Abnormal CD40 Ligand (CD154) Expression in Human Immunodeficiency Virus-Infected Children

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The CD40 ligand (CD154), expressed primarily on activated CD4-positive T cells, is a costimulatory molecule involved in B-cell proliferation, germinal center formation, and immunoglobulin class switching. Since B-cell abnormalities including hypergammaglobulinemia and abnormal antibody-specific immune responses are prominent and occur early during the course of pediatric human immunodeficiency virus (HIV) infection, we measured the baseline levels and the induced levels of expression of CD154 on CD3+ CD8+ (T helper cells) in HIV-infected children and uninfected children born to HIV-positive mothers. The percentage of CD154+ T helper cells activated in vitro and the level of CD154 expressed per T helper cell (mean fluorescent channel [MFC]) were significantly lower in the HIV-infected children than in the uninfected control group (77% ± 3% versus 89% ± 1%, respectively [P < 0.002], and 261 ± 174 versus 415 ± 130 MFC, respectively [P < 0.03]). The levels of CD154 expressed on resting T helper cells in the HIV-infected group were not significantly different from the levels observed in the control group. In the HIV-infected children, the level of CD154 on activated T helper cells correlated with the level of immunodeficiency, as assessed by the CD4 T-cell levels (correlation coefficient [r] = 0.707, P = 0.003), but did not correlate with the viral load or with any of the serum immunoglobulin concentrations measured in this group of HIV-infected children. The baseline level of CD154 expressed on T helper cells did, however, correlate with the concentration of immunoglobulin A in serum. We conclude that HIV-infected children have impaired regulation of CD154 expression which may contribute to the immune dysregulation commonly observed.

B-lymphocyte differentiation and immunoglobulin (Ig) secretion are dependent upon the interaction between the constitutively expressed CD40-cell surface receptor on B cells, the inducible T-cell surface ligand CD154, and cytokines secreted by activated T cells (4, 11, 12, 18, 23, 30, 31, 38). CD154 is a 32- to 39-kDa glycoprotein member of the tumor necrosis factor (TNF) superfamily (5, 17) and is expressed primarily by activated CD4+ T lymphocytes (5, 32) (although it is expressed on a variety of other nonlymphoid cells [5, 9, 15, 17, 29, 34]). Binding of CD154 expressed on activated T helper cells to the CD40 receptor on B cells and other antigen-presenting cells leads to the up-regulation of costimulatory molecules such as CD80 on the antigen-presenting cells, which leads to increased T-cell activation and cytokine secretion (16, 25, 36). Abnormalities in CD40 ligand expression could therefore lead to both B- and T-cell immune abnormalities, as is observed in patients with the common variable immunodeficiency syndrome and the X-linked form of the hyper-IgM syndrome (XHIM-1). Patients with XHIM-1 carry mutations in the gene encoding CD154 (1, 21) and have a profound humoral immune deficiency and a subtle cellular immunodeficiency characterized by normal to elevated levels of IgM in serum with severely decreased levels of IgG, IgA, and IgE in serum (14, 27). Patients with XHIM-1 are prone to recurrent bacterial and opportunistic infections including Pneumocystis carinii pneumonia (21, 27).

Elevated serum Ig levels and increased risk of opportunistic infections, including P. carinii pneumonia, are also common clinical features of pediatric human immunodeficiency virus (HIV) infection (2, 22, 37). Although HIV-infected children are hypergammaglobulinemic, they have impaired T-cell-dependent B-cell responses (3, 35), as witnessed by their abnormal humoral responses to vaccine antigens (20, 33). We hypothesize that aberrant CD154 expression on T cells of children infected with HIV type 1 (HIV-1) may contribute to their immune deficiency. In HIV-infected adults, the level of CD154 expression on activated T cells has been reported to be decreased in one study (35) and normal in another (7). Using a recently developed three-color flow cytometric assay (28), CD154 expression was measured on resting and in vitro activated T helper cells obtained from HIV-infected children and uninfected children born to HIV-infected mothers. The relationship between the level of CD154 expressed on T helper cells and the results of common laboratory tests including CD4 T-cell levels, plasma HIV-1 loads, and serum immunoglobulin concentrations was also examined.

MATERIALS AND METHODS

Study subjects. Sodium heparin-anticoagulated peripheral blood was obtained from 15 HIV-infected children (age range, 1.1 to 4.7 years; mean age, 3.1 years) and 10 uninfected children born to HIV-infected mothers (age range, 0.3 to 3.3 years; mean age, 1.4 years). HIV infection was confirmed in all individuals by a quantitative HIV-1 reverse transcription-PCR assay (Roche Diagnostic Systems Inc., Branchburg, N.J.). All patients included in this study were on antiretroviral

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therapy; the specific therapy at the time of the study for each patient is listed in Table 1. This study was approved by the Institutional Review Board of the Children’s Memorial Institute for Education and Research.

**Laboratory evaluation.** Ig levels were measured in the sera of all HIV-infected children by rate nephelometry in strict accordance with the manufacturer’s recommendations (Array 360 System; Beckman Instruments Inc., Brea, Calif.). Hypergammaglobulinemia was defined as concentrations of IgG, IgA, or IgM in serum greater than 2 standard deviations above the mean age-specific levels defined in the diagnostic immunology laboratory at the Children’s Memorial Hospital, Northwestern University, Chicago, Ill. Peripheral blood immunophenotyping was performed by a standard whole-blood methodology with monoclonal antibodies (Ortho, Raritan, N.J.) and an Ortho Cytoron Absolute flow cytometer, in accordance with recommended guidelines (10). Absolute CD4 counts were calculated as the product of the CD4+ T-lymphocyte percentage and the absolute lymphocyte count obtained on an automated hematology instrument (CellDyne 1600; Abbott Laboratories, Abbott Park, Ill.). Plasma HIV-1 loads were measured by a quantitative HIV-1 reverse transcription-PCR assay (Roche Diagnostic Systems Inc.) and are expressed as the number of HIV RNA copies per milliliter of blood.

**Evaluation of CD154 (CD40L) expression.** A three-color whole-blood flow cytometric assay originally developed in our laboratory as a diagnostic screening test for XHIM-1 was used to study CD154 expression on resting and activated peripheral blood T cells (28). Briefly, heparin-anticoagulated whole blood was diluted with culture medium alone (RPMI) or culture medium containing phorbol myristate acetate (PMA; 15 ng/ml; Sigma, St. Louis, Mo.) and calcium ionophore (300 ng/ml; Sigma). PMA and ionomycin induce the rapid up-regulation of CD154 on T helper cells. The samples were incubated at 37°C for 4 h in a humidified atmosphere of 5% CO₂. After incubation, the samples were washed and resuspended in Ca²⁺- and Mg²⁺-free phosphate-buffered saline. Aliquots (100 μl) from the stimulated and unstimulated (RPMI only) samples were added to three tubes containing the following combinations of monoclonal antibodies: tube 1 (the negative isotype control) contained CD8-FITC, IgG1-phycoerythrin (PE), and CD3-CyChrome; tube 2 contained CD8-FITC, CD154-PE, and CD3-CyChrome; and tube 3 (in vitro stimulation control) contained CD3-FITC and CD69-PE. All monoclonal antibodies except CD69-PE were obtained from PharMingen (San Diego, Calif.); CD69-PE was purchased from Becton Dickinson (San Jose, Calif.). Samples were labeled in the dark at room temperature for 20 min, after which the

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**FIG. 1.** Three-color flow cytometric gating strategy used to evaluate expression of CD154 on T helper cells (i.e., CD3+ CD8+ T cells). An electronic analysis gate was drawn around the lymphocyte cluster (gate R1) on the basis of the forward and right-angle light scatter parameters (A). The contents of gate R1 are sent to a second dot plot of FL1 (CD8-FITC) versus FL3 (CD3-CyChrome), and a second gate (R2) is drawn around the cluster of T cells which are CD3+ CD8+, i.e., T helper cells (B). The expression of CD154 is measured in a third histogram of FL2 (CD154-PE), which contains only the events in R1 and R2 (C). The results are expressed as the percentage of CD3+ CD8+ T lymphocytes expressing CD154 or as the MFC of CD154 expression. The expression of CD154 on in vitro activated T cells is displayed as the filled histogram, and CD154 expression on resting T helper cells is displayed as the dotted line.
erythrocytes were lysed (FACSlyse; Becton Dickinson) and the remaining cells were washed twice, resuspended in 1% parafomaldehyde, and analyzed by flow cytometry.

Flow cytometric analysis. CD154 analysis was performed with a Becton Dickinson FACScalibur flow cytometer equipped with an air-cooled argon ion laser and the ability to detect two light scatter and four fluorescence parameters. The flow cytometer was calibrated daily for fluorescence sensitivity and spectral overlap. A total of 2,500 gated events (see below) per sample were acquired in list mode and analyzed with CELLQuest 3.1 computer software. CD154 expression on T helper lymphocytes was evaluated by using a gating algorithm which included both lymphocytes (using forward and side scatter parameters) and CD3+ CD8+ T lymphocytes (using fluorescence parameters), as presented in Fig. 1. This gating strategy allowed the evaluation of CD4+ T lymphocytes (contained in the population of CD3+ T cells which are CD8 negative) and was necessary because the CD4 molecule itself is down-modulated following stimulation with PMA and ionomycin. Results are expressed as either the percentage of CD3+ CD8+ T lymphocytes expressing CD154 or the level of CD154 expressed per T helper cell (mean fluorescent channel [MFC]). Positive fluorescence was defined as any event which generated a fluorescence signal above the positive-negative discriminator, which was set by using the IgG1-PE isotype control (tube 1). The tube containing CD3 and CD69 (tube 3) was used as the in vitro stimulation control. As a quality control parameter for the procedure, a sample from a healthy adult was run, and the percentage of in vitro activated T cells (CD3+) expressing CD69 had to be greater than 95% or the test was repeated.

Statistical evaluation. Statistical analysis was conducted with Statview (SAS) software for the Macintosh computer. Comparisons were evaluated by an unpaired t test. Correlations (r) were examined by linear regression. The alpha level was predetermined to be 0.05.

RESULTS

Flow cytometric analysis of CD154 expression. The percentage of in vitro activated T helper cells (CD3+ CD8− T lymphocytes) expressing CD154 was significantly lower in the 15 HIV-infected children than in the 10 uninfected children (77% ± 3% versus 89% ± 1%, respectively [P < 0.002]). Abnormal levels of CD154 expression in HIV-infected children was also evident on a per-cell basis. The mean level of CD154 expressed per activated CD3+ CD8− T cell in the HIV-infected children (expressed as MFC) was 261.1 ± 174, whereas it was 414.8 ± 130 in the uninfected children (P = 0.03) (see the results for individual patients and controls in Fig. 2A and B). The percentage of resting CD3+ CD8− T lymphocytes expressing CD154 and the level of CD154 per cell was higher in the HIV-infected children than in the uninfected children (18% ± 6% versus 7% ± 1%, respectively, and 9 ± 2 versus 5 ± 1 MFC, respectively); however, these differences were not significant. CD69 expressed on CD3-positive T cells was used as an in vitro activation control. Following activation the percentage of CD69 expressing T cells increased slightly lower in the HIV-infected children than in the uninfected children (98.6% ± 1.2% versus 99.8% ± 0.1%, respectively [P = 0.005]) and the level of fluorescence per cell was slightly higher (1,610 ± 138 versus 1,480 ± 137 MFC, respectively [P = 0.53]).

Relationship between level of CD154 expressed and other clinical parameters. The concentrations of IgG, IgM, and IgA in serum in the HIV-infected children ranged from 678 to 6,990, 47 to 320, and 15 to 230 mg/dl, respectively (Table 1). Fourteen of the 15 HIV-infected children were hypergammaglobulinemic for at least one class of immunoglobulin (Table 1). At the time of the study all HIV-infected children had detectable HIV loads which ranged from 0.07 × 107 to 78.40 × 105 HIV RNA copies/ml of blood (Table 1). CD4+ T-lymphocyte percentages and absolute CD4 counts are also summarized in Table 1. The relationship between CD154 expression and serum immunoglobulin concentrations, viral load, CD4+ T-lymphocyte percentage, and absolute CD4 count was evaluated by linear regression, as presented in Table 2. The percentage of CD154-positive T helper cells correlated with the CD4+ T-lymphocyte percentage (r = 0.707, P = 0.003) and absolute CD4 count (r = 0.533, P = 0.041) in the HIV-infected children. No other significant relationships were observed between the percentage of CD154+ T helper cells and any of the other clinical parameters evaluated. Of note, the percentage of resting CD3+ CD8− T cells expressing CD154 correlated with the concentration of IgA in serum (r = 0.736, P < 0.0002).

DISCUSSION

Interactions of T cells and B cells via CD154 and CD40, respectively, lead to B-cell proliferation, differentiation, Ig iso-
type class switching, germinal center formation, T-cell activation, and T-cell cytokine secretion (16, 25, 36). Abnormal CD40 ligand expression leads to profound humoral and cellular immune abnormalities, as evidenced in patients with XHIM-1 (14, 27).

We observed abnormalities in the induced expression of CD154 on in vitro activated T helper cells of HIV-infected children. Both the percentage of T helper cells (CD3+ CD8−) expressing CD154 and the amount of CD154 expressed per cell were significantly lower than the those observed in uninfected children born to HIV-infected mothers. Although the level of CD154 (as the percentage of positive cells and the MFC) expressed on resting T helper cells was higher in the HIV-infected children, the differences were not significant.

The percentage of activated T cells expressing CD154 was inversely correlated with the level of immunodeficiency measured as either the percentage or the absolute number of T helper cells circulating in the peripheral blood. Brugnoni et al. also reported a positive correlation between the percentage of CD154+ peripheral blood mononuclear cells (PBMCs) and the percentage of CD4+ PBMCs (8). Since CD154 is expressed primarily on activated T helper cells and since the percentage of T helper cells decreases with progressive infection, the latter study (8) cannot rule out the possibility that the percentage of CD154+ PBMCs is decreased simply because the percentage of CD4+ PBMCs is decreased. As opposed to PBMCs as a whole, we measured CD154 expression on the CD3+ CD8− T-cell subset, which is composed primarily of T helper cells. Within this subset both the percentage of cells and the level of CD154 expressed per cell were reduced in children with HIV infection. Furthermore, the level of expression of CD154 on T helper cells correlated with both the percentage and the absolute number of T helper cells in the peripheral blood. Decreased levels of CD154 expression during adult HIV infection have been demonstrated previously (7, 24, 35), but this study is the first to document aberrant CD154 expression in an HIV-1-infected pediatric population and is also the first to evaluate CD154 expression directly on CD4 T cells (as the predominant population) in whole blood.

Decreased levels of CD154 expression could be due to a generalized cell-associated abnormality or could be due to the specific loss of a subpopulation of T helper cells which, when stimulated, expresses higher levels of CD154. For example, it has been reported that CD154 is expressed at higher levels on stimulated, expresses higher levels of CD154. For example, it has been reported that CD154 is expressed at higher levels on activated memory T helper cells (19); therefore, the loss of memory cells would result in an overall reduction in the level of CD154 on T helper cells. In HIV-infected children, the levels of memory T helper cells are not disproportionately reduced; in fact, the relative proportion of memory T helper cells increases and the disease progresses (6, 8), which reduces the likelihood that the abnormality in CD154 expression is due to an underrepresentation of this subset.

A possible confounder in this study is that CD154 expression was evaluated on CD3+ CD8− T lymphocytes and not on the CD4+ T cells themselves. Although this gating strategy leads to the inclusion of all CD4+ T helper cells, this gate also contains CD3+ cells which do not express CD4 or CD8 (double-nega-

## Table 1. Clinical data for the HIV-infected children

| Subject no. | Age at time of study (yr) | Therapy at time of study | HIV-1 load (10^4) | % CD4+ T cells | Absolute no. of CD4+ T cells (per μl) | HIV-1 load (10^4) | IgG concn (mg/dl) | IgM concn (mg/dl) | % CD4+ cells resting | CD154+ MFC | % CD154+ cells activated | CD154+ MFC |
|-------------|--------------------------|--------------------------|-------------------|----------------|--------------------------------------|-------------------|-------------------|-------------------|---------------------|----------|--------------------------|----------|
| 1           | 4.00                     | D4T                      | 0.88              | 8.0             | 115                                  | 0.88              | 1.110             | 0.28              | 5.2                 | 4        | 46.64                    | 223      |
| 2           | 4.33                     | DDI                      | 2.48              | 33.0            | 1,214                                | 2.48              | 768               | 47                | 9.9                 | 3        | 70.92                    | 52       |
| 3           | 1.42                     | D4T, RTV                 | 55.50             | 20.6            | 950                                  | 55.50             | 2,880             | 284               | 168                 | 3        | 71.73                    | 287      |
| 4           | 3.67                     | AZT, 3TC                 | 78.40             | 29.2            | 603                                  | 78.40             | 905               | 275               | 71                  | 4        | 76.32                    | 110      |
| 5           | 4.67                     | DDI, D4T                 | 1.34              | 39.0            | 1,357                                | 1.34              | 1,629             | 106               | 144                 | 10.6     | 76.44                    | 78       |
| 6           | 3.92                     | AZT, 3TC                 | 0.07              | 20.0            | 496                                  | 0.07              | 1,130             | 86                | 14.0                | 12       | 76.75                    | 72       |
| 7           | 1.92                     | AZT, 3TC                 | 3.08              | 28.9            | 766                                  | 3.08              | 1,210             | 54.9              | 15                  | 3        | 76.77                    | 175      |
| 8           | 4.50                     | AZT, 3TC                 | 8.11              | 39.7            | 1,064                                | 8.11              | 1,690             | 108               | 69                 | 51.8     | 77.87                    | 460      |
| 9           | 4.00                     | DDI, D4T                 | 2.54              | 37.0            | 784                                  | 2.54              | 6,690             | 53.9              | 35                  | 5        | 80.91                    | 300      |
| 10          | 2.67                     | D4T, 3TC                 | 1.32              | 26.2            | 646                                  | 1.32              | 1,710             | 127               | 212                 | 14.9     | 82.54                    | 551      |
| 11          | 3.25                     | D4T, 3TC, RTV, SQV        | 11.80             | 23.7            | 1,029                                | 11.80             | 951               | 320               | 270                 | 9.6      | 82.84                    | 398      |
| 12          | 2.08                     | AZT, 3TC, RTV, SQV        | 17.00             | 34.6            | 1,138                                | 17.00             | 3,000             | 205               | 467                 | 94.4     | 83.20                    | 149      |
| 13          | 3.17                     | AZT, 3TC, RTV            | 2.90              | 29.6            | 1,357                                | 2.90              | 849               | 60.4              | 84                  | 26.6     | 83.45                    | 318      |
| 14          | 1.75                     | AZT, DDI                 | 19.40             | 50.2            | 3,049                                | 19.40             | 1,300             | 71.6              | 21                  | 5.7      | 84.30                    | 155      |
| 15          | 1.08                     | D4T, 3TC                 | 0.33              | 46.2            | 1,615                                | 0.33              | 1,270             | 54.2              | 62                  | 3.3      | 90.76                    | 587      |

a Abbreviations for antiretroviral drugs: AZT, zidovudine; 3TC, lamivudine; D4T, stavudine; RTV, ritonavir; SQV, saquinovir; DDI, didanosine.

b Percentage or mean fluorescence of CD154 expressed on resting peripheral blood CD3+ CD8− T cells.

c Abbreviation for CD154: MFC, mean fluorescence count.
d Serum immunoglobulin levels were above the normal age-associated range.

**Table 2. Relationship of T-lymphocyte expression of CD154 to various clinical parameters**

| Clinical parameter | r value | P value |
|--------------------|---------|---------|
| CD4+ T-cell percent | 0.707 | 0.003 |
| Absolute CD4 count (per mm3) | 0.533 | 0.041 |
| Serum IgG concn (mg/dl) | 0.126 | 0.655 |
| Serum IgM concn (mg/dl) | 0.060 | 0.832 |
| Serum IgA concn (mg/dl) | 0.091 | 0.796 |
| HIV-1 load | 0.036 | 0.900 |

a All comparisons were completed with the percentage of CD154-positive CD3+ CD8− T-cell data generated for the in vitro activated samples obtained from the HIV-infected children. All clinical data used for the table are listed in Table 1. r values were calculated by linear regression analyses, and the P values were obtained by analysis of variance.
tive T cells). The majority of these double-negative T cells express the gamma-delta T-cell receptor, the levels of which have been reported to be increased in HIV-infected individuals (13, 26). Although we have observed CD154 expressed on activated gamma-delta-positive T cells (data not shown), we cannot rule out the possibility that an overrepresentation of this subset or any other CD3+ non-T helper cell could lead to the CD154 abnormalities observed. The impaired induction of CD154 on T cells did not appear to be due to a generalized in vitro activation abnormality in the HIV-infected patients since the percentage of in vitro activated T cells expressing CD69 in HIV-infected patients differed by less than 2% from that in the uninfected patients.

There did not appear to be a relationship between the viral load and CD154 expression, suggesting that the children with the highest viral loads were not necessarily the same children with the most profound defects in CD154 expression. All of the patients were on antiretroviral therapy, and there was no appreciable relationship between the various medications and the abnormality in CD154 expression. Although 14 of the 15 HIV-infected children evaluated in this study were hypergammaglobulinemic, there was no significant relationship between the level of CD154 expressed on activated T helper cells and the concentration of any of the Ig classes measured. The relationship between the percentage of CD154+ resting T helper cells and Ig levels was also assessed, and a significant correlation with the serum IgA concentration was observed. Increased baseline levels of expression of CD154, a member of the TNF alpha (TNF-α) receptor family (16), and the associated elevated IgA levels may be related to the well-documented elevations in TNF-α levels observed in HIV-infected individuals. Although TNF-α levels were not measured in this study, this possible association deserves further attention. A second potential confounder in this study exists due to the temporal relationships between T helper cell CD154 expression and serum Ig concentrations. These relationships should be interpreted with caution due to the extended half-life of Ig in serum. Measurement of the levels of spontaneously secreted Ig may provide further information regarding the relationship between B-cell abnormalities and CD154 expression on T helper cells, although Brugnoni et al. (8) observed no correlation between the levels of spontaneously secreted Ig and the proportion of CD154+ PBMCs.

In conclusion, we observed that children infected with HIV have an impaired ability to up-regulate cell surface CD154 compared with that of non-HIV-infected children born to HIV-infected mothers. The abnormality was manifested as both a reduced percentage of cells expressing CD154 and a decrease in the level of CD154 expressed on a per-cell basis. Since maternal carriers of the CD154 mutation have a decrease in the percentage of cells expressing CD154 with normal levels on a per-cell basis, we believe that the latter abnormality in HIV-infected children may be related to the immunopathogenesis observed in this infection. Impairment of CD154 up-regulation is related to the degree of immunodeficiency, as measured by T helper cell levels, and does not appear to be related to the antiretroviral medication. The abnormal CD40 ligand expression observed in HIV-infected children may play a role in the immunopathogenesis of HIV infection. Further studies are needed to (i) delineate the mechanisms that lead to abnormal CD154 up-regulation on T helper cells, (ii) investigate the specific abnormalities resulting from abnormal CD154 up-regulation, and (iii) determine if the abnormality can be reconstituted with effective antiretroviral therapy.

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