation of B cells.²⁷ Differentiation of B cells into IgA-secreting cells is a complex process involving isotype switching with differentiation into surface IgA-bearing B cells and terminal differentiation of these cells into IgA-producing B cells.^{28, 29} This process is poorly understood, but current evidence indicates that it is directed and regulated by a host of cytokines.¹² Serum levels of IL-7 in adult patients with IgA deficiency are similar to those in control subjects, and TGF- β is reduced; whereas detectable IL-4 and IL-6 levels are observed in patients but not in healthy subjects.¹² This is in keeping with our observations because production of IL-6 and IL-4 by PBMCs of children with IgA deficiency was similar to that of children in the control group. IL-10 synthesis was also comparable. These factors taken together indicate that isolated IgA deficiency cannot be linked with insufficient production of lymphokines, which control final differentiation of B cells and isotype switch.¹⁰ However, the production of TNF- α , but not TNF- β , and IL-10, by patients' PBMCs was increased. This is in contrast to THI, in which the release of all these cytokines was enhanced.

The reason for upregulation of TNF and IL-10 genes in the patients is unclear. The effect of infections should be considered. However, this is not very likely because similar infections were observed in both groups of patients and in the control group, which in fact was formed of children with similar clinical symptoms in whom immunodeficiency was ruled out. Furthermore, spontaneous release of cytokines was comparable in all studied groups. Finally, in IgA deficiency only TNF- α

was upregulated; whereas in THI TNF- α , TNF- β , and IL-10 were upregulated. It is unlikely that an infection or infections would have such selective effect.

In summary, this study demonstrated upregulation of some cytokine production in THI and isolated IgA deficiency. These conditions may be added to the growing list of other immunodeficiency diseases in which aberrant cytokine production is observed.³⁰⁻³⁵ However, further studies are needed to understand the complex interplay between various cytokines in regulating immunoglobulin production.

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