Human Immunodeficiency Virus (HIV)-Specific Gamma Interferon Secretion Directed against All Expressed HIV Genes: Relationship to Rate of CD4 Decline

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Immune responses to human immunodeficiency virus (HIV) are detected at all stages of infection and are believed to be responsible for controlling viremia. This study seeks to determine whether gamma interferon (IFN- γ)-secreting HIV-specific T-cell responses influence disease progression as defined by the rate of CD4 decline. The study population consisted of 31 subjects naïve to antiretroviral therapy. All were monitored clinically for a median of 24 months after the time they were tested for HIV-specific responses. The rate of CD4⁺-T-cell loss was calculated for all participants from monthly CD4 counts. Within this population, 17 subjects were classified as typical progressors, 6 subjects were classified as fast progressors, and 8 subjects were classified as slow progressors. Peripheral blood mononuclear cells were screened for HIV-specific IFN- γ responses to all expressed HIV genes. Among the detected immune responses, 48% of the recognized peptides were encoded by Gag and 19% were encoded by Nef gene products. Neither the breadth nor the magnitude of HIV-specific responses correlated with the viral load or rate of CD4 decline. The breadth and magnitude of HIV-specific responses did not differ significantly among typical, fast, and slow progressors. These results support the conclusion that although diverse HIV-specific IFN- γ -secreting responses are mounted during the asymptomatic phase, these responses do not seem to modulate disease progression rates.

Human immunodeficiency virus (HIV) infection is characterized by robust virus-specific CD8⁺ cytotoxic T-lymphocyte (CTL) responses that are believed to play a role in the control of viral replication and dissemination. These responses are thought to be responsible for the reduction of viremia in primary infection (8, 35), maintenance of the viral set point during the asymptomatic phase (53), and control of viremia in long-term nonprogressors (27). The most direct evidence supporting a role for CD8⁺ CTL in viral control comes from an animal model for HIV infection, i.e., macaques infected with the simian immunodeficiency virus (SIV). In this model, depletion of CD8⁺ T cells results in either increased viremia that remains high until the CD8⁺ T cells are reconstituted or uncontrolled viremia and rapid disease progression (31, 59). CD8⁺ CTLs are believed to exert a selective pressure on the virus sequences they recognize, as demonstrated by the preferential emergence of viral escape mutations in sequences corresponding to CTL epitopes in SIV-infected macaques (2, 33, 52) and in HIV-infected humans (23, 24, 34, 55). However, despite the presence of HIV-specific CTLs at all stages of the infection, CTLs are unable to clear infection (40).

HIV-specific CD8⁺ T cells can suppress viral replication by several mechanisms. They can lyse HIV-infected targets before mature virions can be produced, secrete factors such as MIP-1 α , MIP-1 β , or RANTES, which bind the CCR5 coreceptor

and prevent HIV entry into target cells, and secrete factors that control viral replication (38, 65). HIV-specific CD8⁺ and CD4⁺ T cells also secrete the antiviral cytokine gamma interferon (IFN- γ) upon activation (30). The IFN- γ enzyme-linked immunospot assay (ELISPOT assay) has come into common use as a high-throughput, sensitive technique for measuring the frequency of cells able to secrete a cytokine, such as IFN- γ , upon antigen stimulation (50, 58). The availability of peptide sets corresponding to all expressed HIV genes has led to the development of methods that permit the comprehensive screening of peripheral blood mononuclear cells (PBMCs) for responses to HIV.

Several reports have used peptide pool matrix arrays that include sequences corresponding to all expressed HIV genes to screen for the breadth, magnitude, and specificity of HIV-specific effector function in diverse patient populations. Conflicting results on whether an association exists between the breadth and/or magnitude of HIV-specific IFN- γ secretion and viral load have been reported (1, 7, 12, 17, 51). Furthermore, the cross-sectional design of these studies precluded the assessment of whether breadth, magnitude, and specificity of HIV-specific immune responses predicted the subsequent rate of CD4⁺-T-cell loss. It is well established that the plasma viral load set point strongly predicts the rate of CD4⁺-T-cell decline, progression to AIDS, and death (47). While direct cytopathic effects of HIV on CD4⁺ T cells likely plays a role in CD4⁺-T-cell depletion, most of the cells in this compartment that die are not infected (18, 20). Indirect effects of viral replication, such as immune activation causing increased cell turnover, have been postulated to contribute to the progressive

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TABLE 1. Descriptive characteristics of the 31 subjects included in the study at the time of ELISPOT evaluation^a

Progressor category and subject no.	Age (yr)/sex	HLA locus			Absolute no. of T cells/ μ l		CD4 slope (cells/ μ l/yr)	Plasma viral load (HIV- 1 RNA copies/ml)
		A	B	C	CD4	CD8		
Typical								
1	34/M	2, 11	14, 39	7, 8	425	935	88.7	5,741
2	25/M	2, 3	7, 14	7, 8	725	1450	98	982
3	34/M	2, 11	7, 14	7, 8	999	1,377	57	1,310
4	26/M	2, 26	15, 38	3, 12	504	2,412	34	3,952
5	27/M	3, 30	35, 40	2, 4	600	1,488	45	683
6	23/M	23, 26	15, 38	2, 12	504	1,568	61	12,420
7	42/M	2, 26	35, 40	2, 4	682	880	46	865
8	37/M	2, 36	15, 53	2, 4	510	1,700	77	1,494
9	44/M	2	43, 44	2, 5	660	1,034	35	72
10	18/M	2, 4	13, 15	3, 6	620	1,116	77	4,371
11	35/F	2, 11	27, 52	2, 12	589	798	79	119
12	36/M	2, 26	27, 62	1, 3	520	1,000	35.7	587
13	42/M	1, 2	44, 57	6, 16	840	1,428	98	638
14	33/M	2, 31	40, 44	3, 5	442	986	97.7	729
15	18/M	2	40, 47	2, 15	780	720	78	1,796
16	29/M	1, 2	8, 18	7	552	1,344	35	839
17	31/M	2, 24	15, 40	3	525	1,134	44	1,452
Slow								
18	37/M	3	14	8	588	1,540	0	522
19	33/M	2, 24	18, 44	4, 5	660	1,144	0	564
20	40/M	1, 2	27, 44	2, 5	561	1,782	29	1,476
21	27/M	11, 29	27, 44	1, 16	924	980	0	913
22	38/M	33, 68	15, 35	3, 15	741	2,067	0	5,865
23	36/M	2, 24	7, 27	1, 7	429	689	28	17,700
24	29/M	2, 11	7, 35	4, 5	572	1,352	23	1,192
25	27/M	2, 33	14, 40	2, 8	630	735	9	20,876
Fast								
26	28/M	2, 29	15, 27	1, 2	572	1,222	177	2,217
27	38/M	3, 26	7, 15	3, 7	567	1,029	128	6,395
28	39/M	1	18, 44	5, 6	572	1,034	140	6,425
29	27/M	1, 3	7, 57	7	494	912	150	22,065
30	51/M	1, 3	18, 57	5, 6	600	1,590	132	529
31	42/M	1, 24	8, 15	3, 7	374	1,342	125	6,304

^a M, male; F, female.

loss of CD4⁺ T cells (28, 46). Immune activation is often measured by expression levels of CD38 on CD8⁺ T cells (21, 26). The predictive value of this marker of immune activation for HIV disease progression, including rate of CD4⁺-T-cell decline, was shown to be stronger than and independent of the plasma viral load set point, demonstrating that immune activation is also an important factor in the pathogenic process (37, 41). Therefore, previous studies that assessed associations between total HIV-specific immune responses measured in IFN- γ ELISPOT assays and viral load evaluated correlations between these responses and one factor contributing to rate of CD4⁺-T-cell decline.

In this report, we used an IFN- γ ELISPOT assay to screen PBMCs from 31 antiretroviral therapy-naïve chronically infected subjects with known rates of CD4⁺-T-cell decline calculated from CD4⁺-T-cell counts measured monthly for a minimum of 1 year from the time point used for immune response assessments. The hypothesis being tested was that if HIV-specific IFN- γ secretion is a correlate of protection from disease progression, subjects with slower kinetics of CD4⁺-T-cell loss should have more intense and/or broader responses than those with typical or fast disease progression rates.

We found that neither the magnitude nor the breadth of HIV-specific recognition was correlated with CD4⁺- or CD8⁺-T-cell counts, viral load, or rate of CD4⁺-T-cell decline in this untreated population in the chronic phase of HIV infection. These conclusions also applied to comparisons made among three subgroups selected from among the larger population, namely: a subgroup with a typical rate of CD4⁺-T-cell loss, a fast progressor group, and a slow progressor group with a rate of CD4⁺-T-cell decline similar to that seen in uninfected subjects. The three subgroups exhibited similar breadth and magnitude of HIV-specific IFN- γ secretion to all expressed HIV genes.

MATERIALS AND METHODS

Study population. We studied 31 HIV-infected subjects (26 males and 1 female infected through sexual exposure, 3 hemophilia patients, and 1 intravenous drug user infected parenterally) in the chronic phase of infection. All subjects were asymptomatic and naïve to antiretroviral therapy and were monitored clinically for a median of 24 (range, 12 to 24) months (Table 1). Monthly CD4 counts taken within this period were used to calculate the rate of CD4 decline for each individual from the test date. Because monthly CD4 counts were available for these individuals, we were able to identify three subgroups from this population, based on rates of CD4⁺-T-cell decline, for additional analyses. In-

TABLE 2. Clinical data on subgroups stratified by rate of CD4 decline at the time of ELISPOT evaluation

Parameter (median)	Result (range) for progressor type indicated			P value (ANOVA)
	Typical (<i>n</i> = 17)	Fast (<i>n</i> = 6)	Slow (<i>n</i> = 8)	
CD4 count (cells/ μ l)	589 (425–999)	570 (374–600)	609 (429–924)	NS
CD8 count (cells/ μ l)	1134 (720–1700)	1128 (912–1590)	1248 (689–2067)	NS
Viral load (RNA copies/ml)	982 (72–12420)	6350 (529–22065)	1334 (522–20876)	NS
Rate of CD4 decline (cells/ μ l/yr)	61 (34–98)	136.1 (125–177)	4.4 (0–29)	<0.0001

formed consent was obtained from all study subjects, and the research conformed to all ethical guidelines of the authors' institutions.

Laboratory testing. Plasma viremia was measured by using an HIV RNA multiplex b-DNA assay, version 2.0. Those undetectable by this assay were retested with the ultrasensitive Chiron b-DNA assay (version 3.0; Chiron Corp., Emeryville, Calif.) with a detection limit of 50 HIV type 1 (HIV-1) RNA copies/ml of plasma. Flow cytometric analysis using fluorescein isothiocyanate-conjugated anti-CD3–phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD3–phycoerythrin-conjugated anti-CD8 (Simultest; Becton Dickinson, Palo Alto, Calif.) was used to assess absolute CD4⁺ and CD8⁺-T-cell counts. Complete white blood cell counts were done on whole blood with an Advia 120 instrument (Bayer, Tarrytown, N.Y.).

Cells. PBMCs were isolated from blood by density gradient centrifugation (Ficoll-Paque; Pharmacia, Uppsala, Sweden) and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Mo.) with 90% fetal calf serum (FCS; Canadian Life Technologies, Burlington, Ontario, Canada).

HLA. Subjects were typed for major histocompatibility complex (MHC) class I antigen expression by amplification refractory mutation system-PCR with 95 primer sets amplifying defined MHC class I alleles (ABC SSP Unitray; Pel-Freez Clinical Systems, Brown Deer, Wis.) (11). Genomic DNA for molecular HLA typing was prepared from either fresh blood or Epstein-Barr virus-transformed B-cell lines by using a QIAamp DNA blood kit (QIAGEN, Inc., Mississauga, Ontario, Canada).

Design of peptide pool matrices. The HIV peptide sets used for stimulation were 15 amino acids (aa) with 11-aa overlaps (Gag, Env, Nef, Tat, Rev, Vpr, Vpu, and Vif) or 20 aa with 10-aa overlaps (Pol). The peptides were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, Md.). Lyophilized peptides (*n* = 620) spanning all HIV-1 gene products were dissolved to a final concentration of 10 mg/ml in DMSO and stored at -70°C . These samples included 100 Pol 20-mers and 122 Gag 15-mers corresponding to the HIV-1_{HXB2} clade B isolate; 49 Nef, 27 Rev, 23 Tat, 46 Vif, 22 Vpr, and 19 Vpu 15-mers corresponding to consensus HIV (Vpu) or consensus clade B sequence (Nef, Rev, Tat, Vif, and Vpr); and 212 Env 15-mers corresponding to the HIV_{MN} clade B isolate. Pools containing 2 to 15 peptides were prepared and organized into matrices of Gag, Pol, Nef, Env, and accessory gene peptide pools such that each peptide was present in two pools within each peptide matrix.

Matrix assay for single-cell IFN- γ release. IFN- γ secretion by HIV-specific cells was quantified by using an ELISPOT assay. Cells were resuspended in RPMI containing 10% FCS, 2 mM L-glutamine (ICN Biomedicals, Costa Mesa, Calif.), 50 μ M β -mercaptoethanol (Sigma-Aldrich), 50 IU of penicillin/ml, and 50 μ g of streptomycin (ICN Biomedicals)/ml. Cells (70,000 to 100,000) were plated in 96-well polyvinylidene difluoride-backed plates (MAIPS 45; Millipore, Bedford, Mass.) precoated overnight at 4°C with 100 μ l of the anti-IFN- γ monoclonal antibody 1-DK (5 μ g/ml; Mabtech, Stockholm, Sweden). PBMCs were stimulated with peptide pools such that the final concentration of each peptide within a pool was 2 to 4 μ g/ml. Medium alone with concentrations of DMSO equivalent to that in peptide pools was used as a negative control, and anti-CD3 antibody (Research Diagnostics, Inc., Flanders, N.J.) was used as a positive control stimulus. A pool of 23 peptides (NIH AIDS Research and Reference Reagent Program), derived from cytomegalovirus, Epstein-Barr virus, and influenza virus, restricted to common MHC class I alleles, was also used as a positive control stimulus (14). Sixty-one percent of the individuals tested responded to this pool with an average magnitude of 342 spot-forming cells (SFCs)/ 10^6 PBMCs. The plates were incubated at 37°C in 5% CO_2 overnight. Cells were washed away with phosphate-buffered saline supplemented with 0.05% Tween 20. Spots corresponding to the footprint of IFN- γ -secreting cells were developed by sequential addition followed by washing with phosphate-buffered saline of biotinylated anti-IFN- γ monoclonal antibody 7-B6-1 (Mabtech) diluted to 0.5 μ g/ml for 3 h at room temperature (RT), alkaline phosphatase conjugated to streptavidin (Mabtech) at a dilution of 1:2,000 for 2 h at RT, and of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Bio-Rad Laboratories,

Hercules, Calif.) for 10 to 15 min at RT. Spots were counted with a stereomicroscope (Carl Zeiss, London, Ontario, Canada). Results are expressed as SFCs/ 10^6 PBMCs following the subtraction of negative controls. The criteria used to identify positive responses were obtained by testing 10 low-risk HIV-uninfected individuals with the same peptide pool matrix array. Uninfected individuals produced a mean of 18.3 ± 20.2 SFCs/ 10^6 PBMCs to the stimulatory peptide pools. A positive response was defined as being 3 standard deviations above that seen for uninfected subjects, i.e., >79 SFCs/ 10^6 PBMCs and at least threefold greater than the autologous negative control wells.

Confirmation of peptide specificity. The stimulatory capacity of candidate peptides identified in the peptide pool matrix ELISPOT assay was confirmed in a second experiment with cells from the same time point stimulated with individual peptides that were common to two stimulatory peptide pools in the initial ELISPOT screen. For single peptide verification experiments, cells were plated in triplicate with 4 μ g of individual candidate peptides/ml. The criteria used for the original peptide pool matrix experiment were also used for the verification assay to identify positive responses. Responses to two adjacent overlapping peptides were counted as a single response. The higher response of two adjacent overlapping peptides was used to estimate the total magnitude. Therefore, analyses that include measures of breadth are based on a minimum estimate, as it is possible that peptides and sequential peptides contain more than one epitope. CD8 depletion was performed by using immunomagnetic beads (Miltenyi Biotec, Auburn, Calif.) in order to determine the cell population responsible for the peptide-specific response. Ninety percent of the CD3⁺ CD8⁺ cell population was depleted, as determined by flow cytometry. Among 20 peptide-specific responses detected for three individuals, all were attributed to CD3⁺ CD8⁺ lymphocytes.

Statistical analysis. Statistical analysis and graphical presentation were performed by using GraphPad InStat 3.05 and Excel 2002. CD4⁺-T-lymphocyte slopes were obtained by fitting a linear regression model by the least-squares method for each patient. Analysis of variance (ANOVA) and Student's *t* test were used to assess differences in age, CD4⁺-T-cell count, CD8⁺-T-cell count, and viral load magnitude and breadth among the groups. Pearson's correlation analysis was used to study the relationships between the breadth and magnitude of HIV-specific responses and clinical parameters, such as viral load and rate of CD4⁺-T-cell decline. *P* values of less than 0.05 were considered significant.

RESULTS

Study population. Table 1 provides information on the 31 highly active retroviral therapy-naïve chronically infected subjects studied. At the time point used for screening for HIV-specific IFN- γ secretion, the study population was a median of 34 (range, 18 to 51) years old and had a median CD4⁺-T-cell count of 572 (range, 374 to 999) cells/ μ l, a median CD8⁺-T-cell count of 1,144 (range, 720 to 2,412) cells/ μ l, and a median viral load of 1,452 (range, 72 to 22,065) HIV-1 RNA copies/ml. Monthly absolute CD4⁺-T-cell count values taken over a median of 24 (range, 12 to 24) months were used to determine the rate of CD4⁺-T-cell loss for this population of a median of 57 (range, 0 to 177) cells/ μ l/year. From this study population, three subgroups were constituted based on their rates of CD4⁺-T-cell decline. Subjects in the first subgroup were designated typical progressors (*n* = 17), those in the second subgroup were designated fast progressors (*n* = 6), and those in the third subgroup were designated slow progressors (*n* = 8) (Table 2). The rate of CD4⁺-T-cell loss in the slow progressor

group was not significantly different from that seen in low-risk control HIV-uninfected subjects ($n = 14$) monitored for a median time of 36 (range, 24 to 108) months and who lost a median of 7.9 (range, 0 to 58) cells/ μ l/year (P values were not significant [NS]; Student's t test). All subgroups had a similar median age, CD4⁺-T-cell count, CD8⁺-T-cell count, and viral load at the time points tested (P values was NS; ANOVA). CD4⁺-T-cell slopes for the follow-up period after testing differed significantly among the three groups ($P < 0.001$; ANOVA) (Table 2).

Characteristics of HIV-specific IFN- γ secretion. A peptide pool matrix IFN- γ ELISPOT assay was used to screen PBMCs from the 31 treatment-naïve subjects in the chronic phase of infection for HIV-specific immune responses to all expressed HIV genes. Of the 620 peptides used for screening, 104 stimulated a response. The position of the 104 peptides (35 Gag, 19 Pol, 19 Nef, 16 Acc, and 15 Env) with respect to their location on the HIV sequence is shown in Fig. 1A. The median number of peptides recognized by this study population was 5 (range, 1 to 11), with a median cumulative magnitude of 1,933 (range, 100 to 9,176) SFCs/ 10^6 PBMCs (Fig. 1B). The relative contribution of each gene product to the total breadth and magnitude is shown in Fig. 1C and D, respectively. Determination of these values took into consideration the relative length of each gene product. Gag was the gene product most frequently recognized, with 48% of all responses and 53% of the cumulative magnitude targeting this protein (Fig. 1C and D). The magnitude and breadth of the responses targeting Gag were significantly higher than those for all other gene products ($P < 0.001$; ANOVA). Among Gag subunits, p24- and p17-derived peptides contributed most of the stimulatory capacity attributed to the Gag gene product. All 31 individuals responded to at least one Gag peptide (Fig. 1B). Nef was the second most frequently recognized protein; 19% of all responses detected were directed at Nef-derived peptides, whereas 21% of the cumulative magnitude targeted this gene product (Fig. 1C and D).

Rate of CD4 decline and viral load does not correlate with the breadth and magnitude of HIV-specific IFN- γ -secreting responses. Because at least 12 months of clinical follow-up information was available for this study population after the time of immune response assessment, we were able to determine whether either the breadth or the magnitude of HIV-specific IFN- γ secretion correlated with the subsequent rate of CD4⁺-T-cell decline. No statistically significant correlation was observed between clinical parameters, such as the rate of CD4⁺-T-cell decline or viral load, and the breadth or the magnitude of the immune responses to HIV (Fig. 2A to D) (P values were NS for all correlations; Pearson's correlation coefficient). Furthermore, no significant correlations were found between these clinical parameters and the breadth or magnitude of responses directed to individual HIV gene products (data not shown).

Analysis of HIV-specific IFN- γ -secreting responses in three groups stratified by rate of CD4 decline. Additional comparisons were made among three subgroups of the study population, which exhibited different rates of CD4⁺-T-cell loss. No differences among the groups in the magnitude or breadth of HIV-specific responses to all expressed HIV genes or to individual HIV gene products were detected (P values were NS for all comparisons; ANOVA) (Fig. 3A and B).

DISCUSSION

We present here data on total HIV-specific immune responses measured by IFN- γ ELISPOT for 31 treatment-naïve subjects in the chronic phase of infection for whom a median of 24 (range, 12 to 24) months of follow-up information is available. We found that neither the breadth nor the magnitude of HIV-specific responses correlated with viral loads or with CD4⁺- or CD8⁺-T-cell counts at the time points tested. All subjects remained untreated for up to 24 months from the time they were screened for immune responses, during which monthly CD4⁺-T-cell counts were assessed. We used these serial CD4⁺-T-cell counts to calculate the rate of CD4⁺-T-cell loss during the 12 to 24 months immediately after the immune screening. The slope of CD4⁺-T-cell decline is a powerful surrogate marker for the prediction of disease progression rates (43). We found that neither the breadth nor the magnitude of HIV-specific IFN- γ secretion correlated with the rate of CD4⁺-T-cell decline. Slow, typical, and rapid progressors were selected from the study population based on rates of CD4⁺-T-cell loss and compared by the above immune response parameters. No significant differences among the groups with respect to breadth or magnitude of HIV-specific IFN- γ secretion were seen, suggesting that this function of HIV-specific cells did not play a strong role in modulating the rate of disease progression.

The rate of disease progression in individuals infected with HIV-1 is highly variable. The median time from infection to AIDS is 8 to 10 years, and the average annual decline in CD4⁺-T-cell count is 60 cells/ μ l/year (10, 22). However, time to AIDS can vary from as few as 2 to 3 years to 15 or more years (10). Previous studies have demonstrated the prognostic value of plasma viral loads, absolute CD4⁺-T-cell counts, and immune activation as predictors of HIV disease progression rate (42, 43, 48). However, the use of HIV-specific immune responses as prognostic markers for disease outcome has not been addressed in the past.

At the time the HIV-infected population studied here was assessed for HIV-specific immune responses, the subjects were fairly homogeneous and healthy in terms of CD4 count and viral load. Only five subjects had CD4 counts below 500, and four had viral loads of over 10,000 HIV copies/ml of plasma. Despite these characteristics, we were able to identify three subpopulations based on the rates of CD4⁺-T-cell loss; these subpopulations did not differ from one other in CD4 counts and viral loads at the initial immune response sampling date. In this population, the effects of viral load set point, CD4 count, and antiretroviral therapy on disease progression were minimized. Should the breadth and magnitude of HIV-specific IFN- γ secretion have an effect on CD4⁺-T-cell decline, it would be expected to be easier to detect in such a population.

CTLs specific for MHC class I restricted viral epitopes are thought to be crucial for controlling several viral infections, including HIV (8, 35, 36, 44, 56, 57, 59, 68). The evidence for this idea comes from SIV-infected macaque models, where depletion of CD8⁺ T cells results in uncontrolled viremia. In addition, mutation in CTL epitopes leads to escape from CTL control and vaccination strategies that induce CTL slow disease progression once macaques become infected with a pathogenic challenge virus (2, 3, 5, 6, 31, 59, 60). The temporal

FIG. 1. Survey of HIV-specific IFN- γ -secreting responses in 31 untreated subjects during chronic HIV infection. (A) Location of each stimulatory peptide on a map of the HIV genome. The y axis shows the percentage of individuals recognizing each stimulatory peptide. The sequence of frequently recognized peptides is shown. (B) Cumulative frequency of HIV-specific T-cell responses per million PBMCs for each individual tested. Each stack in the bar represents the number of SFCs/10⁶ PBMCs recognizing separate HIV gene products. The number over each bar is the number of peptides derived from all expressed HIV-1B genes that stimulated above-background levels of IFN- γ secretion. (C) Relative contributions of individual HIV gene products to the cumulative breadth of the HIV-specific response. (D) Relative contributions of individual HIV gene products to the cumulative magnitude of the HIV-specific response.

association between induction of CTLs and reduction in viral load in primary infection and the observation that disease progression rates are associated with certain MHC class I alleles or grouping of these alleles into supertypes supports a role for CTLs in controlling HIV in humans (8, 32, 35, 62). However, CD8⁺ T cells have multiple antiviral functions, including CTL activity and secretion of factors such as IFN- γ ,

chemokines, and other molecules that modulate HIV spread (4, 30, 38, 65). The relationship between lytic activity and IFN- γ secretion was established at the level of single cells that secrete IFN- γ upon antigen stimulation and also develop into cells with lytic activity (16). Other studies have also reported associations between these two functions, which led to the adoption of the IFN- γ ELISPOT assay as a high-throughput

FIG. 1—Continued.

quantitative methodology for enumerating and characterizing HIV-specific effector activity (25). However, the correlation between lytic and IFN- γ -secreting functions of CD8⁺ T cells has been questioned recently. The distribution of HIV-specific memory T-cell subsets in HIV-infected individuals is skewed compared to those recognizing non-HIV antigens, such as cytomegalovirus. The development of terminally differentiated CD45RA⁺ CCR7⁻ effector cells appears to be blocked at the CD45RA⁻ CCR7⁻ memory effector stage. Although cells in both of these memory compartments secrete IFN- γ , only the terminally differentiated cells produce high levels of perforin, a marker for

cells with lytic capacity (13). The discordance between IFN- γ secretion and the ability to lyse HIV-infected target cells efficiently in preterminally differentiated memory cells, which are the predominant effector population in untreated chronically HIV-infected subjects, may contribute to the lack of an association between the breadth and magnitude of HIV-specific IFN- γ secretion and rate of CD4⁺-T-cell loss. This conclusion is further supported by the observation that slow progressors do not have higher and broader HIV-specific IFN- γ -secreting responses than do typical or rapid progressors.

Because assays measuring lytic activity are cumbersome and

FIG. 2. Correlation between the magnitude and breadth of HIV-specific responses with rate of CD4⁺-T-cell decline and plasma viral load among 31 chronically infected individuals. The associations between plasma viral load and cumulative magnitude (A), plasma viral load and cumulative breadth (B), the rate of CD4⁺-T-cell decline and cumulative magnitude (C), and the rate of CD4⁺-T-cell decline and cumulative breadth (D) of HIV-specific responses are shown.

do not lend themselves to screening for responses to large numbers of peptides in a high-throughput format, such as an IFN- γ ELISPOT assay, quantitative measures of lytic activity have not been done on large study populations. Although HIV-specific CTLs are believed to control viremia, IFN- γ secretion may not be the most potent mechanism through which this control is mediated. During chronic viral infections, such as lymphocytic choriomeningitis virus infection in mice, an antigen dose-driven gradual impairment of T-cell function occurs. Secretion of effector molecules, such as IL-2, TNF- α , and IFN- γ , is progressively lost, with IFN- γ being the most resistant to exhaustion (63, 64). Lichterfeld et al. have shown that HIV-specific cytotoxicity is best mediated by a subset of CD8⁺ T cells which maintains secretion of both TNF- α and IFN- γ (39). Furthermore, several studies have suggested that maintenance of HIV-specific IL-2 secretion and proliferation are associated with viral control in long-term nonprogressors and individuals who start effective highly active antiretroviral therapy in early infection (29, 49, 66). Since IFN- γ secretion is the function of CD8⁺ T cells that is maintained longest in the context of HIV viremia, it is likely to detect the broadest range

of peptide-specific responses. However, as shown here, the breadth and magnitude of HIV-specific IFN- γ secretion is not associated with viral control and disease progression.

Others have used a similar comprehensive screening approach to test for HIV-specific IFN- γ secretion in HIV-infected individuals (1, 7, 15, 19, 45, 51). These studies were ideal for obtaining a global picture of immune responses to HIV recognized in Caucasian and non-Caucasian populations infected with clade B or C virus. They were also suited to identifying the regions of HIV that are targeted in the largest cross-section of the populations studied and the features of HIV sequence that are likely to harbor immunogenic epitopes (19, 67). Although data on viral load and CD4⁺-T-cell counts were presented at the time point studied, follow-up information was not available. Therefore, no conclusion could be drawn as to the predictive power of HIV-specific IFN- γ secretion for disease progression. Furthermore, any correlations observed between immune responses and viral load cannot be used to infer a relationship between these immune responses and the rate of disease progression, as viral load set point is only one factor that contributes to HIV disease progression.

FIG. 3. Comparison of the breadth and magnitude of HIV-specific responses between typical, fast, and slow progressors. Seventeen typical progressors, six fast progressors and eight slow progressors were screened for HIV-specific T-cell responses. The frequency (A) and breadth (B) of HIV-specific responses, classified by the rate of disease progression, are shown for each individual tested. The mean frequency and breadth of HIV-specific T-cell responses for each group is represented by a line.

Both direct viral cytopathic effects and indirect effects of viral replication, such as immune activation, contribute to the rate of CD4⁺-T-cell decline and immunologic progression (26). Studies of two natural hosts of SIV, sooty mangabeys and African green monkeys, illustrate the dichotomy between viral load set point and disease progression. Despite high viremia, they exhibit low levels of immune activation and do not progressively lose CD4⁺ T cells (9). Additional observations of patients infected with HIV-1 and HIV-2 have been made. Patients infected with either virus displayed similar degrees of CD4 depletion and immune activation despite significant differences in plasma viral load (61). Taken together, these results show that viremia contributes only partially to the subsequent degree of CD4⁺-T-cell loss.

Our results confirm those generated by others which showed that HIV Gag and Nef are the most frequently targeted HIV gene products in chronically infected subjects (1, 15, 19). Furthermore, similar to the results of these studies, we did not find a correlation between the detected immune responses and viral load. In one study, longitudinal data for clinical follow-up was available and immune response testing was performed at the end of the follow-up period. In this case, conclusions were drawn on the combined effect of the rate of CD4⁺-T-cell decline and viral load on the breadth and magnitude of HIV-specific IFN- γ secretion (54). The present report is the first that comprehensively screened for HIV-specific IFN- γ responses in a group of chronically infected, treatment-naïve subjects monitored for up to 24 months after determination of immune response parameters. We conclude that although HIV-specific IFN- γ -secreting responses are present in all of those tested, neither their breadth nor their magnitude correlates with viral control or the rate of CD4⁺-T-cell decline.

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