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J Immunol (2004) 173 (8): 5121–5129.
https://doi.org/10.4049/jimmunol.173.8.5121

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The Role of CTLs in Persistent Viral Infection: Cytolytic Gene Expression in CD8$^+$ Lymphocytes Distinguishes between Individuals with a High or Low Proviral Load of Human T Cell Lymphotropic Virus Type 1

Alison M. Vine,[1]* Adrian G. Heaps,[1]* Lambrini Kaftantzi,* Angelina Mosley,* Becca Asquith,* Aviva Witkover,* Gillian Thompson,* Mineki Saito,* Peter K. C. Goon,* Laura Carr,‡ Francisco Martinez-Murillo,‡ Graham P. Taylor,† and Charles R. M. Bangham[2]*

The proviral load in human T cell lymphotropic virus type 1 (HTLV-1) infection is typically constant in each infected host, but varies by >1000-fold between hosts and is strongly correlated with the risk of HTLV-1-associated inflammatory disease. However, the factors that determine an individual's HTLV-1 proviral load remain uncertain. Experimental evidence from studies of host genetics, viral genetics, and lymphocyte function and theoretical considerations suggest that a major determinant of the equilibrium proviral load is the CD8$^+$ T cell response to HTLV-1. In this study, we tested the hypothesis that the gene expression profile in circulating CD8$^+$ and CD4$^+$ lymphocytes distinguishes between individuals with a low proviral load of HTLV-1 and those with a high proviral load. We show that circulating CD8$^+$ lymphocytes from individuals with a low HTLV-1 proviral load overexpressed a core group of nine genes with strong functional coherence: eight of the nine genes encode granzymes or other proteins involved in cell-mediated lysis or Ag recognition. We conclude that successful suppression of the HTLV-1 proviral load is associated with strong cytotoxic CD8$^+$ lymphocyte activity in the peripheral blood. ***The Journal of Immunology,*** **2004, 173: 5121–5129.**

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Human T lymphotropic virus type 1 (HTLV-1)[3] is an exogenous human retrovirus that causes two distinct types of disease: adult T cell leukemia (1) and a range of chronic inflammatory diseases, most notably HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (2, 3). In vivo HTLV-1 chiefly infects CD4$^+$ T cells; ~10% of the proviral load is carried by CD8$^+$ cells (4, 5).

Until recently HTLV-1 was thought to be transcriptionally silent in most naturally infected lymphocytes. However, this conclusion was inconsistent with a number of observations. First, in most HTLV-1-infected individuals there are abundant, chronically activated CTL in the circulation, specific to HTLV-1 Ags (6–8), suggesting that there is persistent HTLV-1 Ag expression in vivo. Second, in some individuals HTLV-1 is subject to positive selection in vivo (9): such selection would not be evident in the absence of HTLV-1 replication. In addition, there is now evidence that lymphocytes naturally infected with HTLV-1 contain persistently replicating virus (10); spontaneously express HTLV-1 Ags ex vivo (5, 11); and spontaneously transmit complexes of HTLV-1 Gag proteins and the viral genome to other cells via a "viral synapse" (12).

The proviral load of HTLV-1 is typically high, often between 0.1 and 15% of PBMCs. In one infected person, the proviral load of HTLV-1 is usually stable over time within a factor of 2–4, but this equilibrium value or "set point" of proviral load can differ by >1000 times between infected subjects. As in other persistent viral infections such as HIV-1 and hepatitis C virus (HCV) infection, the risk of disease in HTLV-1 infection is strongly correlated with the set point of the proviral load (13). However, the factors that determine this set point in each individual are not well understood. In particular, it has been difficult to establish whether the cell-mediated immune response plays a material part in controlling the replication rate and therefore the set point of both HTLV-1 and other persistent viruses. HTLV-1 has certain advantages over other viruses in the study of the dynamics and the role of the immune response in persistent viral infections. Unlike HIV-1, infection with HTLV-1 does not cause severe immune suppression (in the absence of leukemia), and the proviral load of HTLV-1 is more stable over time than the plasma viral load of HIV-1. Moreover, the frequency of HTLV-1-specific T cells (both CD4$^+$ and CD8$^+$) is typically higher than the frequency of HCV-specific T cells (14), and the chief host cell infected by HTLV-1 is accessible in the peripheral blood, unlike the hepatocytes infected by HCV.

CD8$^+$ T cells are essential for the elimination of most acute viral infections, but their importance in persistent viral infections remains uncertain. HTLV-1-specific CD8$^+$ T cells are typically abundant and chronically activated (reviewed in Refs. 8 and 15). The dominant Ag recognized is the viral transactivator protein Tax (6, 7, 16, 57). We therefore suggested (17) that the strong CD8$^+$ T cell response to Tax caused the observed positive selection on the *tax* gene (9). This hypothesis was later supported by two observations. First, possession of either of two class 1 HLA alleles,

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Departments of *Immunology and †Infectious Diseases, Wright-Fleming Institute, Imperial College, Norfolk Place London United Kingdom; and ‡Johns Hopkins Medical Institute Microarray Core, Broadway Research Building, Johns Hopkins University, 733 North Broadway, Baltimore, MD 21205

Received for publication March 31, 2004. Accepted for publication August 17, 2004.

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[1] A.M.V. and A.G.H. contributed equally to this work.

[2] Address correspondence and reprint requests to Dr. Charles R. M. Bangham, Department of Immunology, Wright-Fleming Institute, Imperial College, Norfolk Place, London W2 1PG, United Kingdom. E-mail address: c.bangham@imperial.ac.uk

[3] Abbreviations used in this paper: HTLV-1, human T cell lymphotropic virus type 1; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; HCV, hepatitis C virus; AC, asymptomatic carrier; qPCR, quantitative PCR.

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0022-1767/04/$02.00

*HLA-A*02* or *HLA-Cw*08*, was correlated with a lower proviral load of HTLV-1 and a lower risk of HAM/TSP in an endemically infected population in Japan (18–20). This dominant protective effect associated with HLA class 1 alleles suggested that class 1-restricted T cells play an important part in controlling HTLV-1 proviral load in vivo. Second, $CD8^+$ T cells efficiently killed autologous Tax-expressing lymphocytes in fresh PBMCs (5, 11).

The observation that the frequency of HTLV-1-specific $CD8^+$ T cells is positively correlated with the proviral load of HTLV-1 in both patients with HAM/TSP (21) and asymptomatic carriers (ACs) (21, 22) raises the question whether the frequency of these cells play an active role in controlling HTLV-1 proviral load, or whether the frequency merely passively reflects the proviral (Ag) load. A theoretical model (23) showed that an individual with an "efficient" $CD8^+$ T cell response to a persistently replicating virus would have a lower set point of viral load than an individual with an inefficient T cell response, but that the equilibrium frequency of virus-specific $CD8^+$ T cells could be approximately the same in the two individuals. In this model (23), the "efficiency" of the $CD8^+$ T cell response was defined by two parameters: the rate of proliferation of specific $CD8^+$ T cells in response to a given Ag load and the rate at which these cells killed virus-infected target cells.

The roles of $CD4^+$ T cells and NK cells in HTLV-1 infection have been less studied. The frequency of HTLV-1-specific $CD4^+$ T cells appears to be significantly greater in patients with HAM/TSP than in ACs with the same proviral load (24, 25). This observation raises the possibility that such $CD4^+$ T cells contribute to the pathogenesis of the inflammatory disease; their role in defense against HTLV-1 infection is not known. Both the frequency and the activity of NK cells appear to be significantly lower in patients with HAM/TSP than in ACs (26, 27), but again the functional significance of these observations is incompletely understood.

In this study, we used DNA microarray analysis to test the hypothesis that a difference between individuals in the proviral load of HTLV-1 is reflected in the gene expression profile of circulating T lymphocytes.

Materials and Methods

Subjects and cells

Fifty milliliters of peripheral venous blood was obtained from HTLV-1-infected ACs, ethnically matched uninfected controls, and uninfected laboratory control subjects, with fully informed consent. This study was approved by the St. Mary's Hospital Local Research Ethics Committee. Genomic DNA was extracted and the proviral load was assayed by real-time PCR as previously described (28). ACs were placed into one of two groups: 1) high viral load ACs, AC^{high} (>1 proviral copy/100 PBMCs) and 2) low viral load ACs, AC^{low} (<0.1 proviral copy/100 PBMCs). Individuals with an intermediate HTLV-1 proviral load (0.1–1 proviral copies/100 PBMCs) were omitted from the study to increase the power of the study to detect a systematic difference in gene expression between subjects with a high load of HTLV-1 and those with a low load. This procedure is directly analogous to extreme discordant sibling pair analysis in genetic studies of quantitative traits (29). Patients with HAM/TSP were similarly classified as either low proviral load or high proviral load. Note that, since the proviral load is typically higher in HAM/TSP patients than in ACs, the mean proviral load in the HAM^{low} group exceeded the mean proviral load in the AC^{high} group (Table I). After positive selection of $CD4^+$ and $CD8^+$ cells, total RNA from individual patients was pooled into the above groups for microarray hybridization. A total of 29 individuals were studied: 10 patients with HAM/TSP, 10 asymptomatic HTLV-1 carriers, and 9 uninfected controls (Table I). In each of three main experiments (Table I), we compared gene expression in $CD4^+$ and $CD8^+$ cells, respectively, between individuals with a high proviral load of HTLV-1, individuals with a low proviral load, and HTLV-1-seronegative controls. Each experiment therefore involved the use of six microarray chips.

Cell separation and RNA processing

PBMCs were isolated from whole blood using a Ficoll-Histopaque gradient (Sigma-Aldrich, Dorset, U.K.). $CD4^+$ and $CD8^+$ cells were positively selected on a MACS column (Miltenyi Biotec, Surrey, U.K.) using magnetic microbeads according to the manufacturer's instructions. Purity was confirmed by flow cytometry to be >90% in most cases. Total RNA was extracted from freshly isolated cells using the RNeasy Method (Qiagen, West Sussex, U.K.). RNA was reverse-transcribed using an HPLC-purified oligo(dT) primer with a T7 RNA pol site attached to the 5′ end (Transgenomic Bioconsumables, Glasgow, U.K.), with the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen Life Technologies, Paisley, U.K.).

Table I. *Summary of characteristics of three experiments*

Expt.	T Cell Phenotype	Array	Subject Group[a]	Anonymized Codes of Individual Subjects (RNA pooled on the array)	Mean (SEM) Proviral Load (copies/100 PBMCs)	No of Genes in Array That Pass: Expression threshold filter (raw intensity > 1200)	No of Genes in Array That Pass: 2-fold expression ratio filter (normalized data)[b]
AC1	$CD4^+$	01	AC^{high}	HT, HS, HAY	3.83 (2.05)	4224	252
		02	AC^{low}	HAK, HAO, HAE, HX	0.05 (0.02)		
		03	Un	U1, U2, U3	—		
	$CD8^+$	04	AC^{high}	HT, HAW, HS	1.83 (0.29)		
		05	AC^{low}	HAK, HAO, HAE	0.07 (0.01)		
		06	Un	U1, U2, U4	—		
AC2	$CD4^+$	07	AC^{high}	HT, HS, HAY	4.10 (1.51)	570	102
		08	AC^{low}	HX, HY, HBD, HAE	0.02 (0.02)		
		09	Un	UA, UB, UC, UD, UE	—		
	$CD8^+$	10	AC^{high}	HT, HS, HAY	4.10 (1.51)		
		11	AC^{low}	HAE, HY, HBD	0.03 (0.02)		
		12	Un	UA, UC, UD, UE	—		
HAM	$CD4^+$	13	HAM^{high}	TW, TBC, TAE	18.23 (11.8)	565	54
		14	HAM^{low}	TBB, TAA, TBA, TAU, TAC	7.02 (2.11)		
		09	Un	UA, UB, UC, UD, UE	—		
	$CD8^+$	15	HAM^{high}	TBC, TAE, TAL	16.23 (12.6)		
		16	HAM^{low}	TAW, TBB, TAA, TAU, TAC	5.44 (2.15)		
		12	Un	UA, UC, UD, UE	—		

[a] AC, HTLV-1^+ AC; HAM, HAM/TSP patient; Un, uninfected control. Uninfected controls U1–U4 were not ethnically matched with HTLV-1-infected subjects; all other uninfected controls were ethnically matched.
[b] Used in cluster analysis.

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In experiment AC1, individual cDNAs were used in an in vitro transcription reaction in the presence of biotinylated nucleotides (Enzo Bioarray High Yield RNA transcript labeling kit; Enzo Diagnostics, Farmingdale, NY) and T7 polymerase. The cRNAs were then pooled into their appropriate groups. The same procedure was used in experiment AC2, except that the samples were pooled at the cDNA stage before the in vitro transcription. The resulting biotinylated cRNAs were cleaned twice with an RNeasy Mini kit (Qiagen).

cRNA hybridization and scanning

These procedures were conducted at Research Genetics (Birmingham, AL) and the Johns Hopkins Medical Institute Microarray Core Hybridization Unit (Baltimore, MD) following standard Affymetrix protocols (Affymetrix, San Jose, CA). Labeled cRNA was fragmented (94°C for 35 min) and then hybridized to the Human U95A GeneChip array (http://www.affymetrix.com/support/technical/datasheets/hgu95_datasheet.pdf) (45°C for 16 h) with constant rotation at 60 rpm. The chips were then washed using the Affymetrix Fluidics Station 400 to remove nonhybridized target, followed by incubation with a streptavidin-PE conjugate to stain labeled cRNA. Staining was then amplified with goat IgG as a blocking reagent and biotinylated anti-streptavidin Ab (goat), followed by a second staining step with streptavidin-PE conjugate. Genechip image analysis was performed through fluorescence detection using an Agilent GeneArray scanner and Affymetrix Microarray Suite (MAS) version 5.0.

Data analysis

The fluorescence intensity of each probe set was captured from the scanned images using Affymetrix MAS 5.0 Genechip software according to the manufacturer's instructions. The data were then converted to text (.txt) format and exported in Affymetrix Pivot table format into GeneSpring version 5.0.2 (Silicon Genetics, CA), which was used for analysis.

Data normalization

To conduct comparisons between different experimental conditions and to eliminate variation caused by the measurement process, two successive stages of normalization were used: 1) per chip: each measurement was divided by the 50th centile of all measurements in that sample and 2) per gene: each gene was divided by the median of its measurements in all samples.

Raw intensity measurements below 0 were set to 0. Other methods of normalization were also tested, including normalization to positive and negative control genes and normalization to the expression level in the control samples. Normalization was also applied using the Bioconductor (http://www.bioconductor.org) "affy" package (31, 51) using the RMA (robust multichip average) expression. Details of the normalization techniques applied by RMA and MAS5.0 are compared in the study by Bolstad et al. (30).

Gene filtering

Initially, raw data were filtered to remove transcript profiles where no significant gene expression was apparent using a minimum intensity value of 1200 in at least one of the six chips in each respective experiment. Data were filtered again to select genes whose normalized expression ratio varied by 2-fold or more between any of the six chips.

Cluster analysis

K-means clustering (32) was used to identify similar expression profiles within the filtered gene lists. The Pearson correlation coefficient was used to define distance in the clustering analysis. Each filtered gene list was extensively clustered using various cluster group sizes and numbers of iterations. Filtered gene profiles were also grouped with Pearson and Gaussian clustering techniques using ArrayMiner 3.2 (Optimal Design, Brussels, Belgium).

Genes were selected for further investigation only if they were flagged "P" (present) and passed both filters in at least three of the six microarrays in the respective experiment.

Quantitative PCR

Gene-specific primers (20–21 bases long) were designed using sequences deposited in GenBank and obtained from Transgenomic Bioconsumables (Galsgow, UK) and Invitrogen Life Technologies (Paisley, U.K.). PCR primer sequences are available from the authors on request. Preliminary experiments were done with each primer pair and reference cDNA to determine the annealing temperature that gave the greatest yield of specific product with a melting temperature (T_m) distinct from that of the primer dimer.

Dilutions of the reference cDNA (1/100, 1/500, 1/2,500, 1/12,500, 1/62,500) were used to construct a standard curve. Real-time kinetic PCR was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I kit as recommended by the manufacturer. The cDNA templates were the same preparations that were used (after pooling) for in vitro transcription and microarray analysis. However, quantitative PCR (qPCR) was conducted on the individual (unpooled) cDNAs to verify that pooling of samples did not introduce artifacts. Amplification was conducted in a total volume of 20 μl containing primers at 1.0 μM final concentration, 1.6 mM $MgCl_2$, 2 μl of LightCycler FastStart Reaction SYBR Green I mix (containing FastStart TaqDNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and 10 mM $MgCl_2$; Roche Diagnostics), 12.4 μl of H_2O, and 2 μl of the respective cDNA dilution.

The amplification program consisted of 1 cycle of 95°C with a 600-s hold ("hot start") followed by 45 cycles as follows: 95°C (denaturation) for 10 s; specified annealing temperature (ranging from 60 to 68°C depending on the GC content of each primer pair) for 5 s; 72°C (extension) for t seconds, where $t = L/25$ and $L =$ length of amplicon in bp. All temperature transitions were made at 20°C/s. Fluorescence was acquired after heating at 20°C/s to a temperature 4–6°C below the product melting temperature and holding for 2 s.

Melting curve analysis of amplification products was performed at the end of each PCR by cooling the samples to a temperature between 65 and 72°C (usually 5°C higher than the annealing temperature at the amplification program) and then increasing the temperature to 95 at 20°C/s. Fluorescence was acquired every 0.1 s. Duplicate reactions were prepared for each dilution and a negative control without cDNA template (H_2O control) was run with every assay to assess the overall specificity. Unless otherwise mentioned, each assay was repeated once.

Results

The phenotype we sought to correlate with gene expression was the proviral load of HTLV-1. To accentuate the distinction between subjects with a high proviral load and those with a low proviral load, we selected ACs of HTLV-1 in whom two separate assays of proviral load were <0.1% PBMCs (AC^{low}) or >1.0% PBMCs (AC^{high}), omitting those with an intermediate proviral load. Patients with HAM/TSP were similarly divided into HAM^{high} and HAM^{low} groups (Table I; see *Materials and Methods*). To minimize the effects of individual variation, we pooled the nucleic acid from three to four individuals in each group before hybridization to the microarray (see *Materials and Methods*).

Three main experiments were done. In the first experiment (AC1), we compared three groups of subjects: ACs with a low proviral load of HTLV-1, ACs with a high proviral load, and uninfected control subjects (not matched for ethnic origin). In experiment 2 (AC2), we tested whether the results obtained in asymptomatic HTLV-1 carriers were reproducible. Since certain subjects were not available for resampling, some new subjects were included in experiment AC2. The control group in experiment AC2 consisted of ethnically matched subjects (of Afro-caribbean origin). In experiment 3 (HAM), we compared HAM/TSP patients with a high proviral load and HAM/TSP patients with a low proviral load, with the ethnically matched uninfected controls. Table I shows the number, coded identity, and mean proviral loads of subjects in each group.

In each experiment, pooled RNA from $CD4^+$ and $CD8^+$ cells of each of the subject groups (high proviral load, low proviral load, uninfected controls) was hybridized to Affymetrix U95A microarrays. A summary of the procedure used to analyze the data is shown in Fig. 1. K-means cluster analysis was performed on genes whose expression level (i.e., fluorescence intensity on the array) exceeded a threshold value of 1200 (in raw data) and whose normalized intensity varied by ≥2-fold between at least two of the six arrays in each experiment. Selection on these criteria identified between 54 and 252 genes in the three respective experiments (Table I, right-hand column).

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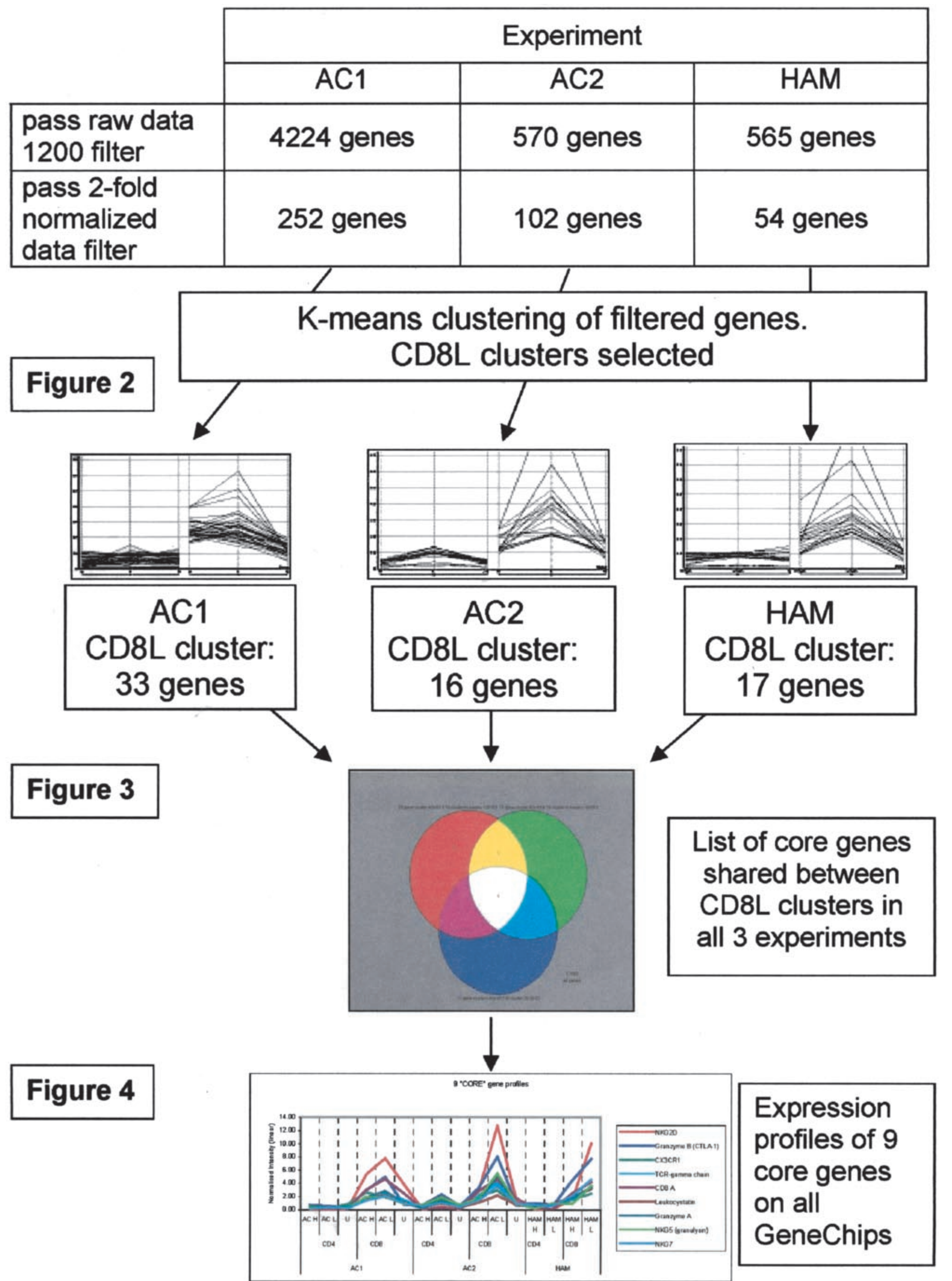

FIGURE 1. Schematic representation of analysis of data. In each experiment, data were analyzed from six microarrays. The numbers of genes that passed each successive stage of the analysis in each experiment are shown; further details of each stage are given in the respective figure legends (see also *Materials and Methods*).

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K-means cluster analysis revealed a small group of genes up-regulated in $CD8^+$ cells in subjects with a low proviral load

First, to determine the optimal number of clusters, K-means cluster analysis was conducted on the filtered and normalized data with either 3, 5, 9, 15, 30, or 60 clusters. The results (not illustrated) showed that approximately nine clusters accounted for the maximum proportion of observed variation in gene expression profile. To ensure that the results were robust, we compared the results of cluster analysis using either the selected genes alone or all expressed genes (Fig. 1). We also compared different methods of normalization of gene expression intensities, different clustering algorithms (using different software packages) and metrics, and different numbers of clusters (see *Materials and Methods*).

Following cluster analysis, a single cluster appeared in each experiment with a consistent profile of mRNA expression levels across the six arrays, regardless of the algorithm used for the clustering, the method of normalization and the number of genes included. This expression profile revealed selective up-regulation of a cluster of genes in the $CD8^+$ cells of subjects with a low proviral load. We refer to this as the "CD8L" ($CD8^+$ low load) cluster. The numbers of genes shared between the respective CD8L clusters in each of the three experiments are shown in Fig. 3. The expression intensities of genes in the CD8L cluster in uninfected control subjects were uniformly low in both the ethnically matched and non-ethnically matched control subjects (Fig. 2).

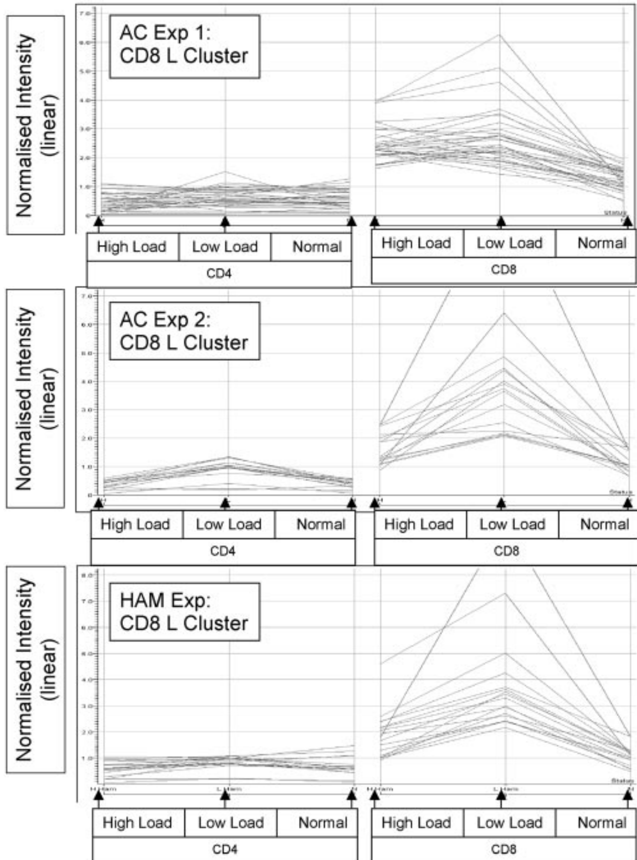
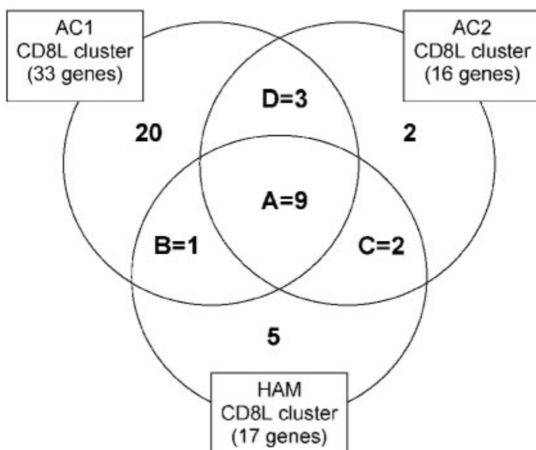


FIGURE 2. Normalized expression intensity of genes in the CD8L cluster in each experiment. The results show uniformly low expression of the CD8L genes in the $CD4^+$ cells. In the $CD8^+$ cells, there was a characteristic rank order of expression intensity: highest in individuals with a low proviral load of HTLV-1, intermediate in those with a high proviral load, and lowest in the uninfected control subjects.

The size of the CD8L cluster varied between 17 and 40 genes, depending on the experiment, the algorithm, and the metric used in the cluster analysis. The results of a representative cluster analysis of the three experiments are shown in Fig. 2. Examination of the genes present in these three clusters revealed a core group of nine genes that were present in each respective cluster (Fig. 3).

The identities of genes in this core group and the genes shared between the CD8L clusters in two of the three experiments are shown in Fig. 3. Remarkably, four of the nine core genes encode proteins that are directly involved in cell-mediated lysis: granzyme A, granzyme H, granulysin, and NKG2D (KLRK1). NKG7, a protein of unknown function, is also present in the lytic granules of cytotoxic lymphocytes. Furthermore, two genes encode proteins concerned with T cell activation during Ag recognition: TCRγ and CD8α. The expression profiles of the core group genes in the three experiments are summarized in Fig. 4.

A strong functional coherence was also evident among the genes present in the CD8L clusters in two of the three experiments (Fig. 3, gene groups B–D). In particular, group C contained another two lysis-related genes, those for granzyme K and perforin; and group D contained the granzyme B precursor. The remaining two genes in these groups were CCL5 (RANTES; two probe sets, Fig. 3,



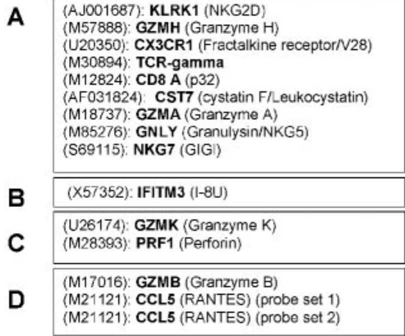


FIGURE 3. Venn diagram showing the number of genes that occurred in each CD8L cluster and the number and identity of genes that occurred in two or more CD8L clusters. In the three experiments, 27, 56, and 53% of genes present in the respective CD8L cluster occurred in this cluster in all three experiments: these shared genes are shown in the central white "core" group ($n = 9$ genes). In both this core group and the genes ($n = 6$) shared by two experiments (gene groups B–D), there was a strong predominance of genes that encode proteins that mediate cellular cytotoxicity (granzymes, perforin, granulysin), T cell Ag recognition (TCRγ, CD8a, NKG2D). Of the five remaining probe sets that appeared in the CD8L cluster in two of the three experiments, two (RANTES, NKG7) are also associated with the lytic granule of cytotoxic lymphocytes.

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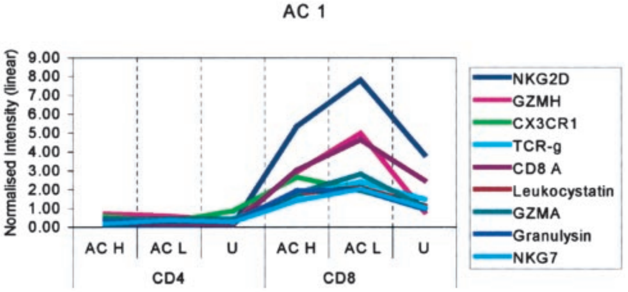

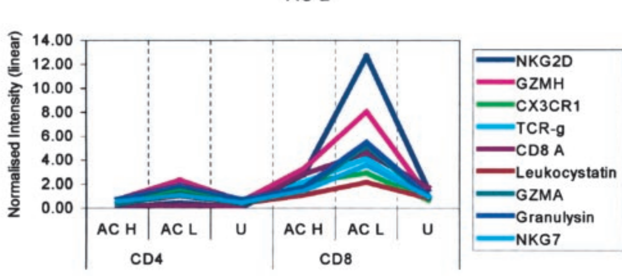

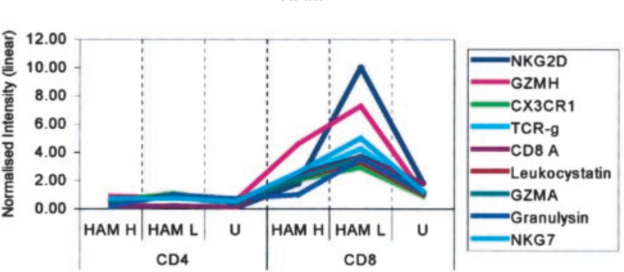

FIGURE 4. Normalized gene expression intensity profiles of the nine genes in the CD8L core cluster displayed in experiments AC1, AC2, and HAM (see Fig. 3, group A).

group D), a chemokine whose release is associated with CD8$^+$ T cell degranulation (33); and IFITM3, also known as 1–8U, is an IFN-inducible gene that is associated with inhibition of tumor cell growth by IFNs (both type 1 and type 2) (34). Brem et al. (34) reported that 1–8U colocalizes with MHC class 1 molecules, and immunoelectron microscopy showed the presence of 1–8U in large intracellular vesicles that resembled exosomes.

These results show that the expression levels of lymphocyte lysis-associated genes in each experiment was greater in ACs with a low proviral load of HTLV-1 than in those with a high proviral load. Although the median proviral load is higher in patients with HAM/TSP (13), within this patient group the same cluster of lymphocyte lysis-associated genes was up-regulated in patients with a lower proviral load.

Consistent clusters were not observed in the CD4$^+$ arrays

Following normalization, filtering, and cluster analysis (as above), neither K-means nor Gaussian cluster analysis identified a cluster of genes in the CD4$^+$ arrays that showed a consistent profile of gene expression or a consistent core of genes that distinguished the low proviral load group from the high proviral load group in any of the three experiments. Similarly, no gene cluster was evident that distinguished patients with HAM/TSP from healthy HTLV-1 carriers, either in the CD4$^+$ arrays or the CD8$^+$ arrays (data not shown).

qPCR analysis corroborated the up-regulation of lysis-related genes in the CD8L cluster

We wished to ensure that the results obtained did not depend on the methods used in mRNA quantification, in particular the in vitro transcription reaction and the pooling of cDNA samples from different individuals. We used real-time qPCR to amplify selected genes from the individual CD4$^+$ and CD8$^+$ lymphocyte cDNAs that were used to prepare the RNA for hybridization to the Affymetrix arrays in experiment AC2. To normalize the mRNA (cDNA) levels in each sample, we amplified cDNA of two "housekeeping" genes, *NPAT* and *β-actin*.

Whether the qPCR products were normalized to the levels of NPAT or β-actin, the results in each case (Table II) corroborated the microarray data. Furthermore, amplification of the individual cDNAs confirmed that the observed CD8L core gene up-regulation was not due to a single highly divergent sample (not illustrated). Thus, granzyme A and NKG2D were up-regulated in the low proviral load subjects compared with the controls. In contrast, the expression of these genes in the high proviral load subjects was similar to that in the uninfected control subjects. These results (Table II) also confirmed the up-regulation of mRNA of both perforin and RANTES in the low load subjects (Fig. 3, gene groups C and D).

Discussion

The high proviral load characteristic of HTLV-1 infection is the strongest correlate of the risk of HTLV-1-inflammatory diseases such as HAM/TSP (20, 35). However, the factors that determine an individual's set point of proviral load, which can differ between individuals by >1000-fold, are not yet understood. Both experiment (36) and theory (37) indicate that, at equilibrium, the proviral

Table II. *Quantification of mRNA expression levels by real-time qPCR*[a]

Gene	Normalized to	Ratio of mRNA Expression Intensity		
		AC^{low}/AC^{high}	AC^{low}/Controls	AC^{high}/Controls
Granzyme A	NPAT	4.55	3.03	0.67
	β-actin	3.34	2.67	0.80
NKG2D	NPAT	3.39	3.29	0.97
	β-actin	2.66	2.63	0.99
Perforin	NPAT	2.81	1.99	0.71
	β-actin	1.88	1.74	0.93
RANTES	NPAT	3.88	3.12	0.80
	β-actin	2.79	2.74	0.98

[a] The values shown are the mean ratios of mRNA levels of the indicated genes in ex vivo CD8$^+$ cells from the respective subject groups normalized to the level of either NPAT mRNA or β-actin mRNA. The results confirm consistent up-regulation of the proteins associated with CD8$^+$ cell-mediated lysis. RANTES is associated with the lytic granule in CD8$^+$ cells.

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load of HTLV-1 is maintained mainly by mitosis of provirus-containing lymphocytes. In principle, HTLV-1 infection could therefore be maintained in one host in the absence of HTLV-1 gene expression. However, several observations indicate that there is persistent expression of HTLV-1 genes (see Introduction). Furthermore, $CD8^+$ T cells efficiently suppress HTLV-1 Ag expression in fresh PBMCs by killing HTLV-1-infected cells (5, 11), which suggests that the abundant activated HTLV-1-specific $CD8^+$ cells in the circulation play a part in limiting the proviral load in vivo. This interpretation is supported by the observation that possession of either of two class I HLA alleles (*HLA-A*02* or *HLA-Cw*08*) was associated with a lower proviral load and a lower risk of HAM/TSP in an endemically infected population in southern Japan (18–20).

We have previously shown (23) that a persistently replicating virus may reach a dynamic equilibrium with the immune response such that the equilibrium proviral load differs widely between individuals, whereas there is little or no difference in the frequency of virus-specific CTLs. In this theory we postulated (23) that individuals differ in one or both of two parameters of the efficiency of antiviral CTLs: the rate of division of CTLs in response to a given Ag load and the rate at which the CTLs lyse virus-infected target cells.

In this study, we wished to test the hypothesis that individuals who efficiently control HTLV-1 replication (i.e., those with a low proviral load of HTLV-1) selectively overexpress, in circulating T cells, genes concerned with either cell division or cell-mediated lysis (or both). Ag-specific lymphocytes were not selected, because there is no current method of isolating all T cells specific to a given pathogen or Ag from ex vivo PBMCs without altering their gene expression. Class 1 tetramers can be used to select $CD8^+$ cells specific to individual epitopes; however, few class 1-restricted T cell epitopes in HTLV-1 have been defined at the optimal length, and binding of class 1 MHC tetramers may alter gene expression in the $CD8^+$ cell.

The results demonstrate a small, robust group of genes that are up-regulated in the total circulating $CD8^+$ cell population in individuals with a low proviral load of HTLV-1, regardless of the presence or absence of HAM/TSP. Remarkably, the majority of these genes encode proteins that participate in cell-mediated cytotoxicity.

The nine genes present in the core CD8L cluster (Fig. 3, group A) encode three secreted effector proteins involved in lymphocyte-mediated cytotoxicity: granzyme A, granzyme H, and granulysin. Granzyme K and perforin were also present in the CD8L cluster in two of the three experiments (Fig. 3, group C). NKG2D (Fig. 3, group A) is a cell surface molecule expressed not only by $CD8^+$ CTLs (both $\alpha\beta$ and $\gamma\delta$), in which it reduces the threshold of TCR-mediated CTL triggering and lysis (38), but also NK cells, in which it is itself a primary recognition structure whose ligation triggers cytotoxic effector function (39–41). The mRNA for CD8α was also present in the core CD8L cluster. CD8α forms a heterodimeric coreceptor for T cells that reduces the threshold of T cell activation (42). It is also expressed on subsets of NK cells and $\gamma\delta$ T cells, frequently as a CD8$\alpha\alpha$ homodimer whose function is still poorly understood but which may bind the nonclassical class 1 MHC ligand TL and modify TCR signaling (43).

The significance of high-level expression of the TCRγ chain gene in the CD8L cluster is unclear. TCRγ is expressed in $\gamma\delta$ T cells and certain subsets of "unconventional" TCR$\alpha\beta$ T cells (44): the function of each of these subsets in host defense is uncertain. The finding that the two genes *NKG2D* and *TCRγ* were expressed at a high level in the CD8L cluster raises the interesting possibility that the innate immune system, in the form of NK-like cells or "unconventional T cells," plays a significant role in host defense against HTLV-1.

The fractalkine receptor CX3CR1 is characteristically expressed at a high level on competent cytotoxic lymphocytes, both CTLs and NK cells (45). There is recent evidence that CX3CR1 influences the efficiency of HIV-1-specific CTLs (46). Intriguingly, CX3CR1 is also expressed on human neurons and microglia (47), although its function in the CNS is unknown. The functions of the remaining genes in the core group are less well characterized. Leukocystatin is a cysteine protease inhibitor which does not appear to have been specifically associated with active cytotoxicity in lymphocytes. Cysteine protease activity is present in $CD8^+$ T cells in cathepsins, which have cytotoxic activity, and in dipeptidyl peptidase I, which cleaves granzyme precursors to release their active forms. NKG7 (also known as GMP-17, TIA-1, or GIGI) is associated with the membranes of cytotoxic granules in NK cells and T cells (48, 49). Although its precise function is not known, translocation of NKG7 from granules to the plasma membrane is associated with active degranulation of the lymphocyte (48).

The frequency of HTLV-1-specific $CD8^+$ T cells shows a zero or weakly positive correlation with proviral load (21, 22). This contrasts with the negative association observed here between $CD8^+$ cell lytic gene expression intensity and proviral load, and the negative correlation observed between the rate of $CD8^+$ cell-mediated lysis and proviral load.[4] We have found that the median frequency of HTLV-1-specific $CD8^+$ T cells is typically 2- to 4-fold higher in HAM/TSP patients than in ACs whether the cells are quantified by limiting dilution analysis (50), class 1 MHC tetramer binding (18). Recently, using a IFN-γ ELISPOT assay (57), we found no significant difference in the frequency of HTLV-1-specific $CD8^+$ T cells between a group of asymptomatic HTLV-1 carriers and patients with HAM/TSP. Others (52, 53) have reported a greater difference in frequency between HAM/TSP patients and ACs. However, both experiment (54–56) and theory (22, 23) show that the frequency of virus-specific $CD8^+$ T cells in the circulation is not a reliable index of the effectiveness of the T cell response to a persistent virus at equilibrium. We conclude that the effectiveness or efficiency of the $CD8^+$ T cell response should be measured by molecular markers of lytic gene expression, as in the present study, or by assays of function such as the rate of $CD8^+$ T cell-mediated lysis.

A low expression intensity of lytic genes could be an effect, rather than a cause, of a high proviral load of HTLV-1. However, three arguments favor our interpretation that CTLs suppress HTLV-1 replication. First, removal of $CD8^+$ cells from PBMCs causes a large and reproducible rise in HTLV-1 expression (5, 11). Second, lytic gene expression was greater in patients with HAM/TSP than in asymptomatic HTLV-1 carriers at a given HTLV-1 load (Fig. 4). Finally, the median proportion of HTLV-1-specific $CD8^+$ cells that are infected with HTLV-1 was $<9\%$ (data from Ref. 5); therefore, the observed differential gene expression cannot be explained by HTLV-1 infection of the specific CTLs themselves.

The proviral load in HAM/TSP patients was systematically higher than in ACs at a given level of lytic gene expression (Fig. 4; data not shown). The reason for this is not yet known: one possibility is that HAM/TSP patients' HTLV-1-infected $CD4^+$ T cells proliferate faster than those of the carriers.

[4] B. Asquith, A. J. Mosley, A. Vine, A. Heaps, P. Goon, E. Hanon, A. Barfield, G. P. Taylor, and C. R. M. Bangham. $CD8^+$ cell lytic efficiency is a major determinant of HTLV-1 proviral load. *Submitted for publication.*

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In this study, the mRNA expression levels of lysis-related genes were elevated in the total $CD8^+$ population. This elevation is unlikely to result from a generalized activation of $CD8^+$ cells by HTLV-1, because individuals with a higher HTLV-1 load had lower lytic gene expression levels. The high lytic gene expression could occur in all $CD8^+$cells in such individuals or could be attributable to HTLV-1-specific cells alone, because the frequency of such cells in the blood is high (57).

Is the mRNA expression intensity of granzyme genes a physiologically meaningful correlate of CTL function? Granzyme mRNA expression, usually assayed by qPCR, is increasingly used as a correlate of acute rejection of allogeneic transplants, in which CTLs play a critical role. Increased granzyme mRNA levels can be detected during rejection both in the graft itself (58, 59), in the blood (60, 61), and even in the urine (62). Measurement of granzyme mRNA is preferable to immunostaining of granzyme proteins as a correlate of CTL activity, because the granzyme proteins do not accumulate in the cell but are rapidly and continually discharged in the lytic granules.

Zhang et al. (63) concluded that impaired CTL-mediated lysis may be a general feature of chronic viral infections, in particular HIV-1, CMV, and EBV. This conclusion was based on an analysis of perforin expression (assayed by flow cytometry) and certain cell surface markers of lymphocyte phenotype. Our results indicate that in the case of HTLV-1 infection, strong expression of genes that express proteins involved in CTL-mediated lysis is associated with a low proviral load in vivo.

The present results demonstrate that, despite the lower frequency of circulating HTLV-1-specific $CD8^+$ T cells in individuals with a low HTLV-1 proviral load, these cells express greater levels of genes that encode granzymes (and other lytic proteins) than the corresponding cells in individuals with a high proviral load. We conclude that the CTL response to HTLV-1 plays a dominant role in determining an individual's set point of HTLV-1 proviral load.

Acknowledgments

We are grateful to the volunteers who participated in this study and to Sara Marshall for critical reading of this manuscript.

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