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Type 1 and Type 2 Cytokine Profiles in Children Exposed to or Infected with Vertically Transmitted Human Immunodeficiency Virus

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Received 7 March 1996/Returned for modification 22 April 1996/Accepted 24 May 1996

In human immunodeficiency virus (HIV)-infected adults, cytokine production profiles switch from predominantly type 1 (interleukin-2 [IL-2] and gamma interferon [IFN-γ]) to type 2 (IL-4 and IL-10) cytokines with disease progression. To test this hypothesis in vertically HIV-infected children, we measured cytokine transcription and production in rapid progressors (RPs), seroreverters (SRs), and those children exposed to HIV in utero (P0s). Production of type 1 and type 2 cytokines was measured in peripheral blood mononuclear cell cultures of 8 SR, 25 P0, and 11 RP children. Unstimulated cultures, irrespective of infection and stage of disease, produced similar levels of IL-2, IFN-γ, IL-4, and IL-10. Upon stimulation with phytohemagglutinin (PHA) plus phorbol-12-myristate-13-acetate (PMA), RP children produced less IL-2 (P < 0.01) and IFN-γ (P < 0.02) than SR children and also expressed significantly less IFN-γ mRNA (P < 0.01) than P0 children. RP children expressed significantly higher levels of IL-4 mRNA than P0 children (P < 0.03). There were no differences in the production of IL-10 by PHA-PMA-stimulated peripheral blood mononuclear cell cultures among the three groups of children. Our data with these pediatric patients suggest that a deficiency in mitogen-stimulated type 1 cytokine production and excess type 2 cytokine (IL-4) transcription correlate with disease progression. Additional studies with larger sample sizes are needed to test further the hypothesis of the type 1-to-type 2 cytokine switch in children infected with HIV.

An estimated 1 million children worldwide are currently infected with the human immunodeficiency virus (HIV) (7). Vertical transmission of HIV was the means of transmission for 90% of the more than 6,600 children (less than 13 years old) with AIDS who were reported to the Centers for Disease Control and Prevention through June 1995 (7). In general, children with vertically acquired HIV infection have shorter clinical latent periods and more rapid disease progression than other individuals with HIV infection (15, 23, 36). However, there is bimodal disease expression, with a late onset of symptoms and long-term survival in some patients (2, 20). At the Pediatric AIDS Clinical Trials Unit in Houston, more than two-thirds of children with vertically acquired HIV infection survive to age 5 years or older (21).

In HIV-infected adults certain cytokine production patterns, termed type 1 and type 2, are thought to be important in disease progression. Type 1 cytokines include interleukin-2 (IL-2) and gamma interferon (IFN-γ), and type 2 cytokines include IL-4 and IL-10. In a study of type 1 and type 2 cytokine production, Clerici et al. (11) have shown that a decrease in recall antigen-stimulated IL-2 production is accompanied by an increase in IL-4 production in HIV-positive adults. In pediatric HIV-positive children, aged between 44 months and 12 years, Vigan’o et al. (38) reported significantly less IL-2 production and significantly more IL-4 production than the levels of production in healthy control subjects. These observations are consistent with the earlier hypothesis of Clerici and Shearer (13) suggesting that a switch in production from a type 1 to a type 2 cytokine profile is associated with disease progression. Furthermore, Gruters et al. (17) have shown that T-cell proliferation and anti-CD3-induced generation of effector cytotoxic T lymphocytes (CTLs) are lost in HIV-infected symptomatic persons because of a decreased capacity for producing IL-2. In HIV-positive children, deficient IL-2 production is associated with decreased T-cell responses to recall antigens and decreased CTL activity (29). Hence, we hypothesized that children with rapid disease progression will have less type 1 cytokine production than HIV-negative children and will tend to lose cellular immunity, whereas less symptomatic patients will have more type 1 cytokine production and stronger CTL activity than rapid progressors. Because little is known about type 1 and type 2 cytokine production in children exposed to or infected with vertically transmitted HIV in infancy and in children early after infection, we measured in vitro cytokine transcription and production in mitogen-stimulated cultures of peripheral blood mononuclear cells (PBMCs) from children exposed to or infected with HIV. Our objective was to determine whether the neonatal profile of type 1 and type 2 cytokines correlates with the clinical outcome in these children.

(Preliminary results of these findings were presented at the International Symposium on Clinical Immunology, San Francisco, Calif., 21 to 23 July 1995.)

MATERIALS AND METHODS

Patient groups. Forty-four blood samples were drawn from children vertically exposed to or infected with HIV. The clinical status of the children was defined according to the Centers for Disease Control and Prevention classification for children on the basis of all symptoms evident at the time of study (5, 6). The diagnosis of HIV type 1 (HIV-1) infection was based on the presence of HIV p24 antigen in serum or plasma detected by standard enzyme-linked immunosorbent assay (ELISA), DNA PCR for proviral gag sequences, and virus isolation (18). At
TABLE 1. Characteristics of study groups exposed to or infected with HIV

<table>
<thead>
<tr>
<th>Study group</th>
<th>No. of children</th>
<th>Median age (mo)</th>
<th>Mean ± SEM CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>25</td>
<td>&lt;1</td>
<td>50.9 ± 2.4 CD4</td>
</tr>
<tr>
<td>PPs</td>
<td>11</td>
<td>21</td>
<td>17.3 ± 2.9 CD4</td>
</tr>
<tr>
<td>SRs</td>
<td>8</td>
<td>17</td>
<td>38.6 ± 2.2 CD4</td>
</tr>
</tbody>
</table>

* Each child was studied once.

\( P = 0.001 \) for RP children versus SR and P0 children.

\( P = 0.01 \) for P0 children versus SR children.

In vitro production of type 1 and type 2 cytokines by PBMC cultures. Whole blood was drawn by venipuncture from these HIV-exposed or -infected children and was collected in Vacutainer tubes (Becton-Dickinson, Mountain View, Calif.) containing heparin as the anticoagulant. PBMCs were separated by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) density gradient centrifugation (3) and were washed twice with phosphate-buffered saline solution. PBMCs were suspended at a concentration of 2 \( \times 10^6 \) cells per ml in RPMI 1640 medium containing 5% penicillin and 0.5% streptomycin supplemented with 10% human type AB plasma. The viabilities of the cells were determined by trypan blue exclusion.

The abilities of the PBMCs to secrete cytokines IL-2, IFN-\( \gamma \), IL-4, and IL-10 in vitro were determined by culturing each 4 \( \times 10^5 \) PBMCs without or in the presence of specific cytokines (PharmaCytometrica, St. Louis, Mo.) at a concentration of 100 U/ml plus 0.1 \( \mu \)g of phosphatase-pathway-activating protein (PMA; Sigma Chemical Co., St. Louis, Mo.) per ml for 48 h. The supernatants were collected, the cell pellet was harvested, and both the supernatant and cell pellet were stored at -70°C.

The supernatants were assayed for the presence of type 1 cytokines (IL-2 and IFN-\( \gamma \)) by using Quantikine ELISA kits (R & D Laboratories, Minneapolis, Minn.) and were assayed for the presence of type 2 cytokines (IL-4 and IL-10) by using Cytoscreen ELISA kits (Biosource International, Camarillo, Calif.). The minimum detectable levels of IL-2, IFN-\( \gamma \), IL-4, and IL-10 with the ELISA kits mentioned above were 10, 5, 2, and 5 pg/ml, respectively. Net cytokine production was calculated as the level of cytokine in stimulated culture supernatants minus the level measured in unstimulated cultures.

Expression of mRNA for type 1 and type 2 cytokines. (i) RNA isolation. RNA was extracted from the cell pellet by the RNazol B method (Cinna/Biotex Labs, Friendswood, Tex.), a modification of the standard guanidinium thiocyanate method (9). The manufacturer's procedure was followed with an additional RNase-free DNase (Promega Corp., Madison, Wis.) treatment at 37°C for 30 min to remove any residual DNA in the RNA extracts. The RNA was then extracted with phenol-chloroform and then chloroform. This was followed by ethanol precipitation. The RNA pellet was washed with 70% ethanol and was dissolved in diethylpyrocarbonate-treated water, and its concentration was assessed by measuring the A_{260} with a spectrophotometer.

(ii) Reverse transcription of RNA into cDNA. Reverse transcriptase (RT)-PCR was performed with a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, Calif.). After denaturation of the freshly prepared RNA at 68°C for 10 min, single-stranded cDNA was reverse transcribed with RT at 42°C for 60 min in a final volume of 20 \( \mu \)l containing 1 \( \mu \)g of total cellular RNA, 2.5 mM random hexamer, 1 mM each (d)deoxynucleoside triphosphate (dNTP), 5 mM MgCl\(_2\), 20 U RNase inhibitor, 1\% PCR buffer II (Applied Biosystems), and 50 U of murine leukemia virus RT. After heating at 95°C for 5 min, the cDNA was used for the subsequent PCR or was stored at -20°C.

(iii) PCR amplification of cDNA. Five microliters of the PBMC-derived cDNA was used for each PCR. The PCR mixture contained 0.125 \( \mu \)M upstream and downstream primers (Clontech Laboratories, Inc., Palo Alto, Calif.), 0.2 mM (each) dNTP, 2.5 U of AmpliTaq DNA polymerase, and PCR buffer containing 5 mM MgCl\(_2\), 2.5 U of AmpliTaq DNA polymerase, and PCR buffer containing 5 mM MgCl\(_2\) and 1 mM Tris-HCl (pH 8.3) in a total volume of 50 \( \mu \)l. For IL-2, IFN-\( \gamma \), and IL-4, the cDNA was amplified for 35 cycles by denaturing at 94°C for 30 s, primer annealing at 60°C for 30 s, and extending at 72°C for 30 s in a thermal cycle (model 9600; Perkin-Elmer Cetus, Norwalk, Conn.). A 10-fold serially diluted positive-standard cDNA derived from PHA-PA-stimulated PBMCs isolated from an HIV-negative control subject was amplified and was used as a reference for each cytokine PCR assay. The message for IL-10 was not determined.

(iv) Analysis of PCR products. One-fifth of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel that was subsequently stained with 0.5 \( \mu \)g of ethidium bromide per \( \mu \)l and visualized with a UV transilluminator. The amplified DNA products were then analyzed by Southern blotting and hybridization with oligonucleotide probes. The probes were selected to hybridize to a portion of the amplified segment between the primers, which ensured the specificity of the segment that was amplified. The probe was 3’ ends labeled with \( [\gamma-^32P] \)ATP (Amersham, Arlington Heights, Ill.) by using T4 polynucleotide kinase and the accompanying recommended procedure (Promega Corp.). The membranes were prehybridized at 54°C for 2 h and hybridized to the probe at the same temperature overnight. To reduce the nonspecific signal, the blot was washed by the procedure of Sambrook et al. (35) and was then exposed to a storage phosphor screen (Eastman Kodak Co., Rochester, N.Y.). The specific signals on the screen were scanned and quantified with a PhosphorImager programmed with ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

The relative amount of RT-PCR products generated from the RNA of the unstimulated and PHA-PA-stimulated PBMCs was determined by measuring the signal ratio of the sample to that of the external standard on the Southern blots. This procedure allows for the relative quantitation of mRNA for each cytokine but does not permit exact measurement of the amount of target cytokine mRNA in each sample (40). The level of cytokine mRNA in the unstimulated PBMC cultures was subtracted from that in the PHA-PA-stimulated cultures.

Statistical analysis. Nonparametric Mann-Whitney statistical tests were used to analyze the differences in cytokine transcription and production between patient groups.

RESULTS

Constitutive production of type 1 and type 2 cytokines by PBMC cultures. The unstimulated cultures of PBMCs from all children produced negligible amounts of type 1 and type 2 cytokines (Table 2). There were no significant differences in IL-2 production between groups. Similarly, we did not record any significant differences between groups with respect to the constitutive production of IFN-\( \gamma \). IL-4 production was undetectable in unstimulated culture supernatants of PBMCs from SR and RP children and only marginally detectable in culture supernatants of PBMCs from P0 children. Despite the wide range of IL-10 responses among all children, we did not detect any significant differences between groups.

Production of type 1 and type 2 cytokines by PHA-PA-stimulated PBMC cultures. The mean ± standard error of the mean (SEM) net production of IL-2 in PHA-PA-stimulated cultures was highest for the SR group (1,888 ± 539 pg/ml); this was followed by the P0 (1,859 ± 357 pg/ml) and the RP (358 ± 175 pg/ml) groups (Fig. 1A). In comparison with PHA-PA-stimulated cultures of PBMCs from the SR and P0 groups, the...
difference in IL-2 production seen in the PHA-PMA-stimulated cultures of PBMCs from the RP group was statistically significant \((P < 0.01)\). There was no significant difference in IL-2 production between P0 and SR children. The mean \pm SEM net production of the other type 1 cytokine, IFN-\(\gamma\), in PHA-PMA-stimulated PBMC cultures was highest for the SR group (3,569 \pm 936 pg/ml); this was followed by the RP (1,057 \pm 233 pg/ml) and the P0 (837 \pm 219 pg/ml) groups (Fig. 1B). PHA-PMA-stimulated cultures of PBMCs from the RP and P0 groups produced significantly less IFN-\(\gamma\) than cultures of PBMCs from SR children \((P < 0.02)\).

The mean \pm SEM net production of IL-4 in PHA-PMA-stimulated culture supernatants was highest for the SR group (8.1 \pm 1.9 pg/ml). The RP and the P0 groups had equivalent mean levels of IL-4 production (5.0 \pm 1.0 pg/ml) (Fig. 1C). The mean \pm SEM net production of IL-10 in PHA-PMA-stimulated cultures was highest for the SR group (85.6 \pm 26.5 pg/ml); this was followed by the RP (42.7 \pm 12.1 pg/ml) and the P0 (39.1 \pm 8.2 pg/ml) groups (Fig. 1D). There were no significant differences in the mean levels of net production of IL-4 and IL-10 among any of the groups studied.

Transcription of cytokine messages by PBMC cultures. The mRNA levels of IL-2, IFN-\(\gamma\), and IL-4 expressed relative to the levels expressed by the positive external control were evaluated for a smaller group of children (RPs, \(n = 7\); SRs, \(n = 5\); P0s, \(n = 9\)). The relative level (mean \pm SEM fold increase) of IL-2 mRNA expression was highest for the SR group (1.6 \pm 0.6); this was followed in descending order by the P0 (1.0 \pm 0.6) and the RP (0.9 \pm 0.4) groups (Fig. 2A). Interestingly, there were no statistical differences in IL-2 transcription between any of the study groups. The relative level (mean \pm SEM fold increase) of IFN-\(\gamma\) mRNA expression was highest for the SR group (17.9 \pm 1.6); this was followed in descending order by the P0 (2.2 \pm 0.7) and the RP (1.4 \pm 0.4) groups (Fig. 2B). The
PBMCs from RP and P0 groups expressed significantly fewer IFN-γ messages than the PBMCs from SR children \((P < 0.01)\). The relative level (mean ± SEM fold increase) of IL-4 mRNA expression for the RP group was highest \((20.0 ± 9.0)\); this was followed in descending order by those for the SR \((10.9 ± 1.9)\) and the P0 \((1.7 ± 0.5)\) groups (Fig. 2C). Compared with PBMCs from the P0 group, PBMCs from the RP and the SR groups expressed significantly higher mean levels of IL-4 messages \((P < 0.03)\). PBMCs from the RP and SR groups expressed similar mean levels of IL-4 messages.

To further delineate the differences in cytokine transcription, representative phosphoimaging pictures are provided in Fig. 3; odd- and even-numbered lanes represent RT-PCR products from unstimulated and PHA-PMA-stimulated cell cultures, respectively. Since there were no significant differences between groups with respect to IL-2 mRNA levels following stimulation with mitogen (Fig. 2A), only the RT-PCR analyses for the levels of IFN-γ mRNA (Fig. 3A) and IL-4 mRNA (Fig. 3B) are presented. In each panel, a total of 10 lanes representing five patients from each group are demonstrated. Figure 3A indicates that PHA-PMA-stimulated PBMCs from the SR group had a stronger expression of IFN-γ mRNAs than the stimulated PBMCs from the RP and P0 groups, while Fig. 3B indicates that the stimulated PBMCs from RP and SR groups had a stronger expression of IL-4 mRNAs than the stimulated PBMCs from the P0 group. Thus, our cytokine transcription results demonstrate that mitogen-stimulated cultures of PBMCs from RP children expressed significantly lower levels of IFN-γ mRNA than cultures of PBMCs from SR children and a significantly higher level of IL-4 mRNA than cultures of PBMCs from P0 children.

**DISCUSSION**

Cell-mediated immunity has been suggested to be more protective than humoral immunity against the progression of HIV infection \((12)\). Cell-mediated immunity responses, such as HIV-specific CTL activity, delayed-type hypersensitivity, and cellular proliferation in response to recall antigens, can be augmented through the production of type 1 cytokines, whereas humoral responses that lead to increased levels of antibody production are enhanced through the production of type 2 cytokines. Other evidence suggests that the actions of these two types of cytokines are mutually exclusive and are reciprocally regulated in vivo \((37)\). We therefore indirectly evaluated the relative efficacies of cell-mediated immunity and humoral responses in children exposed to or infected with HIV by measuring the levels of production of type 1 and type 2 cytokines.

The measurement of constitutive cytokine mRNA expression in freshly isolated PBMCs provides a snapshot of the predominant profile of cytokine expression in vivo. We were unable to detect any differences in the constitutive transcription of mRNAs of the three cytokines (IL-2, IFN-γ, and IL-4).
Within the ranges of the percentage of CD4 cells observed in age-matched uninfected children (14, 28).

HIV-1-specific CTLs have been detected in adult patients with early acute HIV-1 infections and may play an important role in limiting primary viremia (22). However, HIV-1-specific CTL activity is less consistently detected in young infants with vertically transmitted HIV-1 infection (25, 26). CTL activity depends on the presence of IFN-γ, which is produced at high levels by antigen-primed memory T lymphocytes but not by naive T lymphocytes (34). The infrequent detection of HIV-specific CTL responses in early infancy may further suggest a delayed capacity of vertically infected infants to generate IFN-γ. Our data from a selected number of patients, showing that PBMCs from SR children expressed higher levels of IFN-γ mRNA (Fig. 3A) and more IFN-γ production (Fig. 2B) than PBMCs from RP children, are also consistent with this hypothesis. Alternatively, the potential exposure to low-dose virus or viral antigens in the presence of maternal antibody may also serve to immunize the infant and give rise to a long-term specific immunity without the production of antibody (33). Recently, Bryson et al. (4) added credence to this hypothesis by reporting on an infant with perinatally acquired HIV infection who was able to clear the infection and to remain free of symptoms and any evidence of infection.

In comparison with mitogen-stimulated cultures of PBMCs from SR children, cultures of PBMCs from P0 children produced significantly less IFN-γ (Fig. 1B). The deficiency in IFN-γ production may be related to the immune competence of neonates (39), which becomes fully developed by 24 months of age (10). For example, the blood samples from the SR group were obtained from children with a median age of 17 months, whereas the majority of samples obtained from the P0 group were cord blood obtained at birth (Table 1). Alternatively, it may be explained by differences in the proportion of CD4+ naïve (CD45RA-1) and memory (CD45RO-1) cells in the circulation. Human neonatal PBMCs and cord blood mononuclear cells had much higher fractions of CD45RA-1 cells and had decreased levels of production of IFN-γ compared with those from adults (24, 31). Moreover, in AIDS patients with an inverted CD4/CD8 ratio, CD8- cells have been shown to be more potent IFN-γ producers than CD4- cells (16). Viganò et al. (38) also observed that HIV-infected children with fewer than 200 CD4- cells per μl were more likely to secrete IFN-γ than IL-2, as was the case for our RP group.

PBMCs from HIV-infected patients with reduced CD4- T-cell counts were expected to produce lower constitutive levels of IL-4 and IL-10 than PBMCs from HIV-infected patients with normal or only slightly depressed CD4- T-cell counts (27, 32). Despite having the lowest percentage of CD4- lymphocytes among all children studied, the RP children produced similar levels of IL-4 and IL-10 after their PBMCs were stimulated with PHA-PMA (Fig. 1C and D). Our data are inconsistent with previously reported significant increases in both IL-4 and IL-10 production in P2 compared with the levels in age-matched healthy children (38). The differences in the results of these two studies may be due to our use of younger cohorts with fewer severe AIDS-defining conditions compared with the cohorts used in the other study (38). Alternatively, variance in the sensitivities of the assay kits for the detection of individual cytokines may explain the differences in our results and those of other investigators (38).

We also observed IL-4 messages to be relatively higher in the RP group than in the SR group (Fig. 2C), even though the levels of IL-4 production were similar in the two groups (Fig. 1C). A possible explanation for this discrepancy in the HIV-infected infants is the ability of the HIV nef protein to inhibit
protein synthesis by interfering with the translational processes (30). Another recent study also illustrates age-dependent increases in the levels of expression of IL-4 and IL-10 messages; these levels of IL-4 and IL-10 expression did not differ significantly between HIV-infected and SR children (19). Two other studies of in vitro-stimulated PBMCs from HIV-infected adults also provide no clear evidence for a definitive switch to the type 2 cytokine responses in HIV-infected subjects or in any phase of HIV infection (16, 27).

In summary, our study of cytokine profiles in mitogen-stimulated cultures of PBMCs demonstrates that type 1 and type 2 cytokine transcription and production can be augmented to regulated cultures of PBMCs demonstrating that type 1 and type 2 cytokines is more dominant in severely symptomatic children. Therefore, we believe that the loss of type 1 cytokines, but not necessarily an increase in the level of type 2 cytokines, is responsible for HIV disease progression. Additional prospective studies are needed to further investigate the possible mechanism(s) responsible for this phenomenon.

ACKNOWLEDGMENTS

We thank Stephen Li and De-Yu Shen for technical assistance, Valerie Nichols and Debra Mooneyham for specimen collection, Beverly Bohnan for data on clinical staging of HIV-infected children, and Jude Richard for editorial comments on the manuscript. The work on which this publication is based was performed pursuant to grants 5 U01 AI 27551 and 5 R01 HD26603-05 from the U.S. Department of Health and Human Services, grant RR 0188 from the General Clinical Research Center, and grant RO1 AI 29131 from the National Institute of Allergy and Infectious Diseases.

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