IMMUNOLOGIC EVALUATION DURING THE FIRST YEAR OF LIFE OF INFANTS BORN TO CYCLOSPORINE-TREATED FEMALE KIDNEY TRANSPLANT RECIPIENTS

ANALYSIS OF LYMPHOCYTE SUBPOPULATIONS AND IMMUNOGLOBULIN SERUM LEVELS

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Background. In rodents, CsA has been shown to affect T-cell development, giving rise to an abnormal production of mature T cells and the absence of many T-cell subsets as well as to autoimmunity. Surprisingly, only a few studies investigated the effect of the immunosuppressive drug on the immune system of the human fetus.

Methods. We examined six infants born to female kidney transplant recipients who had received cyclosporine and methylprednisolone throughout their pregnancies. Peripheral blood was obtained 1 day and 2, 4, 6, and 12 months after birth, and two-color flow cytometric immunophenotyping of lymphocytes was performed.

Results. Total T cells, as well as CD4+ and CD8+ T cells, were low at birth, but normalized thereafter. Among T-cell activation markers, the expression of CD25, the α chain of the interleukin-2 receptor, was below the normal range or low range throughout the study period, and HLA-DR expression was extremely low at birth and failed to increase up to 12 months. The number of total B cells was lower than normal at birth, but steeply increased over time. In contrast, B-cell subset bearing CD5 antigen was severely depleted throughout the first year of life. Total IgG concentration was significantly lower than in controls at 2, 4, 6, and 12 months after birth, and two-color flow cytometric immunophenotyping of lymphocytes was performed.

Conclusions. Continuous exposure to CsA in utero seemingly impairs T-, B-, and NK-cell development and/or maturation, and most of its effects are still apparent at 1 year, which might suggest that conventional vaccinations should be delayed in these infants.

Over the last few years, several reports have detailed the outcomes of pregnancies in kidney transplant recipients who were maintained on cyclosporine A (CsA) before and during pregnancy (1–6). Surprisingly, only a few studies investigated the effect of the immunosuppressive drug on the immune system of the fetus (7–10). Indeed, CsA does cross the placenta and can be measured in fetal blood (11), at concentrations comparable to the doses that caused abnormalities in developing rodent T cells (12–15).

Treatment of pregnant rats with CsA caused a temporary delay in the development of some hemopoietic organs, including the thymus, spleen, liver, and bone marrow (16). In inbred rodents, CsA has been shown to affect T-cell development in the thymus, giving rise to an abnormal production of mature T cells and the absence of many T-cell subsets (12, 17, 18), as well as to autoimmunity (13, 19, 20).

To evaluate whether CsA can elicit underlying abnormalities in the developing immune system of the human fetus, we have analyzed peripheral blood lymphocyte (PBL) populations from six infants, from birth to 12 months of age, who had been exposed to CsA in utero throughout the pregnancy.

PATIENTS AND METHODS

Subjects. We studied six infants born to immunosuppressed mothers after renal transplantation. All mothers had received a transplant from a cadaveric donor after end-stage kidney disease (chronic glomerulonephritis: five cases; chronic pyelonephritis with reflux: one case) and became pregnant a median of 9 years (range: 3–12 years) after transplantation. All of them were receiving CsA and low-dose methylprednisolone (4 mg/day) to maintain immunosuppressive status throughout their pregnancies. Of note, methylprednisolone is not known to cross the placenta. Three out of six mothers had been taking azathioprine until shortly before the planned conception. Kidney function was fairly stable during pregnancy (serum creatinine median: 1.3; range: 1.0–1.5 mg/dl). Four mothers presented with mild arterial hypertension, well controlled.”

Venous blood samples were obtained after informed parental consent from all children on the first day of life, and then 2, 4, 6, and 12 months after birth.

Normal values were established in 20 healthy unexposed neonates (all delivered by cesarean section, 13 of them 4–7 weeks
preterm), and in a total of 76 normal infants in the age range from 2 months to 1 year, coming from the same geographical area (courtesy of the Laboratory of Immunology, University of Bari, Bari, Italy).

**Sample preparation and analysis.** Peripheral venous blood samples were collected in Vacutainer tubes containing 7.5% tripotassium ethylenediaminetetracetate and stored at room temperature for no longer than 2 hr before staining. A complete blood cell count, including an automated differential count, was performed with a Coulter counter (Coulter Hematology, Hialeah, FL). Absolute subset counts were obtained as the product of the absolute lymphocyte count and the percentage of the lymphocyte subset population of interest, as determined by flow cytometry.

Two-color flow cytometric immunophenotyping of lymphocytes was performed by a lysed whole-blood method using matched combinations of murine monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Briefly, 100-μl aliquots of whole blood were incubated at 4°C for 30 min with combinations of the following optimally titrated FITC- and PE-conjugated CD3, CD4, CD5, CD8, CD16, CD19, CD25, CD56, CD57, and HLA-DR antibodies (Bio-D, Valenzano, Bari, Technopolis Novus Or tus, Italy; and Coulter-Immunotech, Hialeah, FL). After incubation the cells were washed, followed by lysis of erythrocytes using ice-cold isotonic NH₄Cl solution (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4), washed twice with PBS/0.01% NaN₃, then resuspended in 500 μl of PBS/0.5% formaldehyde and subsequently analyzed by flow cytometry (Coulter Epics XL-MCL II, equipped with a 1-milliwatt air-cooled 670-nm argon laser). Background fluorescence was determined with FITC- and PE-conjugated mouse IgG of the appropriate isotype. The lymphocyte gate was checked for its purity by use of CD14/CD45 double staining and was regarded to be correct if the gate included at least 95% of all lymphocytes and contained less than 5% contamination. All samples included an aliquot stained with CD45-FITC to determine the total number of white cells as a reference for other staining combinations. Histograms of the fluorescence of at least 10,000 viable lymphocytes were recorded.

**Total IgG, IgA, IgM, and IgG subclass concentrations were determined by rate nephelometry, using a Beckman analyzer.**

**Statistical analysis.** Data are presented as mean ± standard deviation (SD). Data were compared using a two-tailed unpaired t test. P<0.05 was considered significant.

### RESULTS

**T-lymphocyte subsets.** In CsA-exposed newborns, the number of white blood cells expressing CD3, as well as the number of CD4⁺ and CD8⁺ cells, was significantly lower than normal at birth, but normalized thereafter (Fig. 1).

A number of different surface markers can identify activated T cells (naive or memory). Two that are most often used are MHC class II molecules and the α chain of the interleukin-2 (IL-2) receptor (CD25), which are also expressed at low levels on memory T cells (21). In the subjects studied, the number of CD3⁺HLA-DR⁺ T lymphocytes was extremely low at birth and failed to increase up to 12 months (Fig. 2). The number of cells expressing CD25 antigen, a very early T-cell activation marker, was below the normal range or low range throughout the study period (Fig. 2).

**B cells.** Peripheral blood CD19⁺ cells were low in neonates, but increased to even supernormal levels at later time points (Fig. 3, top panel).

CD5 antigen is usually detected on the majority of B cells of neonates and infants up to at least 6 years, slowly decreasing with age (22, 23). In CsA-exposed children, in contrast,
CD19\(^+\)CD5\(^-\) cells were about 5% of total B lymphocytes at birth and reached less than 15% at 1 year (Fig. 3, bottom panel).

Serum immunoglobulin levels. At birth, the serum of normal neonates contains very low levels of IgM and IgA, but near-adult levels of IgG, which is transported across the placenta. The efficiency of transplacental transfer differs among IgG subclasses: transfer of IgG1 and IgG4 is significantly more efficient than that of IgG3 and, even more, of IgG2.

Accordingly, CsA-exposed newborns had normal serum levels of IgG, but low-range levels of IgA and IgM (Table 2). After 2 months, total IgG concentration was significantly lower than in controls, mainly because of subnormal levels of IgG1 and IgG3 subclasses, whereas IgA and, to a lesser extent, IgM were persistently in the low range. Serum Immunoglobulin levels normalized at 4 months, although IgG1 and IgG3 subclasses remained in the low range of controls up to 6 months (Table 2).

Natural killer (NK) cells. NK cells were defined by the presence of CD16 (low-affinity Fc\(\gamma\) receptor) and CD56 (a member of the neural cell adhesion molecule family), without coexpression of CD3. CD57 defined non-MHC-restricted cytotoxic lymphocytes. Infants showed normal numbers of true NK cells throughout the study (Fig. 4). The expression of CD57 antigen, which is barely detectable at birth, failed to increase over time, in both CD8\(^+\) and CD8\(^-\) subsets (Fig. 5).

Clinical course. The infants did not show any symptoms or signs suggestive of a clinically relevant immunodeficient state. In particular, they did not develop any opportunistic or chronic infections and had a normal growth during their first year of life. The apparent phenotypic abnormalities of peripheral blood lymphocytes, as well as the low levels of serum immunoglobulins, suggested to postpone classical vaccinations, and especially those with live, attenuated vaccines at least after the 6th month. With this approach, none of the infants showed adverse events after the administration of live, attenuated vaccines (at the 7th to 8th month, on average): three infants, whose parents accepted a further evaluation, had a normal antibody response at 18 months of age.

DISCUSSION

Our results demonstrate that infants from CsA-treated female kidney transplant recipients exhibit distinct differences in the phenotypic profile of peripheral T cells, B cells, and NK cells, which persist, although to a variable degree, up to 1 year of age.

CD3\(^+\) T lymphocytes, as well as CD4\(^+\) and CD8\(^+\) T-cell subsets, were transiently lower than in controls at birth, whereas activated T cells remained persistently below the normal range throughout the first year of life. Specifically, T cells expressed relatively low levels of CD25, but were markedly deficient in the expression of HLA-DR antigens, a pattern resembling that exhibited by cord blood T lymphocytes and, even more, by T cells in fetuses at 18–20 weeks of gestation (26). Such pattern led the authors to hypothesize that fetal T lymphocytes would undergo an antigen-induced short-term activation, with limited clonal expansion and meager, if any, generation of memory cells (26). Pilarski et al.
(8) analyzed PBL populations from 7 children, whose age ranged from 0 (cord blood) to 6 years, who had been exposed to CsA, with or without azathioprine, in utero. They found a normal proportion of CD3^+^, CD4^+^, and CD8^+^ T cells in children aged 1 year or more; however, significant differences were detected in the expression of CD45RO and CD29, which are both expressed by most antigen-experienced memory and activated T cells. Indeed, children had a substantially higher expression of CD45RA, indicative of a relative antigen inex-perience, a trend toward less CD45RO, and a definitely reduced expression of CD29 on T cells (8). Thus, our data seem to confirm, in a homogeneous population of infants studied throughout the first year of life, that continuous exposure to CsA in utero can induce an alteration or at least a delay in T-cell development and maturation.

The number of B cells, similar to T cells, was significantly lower than normal at birth. Although, in general, the predominant action of CsA is directed against T-lymphocyte subpopulations (27), some evidence exists that it can directly inhibit B-cell proliferation independent of T-cell influence (28–30). Previously, Takahashi et al. (9) reported a severe B-cell depletion in six newborns from female renal transplant recipients who were taking CsA, azathioprine, and methylprednisolone, which persisted at 3 months of life in two subjects, whereas, in Rose and co-workers’ report (7), B-cell numbers were within the normal range in two infants born to female heart-lung transplant recipients taking CsA only.

Then, we found extremely low levels of CD5^+^ B cells throughout the follow-up. CD5^+^ B lymphocytes appear in early development, constituting the vast majority of the total fetal and neonatal B cells (22, 23). Approximately 50–75% of B cells found in human spleen and more than 70% of those

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<th>Table 2. Total IgG, IgA, IgM, and IgG subclass serum concentrations in neonates exposed to CsA in utero</th>
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* Normal range according to age is indicated in parentheses.  
^b Values below the normal range (P<0.05).  
^c Values in the low range of healthy controls.
found in cord blood express CD5 (22, 23). Arguments and data exist both for models in which CD5+ and CD5− B cells arise in fetal life as separate lineages before immunoglobulin gene rearrangement (31) and for models in which CD5+ B cells result from an antigen-driven differentiation process (32, 33). CD5+ population is responsible for the production of most autoantibodies and low-affinity polyreactive antibodies, which probably play a major role as a first line of defense against infections by providing immunity to common bacterial antigens, especially in the immunologically immature host (22). Then, CD5+ B cells have been proposed to repre- sent the precursor of a newly identified normal B/macrophage cell, which combines the restricted anti-self and antibacterial specificities of CD5+ B cells with the plasticity of macrophages, and might be an important component in fetal and neonatal immune repertoires, where CD5+ B cells usually predominate (34). Moreover, CD5 antigen has been sug-gested to play a critical role in shaping the B-cell repertoire: the interaction of CD5, as well as other endogenous antigens or superantigens, with surface Ig would provide the persis- tence signal needed for B-cell maintenance (35). Finally, cord blood B cells lacking CD5 surface antigen, in contrast to CD5+ cells, have been shown to express remarkably low levels of several adhesion molecules, which are necessary for B lymphocytes to perform a broad variety of functions, such as B-T-cell cooperation, B-cell migration and homing, and B-cell interaction with follicular dendritic cells (36). CD5 mRNA is highly expressed in bone marrow progenitor B cells in the absence of detectable surface CD5 expression (35). Inducers of CD5 surface expression include common microbrial superantigens (such as Staphylococcus aureus Cowan strain I) and anti-IgM antibodies (35). Noteworthy, CsA has been shown to inhibit B-cell responses to polyclonal anti-immunoglobulin antibodies, particularly anti-μ (37–39). We speculate that CsA might similarly interfere with the induc- tion of CD5 surface antigen on progenitor B cells. At any rate, a decreased proportion of B lymphocytes bearing the CD5 molecule might impair many aspects of B-cell physiology (36) and reflect a defect of B-cell activation (40) and development (21).

The only relevant abnormality of quantitative serum Ig was a reduction of IgG1 and IgG3 subclasses, apparent at 2 months and persisting, although to a lesser extent, up to 6 months. Antibodies of the IgG1 and IgG3 isotype generally react with protein and viral antigens and are mainly T-cell dependent (41, 42), which might further suggest an alteration of T-cell–B-cell interaction in the infants studied. NK cells are relatively abundant at birth (about 3 times adult levels) but decline rapidly thereafter (23, 43). It has been proposed that NK cells in human neonates go through two different maturational stages: CD16+56− cells would be responsible for the natural cytotoxicity in newborns with mature NK activity, whereas CD16+56− cells would be largely devoid of cytotoxic activity (44). In this respect, in- fants born from CsA-treated mothers exhibited quite normal numbers of CD3− CD16+56− cells, which would indirectly suggest the possibility of normal NK activity. It may also appear interesting that renal transplant recipients treated with CsA only seem to display normal NK cells and functions (45). On the other hand, the infants displayed persistently low levels of CD57 antigen, both in CD8− and CD8+ subsets. CD57 antigen is almost undetectable on normal cord blood lymphocytes (23), but the CD57− fraction gradually expands with time (23), suggesting that CD57 is acquired relatively late in the maturative steps of lymphoid cells with killer activity.

In conclusion, continuous exposure to CsA in utero seem-ingly hampers T, B, and NK cell development and/or func- tion, and most of its effects last up to 1 year of age. This leads to suggest a careful evaluation of lymphocyte subpopulations and antibody production in infants born to renal transplant recipients taking CsA and, possibly, to delay the administra- tion of classical vaccinations, in view of the potential risk of both suboptimal immunologic responses and adverse events after the use of live, attenuated vaccines in these infants. On the other hand, the clinical relevance of such abnormalities remains questionable, as none of the children studied showed clinical evidence of an immunodeficient state, which supports the notion that the human developing immune system is remarkably resilient and adaptable, even in the presence of immunosuppressive drugs.

REFERENCES

8. Pilarski LM, Yacyshyn BR, Lazarovits AL. Analysis of peripheral blood lymphocyte populations and immune function from children exposed to cyclosporine or to azathioprine in utero. Transplantation 1994; 57: 133.


