Recognition of Human T-Leukemia Virus (HTLV-1) Envelope by Human CD4+ T-Cell Lines From HTLV-1 Seronegative Individuals: Specificity and Clonal Heterogeneity

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Because T-helper cells are critical for immune responses in retroviral infections, CD4+ T-cell lines specific for the human T-leukemia virus type 1 (HTLV-1) envelope have been generated from peripheral T lymphocytes of nonimmune donors to study their naive repertoire. Recombinant fragments (RE1, amino acids [aa] 26-200; RE3, aa 165-307; RE5, aa 308-401; and RE6, aa 165-401) of HTLV-1 envelope, whole envelope glycoprotein, and synthetic peptides were used to induce T-cell lines. CD4+ T-cell lines specific for one or more fragments were obtained from seven of eight individuals tested. T-cell lines generated against envelope glycoprotein from five of five donors did not cross-react with the RE fragment.

THE HUMAN RETROVIRUS human T-leukemia virus type 1 (HTLV-1) is responsible for adult T-cell leukemia (ATL).6,7 It is also associated with the neurologic disease tropical spastic paraparesis (TSP). The antibody response to HTLV-1 antigens can be used for the diagnosis of the seropositive status.7 It is also likely that an ongoing immune response (humoral and/or cellular) may be responsible for the slow progression of the associated diseases, or, if properly driven by means of vaccination, it may induce a protective response.8 Neutralizing antibodies and cytotoxic cells are the chief effector mechanisms at work during viral infections.9-12 In both cases, Th cells13 are required for clonal expansion of antibody-secreting B cells and of specific cytotoxic cells.14 Therefore, the identification of Th cells specific for HTLV-1 in the human repertoire is important for the design of candidate vaccines4 that include viral T-helper epitopes. Furthermore, they are useful for the identification of viral antigens that can be used as in vitro probes to test for the presence of HTLV-1 envelope-specific cellular immunity. As shown in the human immunodeficiency virus (HIV) system,15 this may be an additional diagnostic tool in seronegative high-risk individuals.

MATERIALS AND METHODS

Antigens and media. The recombinant fragments of HTLV-1 envelope were purchased from Repligen (Cambridge, MA). They were expressed in Escherichia coli and contained amino and carboxy tails of E. coli origin of 10 to 30 residues. Sequences are based on published data for HTLV-1 envelope.16 Fragment RE1 included residues 26-200, fragment RE3 residues 166-307, fragment RE5 residues 308-401, and fragment RE6 residues 165-401. The preparation and purification of the envelope glycoprotein produced in the baculovirus expression system has been described in detail.17 A panel of synthetic peptides (20-mers overlapping by 10 residues) was produced by P. Neri (University of Siena, Siena, Italy), Figure 1 illustrates the boundaries of the recombinant antigens used in this study.

RPMI 1640 medium (Flow, Irvine, UK) was used for the generation and the maintenance of the lines and for proliferation assays. It was supplemented with 10 mmol/L L-glutamine, 0.05 mmol/L L-mercaptoethanol, and 5% autologous hematized plasma.

Because T-helper cells are critical for immune responses in retroviral infections, CD4+ T-cell lines specific for the human T-leukemia virus type 1 (HTLV-1) envelope have been generated from peripheral T lymphocytes of nonimmune donors to study their naive repertoire. Recombinant fragments (RE1, amino acids [aa] 26-200; RE3, aa 165-307; RE5, aa 308-401; and RE6, aa 165-401) of HTLV-1 envelope, whole envelope glycoprotein, and synthetic peptides were used to induce T-cell lines. CD4+ T-cell lines specific for one or more fragments were obtained from seven of eight individuals tested. T-cell lines generated against envelope glycoprotein from five of five donors did not cross-react with the RE fragment. The lines specific for RE and env were mapped with overlapping peptides. The lines with single peptide (narrow) specificity contained a variety of clones that used different T-cell receptor Vβ genes. These data (1) suggest that most of the normal individuals carry T-helper precursors specific for epitopes on HTLV-1 envelope; (2) indicate that heterogeneity of HTLV-1 envelope-specific T cells can be detected in the naive repertoire; and (3) define optimal antigenic preparations to be used to assess cellular immunity in HTLV-1-infected individuals.

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Proliferation assay. Flat-bottom microtiter plates contained 2 × 10^6 T cells and 10^6 autologous 2,500-rad irradiated PBMCs as presenting cells (previously pulsed with the relevant antigens at 1 μg/mL) in 200 μL culture medium per well. The cultures were pulsed after 36 hours with 0.5 μCi [3H]-thymidine (5 Ci/mmol; Amersham, Amersham, UK) for 6 hours. After harvesting with a Micromate 196 apparatus, the dry filters were counted in a Matrix 96 beta counter (Packard-Canberra, Meridian, CT) without scintillation fluid. The counting efficiency of this instrument is 20% of conventional scintillation counters. Results are given as mean value of duplicate cultures ± SD.

Estimate of specific precursor frequency. Because of the limited number of cells available from the same donors repeatedly bled for the experiments and the lack of primary proliferative response, formal limiting dilution assay could not be performed to determine the frequency of RE and of envelope-specific cells in PBMCs. In addition, because of the low precursor frequency, we could not use conventional frequency analysis in which the highest cell concentration yields 100% positive wells; in fact, greater than 10^6 cells per well should be plated, but this results in overcrowding and no positive wells could be detected in preliminary assays. Thus, we used an alternative analysis to estimate precursor frequency. Positive signals were enhanced in a two-step assay. In the first step, 24 wells of a microtiter plate were seeded with 10^5 antigen-pulsed PBMCs and IL-2 was added 5 days later. In the second step on day 15, each well was split into three round-bottom wells, one to maintain the experiment and the lack of primary proliferative response, for instance, to establish the lines was performed by 2 PCR on reverse transcribed cDNA by specific T-cell lines was performed by PCR on reverse transcribed cDNA by using a panel of oligonucleotide primers and following the procedure described in detail.5

RESULTS

T-cell lines specific for RE fragments. T-cell lines specific for one or more RE fragments were obtained from seven of eight individuals. A specific response can be detected by the second or third restimulation cycle (data not shown) and by the fourth or fifth cycle the lines are established.

Figure 2 summarizes the lines generated from different donors. The lines were tested after four cycles. Four of seven donors gave rise to RE1-specific lines, six of seven to RE3-specific lines, six of seven to RE5-specific lines, and three of seven to RE6-specific lines.

The lines are specific for the fragments used for in vitro immunization, and no recognition of the E. coli sequences shared by the different recombinant fragments that flank the HTLV-1 sequences can be detected. In fact, a representative T-cell line raised against fragment RE1 does not proliferate to RE3 and RE5 (sharing the same E. coli sequences with RE1), but proliferates to RE6 that overlaps with RE1 from residues 165 to 200 (Fig 3). The lack of response to fragment RE3, which overlaps with RE1 from aa 165 to 200 as RE6, is hard to explain. This fragment has no toxic activity in that RE3-specific lines can be generated from certain donors (Fig 2) and it has no inhibitory activity when coadministered with stimulatory fragments RE1 and RE6 (Fig 3).

The fine specificity of two lines derived from the same
donor (AF) was defined by screening with the panel of overlapping peptides. Line RE1 was responsive to peptides 161-180 and 171-190 that overlap from 171 to 180, and line RE6 was responsive to peptide 311-330. Because of their narrow specificity, these lines were examined for clonal heterogeneity (see below).

T-cell lines specific for HTLV-1 recombinant envelope glycoprotein. Lines were induced by in vitro immunization with the envelope glycoprotein in particulate or soluble form from 9 of 10 individuals. Figure 4 shows two representative lines generated from individual FM by using particulate or soluble antigen that respond to the envelope glycoprotein independently of the particulate or soluble form. Peptide mapping showed specificity for the overlapping peptides 201-220 and 211-230 in both lines, suggesting that the response to this in vitro-defined immunodominant region is a constant feature in lines generated independently from the same individual against the same antigen in two different forms. An early response to the recombinant RE1 fragment was detected at the third cycle in the line induced with soluble antigen (data not shown), but it was lost at the fourth stimulation cycle, in apparent association with a restriction in clonal heterogeneity (see below). Figure 4 also shows a line generated from donor LO that responds to fragment RE1 and recognized two nonoverlapping peptides, 91-110 and 141-160.

Estimate of precursor frequency. The frequency of T-cell precursors specific for individual RE fragments was estimated in individual DF (Fig 5A). The frequencies range from 1 to 2\(\times10^6\) unfractionated PBMCs. In the case of envelope-specific precursors (donors FM, DF, and GB; Fig 5B), frequencies are in the same range. These low frequencies support the notion that the lines have been generated from donors that are HTLV-1 naive to the best of our knowledge. By comparison, the frequency of TT- and PPD-specific proliferating cells detected in the same donors reflects the mem-

**Fig 2.** Proliferative response to recombinant RE fragments. Lines were generated from seven individuals by in vitro immunization with the individual RE fragments and tested with the corresponding fragments for proliferative response after four cycles. □ Background proliferation; □ antigen-induced proliferation. RE1-specific lines were obtained from five individuals; RE3-, RE5-, and RE6-specific lines were obtained from six, six, and three individuals, respectively.

**Fig 3.** T-cell line from donor AF generated by in vitro immunization with RE1 fragment. The line induced with fragment RE1 was tested against the other recombinant fragments after the fourth cycle. Cross-response was only seen with RE6, which overlaps with RE1 by 35 amino acids (residues 165-200). The same overlapping sequence is present on peptide fragment RE3, which is nonstimulatory. Fragment RE5 does not stimulate, even though it carries the same E. coli amino terminal tail of RE1, thus confirming that no recognition of the flanking E. coli sequences occurs. 
ory state to these recall antigens (37 and 22/105 with donor DP; 48 and 26/105 with donor FM).

**Clonal heterogeneity of HTLV-1 envelope-specific T cells.** Keeping in mind that recognition of one single peptide does not imply T-cell monoclonality, two T-cell lines from donor AF specific for fragments RE1 (overlapping sequence 171-180) and RE6 (peptide 311-330) were analyzed for use of TCR Vβ gene families. An RNase protection assay was used on mRNA extracted from the T-cell lines. Figure 6 shows the Vβ gene families that were used by the two T-cell lines specific for the recombinant RE fragments. The RE1-specific line includes 11 major Vβ families; in the case of the RE6-specific line, six major Vβ families were used. These results, summarized in Table 1, indicate the presence of a large cohort of clonotypes engaged in the response to narrow regions of RE recombinant fragments.

A PCR assay was also used to follow-up clonal heterogeneity of T-cell lines responsive to peptides 201-220 and 211-230 (211-220 overlap) of the envelope glycoprotein. A discrete number of TCR Vβ gene families was used by the bulk line, with a loss of certain TCR Vβ families after repeated stimulation cycles (Table 2). The association of reduction in clonal heterogeneity and loss of responsiveness to recombinant RE fragments may be coincidental, or some RE responsive clones may belong to the Vβ families that were eventually lost in the bulk culture.

To further define clonal heterogeneity of the same T-cell lines specific for envelope, clones were obtained by limiting dilution and individually examined by PCR to define their Vβ gene usage. All of the clones exhibited the same peptide specificity as the bulk lines from which they were derived. Table 3 shows that most of the clones are monoclonal in nature, whereas 4 of 14 (28%) are not monoclonal, in agreement with the expected theoretical frequency of 25% according to Poisson distribution when limiting dilution is performed at 0.5 cells/well.

**DISCUSSION**

Antibodies and cytotoxic cells specific for HTLV-1 have been detected in infected patients. In addition, extensive information is available on B epitopes defined on envelope glycoprotein with MoAbs and with polyclonal antibodies. Our focus in HTLV-1-specific human T-helper cells stems from the fact that both antibody production and induction of cytotoxic cells are dependent on specific CD4 cells. The goals of our study were (1) to show the presence of specific T-helper cells in the naive repertoire of seronegative individuals not belonging to categories at risk of exposure to HTLV-1; (2) to test their fine peptide specificity; (3) to estimate the frequency of HTLV-1 envelope-specific precursors in the human naive repertoire; (4) to define the clonal heterogeneity of the human primary response in vitro; and (5) to use the T-cell lines and clones as cellular reagents to define recombinant products as appropriate antigens for proliferative assays to test cellular immunity in HTLV-1-positive individuals.

The frequency of Th cell precursor specific for HTLV-1 envelope or recombinant fragments in the naive repertoire is far too low for detection by a conventional proliferation assay, as seen in the case of HIV antigens, but the relevant cells can be expanded in vitro by repeated stimulation with various antigenic preparations. Specific precursors are present in the repertoire of seronegative individuals, and numerous different clonotypes can recognize the retroviral antigens. The estimated frequency matches with the observed frequencies for other primary retroviral antigens (gp120 and p66 of HIV), as opposed to much higher frequencies for the conventional recall antigens tested here. In addition, because the seronegative donors used for this study do not belong to categories at risk (because of sexual practices or blood transfusion) and belong to a geographical region in which the incidence of HTLV-1 seropositivity is negligible (thus ruling out also in utero exposure), these can be considered bona fide primary in vitro responses.
Fig 5. Estimate of precursor frequency. (A) T-cell response to the four RE fragments by donor DF. PBMCs were pulsed with the relevant fragment plated at 10^6/well in 24 wells, as described in the Materials and Methods. After splitting and specific restimulation, the wells were pulsed, harvested and counted. SI greater than 2 was scored as positive. Background proliferation for the individual wells in the absence of antigen ranged from 100 to 500 cpm. The estimated precursor frequencies of specific cells in 10^6 PBMCs were 1.5, 1.0, 1.9, and 1.7 for the four RE fragments. (B) T-cell response to soluble envelope by donors FM, DF, and GB. The estimated frequencies were 2.2, 1.8, and 1.4, respectively.

To estimate the frequency of cells specific for recall antigens, PBMCs were plated after pulsing with TT (5 µg/mL) or with PPD (25 µg/mL) at 2 x 10^4/well in 24 wells (data not shown). Donor DF had a frequency of 37 TT-specific and 22 PPD-specific cells in 10^6 PBMCs. Frequencies for donors FV and GB were 24 and 16 and 48 and 26, respectively.

With respect to specificity, the T-cell lines we have obtained seem to discriminate the molecular context of the relevant epitopes. Data not shown here because they confirm previous findings with other retroviral antigens indicate that, within the naive human repertoire, it is possible to trigger T-helper cells that recognize synthetic peptides, but not whole proteins that contain the same sequence. These are examples of T cells specific for "cryptic" epitopes. It is likely that, if such clones are recruited in vivo by peptide-containing vaccines, they may be of little relevance because of their inability to recognize the same epitope in the context of the whole protein antigen or in the context of the whole virion.

This observation confirms our previous findings in an animal model in which T-cell clones were able to discriminate between native/denatured and intact/fragmented antigen and with human clones specific for HIV epitopes displayed on peptides, proteins, and viral particles.

If discrimination between the various physical forms of a T-cell epitope depends also on antigen processing, in addition to a role for flanking amino acid sequences and for carbohydrate chains, and for fine specificity of T cells, it may be useful to drive processing mechanisms onto highly efficient presenting cells to facilitate presentation of the relevant epitopes independently of the antigenic context. An example of this possibility has been reported elsewhere. In this case, we showed that a T-cell epitope of a retroviral protein cannot be recognized by specific T cells when presented in the context of whole virions by monocytes as antigen presenting cells (APCs), but it was presented by B cells, suggesting that different APCs have different ability in handling complex antigenic structures. Therefore, driving the antigen onto optimal APC (eg, by the appropriate route of administration or by the use of appropriate adjuvants) may favor exploitation of T cells that would be otherwise neglected in a potentially protective response.

In addition, T-helper cells specific for "cryptic" epitopes may be a byproduct of the immune response in case antigenic fragments that become available upon processing and/or degradation activate such T cells unable to respond to the whole antigen. In this case, immunogens containing the stimulatory fragments (but not the nonstimulatory protein) can be linked to B-cell epitopes (T-B vaccines) to enhance antibody production in those instances in which a
high titer of preformed antibodies is desirable, rather than a delayed anamnestic response to the pathogen itself. The different clonotypes defined according to Vβ gene usage in T-cell lines with narrow peptide specificity may underestimate the real clonal heterogeneity. In fact, the weak signals in the RNAse protection assay may indicate clones present at very low frequency within the bulk line. Furthermore, antigen-specific T-cell clones exhibiting the same TCR Vβ gene usage may differ when the VDJ region of the TCR gene is sequenced.

Expansion of specific T cells from a naïve repertoire mimics in vitro the events that take place during the first encounter in vivo between the T-helper compartment and the viral antigens, as in the case of accidental infection or of deliberate vaccination. These events include antigen administration, uptake and processing by the appropriate APCs, and presentation to specific T cells that are present at low frequency in the naïve repertoire. Vaccination has been proposed for HTLV-I.

It has been pointed out that, in animal models of HTLV-1 infection, the antibody response against envelope confers protection. Because HTLV-1 envelope glycoprotein is a candidate target for vaccination, it is useful to gain more information on its immunodominant T-helper epitopes. This may allow the inclusion of universal T-cell epitopes in recombinant or synthetic vaccines, as proposed in other viral systems.

The identification of epitopes that are recognized by most of the individuals in the population (immunodominant and universal) may also suggest useful reagents to detect a previous exposure to the viral antigens in the absence of seroconversion, as it has been shown in the case of contact with HIV-1 in seronegative individuals. The presence of cellular immunity in the absence of seroconversion may be a valuable diagnostic tool for the identification of infected individuals who have not yet developed (or will never develop) an antibody response. To use such an assay for diagnostic purposes,

**Table 1. TCR Analysis of HTLV-1-Specific Lines**

<table>
<thead>
<tr>
<th>T Cells</th>
<th>Probe Set A</th>
<th>Probe Set B</th>
<th>Probe Set C</th>
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<td>RE1</td>
<td>5.1, 17.1, 19.1, 8.2, 6.4, 3.1, 1.1, 13.1, 2.1a, 2.1b, 12.1b, 8.1, 6.3, 5.1</td>
<td>6.4, 3.1, 1.1, 13.1, 2.1a, 2.1b, 12.1b, 8.1, 6.3, 5.1</td>
<td>6.4, 3.1, 1.1, 13.1, 2.1a, 2.1b, 12.1b, 8.1, 6.3, 5.1</td>
</tr>
<tr>
<td>RE6</td>
<td>17.1, 8.2, 19.1, 8.1, 3.1, 1.1, 2.1b, 13.3, 6.3, 13.1, 5.1</td>
<td>6.4, 3.1, 1.1, 13.1, 2.1a, 2.1b, 12.1b, 8.1, 6.3, 5.1</td>
<td></td>
</tr>
</tbody>
</table>

Summary of TCR Vβ gene usage by T-cell lines specific for RE1 and RE6 recombinant fragments, as defined by the RNAse protection assay illustrated in Fig 5.

Fig 6. Vβ gene usage by T-cell lines specific for RE1 and RE6. RNA was prepared from T-cell lines after the fourth restimulation cycle with antigen. We had previously established that naive T-cell lines specific for different retroviral antigens are enriched in specific T cells close to 100%, because all of the clones obtained by mitogenic stimulation at this stage are antigen specific when tested in an antigen-driven proliferation assay. The panels show the RNAse protection assay performed with Vβ probe sets A, B, and C. Lanes 1, 4, and 7 show the RE1-specific line. Lanes 2, 5, and 8 show the RE6-specific line. Lanes 3, 6, and 9 show the positive controls for all of the probes obtained by running the assay with RNA prepared from human thymocytes. The RE1-specific line contains 11 different clonotypes and the RE6-specific line six different clonotypes, as summarized in Table 1. Weak signals were also detected by the Vβ 5.1 probe with the RE1 line and by the Vβ 2.1, 6.4, 6.5, 13.1, and 13.3 probes with the RE6 line.
it is important to define antigenic preparations (whole proteins, recombinant fragments, or peptides) that are recognized by the majority of individuals in the population. The recombinant fragments RE3 and RE5 seem to qualify as such, but the envelope glycoprotein is also a primary immunogen in the majority of the individuals tested so far. With this information, the next step will be to screen the recombinant antigens and the panel of overlapping synthetic peptides by using T cells or T-cell lines and clones generated from individuals who had a previous contact with HTLV-1 to define the antigens that are most frequently recognized, so as to include them in screening protocols for the definition of cellular immunity in seronegative high-risk individuals.

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REFERENCES


The table illustrates the fluctuations in TCR Vβ gene usage by the bulk T-cell line FM specific for HTLV-1 envelope glycoprotein (induced with soluble antigen) after repeated stimulation cycles. Whereas the majority of Vβ genes are maintained, Vβ 4, 6H, 18, and 22 are gradually lost, and Vβ 11 clearly shows up after the fourth cycle of stimulation.
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