Kinetics of cytokine expression during primary human immunodeficiency virus type 1 infection

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ABSTRACT In the present study, we have determined the kinetics of constitutive expression of a panel of cytokines [interleukin (IL) 2, IL-4, IL-6, IL-10, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α)] in sequential peripheral blood mononuclear cell samples from nine individuals with primary human immunodeficiency virus infection. Expression of IL-2 and IL-4 was barely detected in peripheral blood mononuclear cells. However, substantial levels of IL-2 expression were found in mononuclear cells isolated from lymph node. Expression of IL-6 was detected in only three of nine patients, and IL-6 expression was observed when transition from the acute to the chronic phase had already occurred. Expression of IL-10 and TNF-α was consistently observed in all patients tested, and levels of both cytokines were either stable or progressively increased over time. Similar to IL-10 and TNF-α, IFN-γ expression was detected in all patients; however, in five of nine patients, IFN-γ expression peaked very early during primary infection. The early peak in IFN-γ expression coincided with oligoclonal expansions of CD8$^+$ T cells in five of six patients, and CD8$^+$ T cells mostly accounted for the expression of this cytokine. These results indicate that high levels of expression of proinflammatory cytokines are associated with primary infection and that the cytokine response during this phase of infection is strongly influenced by oligoclonal expansions of CD8$^+$ T cells.

The majority (50–70%) of human immunodeficiency virus type 1 (HIV-1)-infected individuals experience a clinical syndrome of variable severity associated with primary infection (1–5). Due to the lack of specificity of the clinical symptoms and to the fact that hospitalization is required in only a minority (10–15%) of individuals with a severe clinical syndrome, primary HIV infection generally goes unnoticed (1–5). Recently, however, substantial progress has been made in the delineation of the virologic and immunologic events associated with primary infection. Virologic parameters including plasma viremia, p24 antigenemia, and HIV RNA expression in peripheral blood mononuclear cells (PBMC) are extremely high during this stage of infection (3–5). The high levels of viremia coincide with the clinical syndrome, and both down-regulation of viremia and disappearance of symptoms generally occur within 4–8 weeks; it is thought that the decrease of virologic parameters and the resolution of symptoms are the result of an HIV-specific immune response (3–5). Both vigorous humoral and cell-mediated immune responses are associated with primary infection (1–11). With regard to the latter, oligoclonal expansions of CD8$^+$, virus-specific, cytotoxic T lymphocytes represent a major component of the primary HIV-specific cell-mediated immune response (8); a similar phenomenon has been observed in the experimental simian immunodeficiency

virus model of acute infection (12). It has been proposed that virus-specific cytotoxic CD8$^+$ T lymphocytes represent the primary mechanism responsible for the dramatic down-regulation of virologic parameters by the elimination of a large number of virus-expressing cells (6–8, 10, 11). With regard to the humoral immune response, the observation that neutralizing antibodies are detected when transition from the acute to the chronic phase of infection has already occurred suggests that this response may have little effect on the initial spreading of virus (6, 9). However, HIV-binding antibodies appear before neutralizing antibodies, and it is conceivable that this former humoral response may contribute to the removal of virus particles from the circulation and thus to the down-regulation of viremia by promoting the formation of immune complexes (virus plus immunoglobulin plus complement) that may be cleared through and trapped in the reticuloendothelial system (10, 11).

Cytokines mediate a number of important immunoregulatory functions and play a critical role in the modulation of the immune response to different pathogens (13). The potential effects of certain cytokines on virus replication and their involvement in the pathogenesis of HIV disease during the chronic phase of infection have been extensively investigated (14–19). However, little information is available on the patterns of cytokines associated with primary HIV infection (20). In the present study, we have determined the kinetics of cytokine expression in a group of HIV-infected individuals with primary HIV infection and have analyzed the relationship of cytokine expression to other components of the primary HIV-specific immune response.

MATERIALS AND METHODS

Patients. All patients were studied prospectively under protocols approved by the institutional review of boards of the University of Alabama (Birmingham); the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD); and Johns Hopkins University (Baltimore). The clinical histories of patients 1, 2, 3, 4, and 6 have been described elsewhere (4, 5, 8); these patients had significant clinical symptoms. Patients 15 and 30 had a clinical syndrome of moderate severity with fever, diarrhea, fatigue, and lymphadenopathy. In patient 17 the clinical syndrome was characterized predominantly by upper respiratory symptoms. Patient 27 had minimal clinical symptoms with a transient skin rash $\approx$4 weeks after exposure. In all patients resolution of symptoms and down-regulation of viremia were observed within 10 weeks from the onset of symptoms. A lymph node biopsy (axillary node) was performed in patient 15 at day 28 from the onset of symptoms.

Isolation of Mononuclear Cells from Peripheral Blood and Lymph Nodes. Blood was obtained by venipuncture. Mononuclear cells were isolated from blood and lymph nodes as described (21–23).

Quantitation of Cytokine Expression. Cytokine expression was determined by a semiquantitative reverse transcriptase-PCR assay as described (17). The cytokines studied included interleukin (IL)-2, IL-4, IL-6, IL-10, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α). Briefly, unfractionated mononuclear cells or sorted T-cell subsets isolated from peripheral blood of HIV-infected individuals were pelleted and total RNA was extracted by the RNAzol method (Tel-Test, Friendswood, TX) (24). As a standard RNA for the determination of cytokine expression by this PCR assay, total RNA extracted from phytohemagglutinin-activated (7 h) PBMC isolated from one HIV-negative individual was used. In preliminary experiments, we determined that under these conditions (stimulation of PBMC for 7 h with phytohemagglutinin), mRNAs specific for the group of cytokines analyzed in this study were consistently and reproducibly detected. RNA (2 μg) was reverse transcribed and aliquots of cDNA corresponding to 1/20th of the reaction of reverse transcription were amplified with primer pairs specific for each cytokine, together with two-fold serial dilutions of the standard cDNA (from 1:50 to 1:6400). The highest dilution (1:6400) of the standard cDNA for any of the cytokines analyzed corresponded to the last dilution of the standard cDNA in which amplified products (positive signal) could be detected. We amplified cDNA specific for the constant (C) region of the α chain of the human T-cell receptor or for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (25) to normalize the amount of RNA present in each sample. Amplification was performed in the presence of 1× PCR buffer (50 mM KCl/10 mM Tris, pH 8.3/1% dimethyl sulfoxide/1% glycerol) (26), 0.4 μM primers (Lofstrand Laboratories, Gaithersburg, MD), 200 μM dNTPs (Pharmacia), TaqStart antibody (Clontech; 2.5 μl of a dilution 1:8.2 of TaqStart antibody to TaqStart dilution buffer), and 2.5 units of *Taq* polymerase (Perkin–Elmer). An amount of antisense primer end-labeled with [γ-^{32}P] adenosine triphosphate (Amersharm) corresponding to 500,000 to 1,000,000 cpm/μl was also added. With regard to magnesium concentration, it was determined that optimal amplification occurred in the presence of 1.5 mM $MgCl_2$ for IL-4, IL-6, IL-10, and TNF-α; in the presence of 2 mM $MgCl_2$ for IL-2; and in the presence of 2.5 mM $MgCl_2$ for IFN-γ. After determination of the number of PCR cycles required to ensure linearity of amplification, 25 cycles were chosen for TNF-α, 28 cycles for IFN-γ and IL-10, and 31 cycles for IL-2, IL-4, and IL-6. Products of amplification were analyzed by electrophoresis in 5% 29:1 polyacrylamide gels and visualized by autoradiography. The intensity of the signal for each cytokine was measured by a PhosphorImager (Molecular Dynamics). A simple regression curve was fitted for the two-fold dilution of the standard cDNA, and the equation obtained was used to determine the relative amounts of target sequence in the patient samples. Results are expressed as fold increase over the positive control (i.e., the last dilution of the standard cDNA in which amplified products could be detected). We have previously shown that constitutive expression of the cytokines analyzed in the present study was generally not detected in PBMC of HIV-negative seronegative donors (17).

Analysis of T-Cell Subsets. T-cell subsets, as determined by the variable (V) region of the β chain of the T-cell receptor (Vβ repertoire) in unfractionated PBMC collected at different times from the onset of symptoms, were analyzed by a semiquantitative PCR as described (8, 27, 28). Expansions in the relative expression of Vβ families over sequential time points by PCR were considered to be significant only if there was at least a two-fold increase in the relative percentage of the Vβ family in question. Briefly, 2 μg of total RNA, extracted by the

RNAzol method, were reverse transcribed using random hexamers (20 μg ml^{-1}; Promega), avian myeloblastosis virus reverse transcriptase (60 units; Life Sciences, St. Petersburg, FL) and dNTPs (Boehringer Mannheim). PCR analysis for the different Vβs used a 5′-specific Vβ primer and a common 3′ constant-domain Cβ primer. 5′ and 3′ Cα primers were included in each PCR as internal controls. To monitor the migration of Vβ and Cα bands, 3′ Cβ and 3′ Cα primers were radiolabeled with [γ-^{32}P]ATP (Amersharm). Primer sequences of Vβ-specific oligonucleotides and of control Cβ and Cα oligonucleotides have been reported (27). One aliquot of each PCR was loaded and separated on 10% polyacrylamide gels containing 7 M urea. Gels were exposed overnight on Kodak storable phosphor screens, and the radioactive signal for each Vβ was quantified using a PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

Analysis of the Changes in Cytokine Expression in HIV-Infected Individuals During Primary Infection. The nine patients included in this study had well-documented historical, clinical, and laboratory parameters of primary HIV infection. All patients experienced a clinical syndrome of variable severity, which generally appeared within 4–6 weeks of the time of exposure to virus; substantial down-regulation of viremia and resolution of symptoms occurred within 6–8 weeks from the onset of symptoms. Kinetics of mRNA expression specific for a panel of cytokines including IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α were performed on PBMC samples collected at different times during primary infection. The first sample of PBMC was obtained 1–4 weeks after the onset of symptoms at a time of peak viremia or when the levels of viremia were still very high; coincident with the appearance of other components of the primary HIV immune response, PBMC samples were generally collected weekly for the first month and monthly thereafter. In one patient (patient 15) a lymph node biopsy was performed at day 28 after the onset of symptoms, and thus levels of cytokine expression were analyzed in mononuclear cells isolated from both blood and lymph node. To avoid the variability that could have been generated by analyzing the expression of cytokine specific mRNA in separate PCRs for different patients, equal amounts of total RNA extracted from sequential PBMC samples of each patient were reverse transcribed and amplified simultaneously. Estimates of the levels of cytokine expression were obtained by comparing the levels of the target sequence in the patient samples with those observed in two-fold serial dilutions of the standard cDNA. Results were expressed as fold increase over the positive control that corresponded to the highest dilution (1:6400) of the standard cDNA in which a positive signal could be detected.

Analysis of the patterns of cytokine expression during primary infection showed substantial differences in the kinetics within the panel of cytokines studied. However, certain cytokines had similar patterns of expression; for these reasons, the data regarding those cytokines with similar kinetics will be examined and discussed together. Expression of IL-2 and IL-4 was generally not detected (Fig. 1*A*) or barely detected (Fig. 1*B*) in PBMC. The results for IL-2 and IL-4 obtained in patient 15 are representative of those observed in other five patients (patients 1, 2, 3, 4, and 6; data not shown). The kinetics of IL-2 and IL-4 in patient 30 (Fig. 1*B*) were similar to those found in patient 27 (data not shown). A substantial increase over time of the levels of IL-2 (Fig. 1*C*), and to a lesser extent of those of IL-4 (Fig. 1*C*), was observed only in patient 17. These results indicate that there is very little or absent expression of IL-2 and IL-4 in circulating CD4$^+$ T lymphocytes, the T cell subset almost exclusively responsible for the production of these two cytokines (13). However, a lymph node biopsy was performed
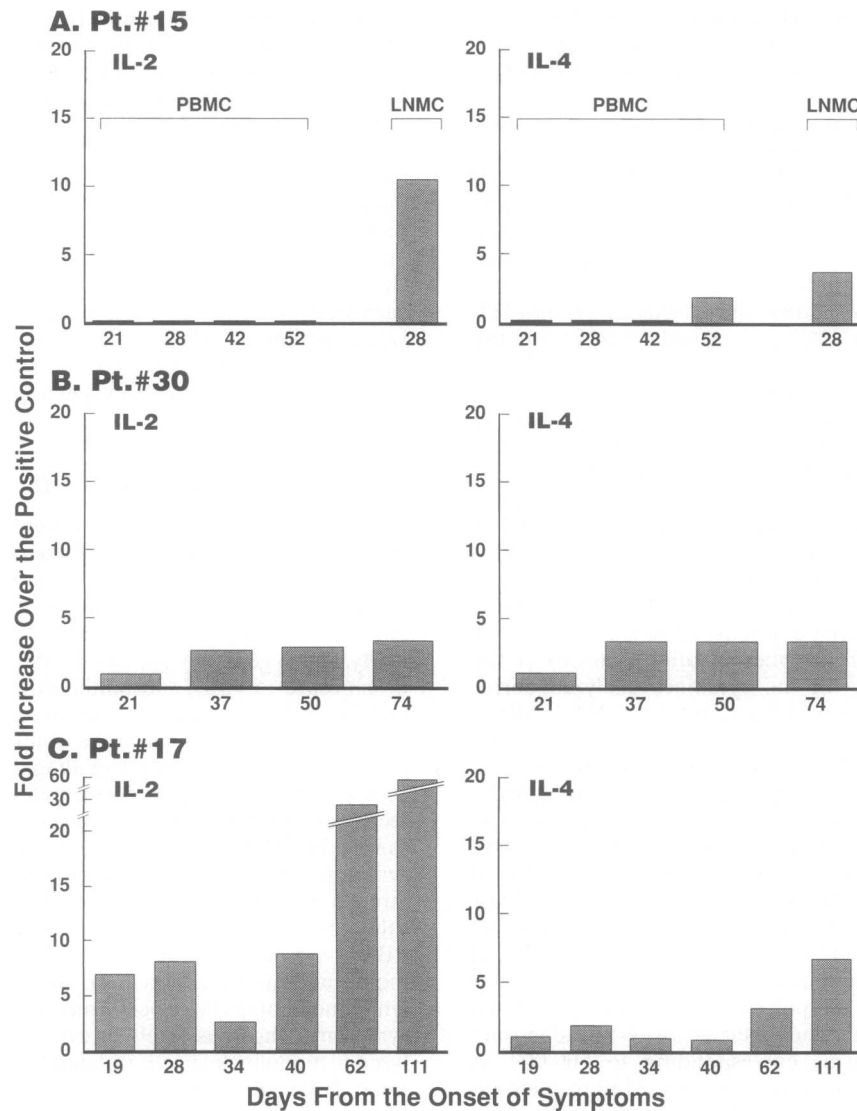
FIG. 1.   Kinetics of IL-2 and IL-4 expression in peripheral blood and lymph node of HIV-infected individuals with primary infection. (*A*) Patient 15: the results obtained in PBMC are representative of those obtained in another five patients. (*B*) Patient 27: similar kinetics were obtained in PBMC of patient 30. (*C*) Kinetics of IL-2 and IL-4 in PBMC of patient 17. Determination of cytokine expression was performed as described. For each cytokine analyzed, samples obtained from peripheral blood and lymph node of the same patient at different time points were amplified in the same PCR reaction. Results are expressed as fold increase over the positive control.

in patient 15 at day 28 after the onset of symptoms, and thus it was possible to compare the levels of expression of IL-2 and IL-4 in peripheral blood versus lymph node in this patient. Of interest, substantial levels of IL-2 (Fig. 1*A*), and to a lesser extent of IL-4 (Fig. 1*A*), were observed in lymph node at a time (day 28) when expression of both cytokines was not detected in peripheral blood (Fig. 1*A*). In addition, the levels of expression of IL-6, IL-10, IFN-γ, and TNF-α were significantly higher (3-fold for each cytokine) in lymph node compared to peripheral blood (data not shown). Therefore, these results indicate that the lack of detection of expression of IL-2 and IL-4 in peripheral blood during primary infection does not necessarily mean that a similar situation exists in other lymphoid compartments.

Expression of IL-6 was not detected in peripheral blood in the majority (6) of patients with primary infection (Fig. 2). However, in three patients (patients 2, 15, and 17) expression of IL-6 was detected in PBMC samples collected late during primary infection (Fig. 2) at a time when down-regulation of viremia had already occurred.

Kinetics of expression of IL-10 and TNF-α were almost identical. Expression of both cytokines was consistently ob-

served in peripheral blood in all nine patients studied (Fig. 3). In four (patients 2, 15, 17, and 27) of nine patients, expression of IL-10 and TNF-α progressively increased over time (Fig. 3*A*), and levels of both cytokines peaked when the transition from the acute to the chronic phase of infection had already occurred. No major changes in the levels of expression of these cytokines between early and late time points were observed in patients 3, 4, and 6 (Fig. 3*B*). A transient increase in the levels of IL-10 and TNF-α was observed in patient 30 during the course of primary infection (Fig. 3*C*); patient 1 was the only individual in whom a peak of both cytokines was detected in the PBMC samples collected at the early time points (Fig. 3*D*).

Similar to IL-10 and TNF-α, expression of IFN-γ was detected in all patients included in this study. Two dominant patterns of IFN-γ expression were observed. Levels of IFN-γ expression were low and stable throughout primary infection, including the transition from the acute to the chronic phase of infection; this pattern was observed in four patients (patients 3, 4, 6, and 27) (Fig. 4*A*). A peak in the expression of IFN-γ was observed in the other five patients (patients 1, 2, 15, 17, and 30) (Fig. 4*B*). In patients 1, 15, and 30, IFN-γ expression peaked in the PBMC samples collected at the early time points

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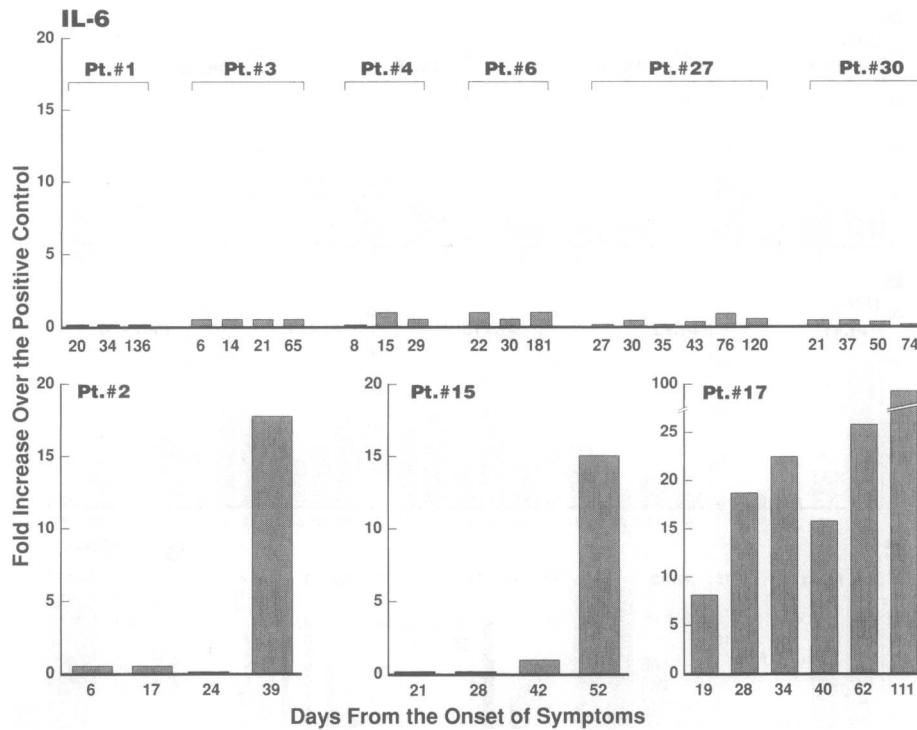
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FIG. 2.   Kinetics of IL-6 expression in peripheral blood of HIV-infected individuals with primary infection. Samples obtained at different times from peripheral blood of the same patient were amplified in the same PCR. Results are expressed as fold increase over the positive control.

and rapidly declined over time (Fig. 4*B*). In patient 2, IFN-γ expression peaked late (day 39) during primary infection (Fig. 4*B*). Of interest, in patient 17 there was a bimodal kinetic of

IFN-γ expression; an early peak (day 19) in IFN-γ expression was followed by a substantial decline (70% reduction by day 40) and by a late increase (80% increase at day 111 compared
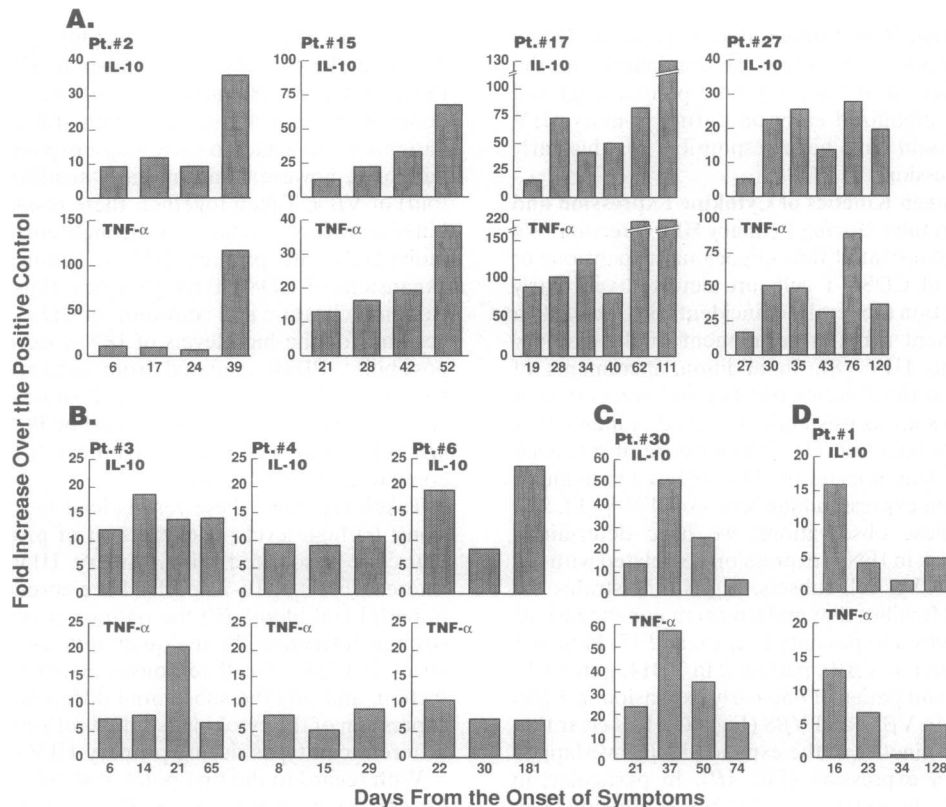

FIG. 3.   Kinetics of IL-10 and TNF-α expression in peripheral blood and lymph node of HIV-infected individuals with primary infection. (*A*) Kinetics of IL-10 and TNF-α expression in PBMC of patients 2, 15, 17, and 27. (*B*) Analysis in PBMC of patients 3, 4, and 6. (*C*) Analysis in PBMC of patient 30. (*D*) Analysis of PBMC in patient 1. Samples obtained at different times from peripheral blood of the same patient were amplified in the same PCR. Results are expressed as fold increase over the positive control.
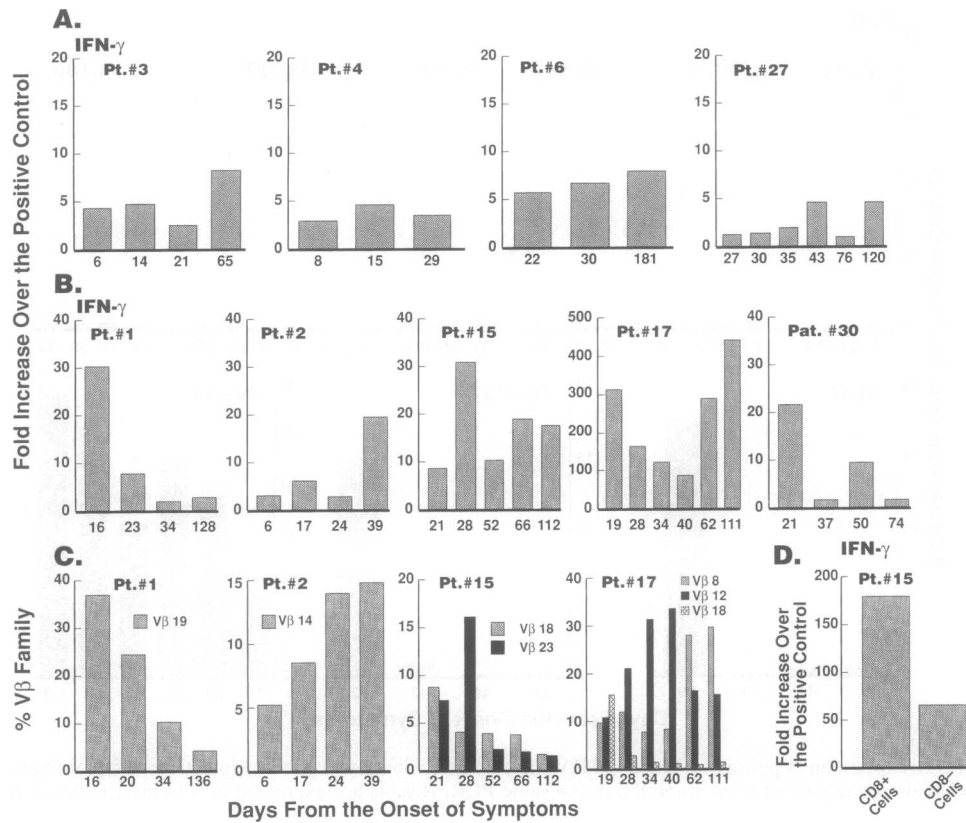
FIG. 4. Kinetics of IFN-γ expression and correlation with changes in the Vβ repertoire in peripheral blood of HIV-infected individuals with primary infection. (*A*) Kinetics of IFN-γ expression in PBMC of patients 3, 4, 6, and 27. (*B*) Longitudinal analysis of IFN-γ expression in PBMC of patients 1, 2, 15, 17, and 30. (*C*) Kinetics of Vβ repertoire in PBMC of patients 1, 2, 15, and 17. (*D*) IFN-γ expression in sorted CD8⁺ and CD8⁻ T-cell populations.

to day 40) (Fig. 4B). The finding that a peak in IFN-γ expression, when present, is found predominantly in the PBMC samples collected at the early time points, suggested that the cell subset mobilized early on during primary HIV immune response could have been responsible for this early peak in IFN-γ expression.

Relationship Between Kinetics of Cytokine Expression and Changes in Vβ Repertoire During Primary HIV Infection. We have previously demonstrated that oligoclonal expansions of certain Vβ subsets of CD8⁺ T cells are detected very early during primary infection (generally coincident with the peak in viremia) and represent a major component of the primary immune response to HIV (8). In addition, increments of variable magnitude in the absolute number and percentage of CD8⁺ T lymphocytes are consistently observed in individuals during primary HIV infection (1). Furthermore, it has been shown that CD8⁺ T lymphocytes of HIV-infected individuals with chronic infection expressed high levels of IFN-γ (17, 29). On the basis of these observations, we have determined whether the early peak in IFN-γ expression correlated with the expansions of CD8⁺ Vβ cell subsets. Fig. 4C illustrates the kinetics of those Vβ families that underwent major expansions during primary infection in patients 1, 2, 15, and 17. Patient 1 had a major expansion in Vβ19, patient 2 in Vβ14, patient 15 in Vβ18 and Vβ23, and patient 17 an early expansion in Vβ18 and late expansions in Vβ12 and Vβ8 (Fig. 4C). It is clear that in these patients the kinetics of the expanded Vβs overlapped with those of IFN-γ expression (Fig. 4B). In particular, in patient 17, who had a bimodal peak of IFN-γ expression (Fig. 4B), both early (Vβ18) and late (Vβ12 and Vβ8) expansions of Vβs coincided with the early and late peaks of IFN-γ. The Vβ repertoire has not been analyzed in patient 30; in this patient, however, there was a major increase in the absolute

number of CD8⁺ T lymphocytes (3959 per μl of blood at day 21; generally the absolute number of CD8⁺ T lymphocytes ranges between 200 and 600 per μl of blood in HIV-negative donors). Among the patients with stable IFN-γ expression, patients 4 and 6 had no early major expansions of Vβ families; patient 3, however, had an early significant expansion (4.5-fold) of Vβ 4. Taken together, these results indicated that the kinetics of IFN-γ expression strongly correlated (in five of six individuals with primary HIV infection) with those of the expansions of CD8⁺ T lymphocytes, thus suggesting that the early mobilization and activation of CD8⁺ T lymphocytes may account for the high levels of IFN-γ expression. To test this possibility, PBMC isolated from patient 15 at day 28 were sorted in CD8⁺ versus CD8⁻ cell subsets, and the levels of IFN-γ expression were determined by PCR. As shown in Fig. 4D, expression of IFN-γ was significantly higher in CD8⁺ cells compared to CD8⁻ cells.

Taken together, these results lead to a number of conclusions: (*i*) high levels of expression of proinflammatory cytokines are associated with primary HIV immune response whereas levels of IL-2 and IL-4 are absent or barely detected in peripheral blood, (*ii*) the pattern of cytokine expression is strongly influenced by the qualitative and quantitative differences in CD8⁺ T-cell responses associated with primary infection, and (*iii*) the substantial differences in the kinetics of expression of the cytokines studied reflects the mobilization of different cell types during primary HIV immune response.

With regard to the first point, it should be underscored that for certain cytokines such as IL-2 and IL-4 peripheral blood may not accurately reflect the situation in other lymphoid compartments; substantial expression of IL-2 was detected in lymph node despite the absent expression in peripheral blood. This is a further indication that certain cytokines may be

produced and may act locally in tissues without entering the circulation.

With regard to the second point, the type of CD8$^+$ T-cell-mediated response (i.e., major oligoclonal expansion) is strongly associated with the presence of the early peak in IFN-γ expression. On the basis of recent studies on the putative soluble suppressor factors produced by CD8$^+$ T lymphocytes (30, 31), it would be important to determine whether the patterns of expression and/or production of these factors are also influenced by qualitative and/or quantitative differences of the CD8$^+$ T cell-mediated response. In this regard, CD8$^+$ T-cell-mediated suppressor activity has been detected in patients with primary infection (32), and certain observations suggest that the early cytokine response may play a primary role in the suppression of virus replication in the large number of infected cells. In particular, we have observed a dissociation between the dramatic downregulation of plasma viremia and the minor changes of viral burden (number of HIV DNA copies in PBMC) during primary infection (5), thus indicating that the decrease in virologic parameters associated with primary infection likely is a process involving multiple mechanisms including elimination of virus-expressing cells by HIV-specific cytotoxic T cells, removal of virus particles from the circulation into the reticuloendothelial system, and suppression of virus replication by cytokines.

Finally, the biphasic response of cytokine expression (early peak in IFN-γ and late peaks in IL-10, TNF-α, and IL-6) that may be detected in certain patients, is a clear indication of the involvement of different cell types in the antiviral immune response. These patterns of cytokine expression are not unique of primary HIV infection; similar cytokine response has been observed in individuals with acute Epstein–Barr virus infection (C.G., unpublished data). Further studies will be needed to determine whether different patterns of cytokine response during primary HIV infection may be associated with a more-versus-less protective immune response and a more effective and sustained control of virus replication over time. In this regard, such studies may provide important information for the development of immune-based therapeutic strategies.

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