Human Immunodeficiency Virus (HIV) Phenotype and Interleukin-2/Interleukin-10 Ratio Are Associated Markers of Protection and Progression in HIV Infection

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Human immunodeficiency virus (HIV) isolability, rate of viral replication, HIV phenotype, type 1 and type 2 cytokine production, and CD4 counts were cross-sectionally analyzed in 63 HIV-seropositive (HIV+) individuals to establish possible correlations between virologic and immunologic markers of protection and progression. We observed that these markers are tightly correlated. Thus, lack or low prevalence of HIV isolability and the presence of nonsyncytium inducing strains are associated with the strongest type 1 cytokine production, the weakest type 2 cytokine production, and highest CD4 counts. Conversely, the isolation of highly replicating syncytium-inducing HIV strains is associated with the weakest type 1 cytokine production, the strongest type 2 cytokine production, and lowest CD4 counts. Additionally, it was determined that the interleukin (IL)-10/IL-2 ratio best discriminates among different virologic scenarios. These data suggest that the virologic and immunologic correlates of disease protection and progression might be associated variables that define two different subsets of HIV+ individuals and lend support to a vitro-immunologic hypothesis of HIV infection.

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MATERIALS AND METHODS

Patients and controls. We analyzed virologic and immunologic parameters in 63 consecutively enrolled HIV+ individuals. Additionally, we analyzed which reciprocal pair of type 1 and type 2 cytokine is better capable to discriminate among HIV+ individuals with different virologic characteristics.

Virus culture. Peripheral blood mononuclear cells (PBMC) from HIV+ healthy individuals were stimulated for 24 to 48 hours with phytohemagglutinin P (PHA, Irvine Scientific, Irvine, CA). Each week, the viral cultures were tested for p24 antigen production using a commercial enzyme-linked immunosorbent assay (ELISA). Once a week, fresh 2-day PHA-prestimulated donor cells were added to the cultures, maintaining a 1:1 ratio. A culture was considered positive if two serial supernatant samples were positive and showed a 10-fold increase in p24 antigen.

Syncytia assay. Each week, the viral cultures were tested for syncytia formation using the MT-2 assay, as described.5 Briefly, 5 × 10³ MT-2 cells were cocultured with an equal number of cells from the PBMC virus cultures in complete RPMI medium in a 96-well microtiter plate and incubated for 3 days. Supernatants were then harvested and tested for cytopathic effect. A culture was considered positive if two serial supernatants showed a cytopathic effect.

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Supported by grants from Istituto Superiore di Sanità (VII Progetto AIDS 1994 and VIII Progetto AIDS 1995) and by Mario Clerici, MD, Cattedra di Immunologia, Università degli Studi di Milano, Via Venezian, 1, 20133 Milano, Italy.

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Table 1. Demographic and Clinical Characterization of the 63 HIV* Patients Studied

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<th>N (%)</th>
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<tr>
<td>Mean age (years ± SD)</td>
<td>34.1 ± 7.3</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>35/28</td>
<td></td>
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<tr>
<td>Risk group:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former IVDU³</td>
<td>35 (55)</td>
<td></td>
</tr>
<tr>
<td>Homosexual</td>
<td>12 (19)</td>
<td></td>
</tr>
<tr>
<td>Heterosexual</td>
<td>16 (26)</td>
<td></td>
</tr>
<tr>
<td>CDC Classification*</td>
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<td></td>
</tr>
<tr>
<td>II-III</td>
<td>58 (92)</td>
<td></td>
</tr>
<tr>
<td>IV A</td>
<td>2 (3)</td>
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<tr>
<td>IV C2</td>
<td>3 (5)</td>
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* According to the CDC 1987 criteria.

well flat-bottom plate in triplicate. Syncytia formation was scored visually after 24 and 48 hours under 40X magnification.

In vitro cytokine production. PBMC were separated on lymphocyte separation medium (Organon Teknika Corp, Durham, NC), washed twice in phosphate-buffered saline (PBS), and the number of viable leukocytes was determined by trypan blue exclusion. PBMC of HIV+ individuals or healthy controls (HC) resuspended at 3 x 10⁶/mL in RPMI 1640 (GIBCO, Grand Island, NY) were either unstimulated or stimulated in vitro for 2 days with PHA (GIBCO) diluted 1:100 at 37°C in a moist, 7% CO₂ atmosphere. Stimulation with a mitogen was chosen because soluble antigens do not induce the production of quantities of IFNγ, IL-4, and IL-10 sufficient to be detectable with current ELISA protocols⁴ and to allow the comparison of these results to those obtained in previous studies.⁴¹⁵ Supernatants were harvested after 48 hours (kinetic studies indicated that 48 hours was the optimal time for assessing PHA-stimulated lymphokines production).⁴* IL-2, IFNγ, IL-4, and IL-10 production was evaluated with commercially available ELISA assays: IL-2: human IL-2 Quantikine (R&D Systems, Minneapolis, MN); IFNγ: human interferon gamma InterTest γ (Genzyme, Cambridge, MA); IL-4: human IL-4 InterTest-4 (Genzyme); IL-10: human IL-10 ELISA (Bender MedSystems, Vienna, Austria) following the procedures suggested by the manufacturers. Cytokine production was calculated from a standard curve of the corresponding recombinant human cytokine.

Statistical analyses. To analyze differences in the HIV replication rate, we used a two-tailed t test for two samples, with equal variance assumed. Differences in immunologic variables between HC and HIV+ individuals classified according to virus isolation and phenotype were analyzed using a distribution-free Kruskal-Wallis test (non-parametric one-way analysis of variance).¹⁶ Possible relationships between immunologic parameters and the increasing severity of virologic results (considering the isolation negative situation the most benign one, and the one in which SI virus is isolated the least favorable one) were evaluated in HIV+ individuals by using a non-parametric Kendall’s rank correlation.¹⁶,¹⁷ Because the informa-

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Fig 1. Kinetics of replication of primary isolates in PBMC of 47 of 63 HIV+ individuals (74.6%) in whom HIV was isolated. Twenty-six HIV+ individuals in whom nonsyncytium-inducing virus was isolated (NSI primary isolates) and 19 HIV+ individuals in whom syncytiu uncertain (SI primary isolates) are shown.
tion provided by different immunologic variables was likely to be interdependent, we performed a discriminant analysis looking for a simple combination of immunologic variables able to separate individuals grouped according to their virologic characteristics. Thus, we searched for the most important variables among IL-2, IFNγ, IL-4, and IL-10 capable to discriminate subjects with different virologic characteristics. A linear discriminant analysis was performed. IL-2, IFNγ, IL-4, and IL-10 were square-root transformed to stabilize their covariance matrices: Box’s M test, P > 1; composition and size of the best subset was established according to Wilks’ criterion (Wilks’ F-to-enter 2.0; discriminant functions have been expressed as functions of the original variables, possibly with integer coefficients, and without irrelevant constant terms.

RESULTS

Virologic analyses. The rate of viral isolation and the cytopathic effect of primary isolates was determined in a consecutive series of 63 HIV+ patients. HIV could not be isolated in 16 of 63 patients (25.4%), and viral isolation was thus possible in 47 of 63 HIV+ individuals (74.6%). SI HIV isolates were present in 19 of 47 (40.4%) individuals in whom viral isolation was possible. HIV-1 primary isolates showed different patterns of viral replication in regard to lag phase of the first positive detection of p24 antigen, and viral titers of the cultures. Thus, NSI isolates showed uniformly lower p24 antigen titers in cultures compared with SI variants at all the points tested. The mean amount of p24 antigen in the cultures was as follows: week 1: NSI = 404 pg/mL; SI = 7,045 pg/mL. (P = 0.03); week 2: NSI = 14,808 pg/mL; SI = 72,114 pg/mL. (P = 0.0005); week 3: NSI = 16,034 pg/mL; SI = 60,413 pg/mL. (P = 0.00002). These results are shown in Fig 1.

Immunologic and statistical analyses. Mitogen-stimulated and unstimulated type 1 and type 2 cytokine production by PBMC was examined in the same patients and in 24 healthy controls. Unstimulated cytokine production was constantly less that 5% of the mitogen-stimulated value for each cytokine and was subtracted from all the results shown. IL-2, IFNγ, IL-4, and IL-10 production is shown as box plots in Fig 2. The results of the analyses and summary statistics are shown in Table 2. According to the results of Kruskal-Wallis tests, important differences between the individuals classified according to HIV positivity, isolation of HIV, and identification of SI variants were observed for all immunologic variables with the exception of β2. Also, a relationship between the severity of the virologic status in HIV+ subjects, as inferred by the isolation of the virus and the presence of SI variants, was evident for all immunologic variables, again with the exception of β2. Thus, mitogen-stimulated production of the type 1 cytokines IL-2 and IFNγ was significantly reduced in parallel with the increasing severity of the viral results, whereas mitogen-stimulated production of the type 2 cytokines IL-4 and IL-10 was significantly increased in parallel with the increasing severity of the viral results and the reduction of CD4 counts (Kendall’s rank correlation). The median production of IL-2 and IFNγ was higher in the healthy controls than in all groups of HIV+ subjects, whereas the median production of IL-4 and IL-10 was lower in HIV+ isolation-negative subjects compared with all other HIV+ individuals.

Discriminating value of type 1 and type 2 cytokines. IL-2 and IL-10 (square-root transformed) resulted in the best discriminating variables. The best discrimination resulted in the direction of the dotted line in Fig 3A, where values of IL-2 were weighted about two times the values of IL-10 (score = √IL-10 - 1.9 √IL-10 = √IL-10 - 2 √IL-2; note the opposite sign assigned to IL-2 and IL-10). However, the best discriminant function in HIV+ individuals is given by simply subtracting √IL-2 from √IL-10 (score = √IL-10 - √IL-2; dashed line in Fig 3A). According to the computed scores, only 56.3% of all subjects and 58.7% of HIV+ subjects were correctly classified. However, ordering the virologic classes from the healthy controls to HIV+ individuals in whom SI HIV variants are isolated (individuals with the most severe virologic status), only two of 87 individuals were misplaced by more than one class. Moreover, both the scores appeared to separate completely HIV+ individuals in whom SI HIV
variants were isolated from HC (Fig 3B). According to a nonrigorous approach, the simple ratio IL-10/IL-2 (or IL-10/IL-2), to avoid 1/0's) appears to separate completely individuals in whom SI HIV variants were isolated (ratios 1.2) from controls (ratios 1.0). The second best combination of two variables was IL-2/IL-4 and IFNγ/IL-10, among all individuals and HIV+ individuals, respectively.

Thus, we suggest that immunological characterization of HIV+ individuals with respect to virologic status should include the evaluation of at least one type 1 cytokine (IL-2 and/or IFNγ) and at least one type 2 cytokine (IL-4 and/or IL-10).

**DISCUSSION**

Because HIV infection was suggested to be an immunovirolelogic disease, we analyzed possible correlations between HIV intrinsic biological properties, such as the rate and the extent of viral replication and viral phenotype and the strength and the quality of the immune response. Our hypotheses were two: (1) a tight correlation exists between the immunologic and the virologic aspects of HIV infection; and (2) the virologically most favorable outcome (inability to isolate HIV from PBMC) is associated with the strongest production of type 1 cytokines, the weakest production of type 2 cytokines, and the highest CD4 counts, whereas the virologically least favorable outcome (isolation of fast-replicating SI HIV variants) is associated with a specular immunologic situation (ie, defective production of type 1 cytokines, strong production of type 2 cytokines, and lowest CD4 counts). Thus, we cross-sectionally analyzed the rate and extent of HIV replication, HIV phenotype, cytokine production, and CD4 counts in 63 consecutively enrolled HIV+ individuals, and we examined cytokine production in 24 HIV+ HC. The results suggest that the virologic and immunologic aspects of HIV infection are likely to be correlated. Therefore, the most dramatic reduction of type 1 cytokine
production, as well as the greatest increment in type 2 cytokine production and the lowest CD4 counts are observed in those HIV+ individuals in whom fast-replicating SI HIV variants are isolated. Additionally, the IL-10/IL-2 ratio is capable of the best correlation with viral parameters, i.e., in vitro viral replication of fully infectious virions and ability to induce a cytopathic effect (SI variants).

Virologic and immunologic correlates of disease activity have been proposed in HIV infection. Analyses of virologic correlates have indicated that the rate and the extent of HIV isolation by PBMC of long-term nonprogressing individuals (LTNP) is difficult, and is in some cases, possible only following depletion of CD8 lymphocytes. Plasma levels of HIV RNA, specific intracellular transcripts, and the number of PBMC containing integrated viral DNA are uniformly reduced in LTNP. Additionally, a correlation exists between the emergence of HIV SI HIV strains and disease progression. Thus, (1) HIV SI variants are isolated in 50% to 60% of HIV+ individuals with AIDS, but in less than 10% of HIV+ asymptomatic individuals; (2) 71% of HIV+ individuals in whom SI HIV variants could be isolated, but only 16% of HIV+ individuals in whom SI variants could not be isolated progressed to AIDS in a 30-month follow-up period; and (3) the isolation of HIV SI variants is associated with increased decline in CD4+ T lymphocytes.

Immunological correlates of disease progression include abnormalities affecting both humoral and cell-mediated immunity. Thus, it was shown that antibodies with better neutralizing ability for laboratory and primary isolates are present in LTNP and that the antibody repertoire of these individuals is directed toward multiple HIV epitopes. We have proposed progressive impairment of type 1 cytokine-driven cell-mediated immunity to be the main immunologic correlate of disease progression. This hypothesis is based on different independent observations: (1) the gradual reduction of the ability of PBMC to produce IL-2, IL-12, and IFNγ (type 1 cytokines) is accompanied in the progression of HIV infection by an increased generation of IL-4, IL-6, and IL-10 (type 2 cytokines)

**Fig 3.** (A) IL-2 and IL-10 production in HIV+ healthy controls (○); HIV+/SI- individuals (*), HIV+/SI+/SI- individuals (†); and HIV+/SI+ individuals (>). The dotted and the dashed lines indicate the direction of the best discriminant function and that of the best discriminant function among HIV+ individuals, respectively. (Note the square-root scales on the X- and Y-axes.) (B) Box plots relative to the scores computed according to the best discriminant function among HIV+ individuals. For each individual, the score was computed according to the function, score = −1/IL-10−IL-2, obtained by discriminant analysis. The scores correspond to measures computed in the direction of the dashed line shown in (A), which in this panel is represented by the axis entitled “score (−1).” The box plot resume the score data as already described in Fig 2; the box covers the range between the lower and the upper quartile, and the central line is at the median. The ‘whiskers’ extend to the 10th and the 90th percentile; more extreme data are plotted as separate points.
variants is the cause of the decreased production of type 1 cytokines, in favor of an increased production of type 2 cytokines, which may be secondary to the different replication requirements of NSI and SI variants. All the patients are enrolled in a longitudinal study that could answer this question.

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