Gmail **Christine Massey <cmssyc@gmail.com>**

FOIA request to CDC re: scientific proof of "HTLV I or II", or purification

Christine Massey <cmssyc@gmail.com> Mon, Oct 31, 2022 at 4:49 PM
To: "FOIA Requests (CDC)" <FOIARequests@cdc.gov>

October 31, 2022

To:
Roger Andoh
Freedom of Information Officer
1600 Clifton Rd NE MS T-01
Atlanta, Georgia 30333
Email: FOIARequests@cdc.gov
Phone: 770-488-6277
Fax: 770-488-6200

Dear Roger,

I require access to general records, as per the *Freedom of Information Act.*

Description of Requested Records:

1. All studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) that scientifically prove the existence of the alleged **"HTLV-I" aka "Human T-Lymphotropic Virus Type I**" or the alleged **"HTLV-II" aka "Human T-Lymphotropic Virus Type II**".

Note:
Scientific proof is NOT

- Opinions
- Speculation
- Review papers
- Descriptive papers

Scientific proof requires

- Use of the scientific method
- Repeatable and falsifiable hypotheses that have been tested using valid, controlled experiments where only 1 variable differs between the experimental and control groups
- In this case, the 1 manipulated variable would be the presence/absence of purified particles suspected of being a "virus"
- Consistent results from valid, controlled experiments (i.e. identical "genomes", consistent in vivo effects)

Records that do not describe the testing of falsifiable, repeatable hypotheses regarding the existence of an alleged "virus" (meaning the existence of the alleged particle and its alleged causation of disease) are disqualified from my request.

2. **If the CDC has no studies responsive to #1 above**, please provide all studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) describing the **purification** of any alleged **"HTLV-I" aka "Human T-Lymphotropic Virus Type I"** or the alleged **"HTLV-II" aka "Human T-Lymphotropic Virus Type II"**, directly from bodily fluid/tissue/excrement or from a cell culture.

Please note that I am not requesting studies/reports where researchers failed to **purify** the suspected "virus" and instead:

- cultured an unpurified sample or other unpurified substance, and/or
- performed an amplification test (i.e. a PCR test), and/or
- produced an in silico "genome", and/or

- produced electron microscopy images of unpurified things.

For further clarity, please note I am already aware that according to virus theory a "virus" requires host cells in order to replicate, and I am **not** requesting records describing the **replication** of a "virus" without host cells.

Further, I am **not** requesting records that describe a suspected "virus" floating in a vacuum; I am simply requesting records that describe its **purification** (**separation** from everything else in the patient sample, as per standard laboratory practices for the purification of other small things).

General Note:
Please also note that my request is **not limited** to records that were authored by the CDC or ATSDR or that pertain to work done at/by the CDC or ATSDR. Rather, my request includes any record matching the above description authored by anyone, anywhere, ever.

Publicly Available Records
If any records match the above description of requested records and are currently available to the public elsewhere, please assist me by providing enough information about each record so that I may identify and access each one with certainty (i.e. title, author(s), date, journal, where the public may access it). Please provide URLs where possible.

Format:
Pdf documents sent to me via email; I do not wish for anything to be shipped to me.

Contact Information:
Christine Massey
Ontario, Canada
Email: cmssyc@gmail.com

Thank you in advance and best wishes,
Christine Massey, M.Sc.

Gmail

Christine Massey <cmssyc@gmail.com>

Your CDC FOIA Request #23-00174-FOIA

MNHarper@cdc.gov <MNHarper@cdc.gov> Fri, Nov 4, 2022 at 9:37 AM
To: cmssyc@gmail.com

November 4, 2022

Request Number: 23-00174-FOIA

Dear Ms. Massey:

This is regarding your attached three Freedom of Information Act (FOIA) requests of October 31, 2022, for:

All studies and/or reports in the possession, custody or control of the people who work at/for the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) and are responsible for the CDC's claim that parasites cause malaria, that describe controlled experiments using any purified parasite to prove causation of malaria.

All studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) that scientifically prove the existence of the alleged "HTLV-I" aka "Human T-Lymphotropic Virus Type I" or the alleged "HTLV-II" aka "Human T-Lymphotropic Virus Type II".

All studies and/or reports in the possession, custody or control of the people who work at/for the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) and are responsible for the CDC's claim that bacteria can cause pneumonia, that describe controlled experiments using purified Mycoplasma pneumoniae, or any other type of purified bacteria, to prove causation of pneumonia..

Please see the attached letter.

Sincerely,
CDC/ATSDR FOIA Office
770-488-6399

4 attachments

Revised Acknowledgement- 23-00174 for three requests.pdf
167K

FOI to CDC re_ proof of any parasite causing malaria.msg
201K

FOIA request to CDC re_ scientific proof of _HTLV I or II__ or purification.msg
103K

FOI to CDC re_ proof of any bacteria causing pneumonia.msg
202K

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

November 4, 2022

Christine Massey
[redacted]
Via email: cmssyc@gmail.com

Dear Ms. Massey:

The Centers for Disease Control and Prevention and Agency for Toxic Substances and Disease Registry (CDC/ATSDR) received your attached three Freedom of Information Act (FOIA) requests dated October 31, 2022. Your request assigned number is 23-00174-FOIA, and it has been placed in our complex processing queue.

In unusual circumstances, an agency can extend the twenty-working-day limit to respond to a FOIA request.

We will require more than thirty working days to respond to your request because we reasonably expect that two or more CDC centers, institutes, and offices (C/I/Os) may have responsive records.

To process your request promptly, please consider narrowing the scope of your request to limit the number of responsive records. If you have any questions or wish to discuss reformulation or an alternative time frame for the processing of your request, you may contact the analyst handling your request Mark Harper at 770-488-8154 or our FOIA Public Liaison, Roger Andoh at 770-488-6277. Additionally, you may contact the Office of Government Services (OGIS) to inquire about the FOIA mediation services they offer. The contact information for OGIS is as follows: Office of Government Information Services; National Archives and Records Administration; 8601 Adelphi Road-OGIS; College Park, Maryland 20740-6001; e-mail at ogis@nara.gov; telephone at 202-741-5770; toll free at 1-877-684-6448; or facsimile at 202-741-5769.

Because you are considered an "Other requester" you are entitled to two hours of free search time, and up to 100 pages of duplication (or the cost equivalent of other media) without charge, and you will not be charged for review time. We may charge for search time beyond the first two hours and for duplication beyond the first 100 pages. (10 cents/page).

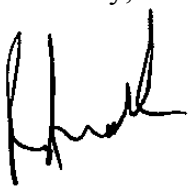
If you don't provide us with a date range for your request, the cut-off date for your request will be the date the search for responsive records starts.

You may check on the status of your case on our FOIA webpage https://foia.cdc.gov/app/Home.aspx and entering your assigned request number. If you have any questions regarding your request, please contact me at 770-488-8154 or via email at wzj6@cdc.gov.

We reasonably anticipate that you should receive documents by January 21, 2023. Please know that this date roughly estimates how long it will take the Agency to close requests ahead of your request in the queue and complete work on your request.

The actual date of completion might be before or after this estimated date.

Sincerely,



Roger Andoh
CDC/ATSDR FOIA Officer
Office of the Chief Operating Officer
(770) 488-6399
Fax: (404) 235-1852

23-00174-FOIA

Gmail

Christine, of the Massey family <cmssyc@gmail.com>

Your CDC FOIA Request #23-00174-FOIA

MNHarper@cdc.gov <MNHarper@cdc.gov> Mon, Dec 5, 2022 at 10:51 AM
To: cmssyc@gmail.com

December 5, 2022

Request Number: 23-00174-FOIA

Dear Ms. Massey:

This is regarding your Freedom of Information Act (FOIA) request of October 31, 2022, for All studies and/or reports in the possession, custody or control of the people who work at/for the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) and are responsible for the CDC's claim that parasites cause malaria, that describe controlled experiments using any purified parasite to prove causation of malaria. All studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) that scientifically prove the existence of the alleged "HTLV-I" aka "Human T-Lymphotropic Virus Type I" or the alleged "HTLV-II" aka "Human T-Lymphotropic Virus Type II". All studies and/or reports in the possession, custody or control of the people who work at/for the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) and are responsible for the CDC's claim that bacteria can cause pneumonia, that describe controlled experiments using purified Mycoplasma pneumoniae, or any other type of purified bacteria, to prove causation of pneumonia.

Please see the attached letter.

Sincerely,
CDC/ATSDR FOIA Office
770-488-6399

7 attachments

- **23-00174 Final Response .pdf**
  131K
- **23-00174 Part 1 .pdf**
  6474K
- **23-00174 Part 2 (12 5 2022).pdf**
  4726K
- **FOIA request to CDC re_ scientific proof of _HTLV I or II__ or purification.msg**
  103K
- **FOI to CDC re_ proof of any parasite causing malaria.msg**
  201K
- **FOI to CDC re_ proof of any bacteria causing pneumonia.msg**
  202K
- **Revised Acknowledgement- 23-00174 for three requests.pdf**
  167K

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

December 5, 2022

Ms. Christine Massey

[redacted]

Via email: cmssyc@gmail.com

Dear Ms. Massey:

This letter is in response to your 3-part consolidated (attached) Centers for Disease Control and Prevention and Agency for Toxic Substances and Disease Registry (CDC/ATSDR) Freedom of Information Act (FOIA) request of October 31, 2022, for:

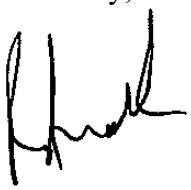
> *1)All studies and/or reports in the possession, custody or control of the people who work at/for the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) and are responsible for the CDC's claim that parasites cause malaria, that describe controlled experiments using any purified parasite to prove causation of malaria*
>
> .
>
> *2) All studies and/or reports in the possession, custody or control of the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) that scientifically prove the existence of the alleged "HTLV-I" aka "Human T-Lymphotropic Virus Type I" or the alleged "HTLV-II" aka "Human T-Lymphotropic Virus Type II".*
>
> *3) All studies and/or reports in the possession, custody or control of the people who work at/for the Centers for Disease Control and Prevention (CDC) and/or the Agency for Toxic Substances and Disease Registry (ATSDR) and are responsible for the CDC's claim that bacteria can cause pneumonia, that describe controlled experiments using purified Mycoplasma pneumoniae, or any other type of purified bacteria, to prove causation of pneumonia..*

A search of our records failed to reveal any documents pertaining to your request for Part 3. However, for parts 2, program provides the attached 13 articles and publications. For Part 1, program offers the attached 5 articles/publications.

You may contact our FOIA Public Liaison at 770-488-6246 for any further assistance and to discuss any aspect of your request. Additionally, you may contact the Office of Government Information Services (OGIS) at the National Archives and Records Administration to inquire about the FOIA mediation services they offer. The contact information for OGIS is as follows: Office of Government Information Services, National Archives and Records Administration, 8601 Adelphi Road-OGIS, College Park, Maryland 20740-6001, e-mail at ogis@nara.gov; telephone at 202-741-5770; toll free at 1-877-684-6448; or facsimile at 202-741-5769.

If you are not satisfied with the response to this request, you may administratively appeal to the Deputy Agency Chief FOIA Officer, Office of the Assistant Secretary for Public Affairs, U.S. Department of Health and Human Services, via the online portal at https://requests.publiclink.hhs.gov/App/Index.aspx. Please mark both your appeal letter and envelope "FOIA Appeal." Your appeal must be electronically transmitted by March 5, 2022.

Sincerely,



Roger Andoh
CDC/ATSDR FOIA Officer
Office of the Chief Operating Officer
(770) 488-6399
Fax: (404) 235-1852

#23-00174-FOIA

JOURNAL OF VIROLOGY, July 1998, p. 6264–6270 Vol. 72, No. 7
0022-538X/98/$04.00+0

Activation of Human T-Cell Leukemia Virus Type 1 *tax* Gene Expression in Chronically Infected T Cells

HSIN-CHING LIN,[1,2] CHARLENE S. DEZZUTTI,[3] RENU B. LAL,[3] AND ARNOLD B. RABSON[1,2,4]*

Viral Pathogenesis Laboratory, Center for Advanced Biotechnology and Medicine,[1] and Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey,[2] Piscataway, New Jersey 08854; HIV/Retrovirus Diseases Branch, Division of HIV/AIDS, STD, and Tuberculosis Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[3]; and Cancer Institute of New Jersey, New Brunswick, New Jersey 08901[4]

Received 29 July 1997/Accepted 17 April 1998

Expression of human T-cell leukemia virus type 1 (HTLV-1) is regulated both by the HTLV-1 Tax transactivator and by cellular transcriptional factors binding to the viral long terminal repeat (LTR), suggesting that cellular signals may play a role in regulating viral expression. Treatment of cells chronically infected with HTLV-1, which express low levels of HTLV-1 RNAs and Tax protein, with phorbol esters (i.e., phorbol12-myristate 13-acetate [PMA]), phytohemagglutinin (PHA), sodium butyrate, or combinations of cytokines resulted in induction of HTLV-1 gene expression. PMA or PHA treatment following cotransfection of HTLV-1 Tax expression plasmids resulted in synergistic activation of HTLV-1 LTR-directed gene expression, apparently involving tyrosine kinase-mediated pathways. These results suggest that cellular activation stimuli may cooperate with HTLV-1 Tax to enhance expression of integrated HTLV-1 genomes and thus may play a role in the pathogenesis of HTLV-1 disease.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATL) (45, 63) and is also associated with a chronic, degenerative neurologic disease known as HTLV-1-associated myelopathy or tropical spastic paraparesis (HAM/TSP) (22, 41), as well as inflammatory arthritis, polymyositis, uveitis, and Sjögren's syndrome. The development of these diseases is associated with long periods of clinical latency following HTLV-1 infection, prior to onset of symptons. The pathogenic mechanisms by which HTLV-1 infection results in these diverse clinical syndromes are still not clear, and only a small percentage of HTLV-1-infected individuals actually develop HTLV-1-related disease processes (for reviews, see references 19 and 58). An important common denominator in the pathogenesis of these diverse syndromes appears to be induction of T-cell proliferation as a result of expression of the HTLV-1 Tax transactivator (for reviews, see references 55 and 64). HTLV-1 gene expression in vivo likely plays an important role in the steps leading to disease induction; however, it is still unclear how viral replication or expression of virally encoded proteins directly contributes to the development of HTLV-1-associated diseases. In HTLV-1-infected, asymptomatic individuals, easily detectable levels of circulating infected cells are seen; however, a further marked increase in the number of circulating infected cells is observed in HAM/TSP patients (12, 23, 30), with levels reported to be as high as one-fifth of the peripheral blood mononuclear cells or randomly cloned T cells (48). These high levels of viral infection are likely due to expansion of infected T cells under the influence of the viral Tax protein (59). These observations suggest that there may exist stimuli which modulate HTLV-1 gene expression in vivo and contribute to HTLV-1 pathogenesis. Furthermore, in situ PCR experiments showed that only approximately 10% of HTLV-1-infected lymphocytes were expressing HTLV-1 RNA (40), and recent studies of HTLV-1-infected T cells cloned randomly from patients demonstrated that most cell clones obtained were not producing detectable HTLV-1 RNAs (48), also suggesting that there may be important cellular controls of viral gene expression.

Transcription of HTLV-1 is regulated both by cellular transcription factors that bind directly to long terminal repeat (LTR) DNA and by a virally encoded transactivator, Tax, which augments HTLV-1 LTR transcription through the Tax response elements (TREs) located in the DNA of the U3 region of the HTLV-1 LTR (9, 43, 54, 56). Tax complexes with the cyclic AMP response element binding proteins (CREB/ATF), affecting the dimerization and binding of Tax-CREB complexes to TRE DNA (5, 44, 57, 65). Binding of CREB proteins to the LTR recruits the transcriptional coactivator, CBP, to the promoter to enhance transcription (31) and allows direct interactions of Tax with the basal transcriptional apparatus (10, 16). Full Tax-mediated activation may also involve other factors that associate with the HTLV LTR (33), such as the Ets proteins (24). Cellular transcriptional factors, such as Ets, Myb, AP1, and AP2, also can bind directly to the HTLV-1 LTR and activate HTLV-1 transcription (8, 15, 24, 29, 37).

Although binding sites for several inducible transcription factors have been mapped in the HTLV-1 LTR, and the expression of these transcription factors has been shown to transactivate the HTLV-1 LTR in vitro, the biological importance of these activation pathways remains to be determined. Furthermore, relatively little is known about activation of integrated HTLV-1 proviral gene expression in HTLV-1-infected cells. Increased expression of HTLV-1 Tax mRNA has been documented following culture of peripheral blood mononuclear cells from HAM/TSP patients and from the skin of ATL patients (52, 53), suggesting the possibility that HTLV-1 gene expression can be induced in vitro. It has also been reported that an HTLV-1 LTR-directed reporter gene transfected into HeLa cells can be activated when transfected cells are cocultured with T cells (such as Hut78 cells), suggesting that factors secreted by T cells may activate HTLV-1 gene expression (4).

* Corresponding author. Mailing address: CABM, Rm. 139, 679 Hoes Ln., Piscataway, NJ 08854. Phone: (908) 235-5368. Fax: (908) 235-4850. E-mail: rabson@rmbcl.rutgers.edu.

Recently, data demonstrating that induction of the cellular stress response may enhance HTLV-1 gene expression have been presented (2, 3). Thus, different cellular signals could play a role in activating expression of HTLV-1 proviruses.

To further study cellular activation stimuli that may affect HTLV-1 gene expression, we have utilized a series of HTLV-1 chronically infected cell lines exhibiting differing levels of expression of HTLV-1 proteins (17, 32). HTLV-1-infected cell lines used in these studies included 1657 cells (derived from an ATL patient), 1996 cells (derived from an asymptomatic carrier), and FS and A212 cells (both derived from HAM/TSP patients) (17, 35). Cells that had been maintained in RPMI 1640 medium with 15% fetal bovine serum (GIBCO-BRL) and 10% recombinant human interleukin-2 (IL-2; Boehringer-Mannheim) were treated with different compounds known to induce gene expression in T cells, including phytohemagglutinin-P (PHA) (5 μg/ml; Sigma), phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma), and sodium butyrate (5 mM; Sigma), for 48 h and then examined for the expression of the HTLV-1 Tax protein by Western blot analysis as previously described (32). HTLV-1-transformed Hut102 cells, derived from an ATL patient (20), were grown in RPMI 1640 medium with 10% fetal bovine serum and were analyzed for induction by the same protocol. Basal *tax* expression was detectable in unstimulated Hut102, 1996, and 1657 cells; however, only very low to undetectable levels of Tax were observed in untreated FS and A212 cells (Fig. 1A, lanes 1). Sodium butyrate, a differentiating agent that enhances cellular gene expression by altering the chromatin structure and causing histone acetylation (49), strongly induced Tax protein expression in all cell lines compared to levels in nontreated cells (Fig. 1A, compare lanes 1 and 4 for each cell line). PMA, a phorbol ester that cooperates in T-cell activation through the protein kinase C (PKC) pathway and has been previously reported to activate the HTLV-1 LTR (47), induced *tax* expression in Hut102, FS, 1996, and 1657 cells but failed to induce expression of this gene in A212 cells (Fig. 1A, lanes 3). Treatment with PHA, a plant lectin that acts as a T-cell mitogen, activating resting T cells through the CD3 molecule on the T-cell membrane (61), strongly increased *tax* expression in FS cells but resulted in only low-level *tax* induction in 1996 cells and caused no apparent induction of expression of this gene in Hut102, 1657, or A212 cells (Fig. 1A, lanes 2).

Since FS cells demonstrated significantly increased HTLV-1 Tax protein expression over background levels in response to all three stimuli initially examined (Fig. 1A), we next examined whether cytokines, as physiologic inducers of different cellular responses, would also induce expression of this protein. FS cells were treated with an array of stimuli, such as PHA, PMA, sodium butyrate, hexamethylene bisacetamide (6 mM; Sigma), lipopolysaccharide (0.1 ng/ml; Sigma), and combinations of IL-1β (2 ng/ml; Boehringer-Mannheim), IL-6 (100 or 1,000 U/ml; Boehringer-Mannheim), tumor necrosis factor alpha (TNF-α) (100 U/ml; Boehringer-Mannheim), and gamma interferon (100 U/ml; Boehringer-Mannheim), for 48 h, and Tax protein expression was detected by Western blot analysis (Fig. 1B). In addition to PMA, PHA, and sodium butyrate, which strongly increased Tax protein expression (three- to fourfold, as determined by densitometric analysis of the Western blot), as shown in the previous experiment, certain cytokine combinations, such as IL-1β plus either IL-6 or TNF-α and gamma interferon plus TNF-α, also resulted in modest (twofold, as determined by densitometry) but detectable induction of Tax protein expression, suggesting that combinations of cytokines may also be able to weakly induce HTLV-1 gene expression from latent states of viral gene expression.

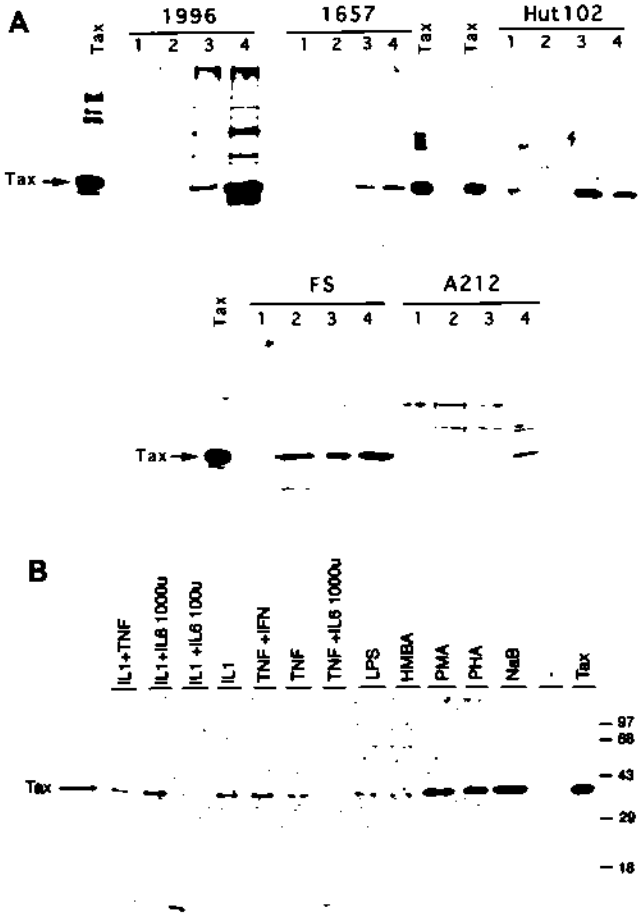


FIG. 1. Expression of Tax protein in HTLV-1 chronically infected cell lines following treatment with various T cell activation stimuli and cytokines. (A) Expression of Tax protein in HTLV-1 chronically infected cell lines following treatment with PHA, PMA, or sodium butyrate. Western blot analysis of whole-cell extracts from HTLV-1 infected patient cell lines (17) and HTLV-1 transformed Hut102 cells was performed with antiserum directed against the HTLV-1 Tax protein. FS, 1996, 1657, A212, and Hut102 cells either were left untreated (lanes 1) or were treated with PHA at 5 μg/ml (lanes 2), PMA at 50 ng/ml (lanes 3), or sodium butyrate at 5 mM (lanes 4) for 48 h, and whole-cell extracts were then prepared in radioimmunoprecipitation assay (RIPA) buffer. Bacterially synthesized Tax protein was used as a positive control. The arrows indicate the position of the Tax protein. (B) Expression of Tax protein in FS cells following treatment with various activation stimuli and cytokines. Western blot analyses of whole cell extracts from HTLV-1 infected FS cells, either untreated or treated with a variety of stimuli, including various differentiation and activation compounds and cytokines, were performed with antiserum directed against the HTLV-1 Tax protein. FS cells were treated as indicated for 48 h, and whole cell extracts were prepared in RIPA buffer. Bacterially expressed Tax protein (Tax) was used as a positive control. The arrow indicates the position of the Tax protein. The numbers on the right indicate the positions of molecular size markers (in kilodaltons). IFN, interferon; LPS, lipopolysaccharide; HMBA, hexamethylene bisacetamide; NaB, sodium butyrate; -, untreated.

Northern blot analysis was performed to further characterize the activation of HTLV-1 gene expression in FS cells. Total cellular RNA (13) was prepared from FS cells treated with the activators 24 h prior to harvest and subjected to Northern blot hybridization (46), using a ^{32}P-radiolabeled HTLV-1 LTR DNA fragment to detect levels of steady-state HTLV-1 RNA in untreated or stimulated cells (Fig. 2A). Untreated FS cells expressed easily detectable levels of full-length (8.4-kb) singly spliced *env* and doubly spliced *tof-rof* RNAs, as well as much lower levels of 1.6- to 2.0-kb *tax-rex* RNAs (14). Treatment of FS cells with PHA, PMA, or sodium butyrate resulted in

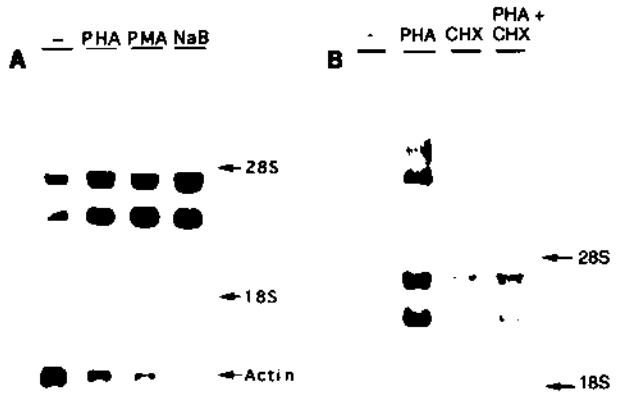


FIG. 2. Induction of HTLV-1 RNA expression in FS cells. (A) FS cells were treated with PHA (5 μg/ml), PMA (50 ng/ml), or sodium butyrate (NaB; 5 mM) for 24 h, and total RNA was prepared by the guanidinium isothiocyanate-acid phenol method (13). Fifteen micrograms of total cellular RNA was electrophoresed through denaturing agarose-formaldehyde gels, and Northern blot analysis was performed with a radiolabeled HTLV-1 LTR DNA fragment (a 500-bp *Xho*I *Hin*dIII fragment from the pU3RCAT plasmid) as a probe. The positions of the 28S and 18S rRNAs are indicated. Hybridization to a mouse actin cDNA probe is shown at the bottom. (B) FS cells were treated with PHA (5 μg/ml), with or without the addition of cycloheximide (CHX; 100 μg/ml), for 24 h, and total RNA was prepared. Northern blot analysis was performed as described for panel A. –, untreated.

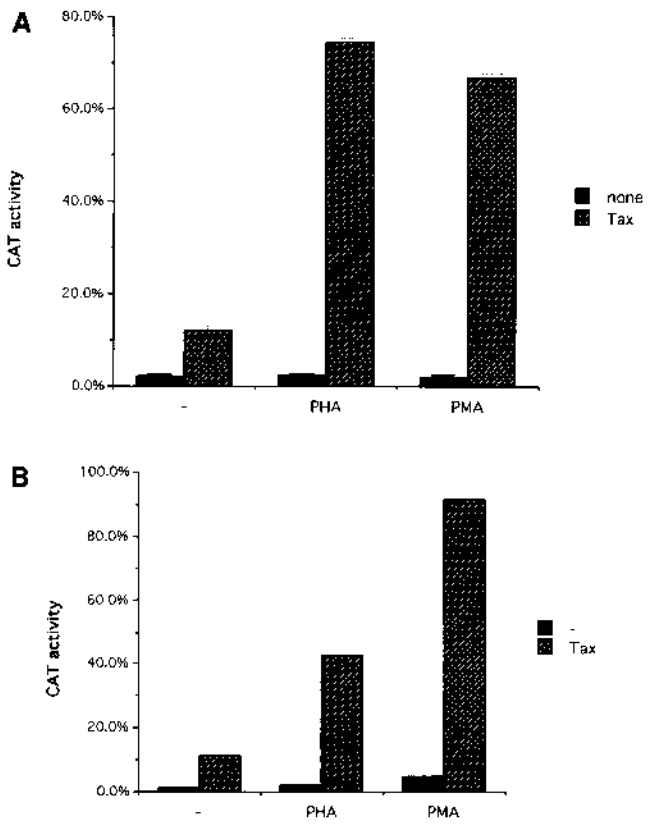


FIG. 3. Synergistic activation of the HTLV-1 LTR by simultaneous stimulation with Tax and either PMA or PHA. (A) The pU3RCAT plasmid (5 μg), containing the HTLV-1 LTR directing the expression of the CAT reporter gene, was transfected into Jurkat cells, with or without an HTLV-1 LTR-driven Tax-expressing vector (pHTLV-1 Tax; 1 μg); 24 h after transfection, the cells were treated with PHA (5 μg/ml) or PMA (50 ng/ml). CAT activities were assayed at 48 h after transfection. Average CAT activities from triplicate transfections are presented as percentages of chloramphenicol converted to the acetylated form, and the standard deviations are indicated by the error bars. (B) The pU3RCAT plasmid, containing HTLV-1 LTR-directed CAT reporter gene, was cotransfected into Hut78 cells, with or without pHTLV-1 Tax, the Tax expression plasmid. Stimuli were added 24 h after transfection. CAT activity was assayed 48 h after transfection.

readily observable increases in the levels of each of the different HTLV-1 RNA species (Fig. 2A), approximately three- to fourfold as quantitated by PhosphorImager analysis (Molecular Dynamics), including induction of the multiply spliced *tax* RNA at from very low to readily detectable levels. These data demonstrate that these stimuli increase the steady-state levels of HTLV-1 RNA, possibly through enhanced LTR transcription. Interestingly, addition of cycloheximide (100 μg/ml) to the PHA treatment regimen (Fig. 2B, lane 4) significantly reduced the activation of mRNA expression in FS cells compared to that resulting from PHA treatment alone (Fig. 2B, lane 2). These data suggest that new protein synthesis is required for PHA-induced activation of HTLV-1 RNA production.

To clarify the possible mechanisms responsible for the increased HTLV-1 gene expression on treatment with T-cell-activating stimuli, transient transfections of T cells (Jurkat cells [Fig. 3A] and Hut78 cells [Fig. 3B]) with an HTLV-1 LTR-driven chloramphenicol acetyltransferase (CAT) reporter plasmid (pU3RCAT [56]) were performed by a DEAE-dextran procedure (18), and at 24 h posttransfection the cells were treated with 5 μg of PHA or 50 ng of PMA per ml. The cells were cultured for another 24 h before being harvested. Equal amounts (2 to 4 μg) of total cellular protein were used for the CAT activity assay (25). To examine possible cooperative effects, the transfections were performed with or without Tax protein coexpression (via cotransfection of pHTLV-1 Tax, a Tax expression vector directed by the HTLV-1 LTR [38]). In both cell lines, PHA or PMA treatment alone did not significantly affect HTLV-1 LTR-directed CAT gene expression (Fig. 3). Cotransfection of an HTLV-1 Tax-expressing plasmid with pU3RCAT resulted in an approximately 6- to 10-fold activation of CAT gene expression. PHA or PMA treatment in conjunction with Tax cotransfection resulted in a further 4- to 6-fold increase in HTLV-1 LTR-driven CAT activity compared with that resulting from Tax cotransfection alone (or 20- to 30-fold activation over that with LTR-CAT alone). Thus, LTR activation by PHA and PMA was Tax dependent and demonstrated synergistic stimulation with Tax transactivation. In these experiments, HTLV-1 Tax expression was directed by the HTLV-1 LTR. Therefore, one possible explanation for the observed results was that PHA or PMA treatment enhanced HTLV-1 LTR-directed Tax expression, thus further augmenting the expression of the HTLV-1 LTR-directed reporter gene through TRE elements in the LTR. Experiments utilizing cotransfection of a Tax expression vector directed by the cytomegalovirus immediate-early promoter also demonstrated synergistic activation of the LTR with PHA treatment (data not shown), suggesting that the effects seen in this experiment were not due simply to activation of the LTR-Tax plasmid but instead represented synergistic activation of HTLV-1 LTR activity by PHA or PMA, plus Tax.

To identify signal transduction pathways that might mediate the activation of the HTLV-1 LTR by PHA-Tax and PMA-Tax, we transfected Jurkat T cells with an HTLV-1 LTR-luciferase reporter plasmid (constructed by inserting the *Xho*I-*Hin*dIII fragment containing the HTLV-1 LTR from

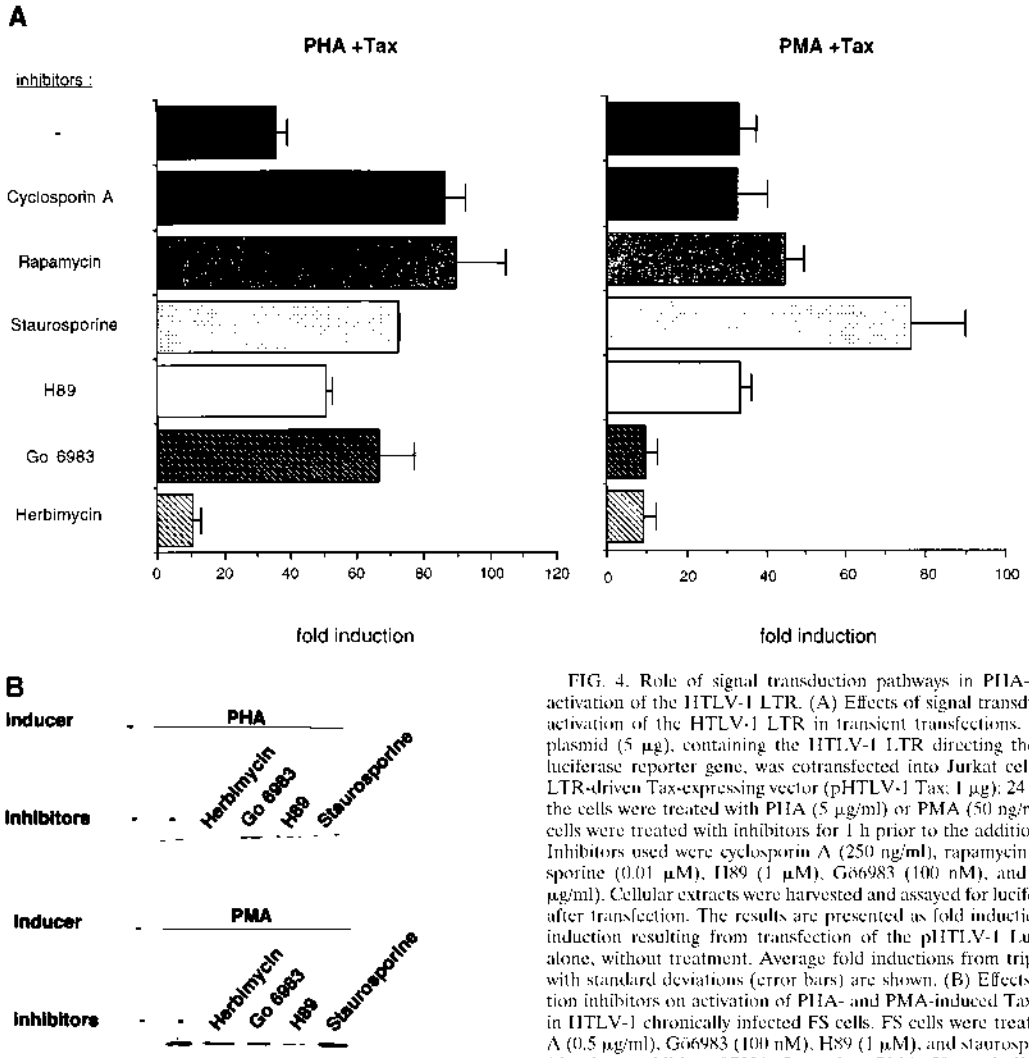


FIG. 4. Role of signal transduction pathways in PHA-Tax and PMA-Tax activation of the HTLV-1 LTR. (A) Effects of signal transduction inhibitors on activation of the HTLV-1 LTR in transient transfections. The pHTLV-1 Luc plasmid (5 μg), containing the HTLV-1 LTR directing the expression of the luciferase reporter gene, was cotransfected into Jurkat cells with an HTLV-1 LTR-driven Tax-expressing vector (pHTLV-1 Tax; 1 μg). 24 h after transfection, the cells were treated with PHA (5 μg/ml) or PMA (50 ng/ml). The transfected cells were treated with inhibitors for 1 h prior to the addition of PMA or PHA. Inhibitors used were cyclosporin A (250 ng/ml), rapamycin (20 ng/ml), staurosporine (0.01 μM), H89 (1 μM), Gö6983 (100 nM), and herbimycin A (0.5 μg/ml). Cellular extracts were harvested and assayed for luciferase activity at 48 h after transfection. The results are presented as fold induction compared to the induction resulting from transfection of the pHTLV-1 Luc reporter plasmid alone, without treatment. Average fold inductions from triplicate transfections with standard deviations (error bars) are shown. (B) Effects of signal transduction inhibitors on activation of PHA- and PMA-induced Tax protein expression in HTLV-1 chronically infected FS cells. FS cells were treated with herbimycin A (0.5 μg/ml), Gö6983 (100 nM), H89 (1 μM), and staurosporine (0.01 μM) for 1 h prior to addition of PHA (5 μg/ml) or PMA (50 ng/ml). Western blot analyses of whole-cell extracts with antiserum directed against the HTLV-1 Tax protein were performed as described in the legend to Fig. 1. −, untreated.

pU3RCAT into the pGL3 luciferase expression vector [Promega]). Twenty-four hours following transfection, the transfected Jurkat cells were divided into aliquots and incubated with a series of signal transduction pathway inhibitors for 1 h prior to the addition of PHA or PMA. Cellular extracts were harvested and assayed for luciferase activity by the use of a luciferase assay system (Promega). The results are shown as fold induction of HTLV-1 luciferase activity over that observed after transfection of the HTLV-1 luciferase plasmid alone (Fig. 4A). The possible toxicities of these treatments were examined by quantifying viable cells by the MTT assay (36), and no significant decrease in cell viability was observed with any treatment compared to that in untreated transfected Jurkat cells (data not shown). The inhibitors used were as follows: the immunosuppressive compounds cyclosporin A (250 ng/ml; Sigma), which functions through the calcineurin pathway, and rapamycin (20 ng/ml; Calbiochem), which inhibits $p70^{s6k}$, $p34^{cdc2}$, and $p33^{cdk2}$; the broad-specificity protein kinase inhibitor staurosporine (0.01 mM; Calbiochem); the protein kinase A-specific inhibitor H89 (1 mM; Calbiochem); the PKC-specific inhibitor Gö6983 (100 nM; Calbiochem); and the protein tyrosine kinase (PTK)-specific inhibitor herbimycin A (0.5 mg/ml; Sigma). Cyclosporin A, rapamycin, staurosporine, and H89 had no inhibitory effects on either PHA-Tax or PMA-Tax activation, suggesting the lack of involvement of the protein kinase A, calcineurin, and p70 S6 kinase-mediated pathways. As expected, Gö6983 inhibited the induction of HTLV-1 LTR by PMA-Tax with no toxicity, indicating the involvement of the PKC pathway in PMA activation of the HTLV-1 LTR (28). Gö6983 failed to inhibit PHA-Tax activation, however, suggesting that the involvement of PKC in PHA-Tax activation is unlikely. In contrast, herbimycin A, a PTK inhibitor, strongly reduced both PHA-Tax and PMA-Tax activation, suggesting the involvement of PTKs in the synergistic activation pathways induced by both PHA and PMA.

We further examined the potential role of signal transduction pathways in the activation of integrated HTLV-1 proviral gene expression. The effects of a series of kinase inhibitors on *tax* induction by PHA or PMA in HTLV-1 chronically infected FS cells were assessed. FS cells were treated with herbimycin A (0.5 mg/ml), Gö6983 (100 nM), H89 (1 mM), and staurosporine (0.01 mM) for 1 h prior to addition of PHA (5 mg/ml) or PMA (50 ng/ml). Cells were lysed 48 h after treatment, and the expression of Tax protein was detected by Western blot anal-

ysis (Fig. 4B). Consistent with the results observed in transient transfection studies, PHA-induced Tax expression was inhibited by herbimycin but not by Gö6983, H89, or staurosporine (Fig. 4B, top panel). Thus, PHA activation of the integrated HTLV-1 provirus in FS cells apparently also involves tyrosine kinase pathways. Somewhat surprisingly, the strong induction of Tax expression by PMA was not significantly inhibited by any kinase inhibitor tested (Fig. 4B, bottom panel), suggesting that the PMA induction pathways in FS cells may be distinct from those in transiently transfected Jurkat cells (Fig. 4A).

In these studies using HTLV-1 chronically infected cell lines, we identified cellular activation stimuli that induced expression of HTLV-1 RNA and Tax protein. These stimuli included both cellular activation and differentiation agents such as PHA, PMA, and sodium butyrate, which strongly induced HTLV-1 gene expression, as well as certain combinations of cytokines which resulted in more modest levels of HTLV-1 induction. Taken together, these results suggest that either direct immune stimulation of infected T cells or immune activation resulting in cytokine secretion could potentially activate HTLV-1 gene expression in infected cells and thus could play a role in HTLV-1 disease pathogenesis. In addition to the immune, differentiation, and cytokine activation pathways that we have described, the induction of cellular stress responses upon either heat stimulation or sodium arsenite treatment has also been shown to activate the expression of HTLV-1 genes from chronically infected cell lines (3). Induction of HTLV-1 expression by these agents was independent of Tax and was mediated by LTR DNA sequences containing the basal promoter without TREs (2) and thus appears to be mechanistically different from either the PMA or PHA stimulation that we have observed.

The mechanisms responsible for HTLV-1 gene activation by the different activating agents used in our studies are likely to be distinct. Sodium butyrate stimulates expression of many cellular and viral genes through the inhibition of histone deacetylases (50), resulting in hyperacetylation of histones, which has been shown to be associated with transcriptionally active chromatin (34). Interestingly, the CBP/p300 proteins associated with CREB/ATF and Tax (31) have been recently shown to be histone acetyltransferases (39); thus, histone deacetylation may represent a common pathway for activation of HTLV-1 gene expression, even by the HTLV-1 Tax protein and associated CREB/ATF complexes. Phorbol esters have been previously shown to activate the HTLV-1 LTR function in combination with HTLV-1 Tax (47) through a 60-bp element that includes the middle HTLV-1 LTR TRE. The TRE itself was not sufficient for tetradecanoyl phorbol acetate induction, suggesting that other cellular LTR-binding transcription factors, such as the phorbol ester-inducible AP1 (c-Jun) and AP2 transcription factors (27, 29, 37), may play a role. Our studies suggest that the PMA-mediated synergistic activation in Jurkat cells likely involves a PKC-mediated signal transduction pathway, although the pathway in FS cells may be somewhat different. Inhibition of the PKC pathway did not, however, block activation of the HTLV-1 LTR pathway by PHA and Tax, suggesting that to mediate HTLV-1 LTR activation, PMA and PHA function at least in part through different signal transduction pathways.

The T-cell mitogen PHA activated HTLV-1 gene expression in FS and 1996 cells but failed to induce HTLV-1 *tax* in A212 and 1657 cells. Many of the effects of PHA stimulation appear to parallel the effects of cross-linking of the CD3 molecule on the T-cell surface, and PHA activation requires an intact T-cell receptor complex (61); therefore, PHA activation of HTLV-1 gene expression may involve T-cell receptor components such as CD3. Fluorescence-activated cell sorter analysis of T-cell surface markers present on the different chronically infected cell lines demonstrated that CD3 expression was restricted to FS and 1996 cells, the two cell lines which exhibited induction of Tax protein expression following PHA treatment, and was not detectable on either A212 or 1657 cells (data not shown), raising the possibility that the effects of PHA on HTLV-1 gene expression may be mediated through CD3 signaling. Herbimycin A, an inhibitor of PTKs, inhibited the synergistic activation by PHA and Tax, raising the possibility that tyrosine kinases involved in T-cell activation, such as Lck, may play a role in the activation process. Herbimycin A also inhibited the synergistic activation induced by PMA and Tax, suggesting that a tyrosine kinase contributes to a downstream step in the synergistic activation of the HTLV-1 LTR, a step common to both the PHA-Tax and PMA-Tax activations of the LTR. Tyrosine kinases could regulate phosphorylation of components of the Tax-CREB/ATF-CBF complex or phosphorylation of downstream targets of this complex, such as Tafs or general transcription factors. It is not clear at this time which cellular transcription factors are required for the synergy with Tax in response to PHA treatment. Cellular Ets transcription factors are one family of transcription factors that may contribute to the activation by PHA and Tax, since transfection of Ets expression plasmids has been previously reported to induce HTLV-1 LTR expression, both alone and in combination with Tax (7, 24, 51); however, other factors, such as Myb or AP-2, could also play a role.

The pathogenesis of HTLV-1-associated disorders is complex, and only a subset of infected individuals progress to ATL, HAM/TSP, or an HTLV-1-associated autoimmune disorder. The time of viral infection apparently is one determinant of pathogenesis; however, it is likely that other host and viral factors also play a role. In particular, given the long latency periods associated with disease appearance, host factors such as the immune response as well as the intracellular regulation of viral gene expression may be important. The observation that immune system activation stimuli such as PHA and PMA as well as certain cytokines and cellular stress signals can induce enhanced HTLV-1 *tax* expression suggests that these stimuli may alter HTLV-1 pathogenesis in vivo. As is clear from our studies, different stimuli activate HTLV-1 gene expression to different degrees in different types of infected cells, possibly reflecting the effects of variable levels of cell surface receptors (such as for PHA) or of different integration sites in which *cis*-acting neighboring cellular DNA sequences might play a role in modulating the LTR autonomous response that we observed in our transfection studies. Despite the potential variability of such activation in vivo, the fact that PHA, PMA, and cytokine-induced stimulation of HTLV-1 gene expression could be readily identified in both transiently transfected as well as chronically infected cells suggests that this mechanism is likely to be operative in at least some of the large numbers of infected cells in vivo. The fact that HTLV-1-infected cells and particles have themselves been reported to induce immune system activation (21, 62) suggests that an autocrine stimulatory pathway, by which induction of HTLV-1 gene expression by immune system, cytokine, or stress activation could result in HTLV-1 production with further T-cell and viral activation, may be in operation.

For HTLV-1 infection, two different scenarios by which enhanced HTLV-1 gene expression may contribute to pathogenesis can be envisaged. In one model, increased HTLV-1 gene expression results in enhanced virus production and spread. This is similar to models proposed for human immunodeficiency virus (6, 42). Newly infected target cells then either

become substrates for transformation into ATL cells or increase the likelihood of infection and proliferation of subsets of T cells, leading to the development of HAM/TSP. This model is unlikely in view of recent observations demonstrating that clonal expansion of HTLV-1-infected cells appears to account for increases in the HTLV-1 proviral load in vivo (11, 59, 60), strongly suggesting that extensive in vivo spread of HTLV-1 through multiple rounds of infection of new target cells may not be required for HTLV-1 pathogenesis (59). An alternative model suggests that the important effect of enhanced HTLV-1 gene expression is increased intracellular levels of the Tax protein. Tax protein expression is clearly associated with the induction of T-cell proliferation; retroviral and herpesvirus vectors expressing Tax can induce sustained proliferation of primary human T cells (1, 26), and the ability of randomly cloned HTLV-1-infected cells to proliferate in vitro is directly correlated with expression of Tax (48). Thus, an important effect of *tax* induction by immune or cytokine stimulation could be direct induction of proliferation of particular infected T cells rather than enhanced viral spread to uninfected cells. These cells activated for proliferation by induction of *tax* might contribute to the pool of cells from which ATL cells or, alternatively, cells responsible for the development of HAM/TSP could arise.

We thank T. Folks for helpful discussions and S. Marriott for purified Tax protein. Antiserum to HTLV-1 Tax was obtained through the NIH NIAID AIDS Research and Reference Reagent Program (contributed by K.-T. Jeang).

This work was funded by a Public Health Service research grant to A.B.R. from the National Cancer Institute (CA-68333) and by the New Jersey Commission on Science and Technology.

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Oncogene (2005) 24, 6005–6015

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Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation

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Over the past 25 years, animal models of human T-lymphotropic virus type 1 (HTLV-1) infection and transformation have provided critical knowledge about viral and host factors in adult T-cell leukemia/lymphoma (ATL). The virus consistently infects rabbits, some non-human primates, and to a lesser extent rats. In addition to providing fundamental concepts in viral transmission and immune responses against HTLV-1 infection, these models have provided new information about the role of viral proteins in carcinogenesis. Mice and rats, in particular immunodeficient strains, are useful models to assess immunologic parameters mediating tumor outgrowth and therapeutic invention strategies against lymphoma. Genetically altered mice including both transgenic and knockout mice offer important models to test the role of specific viral and host genes in the development of HTLV-1-associated lymphoma. Novel approaches in genetic manipulation of both HTLV-1 and animal models are available to address the complex questions that remain about viral-mediated mechanisms of cell transformation and disease. Current progress in the understanding of the molecular events of HTLV-1 infection and transformation suggests that answers to these questions are approachable using animal models of HTLV-1-associated lymphoma
Oncogene (2005) **24,** 6005–6015. doi:10.1038/sj.onc.1208974

Keywords: HTLV-1; animal models; leukemia; lymphoma

Introduction

Human T-lymphtropic virus type 1 (HTLV-1), a member of the deltaretroviruses, is the causative agent of adult T-cell leukemia/lymphoma (ATL) (Yoshida *et al*., 1982), a highly aggressive CD4+ T-cell malignancy. Over the past 25 years, a variety of animal models of HTLV-1, infection and transformation have provided fundamental information about viral and host determinants of this devastating malignancy (Table 1). The virus consistently infects rabbits (Akagi *et al*., 1985; Lairmore *et al*., 1992), some non-human primates (Nakamura *et al*., 1987; Murata *et al*., 1996), and to a lesser extent rats (Suga *et al*., 1991; Ibrahim *et al*., 1994). Viral transmission in mice using typical methods of infection produces inconsistent infections and limited virus expression in tissues (Fang *et al*., 1998; Feng *et al*., 2001; Furuta *et al*., 2002a, b; Nitta *et al*., 2003). Non-human primates have been infected with HTLV-1 and certain species have a natural infection with simian T-lymphotropic virus infection type 1 (STLV-1) (Gessain and Dethe 1996; Takemura *et al*., 2002; Gabet *et al*., 2003). The squirrel monkey has been successfully infected with HTLV-1 and offers an attractive non-human primate model of HTLV-1 for vaccine testing (Kazanji, 2000; Kazanji *et al*., 2001; Mortreux *et al*., 2001a; Sundaram *et al*., 2004). Rats have been infected with HTLV-1-producing cells and offer a tractable model of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/ATL), the neurologic disease associated with the viral infection (Suga *et al*., 1991; Ishiguro *et al*., 1992; Kasai *et al*., 1999; Sun *et al*., 1999; Kannagi *et al*., 2000; Hakata *et al*., 2001). In addition, rats have been used to test the role of cell-mediated immunity to the infection (Kannagi *et al*., 2000; Hasegawa *et al*., 2003). However, controversy exists regarding the reproducibility of the viral infection in rats (Ibrahim *et al*., 1994). Bovine leukemia virus (BLV) infection of sheep offers a reliable model of disease associated with deltaretrovirus infections and insight into viral genetic determinants of tumor induction (Willems *et al*., 2000). This review will focus on current progress in the development of animal models of HTLV-1 infection and transformation.

HTLV-1 replication and mechanisms of carcinogenesis

Understanding HTLV-1 replication forms the basis of development for animal models of the viral-induced

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Table 1 Animal models of HTLV-I infection and disease

Animal[a]	*Advantages*	*Disadvantages*
Rabbit	Low cost and ease of maintenance Easy to induce persistent infection and test immune response, transmission routes, and parameters of infection, for example, viral loads Evaluation of viral genetic determinants of infection using HTLV-I molecular clones	No reliable disease induced following infection Lack of broadly available reagents to study
Rat	Low cost, ease of maintenance, and can be genetically modified Can become persistent infected to test immune response (e.g. cytotoxic T cells) and parameters of infection, for example, viral loads Some strains useful for neurologic disease model Immunologic reagents available	Wide variation in infection in some strains Disease induced following infection does not histologically model human neurologic disease ATL models require immunodeficient rats
Immunocompetent mice	Low cost, ease of maintenance, and can be genetically modified Can test immune response (e.g. cytotoxic T cells) Immunologic reagents available	Limited evidence of *in vivo* spread of infection No apparent disease induced following infection
Non-human primate	Phylogenetic similarity to humans Good model for persistent infection Development of HTLV-like disease using molecular clones reported Immunologic reagents available	Cost and availability Inconsistent disease development after prolonged period of time
Immunodeficient mice (e.g. NOD SCID)	Development of lymphoma following transplantation Used to test therapeutic reagents against tumor Immunologic reagents available	Cost and complicated maintenance Available of ATL cell lines to induce tumors is limited
Transgenic or knockout mice	Induction of lymphoma and other lymphocyte-mediated diseases using individual gene products of HTLV-I Immunologic reagents available Ability to test of host parameters in induction of disease, for example, cytokines	Cost of production and maintenance CD4+ T-cell lymphomas inconsistently produced Overexpression of viral gene products may not be representative of carcinogenesis in HTLV-I infection

[a]Excluding BLV and STLV animal models

malignancy. HTLV-I is a complex retrovirus with type C retroviral morphology. The virus belongs to the deltaretrovirus group along with BLV and other primate lymphotropic viruses including STLV-1. Unlike simple retroviruses, complex retroviruses like HTLV-I carry additional genes to encode for several regulatory and accessory proteins. As a complex retrovirus, HTLV-I encodes regulatory proteins from the pX region (between *env* and 3′-LTR), by alternative splicing from four open reading frames (ORF). ORF IV and ORF III encode regulatory proteins Tax (Transcriptional Activator of pX region) and Rex, respectively (Kiyokawa *et al*., 1985; Seiki *et al*., 1985). Tax, a 40 kDa nuclear protein produced from a doubly spliced mRNA, is transactivator of HTLV-1 gene transcription from the viral LTR (Felber *et al*., 1985; Sodroski *et al*., 1985). Tax forms complexes with DNA-binding proteins, such as CREB, to contact GC-rich sequences flanking the Tax-responsive element 1 (TRE-1) for the transactivation of the LTR (Kimzey and Dynan, 1999; Lenzmeier *et al*., 1999). Tax enhances the binding of cyclic AMP response/activator of transcription proteins to the TRE-1 and several basic leucine zipper proteins to the TRE-2 (Perini *et al*., 1995). In addition, Tax regulates the expression of numerous cellular genes, predominantly by induction of the transcription factors nuclear factor kappa of B cells (NF-κB) and serum response factor, independent of CREB activation (Gatza *et al*., 2003; Jeang *et al*., 2004; Kehn *et al*., 2004). Rex, a nucleolar localizing 27 kDa protein, is responsible for nuclear export of unspliced (*gag/pol*) and singly spliced (*env*) viral RNA to the cytoplasm. Even though Rex is not required for *in vitro* immortalization by HTLV-I, Ye *et al*. (2003) reported that Rex is critical for efficient infection of cells and persistence *in vivo*.

In addition to the regulatory proteins Tax and Rex, the HTLV-I pX genome region encodes four accessory proteins, $p12^{I}$, $p27^{I}$, $p13^{II}$, and $p30^{II}$ from alternatively spliced forms of mRNA in ORF I and II (Berneman *et al*., 1992; Koralnik *et al*., 1992, 1993; Ciminale *et al*., 1995). These proteins are important for *in vivo* viral infectivity, host cell activation, and regulation of gene transcription (Collins *et al*., 1998; Ciminale *et al*., 1999; Trovato *et al*., 1999; Albrecht *et al*., 2000, 2002; Bartoe *et al*., 2000; D'Agostino *et al*., 2000; Zhang *et al*., 2000, 2001; Ding *et al*., 2002; Franchini *et al*., 2003; Kim *et al*., 2003; Michael *et al*., 2004; Nicot *et al*., 2004; Younis *et al*., 2004). The role of these accessory proteins in HTLV-1-mediated transformation has not been

elucidated, but they are clearly important in the ability of the virus to infect and spread *in vivo*.

Adult T-cell leukemia/lymphoma

Animal models of HTLV-1 transformation ideally mimic the variety of forms of ATL- or HTLV-1-associated diseases. Typically, 1–5% of HTLV-1-infected individuals develop ATL after a latent period of 20–30 years (Yamaguchi and Takatsuki, 1993; Cleghorn *et al*., 1995). ATL is an aggressive T-cell malignancy with a leukemic phase characterized by circulating, activated CD4+/CD25+ T cells (Uchiyama *et al*., 1977). Infection early in life is associated with the development of ATL and the estimated lifetime risk is about 5% in individuals infected before the age of 20 years (Cleghorn *et al*., 1995; Wilks *et al*., 1996). The incidence rate is 2–4 per 100 000 person-years and males have higher risk than females (Kondo *et al*., 1989; Cleghorn *et al*., 1995). The clinical features, treatment and prevention of ATL are reviewed in this volume.

The development of ATL has been the focus of many investigations, but the exact mechanism is not completely understood. After infecting CD4−CD25+ T cells, the HTLV-1 provirus is randomly integrated into the host genome, where it persists for years. Although HTLV-1 replicates via reverse transcription during the early stages of infection, the virus genome is effectively replicated during clonal expansion of infected cells (Mortreux *et al*., 2003). Typically, there is a progression from polyclonal to oligoclonal and then to monoclonal proliferation *in vivo*, which is achieved while the cells become interleukin 2 (IL-2) independent (Yoshida *et al*., 1984; Hollsberg *et al*., 1992; Franchini, 1995). Based on the long clinical latency and the low percentage of individuals who develop ATL, T-cell transformation is believed to be the result of a series of cellular alterations and/or mutations. Transformation of infected lymphocytes is believed to be initiated through induction of cellular genes and alterations in critical cellular activation and death pathways by the viral transactivator Tax (Gatza *et al*., 2003).

Intriguingly, despite a strong immune response mounted against the HTLV-1, the virus is able to persist in the host. Several possible mechanisms have been suggested to explain this observation: often the genome is partly deleted, resulting in defective virus that may provide a mechanism for escape from immune surveillance (Bangham 2000, 2003a, b). Recent reports (Asquith *et al*., 2000) suggest that HTLV-1 infection is in a dynamic balance between the immune response and virus replication (Asquith *et al*., 2000). What determines whether an HTLV-1-infected individual will progress to develop ATL is unclear. In the early course of HTLV-1 infection, integration of the provirus into host cell chromatin is random and polyclonal (Mortreux *et al*., 2003). During the long premalignant phase, there is oligoclonal expansion of HTLV-1-infected cells (Wattel *et al*., 1995; Cavrois *et al*., 1996; Etoh *et al*., 1997). Somatic mutations are increased in peripheral blood mononuclear cell (PBMC) DNA from ATL patients compared to asymptomatic carriers (Mortreux *et al*., 2003). Somatic mutations are increased in both the provirus and within the proviral flanking sequences, and these mutations are thought to primarily arise during the clonal proliferative phase rather than during reverse transcription (Mortreux *et al*., 2001b).

Factors that impair the cellular immune response and promote T-cell proliferation are thought to increase genetic instability of the virus and therefore increase the likelihood of development of ATL (Mortreux *et al*., 2003). One such factor is the HTLV-1 Tax protein. Tax expression promotes cellular proliferation and increase genetic instability (reviewed in Yoshida, 2001; Mortreux *et al*., 2003). In general, one of the tenets of malignant progression is that it requires mutations in several genes, including oncogenes, tumor suppressor genes, DNA repair genes, and apoptosis-regulating genes. Tax has been demonstrated to regulate gene expression of the apoptosis-regulating genes, *bcl-xL* (Tsukahara *et al*., 1999) and *bax* (Brauweiler *et al*., 1997), the DNA repair genes, *PCNA* (Ressler *et al*., 1997) and *β-polymerase* (Jeang *et al*., 1990), the cell cycle regulators, *cyclin D2*, *cyclin E*, *E2F1*, *CDK2*, *CDK4*, *CDK6*, *p19 (INK4d)*, and *p27 (Kip1)* (Iwanaga *et al*., 2001), and the tumor suppressor, p53 (de La *et al*., 2003). Therefore, Tax can promote malignant progression not only by increasing cellular proliferation but also by altering the expression of genes known to have the potential to contribute to malignant progression. Thus, over the past two decades, most animal models of ATL have focused on Tax and its role in lymphocyte transformation.

The route of primary HTLV-1 infection is also correlated with the course of clinical disease. Specifically, most cases of ATL occur subsequent to mucosal exposure, whereas most cases of HAM/TSP occur subsequent to intravenous exposure (Osame *et al*., 1990; Kannagi *et al*., 2000). Interestingly, oral inoculation of rats with HTLV-1 generally results in a persistent infection with immune unresponsiveness (Kato *et al*., 1998), whereas intravenous inoculation results in strong antibody and T-cell responses (Kannagi *et al*., 2000). Extrapolating from this, it is currently believed that intravenous exposure in humans also results in the strong immune response typical of HAM/TSP patients, whereas oral (mucosal) exposure allows for an initial diminished immune response and subsequent survival and outgrowth of infected CD4+ T cells. Intravenous exposure initially leads to infection of a large number of circulating T-lymphocytes, whereas mucosal exposure initially leads to infection of macrophage and dendritic cells and only a small number of T-lymphocytes (reviewed in Grant *et al*., 2002). HTLV-1 has the ability to stimulate T-lymphocytes to enter the cell cycle and promote high levels of gene expression, whereas mucosal dendritic cells and macrophages are postmitotic and therefore not likely to produce high levels of virus following infection (Grant *et al*., 2002). As immune response correlates with virus production, the lower levels of viral production by macrophages and dendritic

cells may lead to an initially diminished immune response following oral exposure.

Animal models of HTLV-1 infection and disease

Rabbit models

Rabbits are used extensively as a model of HTLV-1 infection in humans because of the ease and consistency of transmission of the viral infection in this species. Infectivity for rabbits was first demonstrated in the mid-1980s using intravenous inoculations of the MT-2 cell line (Akagi *et al*., 1985), a T-cell leukemia cell line established from a patient with ATL, and with the Ra-1 cell line (Miyoshi *et al*., 1985), a rabbit lymphocyte cell line derived from cocultivation of rabbit lymphocytes with MT-2 cells. Early studies in rabbits verified routes of transmission (e.g. blood, semen, milk) for the virus infection (Kotani *et al*., 1986; Uemura *et al*., 1986, 1987; Hirose *et al*., 1988; Iwahara *et al*., 1990; Kataoka *et al*., 1990). Pioneering studies utilizing the rabbit model of HTLV-1 have provided important clues as to the number of cells capable of transmitting the virus infection (Kataoka *et al*., 1990) and effective means to prevent the transmission of the virus (Takehara *et al*., 1989; Kataoka *et al*., 1990; Sawada *et al*., 1991; Miyoshi *et al*., 1992; Tanaka *et al*., 1994).

The rabbit model has provided important knowledge of the immune response against HTLV-1 infection. Early studies defined the sequential development of antibodies against the virus infection (Cockerell *et al*., 1990), and methods to detect HTLV-1 proviral DNA in infected tissues (Cockerell *et al*., 1990). Inoculation of rabbits with HTLV-1-infected cell lines derived from patients with ATL or HAM/TSP demonstrate the heterogeneity in the biological response to HTLV-1 infection (Lairmore *et al*., 1992). Immunization of rabbits with synthetic peptides verified immunodominant epitopes of the viral envelope protein (Env) (Lal *et al*., 1991; Tanaka *et al*., 1991) and also defined regions of Env important for antibody-dependent cell-mediated cytotoxicity (Chen *et al*., 1991). Soon after, it was demonstrated that peptide immunization with amino acids 190–199 of the Env protein could protect rabbits from subsequent HTLV-1 challenge, opening the possibility for vaccine development (Tanaka *et al*., 1994). More complex synthetic peptides, which use chimeric constructs that mimic native viral proteins, have also been generated and tested in rabbit models (Conrad *et al*., 1995; Frangione-Beebe *et al*., 2000).

Infectious molecular clones of HTLV-1 were first developed in the mid-1990s (Kimata *et al*., 1994; Derse *et al*., 1995; Zhao *et al*., 1995). These molecular clones were used to immortalize human PBMCs to create the ACH cell line, which was then used to infect rabbits (Collins *et al*., 1996). It was demonstrated that the lethally irradiated ACH cell line successfully establishes infection in the PBMCs of rabbits (Collins *et al*., 1996). Subsequently, ACH clones with mutations within the ORF encoding the HTLV-1 accessory proteins, $p12^{I}$, $p13^{II}$, and $p30^{II}$, were generated (Robek *et al*., 1998), and inoculated into rabbits to demonstrate the necessity of these accessory proteins for establishment of infection and maintenance of proviral loads (Collins *et al*., 1998; Bartoe *et al*., 2000; Silverman *et al*., 2004). The necessity of the Rex protein for *in vivo* infection has also recently been demonstrated in the rabbit model (Ye *et al*., 2003).

Establishment of a rabbit model of clinical HTLV-1 disease has been more problematic. In the majority of studies, rabbit infection has paralleled the asymptomatic infection of humans. A few groups have reproduced an 'ATL-like disease' via intraperitoneal or intravenous injection of HTLV-1-transformed cells; however, this required a minimum of 1×10^8 cells in the inoculum, and death occurred within the first few weeks of inoculation (Seto *et al*., 1988; Ogawa *et al*., 1989; Zhao *et al*., 1993). These studies did not demonstrate if the leukemic cells were from the inoculum or generated *de novo*. Sporadic reports of clinical disease in HTLV-1-infected rabbits include uveitis (Taguchi *et al*., 1993), cutaneous lymphoma (Simpson *et al*., 1996; Kindt *et al*., 2000), and thymoma (Zhao *et al*., 2002). In each of these cases, clinical disease developed after 1 year and usually several years after the initial infection.

Rat models

Experimental infection of rats with HTLV-1 was first established in 1991 (Suga *et al*., 1991). Although initial experimental infection was achieved with F344 rats, it was later established that there was considerable differences in the response of various rat strains to HTLV-1 infection (Ishiguro *et al*., 1992; Kushida *et al*., 1993; Ibrahim *et al*., 1994). Wistar–King–Aptekman–Hokudai (WKAH) rats emerged as a model of HAM/TSP. HTLV-1-infected WKAH rats develop spastic paraparesis with degenerative thoracic spinal cord and peripheral nerve lesions several months following inoculation (Ishiguro *et al*., 1992; Kushida *et al*., 1993). The pathology of rat HAM/TSP differs from that seen in humans. Lesions in humans have a marked T-cell infiltration of affected regions, whereas lymphocytes are not seen in the lesions in rats (Yoshiki, 1995). Subsequent studies defined the time periods over which the pathologic changes occur (Ohya *et al*., 1997, 2000), and indicated that apoptosis of oligodendrocytes and Schwann cells is the primary event leading to demyelination (Yoshiki 1995, 1997; Ohya *et al*., 1997, 2000). Macrophages are seen in the lesions of rats in response to the demyelination. Production of HTLV-1 pX mRNA, tumor necrosis factor (TNF) alpha mRNA, as well as altered expression of the apoptosis-modifying genes, *bcl-2*, *bax*, and *p53*, have been identified within the lesions (Ohya *et al*., 1997, 2000; Tomaru *et al*., 2003). In addition, HTLV-1 provirus has been identified in microglial cells and macrophages associated with lesions (Kasai *et al*., 1999).

Development of rat models for clinical ATL has required the use of immunodeficient rats. Ohashi *et al*. (1999) demonstrated that an 'ATL-like lymphoproliferative disease' could be established in adult nude (nu/nu)

rats following inoculation of some, but not all, HTLV-1-immortalized cell lines. This led to studies that examined methods of protection against tumor development, including adoptive transfer of T cells (Kannagi *et al*., 2000) and Tax-specific peptide vaccines (Hanabuchi *et al*., 2001). A protective effect was achieved with each of these systems. Most recently, a protective effect against tumor formation in nude rats was achieved with Tax-specific small interfering RNAs (siRNA) (Nomura *et al*., 2004).

Non-human primate models

Experimental HTLV-1 infection, without disease development, in non-human primates was demonstrated in several monkey species inoculated with MT-2 cells, Ra-1 cells, or autologous HTLV-1-infected cell lines (Miyoshi *et al*., 1984; Yamamoto *et al*., 1984; Nakamura *et al*., 1986). Recently, the squirrel monkey has been established as an experimental model of HTLV-1 infection. Peripheral lymphocytes, spleen, and lymph nodes were verified as major reservoirs for HTLV-1 virus during the early phase of infection (Kazanji *et al*., 1997, 2000; Kazanji, 2000). It was subsequently established that similar to humans, HTLV-1 infection in squirrel monkeys begins through reverse transcription of the virus genome, which is then followed by clonal expansion of infected cells (Mortreux *et al*., 2001a).

Early studies tested for antibodies specific for HTLV-1 membrane antigens in macaques with malignant lymphoma (Homma *et al*., 1984; Kanki *et al*., 1985). An experimentally HTLV-1-inoculated rhesus macaque developed arthritis, uveitis, and polymyositis (Beilke *et al*., 1996). More recently, development of clinical disease was reported in pig-tailed macaques following inoculation with a pig-tailed macaque cell line persistently infected with the ACH HTLV-1 molecular clone (McGinn *et al*., 2002). In this report, pig-tailed macaques died naturally at 35–82 weeks postinoculation with lymphopenia, arthropathy, and diarrhea. Like humans with HTLV-1 infection, macques that survived exhibited various combinations of rash, diarrhea, lymphadenopathy, and lymphopenia.

Vaccine candidates against HTLV-1 infection have been tested in non-human primates as early as 1987 (Nakamura *et al*., 1987). Subsequently, successful passive immunization has been achieved with hyperimmune globulin from healthy donors (Murata *et al*., 1996; Akari *et al*., 1997). Successful immunization against HTLV-1 infection has also been accomplished with recombinant vaccinia virus expressing envelope or gag gene products in combination with DNA vaccines (Ibuki *et al*., 1997; Kazanji *et al*., 2001).

Mouse models of HTLV-1 infection

Mouse models of ATL would be useful in providing a small and inexpensive animal for studies of pathogenesis, treatment, and prevention. Unfortunately, HTLV-1 does not efficiently infect murine cells, and thus, mouse models must be manipulated to establish HTLV-1 infection. HTLV-1 carrier mice can be established by intraperitoneal inoculation of MT-2 cells into C3H/HeJ and BALB/c mice (Kushida *et al*., 1997; Fang *et al*., 1998; Feng *et al*., 1999). Intraperitoneal injection of HTLV-1-producing MT-2 cells into neonatal C3H/HeJ mice did not result in detectable antigen or antibody production, although provirus was detected in spleen, lymph node, and thymus at 15 weeks of age and was integrated in the mouse genome. In splenocytes, there was preferential infection of T-lymphocytes. After 18 months, HTLV-1 provirus was detected in spleen DNA in eight of nine C3H/HeJ mice, with polyclonal integration (Tanaka *et al*., 2001). Within these mice, provirus was identified within CD4+ T cells, CD8+ T cells, B cells, and granulocytes within the spleen (Feng *et al*., 1999). Provirus was also identified within various tissues including thymus, lymph nodes, lung, liver, and kidney, although the cell type containing provirus in these organs was not identified, and the proviral loads were not quantitated (Kushida *et al*., 1997; Fang *et al*., 1998). Interestingly, neither viral mRNA production nor an HTLV-1 antibody response was found in the majority of these mice, thus this model fails to mimic persistently infection humans (Kushida *et al*., 1997; Fang *et al*., 1998). Moreover, similar to the rabbit and rat models, progression to ATL has not been demonstrated in the immunocompetent mouse.

In an attempt to improve the efficiency of infection, a chimeric HTLV-1 virus was used in which the HTLV-1 *env* gene was replaced by the ecotropic Moloney murine leukemia virus *env* gene (Delebecque *et al*., 2002). The chimeric virus was able to replicate in murine cells in culture, but T-cell transformation was not evaluated. Infection of BALB/c, C3H/HeJ, 129Sv, and 129SvIFNAR−/− mice resulted in persistent infection with 500–800 proviral copies/10^5 splenocytes in the majority of mice. In addition, virus was detected in several organs, including lymphoid organs, CNS, lung, and gonads. Importantly, this model demonstrated preferential infection of CD4+ cells and oligoclonal integration of the virus in infected cells. While these mice did not develop lesions, they did have persistent humoral and cellular immune responses.

Tumor transplant models in mice

The severe combined immunodeficiency (SCID) mouse has been a successful model to investigate the proliferative and tumorigenic potential of ATL cells (Ishihara *et al*., 1992; Feuer *et al*., 1993; Kondo *et al*., 1993; Ohsugi *et al*., 1994). SCID mice inoculated with ATL cells succumb to lymphomas, and tumor cells recovered from mice retain the phenotypic and genotypic characteristics of the original tumor cell inoculate (Feuer *et al*., 1993; Kondo *et al*., 1993; Imada *et al*., 1995). Interestingly, HTLV-1-infected cell lines of nonleukemic origin are not tumorigenic following SCID mouse engraftment (Feuer *et al*., 1995; Imada *et al*., 1995; Uchiyama, 1996). Inoculation of HTLV-1-infected

nonleukemic cell lines will form tumors in SCID mice only when natural killer (NK) cell activity has been suppressed by sublethal irradiation or by treatment of animals with antiserum, which transiently abrogates NK activity (anti-asialo-GM1) (Feuer *et al*., 1995). Murine NK cells directly mediate cytolysis of cells harboring active HTLV-1 gene expression, suggesting that the absence of viral gene expression in ATL cells contributes to the ability of these cells to evade immune surveillance in humans (Stewart *et al*., 1996). The absence of viral gene expression in the HTLV-1 leukemias of SCID mice has been confirmed (Imada *et al*., 1995, 1996). The inability of HTLV-1-infected nonleukemic cell lines to induce tumorigenesis has also been recently demonstrated in SCID/bg and nonobese diabetic/SCID (NOD/SCID) mice (Liu *et al*., 2002). Using an SCID/bg mouse model, Richard *et al*. (2001) were able to develop a model for ATL with associated humoral hypercalcemia of malignancy. Interestingly, elevation of parathyroid hormone-related protein in this model was shown to be independent of Tax expression. SCID models of ATL have proven useful in examining treatment strategies for ATL. Variable success in tumor suppression has been achieved with the proteasome inhibitor, PS-341 (Tan and Waldmann, 2002), humanized anti-CD2 monoclonal antibody (Zhang *et al*., 2003b), an NF-κB inhibitor (Dewan *et al*., 2003), and humanized anti-CD52 monoclonal antibody (Zhang *et al*., 2003a). Recently, SCID mice less than 5 weeks old with low NK cell activity developed rapid tumor formation resulting in death (Ohsugi *et al*., 2004). In summary, transplantation models demonstrate unique properties of ATL cells compared to T-lymphocytes immortalized by HTLV-1 in culture, which correlate with limited viral gene expression and resistance to NK cell-mediated cytotoxicity.

Transgenic models

Transgenic mouse models of HTLV-1 have provided an understanding of the role of Tax and Tax-mediated disruption of lymphocyte function or cytokines in HTLV-1-associated lesions. Nerenberg (1990) described neurofibromas in HTLV-1 promoter (LTR)-Tax transgenic mice and used antisense inhibition of NF-κB to demonstrate its role in tumor growth. Lymphocyte-mediated arthropathy developed in Tax transgenic mice controlled through the HTLV-1 promoter (Iwakura *et al*., 1991, 1995; Yamamoto *et al*., 1993; Fujisawa *et al*., 1996). Increased levels of nerve growth factor, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-2 receptor also developed in transgenic mice in which Tax was expressed from the human granzyme B promoter (GzmB) (Grossman and Ratner, 1997). Transgenic mice expressing Tax under the regulatory control of the CD3-epsilon promoter enhancer manifested salivary and mammary adenomas (Hall *et al*., 1998). Overall, these studies provide strong support for the oncogenic capabilities of Tax, although they did not demonstrate mechanisms of carcinogenesis.

To improve Tax expression in lymphoid compartments, various investigators have utilized alternative promoters in transgenic mice. HTLV-LTR-c-myc and immunoglobulin enhancer/promoter (Ig-enh)-Tax transgenic mice lines have been crossed resulting in the development of a variety of tumors (Benvenisty *et al*., 1992). Transgenic mice that specifically target the mature T-cell compartment by using the GzmB promoter develop large granular lymphocytic (LGL) tumor development (Grossman *et al*., 1995). Tumors composed of large granular lymphocytes developed in these mice on the tail, legs, and ears. Another interesting feature of the GzmB-Tax transgenic mice is the presence of osteolytic bone lesions, similar to those found in ATL patients (K Weilbaecher, Washington University, manuscript submitted). This represents a unique animal model with a high frequency of metastatic bone lesions, thus providing opportunities to study pathogenesis and treatment of this common complication of metastatic tumors in humans. Lines of LGL cells cultured from these mice displayed surface markers indicating a pre-NK cell lineage (positive for FcγRII/III, IL-2β, CD44, Thy 1.2, 5E6) (Grossman and Ratner, 1997). Tumors arising in these mice exhibited high levels of NF-κB expression, and expression of NF-κB target genes, including *IL-6*, *IL-10*, and *IL-15*, *GM-CSF*, and *interferon-gamma (IFN-γ)* (Grossman and Ratner, 1997; Portis *et al*., 2001b). Primary tumor cells from these mice expressed IL-1, IL-6, IL-10, and IL-15, GM-CSF, and IFN-γ, but not IL-2, IL-4, or IL-9 (Grossman and Ratner, 1997). In contrast to primary tumor cells, tumor cell lines did not exhibit IL-1 expression, suggesting that IL-1 was expressed from a nonmalignant cell population infiltrating the LGL tumors. IL-1 can promote malignant cell growth and invasiveness, and also induce antitumor immunity (Apte and Voronov, 2002). High levels of GM-CSF in these transgenic mice may initiate neutrophilia, a characteristic finding for this animal model. IFN-γ expression is likely a manifestation of the activated NK cell phenotype and may contribute to angiostatic regulation of these tumors. GzmB-Tax transgenic mice with homozygous deletion of IFN-γ manifested earlier onset of tumors and enhanced angiogenesis, but no significant alteration in CD4+ or CD8+ immune responses (Mitra-Kaushik *et al*., 2004a).

The roles of other cytokines in this model remain to be determined. IL-6 is a pleiotropic cytokine, acting as an acute-phase reactant that regulates differentiation, proliferation, and survival of a wide variety of cell types (Horn *et al*., 2000). IL-10 suppresses inflammatory responses, and regulates the growth of NK and T cells, and other cell types (Scholz *et al*., 1996; Tsuruma *et al*., 1996; Furuya *et al*., 1999; Carvalho *et al*., 2001). Thus, IL-10 could play an immunoevasion role for HTLV-1 infection. The role of overexpressed ILs in HTLV-1 carcinogenesis remains unclear, but the future use of siRNAs and breeding with knockout mice should clarify their individual contributions. Inducible systems to test the requirement of Tax in tumor maintenance and allow differential expression of Tax *in vivo* are needed to verify the role of NF-κB in HTLV-1-mediated lymphoma.

The p53 tumor suppressor and cell cycle regulatory protein is mutated in some HTLV-1-associated lymphomas, and is functionally inactivated by Tax when exogenously expressed *in vitro* (Mahieux *et al*., 2000; Pise-Masison *et al*., 2001; Gatza *et al*., 2003; Jeong *et al*., 2004). In p53 heterozygous mice, tumor onset was the same as p53 homozygous wild-type animals, but the heterozygous animals manifested more rapid tumor progression and death (Portis *et al*., 2001a). The molecular basis for this phenotype remains to be determined.

Therapeutic modalities in HTLV-1 animal models

Animal models of ATL have been quite useful in testing various anticancer therapeutic approaches. Based on the findings of NF-κB activation in ATL, several investigators have utilized these models to block NF-κB expression. The proteasome inhibitor, borteozomib, inhibits the degradation of the inhibitor of NF-κB, IκBα. It was shown to inhibit NF-κB activation in ATL cells and Tax transgenic tumor cells in culture and in mouse transplantation studies (Tan and Waldmann, 2002; Satou *et al*., 2004; Mitra-Kaushik *et al*., 2004b). In several of these studies, the majority of the cell death was found to be due to apoptosis. An inhibitor of NF-κB DNA-binding activity, Bay 11-7082, was also shown to block NF-κB activity and resulted in tumor regression in ATL-transplanted NOD-SCID-gammac knockout mice (Dewan *et al*., 2003).

Treatment of ATL-bearing mice with a humanized anti-CD2 monoclonal antibody led to tumor regression (Zhang *et al*., 2003b). The activity of the mononclonal antibody was most likely due to antibody-dependent cellular cytotoxicity, since expression of Fcγ receptors on neutrophils and monocytes was required for activity. Treatment of ATL-bearing NOD-SCID mice with an α-emitting radionuclide, bismuth 213, conjugated to an antibody to the IL-2 receptor proved to be highly effective in inducing tumor regressions (Zhang *et al*., 2002). The activity of bismuth 213 in this model was greater than that of unconjugated antibody or radionuclide, or antibody conjugated to β-emitting radionuclide, yttrium 90. Flavopiridol, an inhibitor of cyclin-dependent kinases, was tested for its therapeutic efficacy alone and in combination with humanized anti-Tac antibody (HAT), which recognizes CD25, in a murine model of human ATL using MET-1 leukemic cells (Zhang *et al*., 2005). Either flavopiridol, given 2.5 mg/kg body weight daily for 5 days, or HAT, given 100 μg weekly for 4 weeks, inhibited tumor growth and prolonged survival of the leukemia-bearing mice (Zhang *et al*., 2005). Collectively, these studies provide hope that ATL transplant and Tax transgenic models will provide new directions in the development of effective therapies against HTLV-1-induced lymphoproliferative diseases including ATL.

Future prospects

During the past 25 years since the first isolation of HTLV-1 from patients' cutaneous forms of ATL, a variety of animal models of HTLV-1 infection and transformation have expanded the understanding of the role of viral gene products and host factors that determine HTLV-1-mediated lymphocyte transformation. The knowledge gained from the study of animal models of HTLV-1 infection and disease can be extended to understand the pathogenesis and therapeutic intervention strategies of other tumors of hematopoietic origin. Future application of these animal models will also be critical in the development of vaccine approaches to elicit cellular immune responses to key viral proteins that mediated transformation, for example, Tax and to refine pharmacological targets to ablate HTLV-1-transformed T cells. Newer approaches using inducible expression systems (e.g. Cre lox recombinase) will need to be developed to address issues related to the role of viral gene products in the maintenance of the tumor phenotype in ATL. Moreover, recent studies of the HTLV-1 receptor suggest new approaches in using genetically engineered mouse models for HTLV-1 infection.

Acknowledgements

This work was supported by National Institute of Health grants RR-14324 and CA-100730 awarded to Dr Michael Lairmore and CA-70529 and CA-09338 awarded through the Ohio State University Comprehensive Cancer Center.

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Pergamon

Leukemia Research Vol. 19, No. 9, pp. 621–628, 1995.
Elsevier Science Ltd
Printed in Great Britain
0145-2126/95 $9.50 + 0.00

0145-2126(95)00030-5

CHARACTERIZATION OF A HTLV-I-INFECTED CELL LINE DERIVED FROM A PATIENT WITH ADULT T-CELL LEUKEMIA WITH STABLE CO-EXPRESSION OF CD4 AND CD8

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(*Received* 24 *October* 1994. *Accepted* 12 *February* 1995)

Abstract—A long-term T-cell line, termed SP^+, was developed from a human T-cell leukemia virus type I (HTLV-I)-infected patient with adult T-cell leukemia that is dependent on exogenous IL-2 for growth. The SP^+ expresses a full complimentation of HTLV-I-specific viral proteins, and contains replication competent viral particles. Restriction enzyme digestion followed by Southern blot analysis demonstrated the presence of a single integrated proviral copy and limiting dilution analysis confirmed the clonality of the cell line. Interestingly, phenotypically, the SP^+ cell line is $CD2^+$, $CD3^+$ and coexpresses CD4 and CD8, yet lacks TCR$\alpha\beta$ and TCR$\gamma\delta$ expression. Further ontogenetic characterization of the SP^+ cell line demonstrated the lack of thymic T-cell precursor markers, including absence of cell surface expression of CD1, intracellular thymic terminal deoxynucleotidyl transferase (TdT) enzyme, as well as message expression for V(D)J recombinase activating gene-1 (RAG-1). Furthermore, the SP^+ cell did express the message for the CD3δ chain. Taken together, these data suggest that the SP^+ cell line resulted from HTLV-I infection of a mature $CD4^+/CD8^+$ lymphocyte. This cell line can be potentially useful as a model, both for regulation of cellular functions by HTLV-I and for immunologic functions of mature dual CD4/CD8 positive T-cells.

Key words: ATL, cell line, dual CD^+ $CD8^+$ positive.

Introduction

Adult T-cell lymphoma/leukemia (ATL) is a unique type of T-cell malignancy characterized by elevated numbers of circulating lymphocytes, frequent skin lesions, hepatosplenomegaly, lymphadenopathy, and a rapidly fatal terminal clinical course [1]. Isolation of human T-cell leukemia virus type I (HTLV-I) from cell lines derived from patients with ATL and subsequent seroepidemiologic studies established the etiologic role of this virus in the development of ATL [1, 2]. A direct role for HTLV-I in the leukemogenesis of ATL still remains to be determined, since the integration site of the virus is variable and the viral genome does not appear to contain any known oncogene. However, the 3′ end of the HTLV-I genome (*pX*) encodes for a *trans*-activating protein, tax, which not only enhances the transcription of its own long terminal repeat, but also promoters of several cellular genes, in particular those involved in T-cell activation [3].

Biologically, HTLV-I is capable of immortalizing T-lymphocytes *in vitro* although the exact process of malignant transformation is unknown [4, 5]. Most of these cell lines, however, are generated by co-cultivation with activated lymphocytes, since cell-free infection with HTLV-I is often not successful. These transformed cells often have demonstrated altered cellular functions [6]. Moreover, long-term T-cell lines developed from patients infected with HTLV-I, frequently result in altered cell surface phenotypes [7–9]. In order to better understand the phenotypic modulation, we have developed a primary T-cell line from a patient with ATL, which possesses a unique phenotypic characteristic of dual surface CD4 and CD8 positivity. We have further demonstrated that the cell line does not represent an

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immature T-cell lineage, but rather is of mature T-cell lineage.

Materials and Methods

Preparation of thymocytes and PBL

Normal thymus samples from children (less than 1 year old) undergoing corrective cardiac surgery (kindly provided by Dr A. A. Ansari, Emory University, Atlanta, GA, U.S.A.) were washed with phosphate buffered saline and a single cell suspension was obtained by gentle dispersion with the flat end of a 12 ml syringe plunger. The large cell clumps were allowed to settle, and the suspended cells were treated with ammonium chloride to remove residual red blood cells. Peripheral blood lymphocytes (PBLs) were purified from heparinized whole blood by standard Ficoll procedures. Both thymocytes and lymphocytes were cryopreserved and stored in liquid nitrogen until needed.

Establishment of HTLV-I cell line

PBLs from a 46 year old female patient with ATL [SP] [10] were used to generate the cell line. The PBLs were stimulated with 0.1% PHA (Difco Laboratories, Detroit, MI, U.S.A.) for 3 days in RPMI-1640, supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (C-RPMI), and then expanded in C-RPMI containing 10% purified IL-2 (Advanced Biotechnologies Inc., Columbia, MD, U.S.A.). The SP cells were cloned by limiting dilution (10 cells/well) in C-RPMI with 10% IL-2. Two other cell lines derived from HAM patients (EG and JR) have previously been reported and were included for comparison purposes [11].

HTLV-antigen production and Western blot analysis

Culture supernatants were collected for viral antigen and reverse transcriptase (RT) production. The HTLV-I/II $p24^{gag}$ antigen was determined using an antigen capture assay kit (Coulter Immunology) and RT activity was assayed using a Mg^{2+}-dependent RT assay [12]. Western blot analysis was used to analyze viral proteins, essentially as described previously [11].

Southern-blot hybridization and PCR analysis

To establish clonality of the SP cell line, genomic DNA was isolated from the cell line and digested with the restriction endonuclease EcoRI, followed by electrophoresis through a 0.8% agarose gel. DNA was transferred to a nylon filter (Hybond-N, Amersham) and hybridized with ^{32}P labelled HTLV-I LTR probe (pU3RI). The filter was then washed and exposed to X-ray film (XAR-2, Kodak).

Limiting cell dilution followed by polymerase chain reaction (PCR) analysis was performed using HTLV-specific primers SK 110/111 and hybridized to a HTLV-I-specific probe (SK 112) as previously described [13]. The restriction fragment length polymorphism (RFLP) analysis was used to determine the genetic heterogeneity [11].

Phenotypic analysis

Surface phenotypic analysis of the cell line was carried out by flow cytometry analysis (FACScan, Becton Dickinson) using directly labelled antibodies to CD1, CD2, CD3, CD4, CD8, CD25, HLA-DR, TCR$\alpha\beta$ (WT-31) and TCR$\tau\delta$ and (11F2). Cytoplasmic analysis for terminal deoxynucleotidyl transferase (TdT) enzyme was determined by labelling cells with a FITC-conjugated mAb directed against human TdT (Gen Trak, Inc., Plymouth Meeting, PA, U.S.A.) according to the manufacturer's instructions.

Karyotypic analysis of SP cell line

For cytogenetic analysis of SP, colcemid-treated cells were dispersed with a trypsin EDTA solution, placed in hypotonic medium for 8 min, and fixed in methanol:glacial acetic acid (3:1). The cells were centrifuged at 1500 rpm, suspended in a small volume of fixative, and dropped on to cold wet slides. The slides were air-dried and stained with 4% Giemsa solution. Chromosomes were examined and counted to establish ploidy and constitutional aberrations. For trypsin–Giemsa banding of chromosomes, the slides were aged at 60°C on a slide warmer for 18 h, immersed briefly (30 s) in a trypsin EDTA solution, and stained with 4% Gurr–Giemsa solution for 11 min. The slides were washed, dried, and mounted; the metaphase was photographed at 800× magnification with Technical Pan Film (Kodak, Rochester, NY, U.S.A.) and printed on Rapidoprint FP 1-2 (Agfa-Gevaert).

Reverse transcription–polymerase chain reaction (RT–PCR)

The message for human recombinase transactivating gene (RAG-1), CD3δ and β-actin was detected by RT–PCR based assays, using specific primers and probes for the respective genes. For RAG-1 gene expression [14], total cellular RNA was extracted using a RNA Separator kit (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.) and RT–PCR was performed by using the GeneAmp™ RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The primers for RAG-1 were RAG-1F 5′-TGA GGG CGA GGA ATG AGC ACA GGC A (1437–1462) and RAG-1R 5′-TCC ATC AAA GCA GAC ACC AAA GCT (1804–1828). The cDNA was amplified for 40 cycles of 1.5 min at 94°C, 2.0 min at 50°C, and 4.0 min at 72°C. The resulting product (391 bp) was separated by electrophoresis on a 1% agarose gel, transferred on to Hybond-N membrane and probed with a ^{32}P end-labelled RAG-1P probe [5′ATT ATT GAT GGG CTG TCT GGA (1721–1742)]. The blot was exposed for 2 h on a phosphor screen and analyzed on a Phosphoimager using Image Quant™ software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

For β-actin and CD3δ message expression, total RNA was purified through a CsCl gradient. RNA was resuspended in cDNA conversion buffer (1 × RT buffer [5 × RT buffer: 250 mM Tris pH 8.3, 30 mM $MgCl_2$, 200 mM KCl, 50 mM DTT, 0.05% NP-40], 1 μg oligo-dT, 5 μg BSA, 10 U RNasin, 0.5 U AMV-RT, 25 μmol of each dNTP, brought to 50 μl with DEPC-treated water) and incubated for 2 h at 42°C. The resultant cDNA was precipitated overnight and resuspended in water for a final concentration of 250 ng/μl (of the original RNA quantity). PCR amplification was performed using 1 × PCR buffer, 160 μmol of each dNTP, 2.5 U Taq Polymerase, 200 ng of each primer, and brought to 50 μl with water. Five hundred nanograms of cDNA were added and then amplified for 45 cycles of 1 min at 95°C, and 2 min at 65°C. The primers for B-actin were 5′GTG GGG CGC CCC AGG CA CCA and 3′CTC CTT AAT GTC ACG CAC GAT TTC and for CD3 and were 5′CTG GAC CTG GGA AAA CGC ATC and 3′GTA CTG AGC ATC ATC TCG ATC. The amplicons were separated through a 2% agarose gel and photographed.

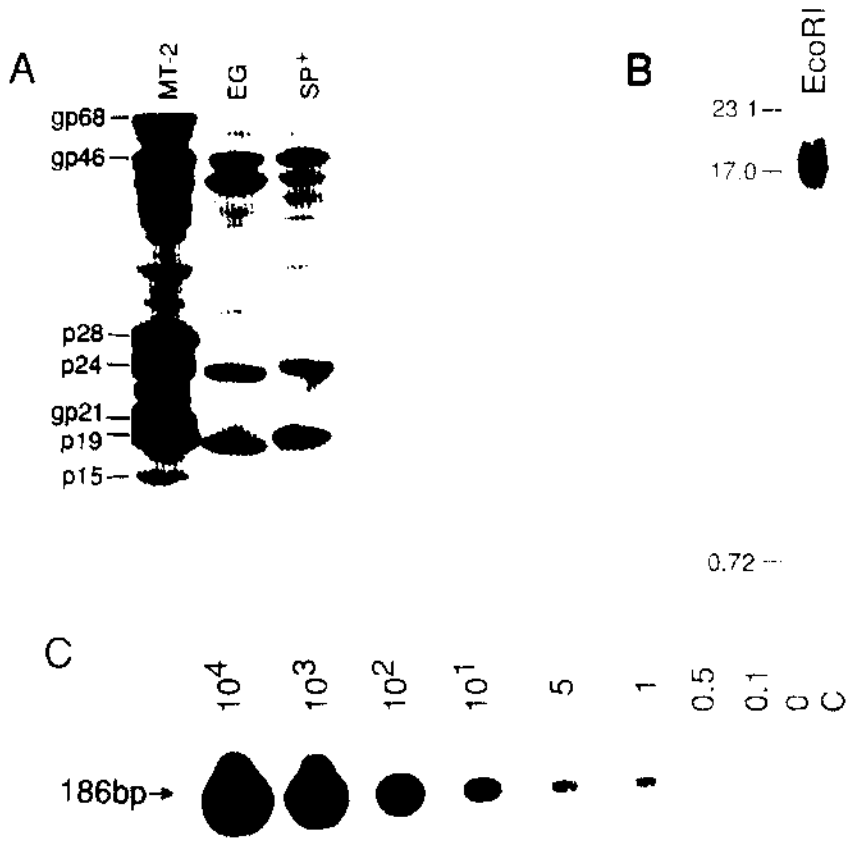

Fig. 1. HTLV-I protein profile and genotypic analysis of SP+ cell line. (A) Western blot analysis of immunoreactive proteins derived from HTLV-I-infected cell lines, MT-2, EG, and SP+. (B) Southern blot analysis of *EcoRI* digested genomic DNA from SP+ demonstrates a single band of 17 Kb. (C) Limiting dilution PCR analysis of SP+ using SK 110/111 primers, followed by hybridization with SK112.

Results

Virologic and genotypic analysis

The cell line SP+ was derived from an activated primary PBL culture isolated from an ATL patient. The cell line is dependent on IL-2 for growth (SP+) and has been maintained in culture for over 4 years. The presence of a replication-competent virus was demonstrated by RT activity of SP+ cultures (concentrated supernatant 100×) which was five-fold over background as compared to other (100×) HTLV-I-infected lines (IR and EG) and produced stable levels of soluble $p24^{gag}$ antigen (data not shown). Analysis of viral proteins demonstrated the presence of both *gag* (p24 and p19) and *env* (gp46) proteins (Fig. 1A), similar to the protein profiles of MT-2 and EG, suggesting a full complementary expression of HTLV-I proteins.

The genomic analysis of the EcoRI digestion of DNA from the SP+ cell line generated a single band approximately 17 Kb in size by Southern blot analysis, which suggests that the SP cell line contained a single integrated HTLV-I gene copy (Fig. 1B). Limiting dilution analysis of the SP+ cell line, followed by PCR, demonstrated the presence of the HTLV-I genome at the single cell level (Fig. 1C), further confirming the clonality of the cell line. The RFLP analysis of the long terminal repeat followed by restriction enzyme analysis demonstrated the presence of *A pal* and *N del* restriction sites (data not shown). In accordance with our previous study [11], this restriction pattern identified SP+ to be of the HTLV-I subtype IV category.

Surface phenotype analysis

The SP+ cell line and two other HTLV-I-positive cell lines (EG and IR) were examined to determine the T-cell lineage specificity. Analysis using T-cell subset monoclonal antibodies demonstrated that while IR and EG

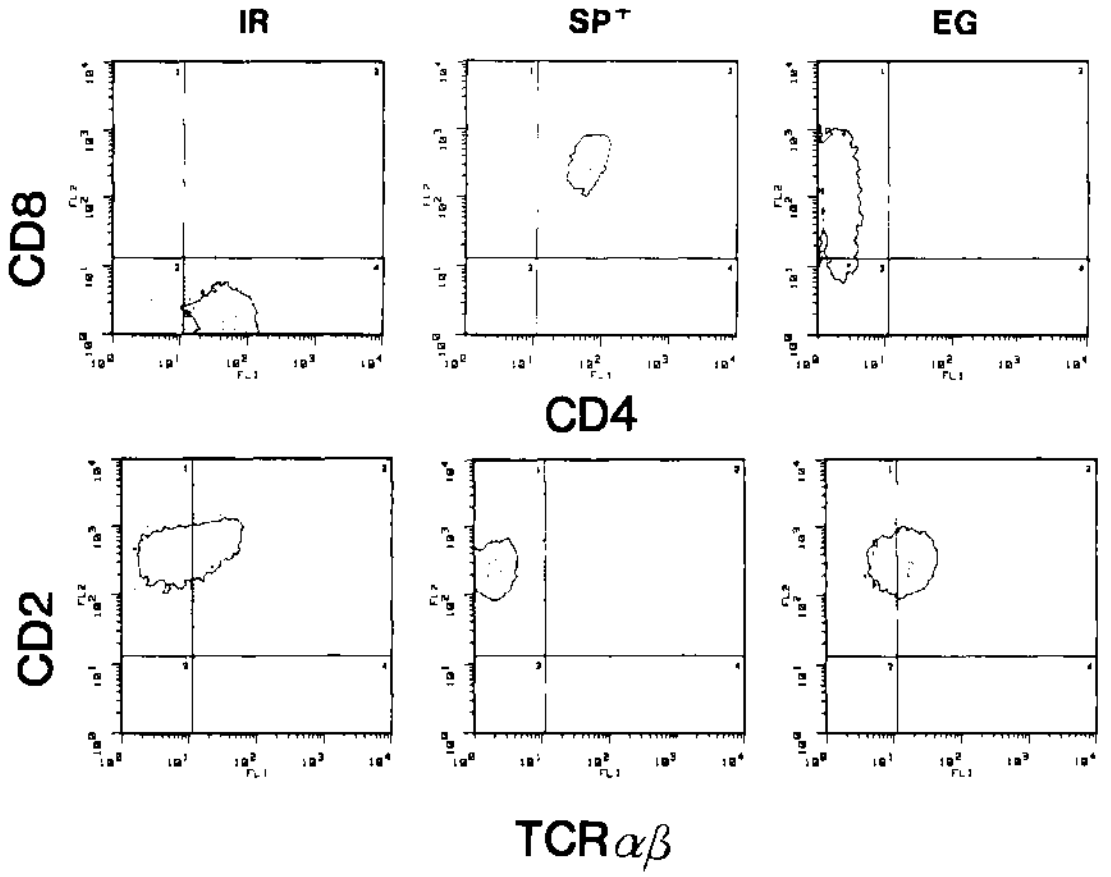

Fig. 2. Dual color analysis of CD4 and CD8 (top panel) and CD2 and TCR$\alpha\beta$ (bottom panel) in HTLV-I-infected cell lines, IR, SP^+ and EG.

were $CD4^+$ and $CD8^+$, respectively, SP^+ demonstrated a unique characteristic of dual $CD4^+/CD8^+$ phenotype (Fig. 2, top panels). While all three cell lines expressed the CD2 marker, T-cell surface receptor (TCR$\alpha\beta$) expression was observed only on IR and EG (Fig. 2, bottom panels); interestingly the SP^+ cell line did not express surface TCR$\alpha\beta$ or TCR$\tau\delta$. All cell lines expressed activation markers HLA-DR and CD25 (data not shown).

Karyotypic analysis of SP

To exclude the possibility that SP was the product of a $CD4^+$ by $CD8^+$ cell fusion, a detailed chromosome analysis was performed. SP was found, for the most part, to be a normal diploid human female (46,XX) cell line (Fig. 3). Within the lower ploidy population, most of the metaphase had 46 paired chromosomes, as shown by the exact chromosome count and karyotyping results. These results were also indicative of some random loss and/or gain of chromosomes from metaphase to metaphase, most likely caused by low level division infidelity.

Ontogenic relationship of SP^+ cell line

The dual expression of CD4 and CD8 which is characteristic of undifferentiated T-cells [15], along with the observation that fetal thymocytes can be infected with HTLV-I [16, 17], led us to examine whether SP^+ cell line might represent an outgrowth of an immature thymic T-cell. As expected, the majority of thymic lymphocytes (95%) are dual CD4/CD8 positive while only a minority of PBL (2.5%) have this phenotype (Fig. 4, top panel). To further explore the possibility that the SP^+ cell line may have been derived from an undifferentiated thymic precursor cell which could have potentially been transformed by HTLV-I, we analyzed SP^+ for the surface expression of CD1 and CD3. While >95% of thymocytes were positive for CD1, neither SP^+ nor PBL expressed this surface marker (Fig. 4, bottom panel). As expected, both SP^+ and PBL demonstrated cell surface expression of CD3 (Fig. 4).

To further exclude the thymic origin of SP^+, we next examined the cell line for the expression of the TdT enzyme and RAG-1 message expression. TdT is involved in the rearrangement of Ig genes for genetic diversity of T- and B-cells, while the RAG-1 gene is

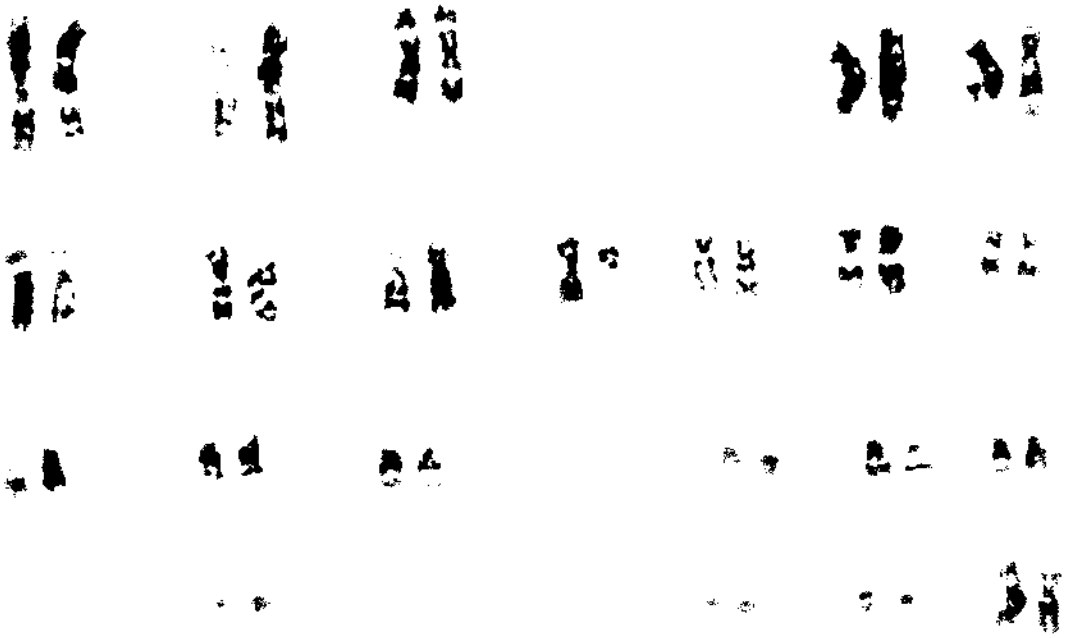

Fig. 3. Karyotypic analysis of SP^+ cell line, demonstrating a normal diploid human female (46,XX) cell line.

required for V(D)J DNA rearrangements at specific loci for T-cell receptor genes [14, 15]. As thymocytes differentiate and mature into CD3-positive T-cells, they lose expression of both TdT and RAG-1 [15]. Cytoplasmic fluorescence staining for TdT enzyme as well as message expression for RAG-1 was only observed in thymocytes (Fig. 5A, B), whereas the SP^+ cell line was negative for both TdT and RAG-1 expression (Fig. 5A, B). As expected, the T-cell line Molt4, which demonstrates thymic markers was positive for both TdT and RAG-1, while PBLs were negative for both. Further analysis for CD3δ transcription by RT–PCR demonstrated that the SP^+ cell line had detectable levels of CD3δ message (Fig. 5C). These data together strongly suggest that enzymatically, phenotypically, and genotypically, SP^+ represents a mature T-cell.

Discussion

We have characterized a HTLV-I-infected cell line derived from a patient with ATL that contains a single integrated full-length copy of the HTLV-I genome and a full complimentary profile of expressed viral proteins. Phenotypically, the cell line is unique in that it dually expresses CD4 and CD8 surface receptors with no detectable TCR$\alpha\beta$ expression. Down modulation of a TCR-CD3 complex on the cell surface has been well documented for ATL [18, 19]. Since only the TCR complex of complete heptamers ($\alpha\beta\tau\delta\varepsilon\varsigma$) can be transported to the cell surface for their expression [20], it is likely that SP^+ cell lines lacks one of the TCR chains, therefore, not allowing their expression. A previous study has demonstrated that loss of the TCR complex from the cell surface of the HTLV-I-infected T-cell line was due to the coordinated down-regulation of CD3 τ, δ, ε, and ς mRNA expression by the inactivation of a T-cell specific CD3 ε enhancer, which was thought to be repressed indirectly by the HTLV-I-encoded *tax* protein [21]. While expression of all TCR chains was not examined, we did find message for CD3δ.

The karyotype analysis has ruled out the possibility that fusion between a $CD4^+$ and $CD8^+$ lymphocyte occurred because the predominant cell possesses a normal diploid karyotype. It has been shown, however, that HTLV-I is capable of inducing genetic changes in infected human T-lymphocytes through chromosome and gene rearrangements [22]. In contrast to these studies, we did not find any major change or deletion of a chromosome in SP^+.

The dual CD4 and CD8 positivity, a phenotype that characterizes the majority of thymic precursors [13], led us to examine where in the ontogeny of the cell line these cells might have become infected with HTLV-I. Early progenitors of T-lymphocytes contain the enzyme TdT, which catalyzes the irreversible addition of

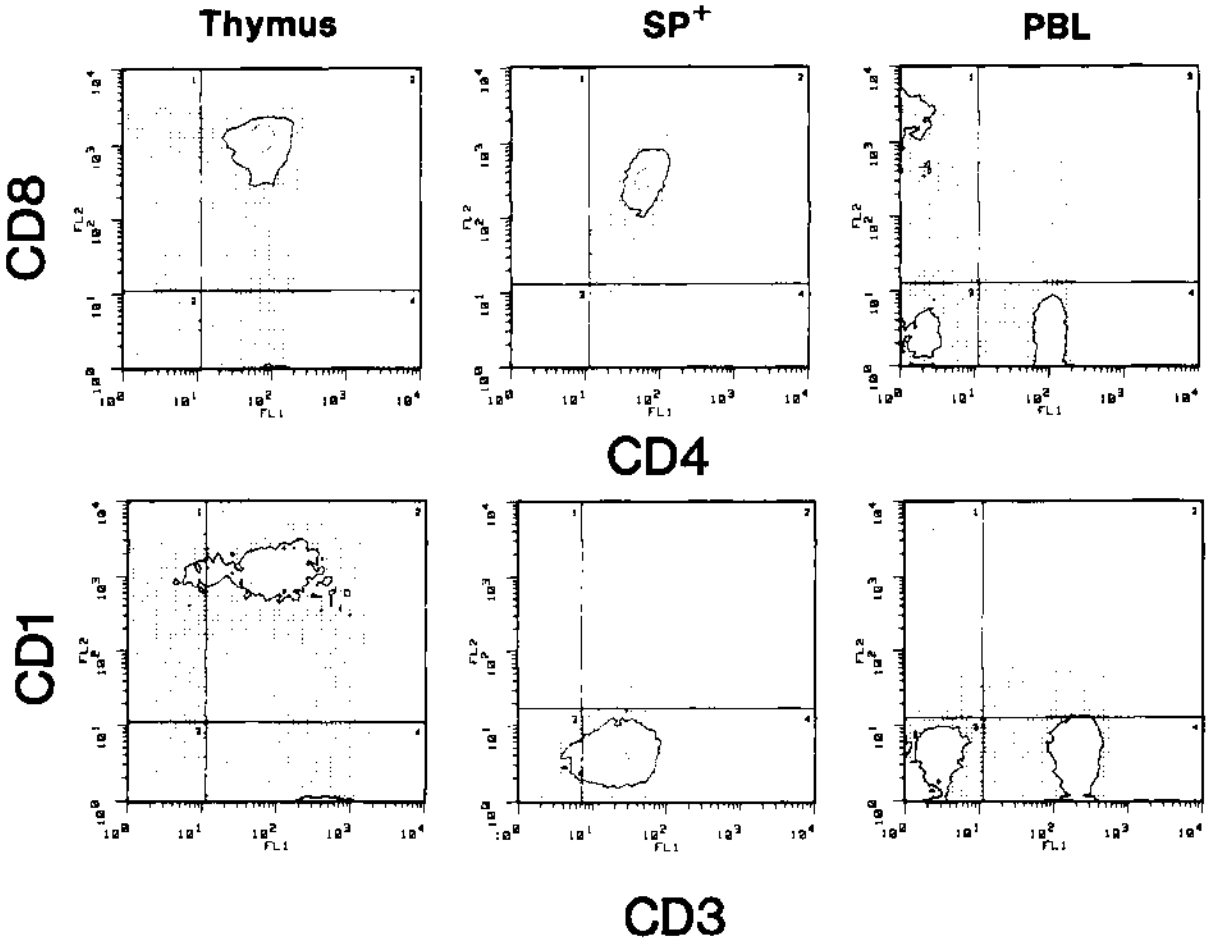

Fig. 4. Dual color analysis of CD4 and CD8 (top panel) and CD1 and CD3 (bottom panel) of human thymus cells, SP^+ cell line and human peripheral blood lymphocytes.

deoxynucleotides to the terminal 3′-hydroxyl groups of DNA. As precursor cells differentiate and emerge from the thymus, they lose TdT enzyme expression. Similarly, early thymocytes also contain the message for RAG-1, which is lost following thymic departure and single CD4 or CD8 expressing cells emerge. During intrathymic T-lymphocyte ontogeny, immature $CD4^+CD8^+$ thymocytes develop into functionally competent $CD3^+CD4^+CD8^-$ or $CD3^+CD4$ $CD8^+$ T-cells after this transient expression of the double positive $CD4^+CD8^+$ phenotype [15]. That SP^+ was derived from an undifferentiated thymic precursor was excluded due to the fact that SP neither expresses the thymus-specific surface marker, CD1, nor does it contain TdT enzyme or message for RAG-1.

Several hypotheses could explain the origin of the dual positive phenotype. The CD4 gene might become derepressed in $CD8^+$ cells and take on a dual CD4/CD8 phenotype in culture especially in some pathologic states. For instance, infection with human herpesvirus 6 leads to coexpression of CD4, and CD8 [23] cells were analyzed for expression of HSV-6 and found to be negative (data not shown). A second possibility is that interleukin-4 (IL-4) can modulate coexpression of CD8 on the surface of $CD4^+$ T-lymphocytes. A recent study [24] has demonstrated that the removal of IL-4, and subsequent culturing in IL-2, resulted in a loss of CD8 expression. We have maintained cultures in both purified and recombinant IL-2 and the cells retained their dual expression of CD4/CD8. Although soluble IL-4 was not detectable in culture supernatants in SP, IL-4 message was detected by RT-PCR (data not shown). In the present study, IL-4 did not appear to induce coexpression since other $HTLV\text{-}I^+$ cell lines also contained message for IL-4 and maintained either $CD4^+$ or $CD8^+$ phenotype (Dezzutti *et al.*, in preparation).

Another possible explanation of dual positivity is that

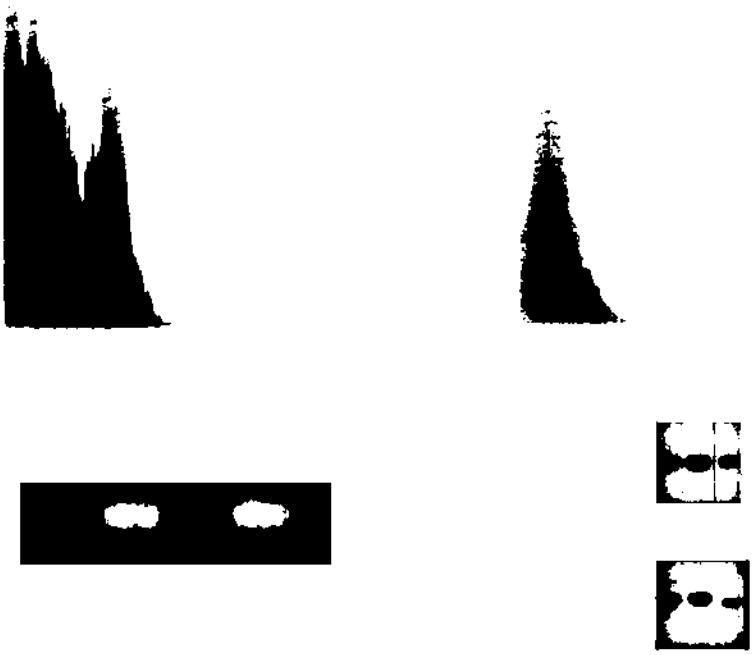

Fig. 5. Detection of intracellular thymic TdT expression and message expression for RAG-1 and CD3δ in SP$^+$ cell line. (A) Analysis of intracellular TdT expression in fetal thymus cells (left panel) and SP$^+$ cell line (right). The horizontal bar (|-|) in left panel illustrates the region of TdT positivity that is 22% of the total population. (B) Southern blot analysis of RT–PCR of RAG-1 message (391 bp fragment) in human PBL, Molt-4, SP$^+$ cell line and fetal thymus. (C) RT–PCR analysis of CD3δ (300 bp) and β-actin (548 bp) in HTLV-I-infected cell line. From left to right: MT-2 (lane 1), SP$^+$ (lane 2) and EG (lane 3).

an original small, undetectable population of dual positive T-lymphocytes was initially HTLV-I infected in the peripheral blood and indeed a small number of CD3$^+$ T-lymphocytes coexpressing CD4 and CD8 exist in the peripheral circulation [25–27]. This possibility, of course, implies that the dual positive cell outgrew other infected and uninfected cells in culture. Recently, it has been observed that HTLV-I is capable of inducing dual CD4$^+$, CD8$^+$ expression in IL-2 dependent T-lymphocytes of some ATL patients [9]. Alternatively, HTLV-I can have a direct effect on the promoters for CD4 and CD8, thereby resulting in up-regulation of either CD4 or CD8 receptors. Infection of purified CD8$^+$ cells by HTLV-I has been shown to induce expression of CD4, resulting in double-positive cells in short-term cultures [28]. Further exploration into either of these avenues needs to be determined.

SP$^+$, which now has been in culture for over 4 years stably co-expressing CD4 and CD8, could prove to be a valuable tool as a model for studying the function of the dual CD4/CD8 population that exists in the peripheral blood. Since SP cells undergo a rapid lysis by HIV (data not shown), this cellular model could prove invaluable in determining the efficacy of pharmaceutical agents against HIV and other diseases which might involve interaction with dual-positive T-cells. Such a cell line is also valuable in studying the regulation of various cellular genes by HTLV-I since it contains a single integrated copy of the virus expressing all of the viral gene products.

Acknowledgements—The authors thank Bill Switzer for technical advice on the generation of primers and probes for RAG-1 message analysis, and Dr W.D. Peterson Jr (NCI contract N01-CP-85645, Children's Hospital of Michigan, Detroit, MI, U.S.A.) for karyotype analysis. Dr Lairmore is

supported by grants from The National Cancer Institute (CA-55185) and the American Cancer Society (IRG-16-30).

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VIRUS 00887

Virus Research

Characterization of human T-lymphotropic virus type I- and II-infected T-cell lines: antigenic, phenotypic, and genotypic analysis

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(Received 20 November, 1992; revision received and accepted 19 January 1993)

Summary

Eighteen long-term T-cell lines were established from peripheral blood mononuclear cells of individuals infected with human T-lymphotropic virus type I (HTLV-I) or II (HTLV-II). These cell lines (10 HTLV-I and 8 HTLV-II), representing diverse pathologic profiles and geographic regions, have been in culture for over 6 months and have constitutively produced $p24^{gag}$ antigen. Antigenic characterization of the cell lines by Western blot analysis demonstrated that all but one produced *gag* (p24) and *env* (gp46 or gp52) structural proteins; one HTLV-I-infected cell line exhibited an aberrant protein profile. Phenotypic analysis of the HTLV-infected cell lines demonstrated phenotypes consistent with activated T-cells ($CD5^+$, $CD25^+$, $HLA\text{-}DR^+$). The HTLV-I-infected cell lines were predominantly $CD4^+$ (IR, FS, A212, SP, 1657, 1742, 3669, 1996, and 3614), whereas EG was $CD8^+$. The HTLV-II-infected cell lines were either $CD4^+$ (H2A, Y17, G12.1), $CD8^+$ (H1H, H2E, Y03, Y06), or both (H1B). Restriction map analysis and subtyping of the viral genomes demonstrated heterogeneity among these isolates. Of the HTLV-I-infected cell lines, six were subtype II, one was subtype III and, on the basis of additional restriction sites, another subtype, tentatively classified as

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subtype IV, could be identified for three of the HTLV-I-infected cell lines. Of the HTLV-II-infected cell lines, six were subtype HTLV-IIa and two were subtype HTLV-IIb. While the majority of the cell lines resemble the prototypic HTLV-I-infected (MT-2) and HTLV-II-infected (MoT) cell lines, the antigenic, phenotypic, and genotypic data collectively demonstrate heterogeneity among viral isolates representing diverse geographic regions.

Human T lymphotropic virus; HTLV; T-cell line; Genotype; Restriction fragment length polymorphism

Introduction

Human T-lymphotropic virus types I and II (HTLV-I and HTLV-II) belong to a family of C-type retroviruses that share extensive structural and functional homologies. HTLV-I is etiologically linked to development of HTLV-associated myelopathy (HAM), also known as tropical spastic paraparesis, and adult T-cell leukemia (ATL). HTLV-I infection is endemic in Japan, the Caribbean, and some parts of Africa (Manns and Blattner, 1991). HTLV-II has not yet been associated with any particular disease, and infections with HTLV-II are endemic in certain indigenous native indians in the Americas (Lairmore et al., 1990). In the United States, infection with HTLV-I or HTLV-II has been documented in injecting-drug users (IDU), female prostitutes, patients attending sexually transmitted disease clinics, volunteer blood donors, and recipients of multiple blood transfusions (Manns and Blattner, 1991; CDC, 1990).

The genomic structure of HTLV-I and HTLV-II is unique. Unlike known acute transforming RNA tumor viruses, they do not contain a typical oncogene (Smith and Greene, 1991). Rather, the 3' end of the proviral genome encodes the *tax* protein, which not only acts as a transcriptional activator of the viral long terminal repeat (LTR) (Felber et al., 1985), but trans-activates several heterologous promoters, in particular the cellular genes involved in T-cell activation and proliferation (Smith and Greene, 1991). Indeed, the ability of HTLV-I to cause proliferation and immortalization of infected cells has been well documented. The HTLV-I-infected cell lines established from cord blood by co-cultivation with virus-producing cell lines or immortalization of antigen-specific T-cell clones have provided a useful model to study the process of HTLV-I-induced leukemogenesis in vitro (Del Mistro et al., 1986; Faller et al., 1988). While not much is known about the functional effects associated with HTLV-II infection, infection with HTLV-I results in both phenotypic and functional changes of T-cells (Faller et al., 1988; Gessain et al., 1990; Yssel et al., 1989).

The genomic analysis of HTLV isolates has demonstrated restricted mutation rates (Paine et al., 1991; De et al., 1991), in contrast to that of human immunodeficiency virus, which exists as a dynamic mixture of quasi-species (Coffin, 1986). Despite the significant sequence conservation among HTLV-I isolates, genomic

variation was observed to be greater between isolates from different geographic regions than between isolates from the same region (Paine et al., 1991; De et al., 1991; Komurian et al., 1991). Recently, point mutations in the nucleotide sequence, resulting in altered restriction endonuclease (RE) fragment sizes, have allowed molecular characterization of various isolates. These altered RE fragment sizes have yielded HTLV-I and HTLV-II isolate subtypes by restriction fragment length polymorphism (RFLP) analysis (Hall et al., 1992; Komurian-Pradel et al., 1992).

As a first step towards defining the phenotypic changes of HTLV-infected cells and determining the effect of HTLV on constitutive expression of genes involved in T-cell proliferation (both the early activation proto-oncogenes and the cytokine genes), we have established cell lines representing diverse geographic isolates from individuals infected with HTLV-I or HTLV-II. In the present investigation, we report the antigenic, phenotypic, and genotypic characterization of these HTLV-infected lines.

Materials and Methods

Study population

Eighteen individuals seropositive for antibodies to HTLV-I/II and whose infections were further confirmed to be HTLV-I ($n = 10$) or HTLV-II ($n = 8$) by polymerase chain reaction (PCR) and synthetic peptide-based assays (Lal et al., 1992b) were included in the study. Three of the four HTLV-I-infected patients were recruited through the CDC HAM/TSP surveillance system; these included one patient from Jamaica (EG, Janssen et al., 1991) and two patients from the United States (FS, McKendall et al., 1991; and IR, Kaplan et al., 1991). The fourth HAM patient was from Cairo, Egypt (A212) who had developed neurological symptoms a year after a blood transfusion (Constantine et al., 1992). Both ATL cases, SP (Ratner et al., 1990) and 1657 were from the United States. Of the four asymptomatic HTLV-I-infected blood donors, one was the concordently infected spouse of 1657 (1742), two were blood donors (3669, 1996) from Japan, and one was a female prostitute (3614) from Peru. Of the eight HTLV-II-infected individuals, four were IDUs (H1B, H1H, H2E, H2A) from the United States, three (Y03, Y06, Y17) were from Mexico, and one (G12.1) was an asymptomatic Guaymi Indian from Panama (Lairmore et al., 1990). The demographics of these individuals are shown in Table 1.

Development of cell lines

Long-term T-cell lines were developed from infected individuals as follows: purified peripheral blood mononuclear cells (PBMC) from HTLV-infected individuals were stimulated with 0.1% phytohemagglutinin (PHA-P, Difco, Detroit, MI) and cultured at 1×10^6 cells/ml in RPMI-1640 supplemented with 15% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml) (C-RPMI), and 10% interleukin-2 (IL-2, Advanced Biotechnologies Inc., Silver

Spring, MD). After 72 h, the HTLV-infected PBMC were co-cultured with an equal number of PHA-P-stimulated PBMC from HTLV seronegative donors and maintained in C-RPMI containing 10% IL-2. The culture medium was changed every 4 days and cells were expanded depending upon their growth. Stable transformed cell lines were established after 4–6 months in culture and some of the cell lines have been in culture for more than 3 years (Table 1). An attempt was made to wean the cell lines off IL-2 in a gradual manner; if the cell lines started to die, exogenous IL-2 was added to maintain the cell growth. Previously established HTLV-I-infected (HuT102 and MT-2) and HTLV-II-infected (MoT) cell lines were maintained in C-RPMI.

HTLV-antigen production

HTLV virus production in the culture supernatants was determined by using a $p24^{gag}$ antigen capture assay (Coulter Immunology, Hialeah, FL) according to the manufacturer's instructions. The monoclonal antibody (mAb) used in the assay recognizes a *gag* antigenic determinant common to both HTLV-I and HTLV-II. The $p24^{gag}$ antigen levels were tested on supernatants collected at day 3 of cultures set at 5×10^5/ml.

Phenotype analysis

Lymphocyte phenotype analysis of the cell lines was performed by Flow cytometry analysis using a FACScan (Becton Dickenson, San Jose, CA). Briefly, 50,000 cells were individually stained with mAbs to the cluster of differentiation (CD) antigens for 30 min at 4°C. The mAbs conjugated with either FITC or phycoerythrin (PE) included: Leu 1 (CD5), Leu 2a (CD8), Leu 3a (CD4), 2A3 (CD25), and L243 (HLA-DR) (Becton Dickinson, Mountain View, CA). The cells were washed twice in cold FACS buffer (phosphate-buffered saline [PBS] with 0.2% sodium azide, 0.1% bovine serum albumin [BSA] and 2% human AB serum), and fixed in 1% paraformaldehyde for 30 min. The fixed cells were analyzed using a FACScan and data analysis was performed using Consort 30 software on a Hewlett Packard computer.

Analysis of viral proteins

Approximately 10^8 cells were lysed in buffer (0.1 M PBS [pH 7.4] containing 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM PMSF) at 4°C for 16 h, and then centrifuged at 1500 rpm for 20 min, followed by airfuging for 30 min. Equal amounts of cell lysates were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene diflouride membrane. The membrane was blocked for 2 h at room temperature (RT) in Tris-buffered saline (TBS) containing 5% BSA, and then incubated with pooled anti-HTLV-I/II-positive human sera overnight at RT. The membrane was then washed six times with TBS containing 0.05% Tween-20 (TBS-T) and incubated with ^{125}I-labeled Protein-G (10^5 cpm/ml; NEN, Boston, MA) for 2 h at RT, followed by three washes in TBS-T and a final wash with TBS-T containing 10 mM EDTA. The membrane was dried and exposed to Kodak X-ray film.

TABLE 1

Demographics and characteristics of the HTLV-I- and -II-infected cell lines

No.	Cell line	Age, gender	Geographic origin	Clinical diagnosis	Cultured cell line		Phenotype	Genotypic subtyping
					Time	$p24^{gag}$(ng/ml)		
PCR-confirmed HTLV-I								
1.	EG	9F	Jamaica	HAM	2 yr	82	CD8	*Apa*I, *Sac*I (subtype II)
2.	FS	41M	US	HAM	3 yr	6	CD4	*Apa*I, *Sac*I (subtype II)
3.	IR	65M	US	HAM	3 yr	ND [1]	CD4	*Apa*I, *Sac*I (subtype II)
4.	A212	45F	Egypt	HAM	1 yr	20	CD4	*Apa*I, *Sac*I (subtype II)
5.	SP [2]	43F	US	ATL	3 yr	14	CD4	*Apa*I (subtype IV)
6.	1657	31F	US	ATL	2.5 yr	>170	CD4	*Apa*I (subtype IV)
7.	1742	31M	US	ASY	2.5 yr	134	CD4	*Apa*I (subtype IV)
8.	3669 [2]	28F	Japan	ASY	2 yr	144	CD4	*Dra*I, *Mae*III (subtype III)
9.	1996	43F	Japan	ASY	1.5 yr	163	CD4	*Apa*I, *Sac*I (subtype II)
10.	3614	Unknown	Peru	ASY	2 yr	78	CD4	*Apa*I, *Sac*I (subtype II)
PCR-confirmed HTLV-II								
11.	H1B	40F	US	ASY	2 yr	23	CD4/CD8 [3]	*Xho*I (Subtype a)
12.	H1H	48M	US	ASY	2 yr	39	CD8 [4]	*Xho*I (Subtype a)
13.	H2E	57M	US	ASY	2 yr	39	CD8	*Xho*I (Subtype a)
14.	H2A	35M	US	ASY	2 yr	16	CD4	*Xho*I (Subtype a)
15.	Y03	22F	Mexico	ASY	6 mo	22	CD8	Subtype b
16.	Y06	33F	Mexico	ASY	6 mo	71	CD8	*Xho*I (Subtype a)
17.	Y17	8M	Mexico	ASY	6 mo	72	CD4	*Xho*I (Subtype a)
18.	G12.1	26F	Panama	ASY	3 yr	ND	CD4	Subtype b

[1] ND: not determined.
[2] SP and 3669 were grown in medium without exogenous IL-2.
[3] Represents 43% CD4 and 57% of CD8 population.
[4] 44% of the $CD8^+$ cells co-express CD4 marker.

Polymerase chain reaction analysisPCR analysis using primer pairs from *pol* and *tax* genes of HTLV-I and HTLV-II was performed with total genomic DNA isolated from the established cell lines, using reaction conditions as described previously (Lal et al., 1992b).

RFLP analysis

For HTLV-I cell lines, primers specific for the LTR^{31-768} region (LTR1, ACC ATG AGC CCC AAA TAT CCC CC; and LTR2, AAT TTC TCT CCT GAG AGT GCT ATA G) were used to amplify a 738-bp product, which was digested with *Apa*I, *Nde*I, *Dra*I, *Mae*III, and *Sac*I RE as described elsewhere (Komurian-Pradel et al., 1992). Similarly, for HTLV-II-infected cell lines, primers from the

transmembrane glycoprotein (gp21; 6011–6705) of HTLV-II were used for nested amplification. The first set of primers were BSEF4 (CAG GCA ATA ACG ACA GAT AA) and FLENV.R1 (AAG CTT AAG CTT ACT GTG GAT GGG TCA ATG GTA GGG G), followed by nested PCR using primers GP21F1 (CTG CAA CAA CTC CAT TAT CCT) and GP21R1 (CTG CAG AAG CTA GCA GGT CTA), resulting in a 630-bp product. Digestion with *Xho*I results in 450-bp and 180-bp products in HTLV-IIa subtype, whereas HTLV-IIb subtype remains undigested (Hall et al., 1992).

Results

Cell line development

A total of 18 cell lines representing diverse geographic regions and the clinical spectrum were developed by co-cultivation (Table 1). Of the 10 cell lines derived from individuals infected with HTLV-I, four were from patients with HAM (EG, FS, IR, A212), two were from patients with ATL (SP, 1657), one was from the asymptomatic spouse of 1657 (1742), two were from asymptomatic blood donors (3669, 1996), and the remaining one was from a prostitute (3614). Of the eight HTLV-II-infected cell lines, four were from IDU (H1B, H1H, H2E, H2A), two were from female prostitutes (Y03, Y06), one was from an asymptomatic 8-year-old son of Y06 (Y17), and one was from an asymptomatic Guaymi Indian from Panama (G12.1). All of the cell lines were dependent on exogenous IL-2 for growth, except for SP and 3669, which are IL-2 independent. All of the cell lines have been in culture for 6 months to over 3 years, with continuous $p24^{gag}$ antigen production in the culture supernatants. The amount of HTLV-antigen ranges from 6 ng/ml to > 170 ng/ml, depending on the cell line (Table 1).

Antigenic characterization

To determine the antigenic profiles of the cell lines, Western blot (WB) analysis was performed on cell lysates, followed by probing with pooled human sera derived from individuals naturally infected with HTLV-I or HTLV-II (Fig. 1). All HTLV-I-infected cell lines tested, except for FS, exhibited virus-specific bands at the expected molecular weight positions both for *gag* (p19, p24) and *env* (gp46); FS demonstrated aberrant protein bands that were of different sizes than expected. A protein profile of IR could not be generated because of low cell viability. HTLV-II-infected cell lines resembled prototypic MoT in that all cell lines had bands at the expected molecular weight positions for *gag* (p24) and *env* (gp52) proteins; as expected, a $p19^{gag}$ protein could not be detected. However, an immunoreactive 21-kDa protein was detected in six of the eight cell lines (Fig. 1) which could either represent the $p19^{gag}$ equivalent in HTLV-II (Kalyanaraman et al., 1985) or could be the transmembrane protein. These results demonstrate that these cell lines, derived from diverse geographic origins, were harboring HTLV isolates that closely resembled the prototypic isolates.

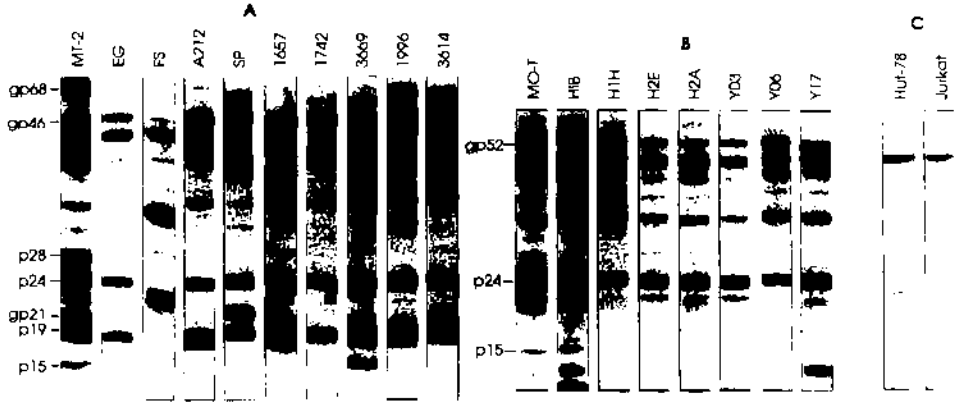

Fig. 1. Immunoreactive antigenic profiles of HTLV-I- and -II-infected cell lines by Western blot analysis. The cell lysates derived from cell lines infected with HTLV-I (A), HTLV-II (B) or uninfected cells (C) were probed with pooled HTLV-I- and HTLV-II-positive human sera. With the exception of FS, all HTLV-I-infected cell lines produced *gag* (p24, p19) and *env* (gp46) immunoreactive proteins. All HTLV-II-infected cell lines produced *gag* (p24) and *env* (gp52) immunoreactive proteins. Uninfected cell lines did not demonstrate any viral specific bands.

Phenotypic analysis

Cell surface phenotype analysis demonstrated that all cell lines were of T-cell origin ($CD5^+$). Among the subset of T-cells, all HTLV-I-cell lines, except for EG, were $CD4^+$, whereas EG was $CD8^+$. Of the HTLV-II-positive cell lines, three were $CD4^+$ (H2A, Y17, G12.1), four were $CD8^+$ (H1H, H2E, Y03, Y06), and one (H1B) represented a mixture of both, with 43% $CD4^+$ and 57% $CD8^+$ cells (Table 1). Interestingly, 44% of the $CD8^+$ cells in cell line H1H co-express $CD4^+$ marker, resulting in a phenotype that is characteristic of undifferentiated T-cells. All cell lines expressed IL-2R (CD25) and HLA-DR on either CD4 or CD8 cells, demonstrating an activated phenotype (data not shown).

Restriction maps and subtyping

Modification of restriction sites as a result of point mutations has recently been used to analyze HTLV polymorphism (Hall et al., 1992; Komurian-Pradel et al., 1992). We therefore sought to determine the limited genomic heterogeneity of the cell lines by restriction mapping. Analysis of the LTR of different HTLV-I isolates demonstrated loss of restriction site *Apa*I ($G^{113} \rightarrow A$) and *Nde*I ($T^{323} \rightarrow C$) in subtype I, loss of *Mae*III ($T^{634} \rightarrow C$) and *Dra*I ($A^{503} \rightarrow G$) in subtype II, and loss of *Sac*I ($G^{503} \rightarrow A$) in subtype III. Restriction enzyme analysis of the HTLV-I-infected cell lines showed that all except 3669 had restriction site for *Apa*I (Fig. 2) and all were positive for *Nde*I (data not shown). Six of the 10 cell lines also had the *Sac*I site (EG, FS, IR, A212, 1996, 3614); on the basis of criteria by Komurian-Pradel et al. (1992), these cell lines were classified as subtype II. One of the Japanese isolates (3669) containing *Mae*III and *Dra*I sites was classified as subtype III (Table 1). Three of the US isolates (SP, 1657, 1742) contained only

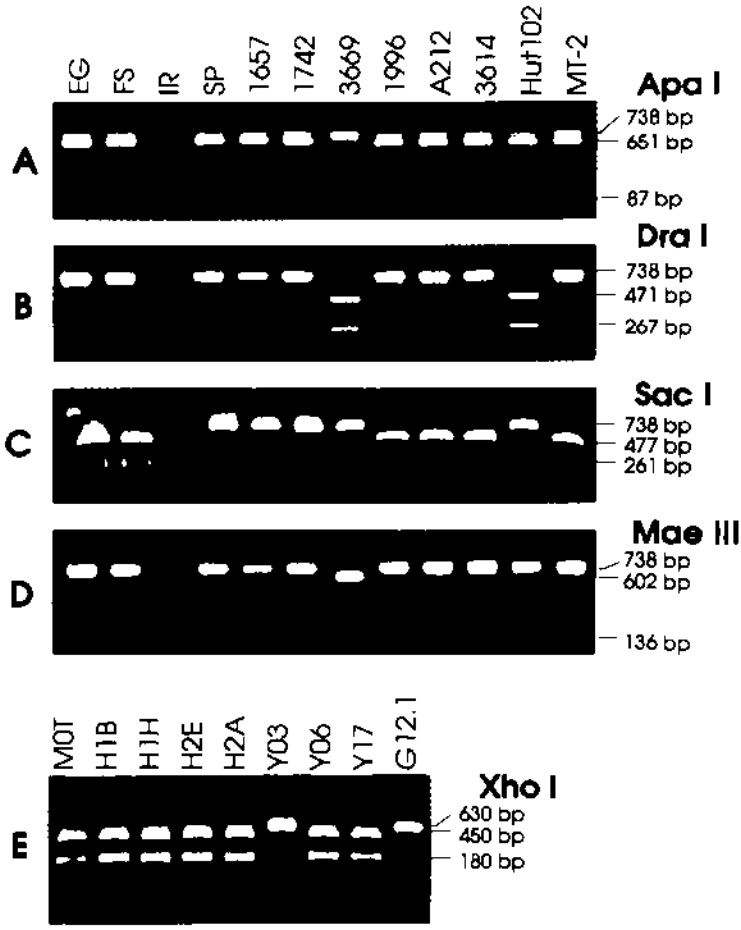

Fig. 2. Genotypic analysis of HTLV-I- and -II-infected cell lines by restriction endonuclease digestion of PCR amplicons. For the HTLV-I-infected cell lines, a 738-bp LTR amplicon was digested with *Apa*I (A), *Dra*I (B), *Sac*I (C), and *Mae*III (D) to give each respective products as shown on the right for each digest. For the HTLV-II-infected cell lines, a 630-bp *env* amplicon was digested with *Xho*I (E).

*Apa*I and did not meet the criteria of the known HTLV-I subtypes, thus representing a new subtype tentatively designated subtype IV.

Similar analysis of the transmembrane region of HTLV-II has demonstrated a *Xho*I site in subtype HTLV-IIa, which is not present in subtype HTLV-IIb (lost because of a single base change from $C^{6209} \rightarrow T$). Analysis of HTLV-II-infected cell lines demonstrated that all cell lines, except for Y03 and G12.1, contained a *Xho*I site, and therefore were typed as subtype HTLV-IIa. Y03 and G12.1 were typed as subtype HTLV-IIb.

Discussion

Studies on the regulation of viral protein expression and of the effects of cellular host factors on viral gene expression are hampered because of limited availability of the PBMC from individuals infected with HTLV-I or HTLV-II. In addition to the extremely low seroprevalence for these viruses (0.02%) (CDC, 1990), the low number of infected PBMC in vivo leads to experimental limitations when one attempts to analyze the effect of HTLV infection upon induction of early T-cell activation markers or oncogene expression. Analyses of the direct effect of these viruses on cellular genes and protein expression have not been suitable, since cell-free infection with HTLVs results in extremely low efficiency of infection (Fan et al., 1992). We therefore generated long-term T-cell lines from geographically diverse individuals infected with HTLV-I or HTLV-II.

Among the HTLV-I-infected cell lines, four were from patients with HAM (two from the United States, one from Jamaica, and one from Egypt), two were from ATL patients (both from the United States), four were from asymptomatic individuals (one from the United States, two from Japan, and one from Peru). Among the HTLV-II-infected cell lines, four were from the United States, three were from Mexico, and one was from Panama. Regardless of their pathological or geographical origin, all cell lines, with the exception of FS, produced viral antigens and expressed similar patterns of structural proteins. FS generated aberrant protein products that may reflect altered mRNA splicing or protein processing. Interestingly, all the HTLV-I-infected cell lines demonstrated a strong immunoreactive band at $p19^{gag}$, which further lends credence to our initial observations that an immunodominant epitope located at the C-terminus of $p19^{gag}$ on HTLV-I is responsible for the type-specific immune responsiveness to this protein in WB assays (Lal et al., 1992a). The equivalent of HTLV-I $p19^{gag}$ in HTLV-II has been shown to be $p21^{gag}$ (Kalyamaraman et al., 1985). Six of the eight cell lines examined contain a 21-kDa protein. Our inability to consistently detect a band at 21 kDa in HTLV-II-infected cell lines presumably reflects the poor immunogenicity of the $p21^{gag}$ protein of HTLV-II. Further, the variable amounts of $p24^{gag}$ antigens in the culture supernatants reflects variation in the integrated viral copy numbers, the number of infected cells, as well as defective virus copies. Neither the integration site nor the copy number per infected cell was determined in the present study.

HTLVs have a preferential tropism for T-cells, although other cell types have successfully been infected with this virus (Sinangil et al., 1985). The phenotypic analysis of the cell lines demonstrated that all of them were activated T-cells ($CD25^+$ and $HLA\text{-}DR^+$), in agreement with the results reported by others (Gessain et al., 1990). While the majority of the HTLV-I-infected cell lines had a $CD4^+$ phenotype, EG, derived from a HAM patient, was $CD8^+$. Among the HTLV-II-infected cell lines, three were $CD4^+$, four were $CD8^+$, and one had both $CD4^+$ and $CD8^+$ cells, suggesting that HTLV-II infects both subsets equally. Recently, however, HTLV-II has been shown to have a preferential tropism for CD8 cells in vivo (Ijichi et al., 1992). Whether co-cultivation of patient PBMCs

with uninfected PBMCs allows HTLV-II-positive $CD4^+$ cells to propagate in culture rather than HTLV-II-infected $CD8^+$ cells remains to be determined. Of greater significance is the finding that almost half of the $CD8^+$ H1H cells co-expressed CD4 on their surface. It is unclear whether the phenotypic differences seen in this cell line were the result of an infection of undifferentiated lymphocyte precursor cells, or the alteration of cell-surface marker expression as a consequence of infection with HTLV-II. Recently, a cell line derived from an ATL patient has similarly been shown to co-express CD4 and CD8 on all of the cells (Rowe et al., submitted).

An association between altered cell surface marker expression, immunologic competence, and leukemogenesis is well documented in HTLV-infected cells (Gessain et al., 1990; Yssel et al., 1989). Indeed, a hallmark of infection with HTLV is the constitutive expression of IL-2Rα (CD25) in the absence of mitogenic or antigenic stimulation (Ballard et al., 1988). All of the cell lines in the present study expressed high levels of IL-2Rα on both CD4- and CD8-positive populations, suggesting a role of not only HTLV-I but also HTLV-II in the dysregulation of IL-2Rα expression in human T-cells. Thus, in accordance with previous studies (Fujisawa et al., 1991; Wano et al., 1988), the aberrant and augmented induction of the IL-2R gene, presumably by the *tax* protein, enables the host T-cells to maintain a highly efficient growth rate possibly by altering the signal transduction mechanisms used by IL-2Rα (Yodoi and Uchiyama, 1992). No difference in the intensity of CD25 was observed among those cell lines that were IL-2-dependent for their growth, compared with those that did not require exogenous IL-2 (SP, 3669). Thus, cellular proliferation perhaps is dependent on expression of *tax* alone or in synergy with other soluble products, including cytokines.

The genomic analysis of different isolates has demonstrated remarkable conservation in the proviral sequences. However, specific mutations resulting in loss of or acquisition of a new restriction site have allowed a simple way to further subtype HTLV-I and HTLV-II isolates from diverse geographic origins (Paine et al., 1991; De et al., 1991). Restriction mapping analysis of the HTLV-I LTR demonstrated that most of the cell lines were subtype II and one (3669) was subtype III. Interestingly, unlike Japanese ATL isolates (Komurian-Pradel et al., 1992), both of the ATL cell lines and the cell line from the spouse of an ATL patient in the present study have lost *Mae*III and *Dra*I sites, and presumably represent a different subtype, tentatively assigned subtype IV. While the mutations in the LTR region of HTLV-I have not been linked to the two distinct pathologies associated with HTLV-I (Paine et al., 1991), the functional analysis of the LTRs carrying small mutations has demonstrated biological differences in the activity levels of promoters in different cellular environments (Gonzalez-Dunia et al., 1992). These results suggest that variations in the HTLV-I promoter might be, in part, responsible for the differential viral expression and cellular tropism. Restriction map analysis of HTLV-II-infected cell lines demonstrated that most were subtype HTLV-IIa, whereas isolates from a Mexican prostitute and a Guaymi Indian were subtype HTLV-IIb. Recently, complete genomic sequencing of the G12.1 HTLV-II isolate has demonstrated several specific mutations and restriction sites, based on

which this isolate has been designated as subtype HTLV-IIc (Pardi et al., submitted).

In conclusion, these T-lymphoid cell lines, which all contain replication-competent virus and appear to be similar to prototypic isolates, at least in the structural proteins, should allow a detailed analysis of functional and biological effects of the virus on cellular gene expression. In addition, the complex interactions between viral and host factors that determine the different outcome of HTLV-I-infection will be facilitated with the cell lines representing HAM, ATL, and asymptomatic isolates from the same geographic area. Furthermore, analysis of the antigenic changes caused by genetic rearrangements and non-synonymous mutations in the viral genome would have strong implications both for diagnostic and protective immune responses.

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VIRUS 00887

Virus Research

Characterization of human T-lymphotropic virus type I- and II-infected T-cell lines: antigenic, phenotypic, and genotypic analysis

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(Received 20 November, 1992; revision received and accepted 19 January 1993)

Summary

Eighteen long-term T-cell lines were established from peripheral blood mononuclear cells of individuals infected with human T-lymphotropic virus type I (HTLV-I) or II (HTLV-II). These cell lines (10 HTLV-I and 8 HTLV-II), representing diverse pathologic profiles and geographic regions, have been in culture for over 6 months and have constitutively produced $p24^{gag}$ antigen. Antigenic characterization of the cell lines by Western blot analysis demonstrated that all but one produced *gag* (p24) and *env* (gp46 or gp52) structural proteins; one HTLV-I-infected cell line exhibited an aberrant protein profile. Phenotypic analysis of the HTLV-infected cell lines demonstrated phenotypes consistent with activated T-cells ($CD5^+$, $CD25^+$, $HLA\text{-}DR^+$). The HTLV-I-infected cell lines were predominantly $CD4^+$ (IR, FS, A212, SP, 1657, 1742, 3669, 1996, and 3614), whereas EG was $CD8^+$. The HTLV-II-infected cell lines were either $CD4^+$ (H2A, Y17, G12.1), $CD8^+$ (H1H, H2E, Y03, Y06), or both (H1B). Restriction map analysis and subtyping of the viral genomes demonstrated heterogeneity among these isolates. Of the HTLV-I-infected cell lines, six were subtype II, one was subtype III and, on the basis of additional restriction sites, another subtype, tentatively classified as

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subtype IV, could be identified for three of the HTLV-I-infected cell lines. Of the HTLV-II-infected cell lines, six were subtype HTLV-IIa and two were subtype HTLV-IIb. While the majority of the cell lines resemble the prototypic HTLV-I-infected (MT-2) and HTLV-II-infected (MoT) cell lines, the antigenic, phenotypic, and genotypic data collectively demonstrate heterogeneity among viral isolates representing diverse geographic regions.

Human T lymphotropic virus; HTLV; T-cell line; Genotype; Restriction fragment length polymorphism

Introduction

Human T-lymphotropic virus types I and II (HTLV-I and HTLV-II) belong to a family of C-type retroviruses that share extensive structural and functional homologies. HTLV-I is etiologically linked to development of HTLV-associated myelopathy (HAM), also known as tropical spastic paraparesis, and adult T-cell leukemia (ATL). HTLV-I infection is endemic in Japan, the Caribbean, and some parts of Africa (Manns and Blattner, 1991). HTLV-II has not yet been associated with any particular disease, and infections with HTLV-II are endemic in certain indigenous native indians in the Americas (Lairmore et al., 1990). In the United States, infection with HTLV-I or HTLV-II has been documented in injecting-drug users (IDU), female prostitutes, patients attending sexually transmitted disease clinics, volunteer blood donors, and recipients of multiple blood transfusions (Manns and Blattner, 1991; CDC, 1990).

The genomic structure of HTLV-I and HTLV-II is unique. Unlike known acute transforming RNA tumor viruses, they do not contain a typical oncogene (Smith and Greene, 1991). Rather, the 3' end of the proviral genome encodes the *tax* protein, which not only acts as a transcriptional activator of the viral long terminal repeat (LTR) (Felber et al., 1985), but trans-activates several heterologous promoters, in particular the cellular genes involved in T-cell activation and proliferation (Smith and Greene, 1991). Indeed, the ability of HTLV-I to cause proliferation and immortalization of infected cells has been well documented. The HTLV-I-infected cell lines established from cord blood by co-cultivation with virus-producing cell lines or immortalization of antigen-specific T-cell clones have provided a useful model to study the process of HTLV-I-induced leukemogenesis in vitro (Del Mistro et al., 1986; Faller et al., 1988). While not much is known about the functional effects associated with HTLV-II infection, infection with HTLV-I results in both phenotypic and functional changes of T-cells (Faller et al., 1988; Gessain et al., 1990; Yssel et al., 1989).

The genomic analysis of HTLV isolates has demonstrated restricted mutation rates (Paine et al., 1991; De et al., 1991), in contrast to that of human immunodeficiency virus, which exists as a dynamic mixture of quasi-species (Coffin, 1986). Despite the significant sequence conservation among HTLV-I isolates, genomic

variation was observed to be greater between isolates from different geographic regions than between isolates from the same region (Paine et al., 1991; De et al., 1991; Komurian et al., 1991). Recently, point mutations in the nucleotide sequence, resulting in altered restriction endonuclease (RE) fragment sizes, have allowed molecular characterization of various isolates. These altered RE fragment sizes have yielded HTLV-I and HTLV-II isolate subtypes by restriction fragment length polymorphism (RFLP) analysis (Hall et al., 1992; Komurian-Pradel et al., 1992).

As a first step towards defining the phenotypic changes of HTLV-infected cells and determining the effect of HTLV on constitutive expression of genes involved in T-cell proliferation (both the early activation proto-oncogenes and the cytokine genes), we have established cell lines representing diverse geographic isolates from individuals infected with HTLV-I or HTLV-II. In the present investigation, we report the antigenic, phenotypic, and genotypic characterization of these HTLV-infected lines.

Materials and Methods

Study population

Eighteen individuals seropositive for antibodies to HTLV-I/II and whose infections were further confirmed to be HTLV-I ($n = 10$) or HTLV-II ($n = 8$) by polymerase chain reaction (PCR) and synthetic peptide-based assays (Lal et al., 1992b) were included in the study. Three of the four HTLV-I-infected patients were recruited through the CDC HAM/TSP surveillance system; these included one patient from Jamaica (EG, Janssen et al., 1991) and two patients from the United States (FS, McKendall et al., 1991; and IR, Kaplan et al., 1991). The fourth HAM patient was from Cairo, Egypt (A212) who had developed neurological symptoms a year after a blood transfusion (Constantine et al., 1992). Both ATL cases, SP (Ratner et al., 1990) and 1657 were from the United States. Of the four asymptomatic HTLV-I-infected blood donors, one was the concordently infected spouse of 1657 (1742), two were blood donors (3669, 1996) from Japan, and one was a female prostitute (3614) from Peru. Of the eight HTLV-II-infected individuals, four were IDUs (H1B, H1H, H2E, H2A) from the United States, three (Y03, Y06, Y17) were from Mexico, and one (G12.1) was an asymptomatic Guaymi Indian from Panama (Lairmore et al., 1990). The demographics of these individuals are shown in Table 1.

Development of cell lines

Long-term T-cell lines were developed from infected individuals as follows: purified peripheral blood mononuclear cells (PBMC) from HTLV-infected individuals were stimulated with 0.1% phytohemagglutinin (PHA-P, Difco, Detroit, MI) and cultured at 1×10^6 cells/ml in RPMI-1640 supplemented with 15% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml) (C-RPMI), and 10% interleukin-2 (IL-2, Advanced Biotechnologies Inc., Silver

Spring, MD). After 72 h, the HTLV-infected PBMC were co-cultured with an equal number of PHA-P-stimulated PBMC from HTLV seronegative donors and maintained in C-RPMI containing 10% IL-2. The culture medium was changed every 4 days and cells were expanded depending upon their growth. Stable transformed cell lines were established after 4–6 months in culture and some of the cell lines have been in culture for more than 3 years (Table 1). An attempt was made to wean the cell lines off IL-2 in a gradual manner; if the cell lines started to die, exogenous IL-2 was added to maintain the cell growth. Previously established HTLV-I-infected (HuT102 and MT-2) and HTLV-II-infected (MoT) cell lines were maintained in C-RPMI.

HTLV-antigen production

HTLV virus production in the culture supernatants was determined by using a $p24^{gag}$ antigen capture assay (Coulter Immunology, Hialeah, FL) according to the manufacturer's instructions. The monoclonal antibody (mAb) used in the assay recognizes a *gag* antigenic determinant common to both HTLV-I and HTLV-II. The $p24^{gag}$ antigen levels were tested on supernatants collected at day 3 of cultures set at 5×10^5/ml.

Phenotype analysis

Lymphocyte phenotype analysis of the cell lines was performed by Flow cytometry analysis using a FACScan (Becton Dickenson, San Jose, CA). Briefly, 50,000 cells were individually stained with mAbs to the cluster of differentiation (CD) antigens for 30 min at 4°C. The mAbs conjugated with either FITC or phycoerythrin (PE) included: Leu 1 (CD5), Leu 2a (CD8), Leu 3a (CD4), 2A3 (CD25), and L243 (HLA-DR) (Becton Dickinson, Mountain View, CA). The cells were washed twice in cold FACS buffer (phosphate-buffered saline [PBS] with 0.2% sodium azide, 0.1% bovine serum albumin [BSA] and 2% human AB serum), and fixed in 1% paraformaldehyde for 30 min. The fixed cells were analyzed using a FACScan and data analysis was performed using Consort 30 software on a Hewlett Packard computer.

Analysis of viral proteins

Approximately 10^8 cells were lysed in buffer (0.1 M PBS [pH 7.4] containing 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM PMSF) at 4°C for 16 h, and then centrifuged at 1500 rpm for 20 min, followed by airfuging for 30 min. Equal amounts of cell lysates were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene diflouride membrane. The membrane was blocked for 2 h at room temperature (RT) in Tris-buffered saline (TBS) containing 5% BSA, and then incubated with pooled anti-HTLV-I/II-positive human sera overnight at RT. The membrane was then washed six times with TBS containing 0.05% Tween-20 (TBS-T) and incubated with ^{125}I-labeled Protein-G (10^5 cpm/ml; NEN, Boston, MA) for 2 h at RT, followed by three washes in TBS-T and a final wash with TBS-T containing 10 mM EDTA. The membrane was dried and exposed to Kodak X-ray film.

TABLE 1

Demographics and characteristics of the HTLV-I- and -II-infected cell lines

No.	Cell line	Age, gender	Geographic origin	Clinical diagnosis	Cultured cell line		Phenotype	Genotypic subtyping
					Time	$p24^{gag}$(ng/ml)		
PCR-confirmed HTLV-I								
1.	EG	9F	Jamaica	HAM	2 yr	82	CD8	*Apa*I, *Sac*I (subtype II)
2.	FS	41M	US	HAM	3 yr	6	CD4	*Apa*I, *Sac*I (subtype II)
3.	IR	65M	US	HAM	3 yr	ND [1]	CD4	*Apa*I, *Sac*I (subtype II)
4.	A212	45F	Egypt	HAM	1 yr	20	CD4	*Apa*I, *Sac*I (subtype II)
5.	SP [2]	43F	US	ATL	3 yr	14	CD4	*Apa*I (subtype IV)
6.	1657	31F	US	ATL	2.5 yr	>170	CD4	*Apa*I (subtype IV)
7.	1742	31M	US	ASY	2.5 yr	134	CD4	*Apa*I (subtype IV)
8.	3669 [2]	28F	Japan	ASY	2 yr	144	CD4	*Dra*I, *Mae*III (subtype III)
9.	1996	43F	Japan	ASY	1.5 yr	163	CD4	*Apa*I, *Sac*I (subtype II)
10.	3614	Unknown	Peru	ASY	2 yr	78	CD4	*Apa*I, *Sac*I (subtype II)
PCR-confirmed HTLV-II								
11.	H1B	40F	US	ASY	2 yr	23	CD4/CD8 [3]	*Xho*I (Subtype a)
12.	H1H	48M	US	ASY	2 yr	39	CD8 [4]	*Xho*I (Subtype a)
13.	H2E	57M	US	ASY	2 yr	39	CD8	*Xho*I (Subtype a)
14.	H2A	35M	US	ASY	2 yr	16	CD4	*Xho*I (Subtype a)
15.	Y03	22F	Mexico	ASY	6 mo	22	CD8	Subtype b
16.	Y06	33F	Mexico	ASY	6 mo	71	CD8	*Xho*I (Subtype a)
17.	Y17	8M	Mexico	ASY	6 mo	72	CD4	*Xho*I (Subtype a)
18.	G12.1	26F	Panama	ASY	3 yr	ND	CD4	Subtype b

[1] ND: not determined.
[2] SP and 3669 were grown in medium without exogenous IL-2.
[3] Represents 43% CD4 and 57% of CD8 population.
[4] 44% of the $CD8^+$ cells co-express CD4 marker.

Polymerase chain reaction analysisPCR analysis using primer pairs from *pol* and *tax* genes of HTLV-I and HTLV-II was performed with total genomic DNA isolated from the established cell lines, using reaction conditions as described previously (Lal et al., 1992b).

RFLP analysis

For HTLV-I cell lines, primers specific for the LTR^{31-768} region (LTR1, ACC ATG AGC CCC AAA TAT CCC CC; and LTR2, AAT TTC TCT CCT GAG AGT GCT ATA G) were used to amplify a 738-bp product, which was digested with *Apa*I, *Nde*I, *Dra*I, *Mae*III, and *Sac*I RE as described elsewhere (Komurian-Pradel et al., 1992). Similarly, for HTLV-II-infected cell lines, primers from the

transmembrane glycoprotein (gp21; 6011–6705) of HTLV-II were used for nested amplification. The first set of primers were BSEF4 (CAG GCA ATA ACG ACA GAT AA) and FLENV.R1 (AAG CTT AAG CTT ACT GTG GAT GGG TCA ATG GTA GGG G), followed by nested PCR using primers GP21F1 (CTG CAA CAA CTC CAT TAT CCT) and GP21R1 (CTG CAG AAG CTA GCA GGT CTA), resulting in a 630-bp product. Digestion with *Xho*I results in 450-bp and 180-bp products in HTLV-IIa subtype, whereas HTLV-IIb subtype remains undigested (Hall et al., 1992).

Results

Cell line development

A total of 18 cell lines representing diverse geographic regions and the clinical spectrum were developed by co-cultivation (Table 1). Of the 10 cell lines derived from individuals infected with HTLV-I, four were from patients with HAM (EG, FS, IR, A212), two were from patients with ATL (SP, 1657), one was from the asymptomatic spouse of 1657 (1742), two were from asymptomatic blood donors (3669, 1996), and the remaining one was from a prostitute (3614). Of the eight HTLV-II-infected cell lines, four were from IDU (H1B, H1H, H2E, H2A), two were from female prostitutes (Y03, Y06), one was from an asymptomatic 8-year-old son of Y06 (Y17), and one was from an asymptomatic Guaymi Indian from Panama (G12.1). All of the cell lines were dependent on exogenous IL-2 for growth, except for SP and 3669, which are IL-2 independent. All of the cell lines have been in culture for 6 months to over 3 years, with continuous $p24^{gag}$ antigen production in the culture supernatants. The amount of HTLV-antigen ranges from 6 ng/ml to > 170 ng/ml, depending on the cell line (Table 1).

Antigenic characterization

To determine the antigenic profiles of the cell lines, Western blot (WB) analysis was performed on cell lysates, followed by probing with pooled human sera derived from individuals naturally infected with HTLV-I or HTLV-II (Fig. 1). All HTLV-I-infected cell lines tested, except for FS, exhibited virus-specific bands at the expected molecular weight positions both for *gag* (p19, p24) and *env* (gp46); FS demonstrated aberrant protein bands that were of different sizes than expected. A protein profile of IR could not be generated because of low cell viability. HTLV-II-infected cell lines resembled prototypic MoT in that all cell lines had bands at the expected molecular weight positions for *gag* (p24) and *env* (gp52) proteins; as expected, a $p19^{gag}$ protein could not be detected. However, an immunoreactive 21-kDa protein was detected in six of the eight cell lines (Fig. 1) which could either represent the $p19^{gag}$ equivalent in HTLV-II (Kalyanaraman et al., 1985) or could be the transmembrane protein. These results demonstrate that these cell lines, derived from diverse geographic origins, were harboring HTLV isolates that closely resembled the prototypic isolates.

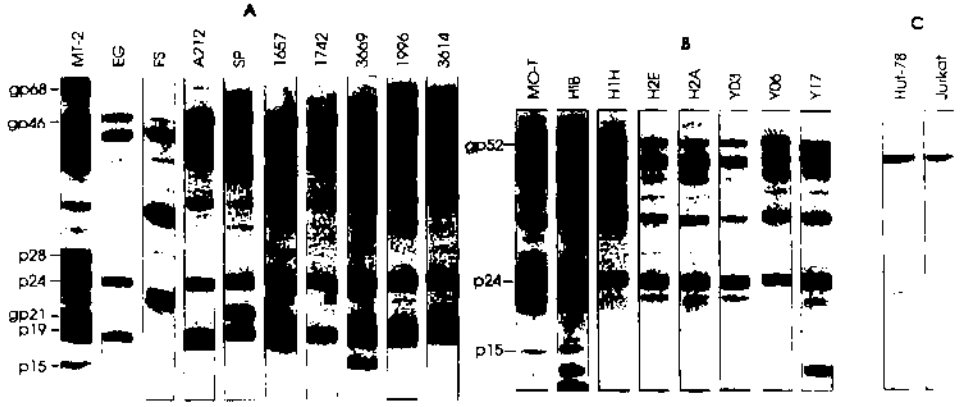

Fig. 1. Immunoreactive antigenic profiles of HTLV-I- and -II-infected cell lines by Western blot analysis. The cell lysates derived from cell lines infected with HTLV-I (A), HTLV-II (B) or uninfected cells (C) were probed with pooled HTLV-I- and HTLV-II-positive human sera. With the exception of FS, all HTLV-I-infected cell lines produced *gag* (p24, p19) and *env* (gp46) immunoreactive proteins. All HTLV-II-infected cell lines produced *gag* (p24) and *env* (gp52) immunoreactive proteins. Uninfected cell lines did not demonstrate any viral specific bands.

Phenotypic analysis

Cell surface phenotype analysis demonstrated that all cell lines were of T-cell origin ($CD5^+$). Among the subset of T-cells, all HTLV-I-cell lines, except for EG, were $CD4^+$, whereas EG was $CD8^+$. Of the HTLV-II-positive cell lines, three were $CD4^+$ (H2A, Y17, G12.1), four were $CD8^+$ (H1H, H2E, Y03, Y06), and one (H1B) represented a mixture of both, with 43% $CD4^+$ and 57% $CD8^+$ cells (Table 1). Interestingly, 44% of the $CD8^+$ cells in cell line H1H co-express $CD4^+$ marker, resulting in a phenotype that is characteristic of undifferentiated T-cells. All cell lines expressed IL-2R (CD25) and HLA-DR on either CD4 or CD8 cells, demonstrating an activated phenotype (data not shown).

Restriction maps and subtyping

Modification of restriction sites as a result of point mutations has recently been used to analyze HTLV polymorphism (Hall et al., 1992; Komurian-Pradel et al., 1992). We therefore sought to determine the limited genomic heterogeneity of the cell lines by restriction mapping. Analysis of the LTR of different HTLV-I isolates demonstrated loss of restriction site *Apa*I ($G^{113} \rightarrow A$) and *Nde*I ($T^{323} \rightarrow C$) in subtype I, loss of *Mae*III ($T^{634} \rightarrow C$) and *Dra*I ($A^{503} \rightarrow G$) in subtype II, and loss of *Sac*I ($G^{503} \rightarrow A$) in subtype III. Restriction enzyme analysis of the HTLV-I-infected cell lines showed that all except 3669 had restriction site for *Apa*I (Fig. 2) and all were positive for *Nde*I (data not shown). Six of the 10 cell lines also had the *Sac*I site (EG, FS, IR, A212, 1996, 3614); on the basis of criteria by Komurian-Pradel et al. (1992), these cell lines were classified as subtype II. One of the Japanese isolates (3669) containing *Mae*III and *Dra*I sites was classified as subtype III (Table 1). Three of the US isolates (SP, 1657, 1742) contained only

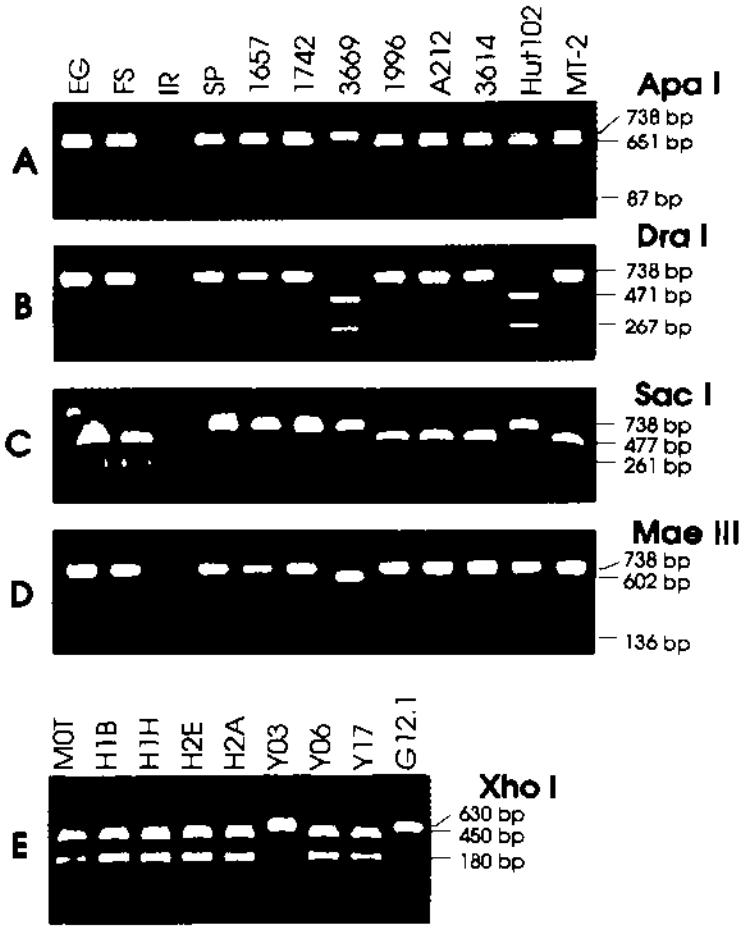

Fig. 2. Genotypic analysis of HTLV-I- and -II-infected cell lines by restriction endonuclease digestion of PCR amplicons. For the HTLV-I-infected cell lines, a 738-bp LTR amplicon was digested with *Apa*I (A), *Dra*I (B), *Sac*I (C), and *Mae*III (D) to give each respective products as shown on the right for each digest. For the HTLV-II-infected cell lines, a 630-bp *env* amplicon was digested with *Xho*I (E).

*Apa*I and did not meet the criteria of the known HTLV-I subtypes, thus representing a new subtype tentatively designated subtype IV.

Similar analysis of the transmembrane region of HTLV-II has demonstrated a *Xho*I site in subtype HTLV-IIa, which is not present in subtype HTLV-IIb (lost because of a single base change from $C^{6209} \rightarrow T$). Analysis of HTLV-II-infected cell lines demonstrated that all cell lines, except for Y03 and G12.1, contained a *Xho*I site, and therefore were typed as subtype HTLV-IIa. Y03 and G12.1 were typed as subtype HTLV-IIb.

Discussion

Studies on the regulation of viral protein expression and of the effects of cellular host factors on viral gene expression are hampered because of limited availability of the PBMC from individuals infected with HTLV-I or HTLV-II. In addition to the extremely low seroprevalence for these viruses (0.02%) (CDC, 1990), the low number of infected PBMC in vivo leads to experimental limitations when one attempts to analyze the effect of HTLV infection upon induction of early T-cell activation markers or oncogene expression. Analyses of the direct effect of these viruses on cellular genes and protein expression have not been suitable, since cell-free infection with HTLVs results in extremely low efficiency of infection (Fan et al., 1992). We therefore generated long-term T-cell lines from geographically diverse individuals infected with HTLV-I or HTLV-II.

Among the HTLV-I-infected cell lines, four were from patients with HAM (two from the United States, one from Jamaica, and one from Egypt), two were from ATL patients (both from the United States), four were from asymptomatic individuals (one from the United States, two from Japan, and one from Peru). Among the HTLV-II-infected cell lines, four were from the United States, three were from Mexico, and one was from Panama. Regardless of their pathological or geographical origin, all cell lines, with the exception of FS, produced viral antigens and expressed similar patterns of structural proteins. FS generated aberrant protein products that may reflect altered mRNA splicing or protein processing. Interestingly, all the HTLV-I-infected cell lines demonstrated a strong immunoreactive band at $p19^{gag}$, which further lends credence to our initial observations that an immunodominant epitope located at the C-terminus of $p19^{gag}$ on HTLV-I is responsible for the type-specific immune responsiveness to this protein in WB assays (Lal et al., 1992a). The equivalent of HTLV-I $p19^{gag}$ in HTLV-II has been shown to be $p21^{gag}$ (Kalyamaraman et al., 1985). Six of the eight cell lines examined contain a 21-kDa protein. Our inability to consistently detect a band at 21 kDa in HTLV-II-infected cell lines presumably reflects the poor immunogenicity of the $p21^{gag}$ protein of HTLV-II. Further, the variable amounts of $p24^{gag}$ antigens in the culture supernatants reflects variation in the integrated viral copy numbers, the number of infected cells, as well as defective virus copies. Neither the integration site nor the copy number per infected cell was determined in the present study.

HTLVs have a preferential tropism for T-cells, although other cell types have successfully been infected with this virus (Sinangil et al., 1985). The phenotypic analysis of the cell lines demonstrated that all of them were activated T-cells ($CD25^+$ and $HLA\text{-}DR^+$), in agreement with the results reported by others (Gessain et al., 1990). While the majority of the HTLV-I-infected cell lines had a $CD4^+$ phenotype, EG, derived from a HAM patient, was $CD8^+$. Among the HTLV-II-infected cell lines, three were $CD4^+$, four were $CD8^+$, and one had both $CD4^+$ and $CD8^+$ cells, suggesting that HTLV-II infects both subsets equally. Recently, however, HTLV-II has been shown to have a preferential tropism for CD8 cells in vivo (Ijichi et al., 1992). Whether co-cultivation of patient PBMCs

with uninfected PBMCs allows HTLV-II-positive $CD4^+$ cells to propagate in culture rather than HTLV-II-infected $CD8^+$ cells remains to be determined. Of greater significance is the finding that almost half of the $CD8^+$ H1H cells co-expressed CD4 on their surface. It is unclear whether the phenotypic differences seen in this cell line were the result of an infection of undifferentiated lymphocyte precursor cells, or the alteration of cell-surface marker expression as a consequence of infection with HTLV-II. Recently, a cell line derived from an ATL patient has similarly been shown to co-express CD4 and CD8 on all of the cells (Rowe et al., submitted).

An association between altered cell surface marker expression, immunologic competence, and leukemogenesis is well documented in HTLV-infected cells (Gessain et al., 1990; Yssel et al., 1989). Indeed, a hallmark of infection with HTLV is the constitutive expression of IL-2Rα (CD25) in the absence of mitogenic or antigenic stimulation (Ballard et al., 1988). All of the cell lines in the present study expressed high levels of IL-2Rα on both CD4- and CD8-positive populations, suggesting a role of not only HTLV-I but also HTLV-II in the dysregulation of IL-2Rα expression in human T-cells. Thus, in accordance with previous studies (Fujisawa et al., 1991; Wano et al., 1988), the aberrant and augmented induction of the IL-2R gene, presumably by the *tax* protein, enables the host T-cells to maintain a highly efficient growth rate possibly by altering the signal transduction mechanisms used by IL-2Rα (Yodoi and Uchiyama, 1992). No difference in the intensity of CD25 was observed among those cell lines that were IL-2-dependent for their growth, compared with those that did not require exogenous IL-2 (SP, 3669). Thus, cellular proliferation perhaps is dependent on expression of *tax* alone or in synergy with other soluble products, including cytokines.

The genomic analysis of different isolates has demonstrated remarkable conservation in the proviral sequences. However, specific mutations resulting in loss of or acquisition of a new restriction site have allowed a simple way to further subtype HTLV-I and HTLV-II isolates from diverse geographic origins (Paine et al., 1991; De et al., 1991). Restriction mapping analysis of the HTLV-I LTR demonstrated that most of the cell lines were subtype II and one (3669) was subtype III. Interestingly, unlike Japanese ATL isolates (Komurian-Pradel et al., 1992), both of the ATL cell lines and the cell line from the spouse of an ATL patient in the present study have lost *Mae*III and *Dra*I sites, and presumably represent a different subtype, tentatively assigned subtype IV. While the mutations in the LTR region of HTLV-I have not been linked to the two distinct pathologies associated with HTLV-I (Paine et al., 1991), the functional analysis of the LTRs carrying small mutations has demonstrated biological differences in the activity levels of promoters in different cellular environments (Gonzalez-Dunia et al., 1992). These results suggest that variations in the HTLV-I promoter might be, in part, responsible for the differential viral expression and cellular tropism. Restriction map analysis of HTLV-II-infected cell lines demonstrated that most were subtype HTLV-IIa, whereas isolates from a Mexican prostitute and a Guaymi Indian were subtype HTLV-IIb. Recently, complete genomic sequencing of the G12.1 HTLV-II isolate has demonstrated several specific mutations and restriction sites, based on

which this isolate has been designated as subtype HTLV-IIc (Pardi et al., submitted).

In conclusion, these T-lymphoid cell lines, which all contain replication-competent virus and appear to be similar to prototypic isolates, at least in the structural proteins, should allow a detailed analysis of functional and biological effects of the virus on cellular gene expression. In addition, the complex interactions between viral and host factors that determine the different outcome of HTLV-I-infection will be facilitated with the cell lines representing HAM, ATL, and asymptomatic isolates from the same geographic area. Furthermore, analysis of the antigenic changes caused by genetic rearrangements and non-synonymous mutations in the viral genome would have strong implications both for diagnostic and protective immune responses.

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Int. J. Cancer: **50,** 124–130 (1992)

Publication of the International Union Against Cancer
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COMPARATIVE BIOLOGICAL RESPONSES OF RABBITS INFECTED WITH HUMAN T-LYMPHOTROPIC VIRUS TYPE I ISOLATES FROM PATIENTS WITH LYMPHOPROLIFERATIVE AND NEURODEGENERATIVE DISEASE

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An experimental rabbit model was used to determine host responses to infection by various human T-lymphotropic virus type-I (HTLV-I) strains. Seven groups of 4 to 5 rabbits each were inoculated with lethally-irradiated HTLV-I-infected cell lines derived from patients with adult T-cell leukemia/lymphoma or from patients with HTLV-I-associated myelopathy. Four separate control groups of 2 rabbits each were inoculated with similarly prepared HTLV-I-negative cells derived from rabbits or humans. Anti-viral antibody responses were assessed by immunoblot assay and hematologic parameters were measured using automated cell counters and cytologic staining. The virologic status of challenged rabbits was determined by co-culture and HTLV-I antigen capture assay, as well as by polymerase chain reaction (PCR) amplification of HTLV-I DNA from peripheral blood mononuclear cells (PBMC) or tissues. The HTLV-I inocula could be separated into groups based upon their infectivity to rabbits: highly infectious strains elicited intense serologic responses and were detected frequently in tissues by antigen and PCR assays, while other strains were moderately to poorly infectious, induced weak antibody responses and were infrequently detected by antigen and PCR assays. Overall, PBMC appeared to have the greatest quantity of HTLV-I containing cells, while bone marrow was a poor source of virus. No clinical or hematologic abnormalities were evident during the 24-week course of infection. Taken together, our results suggest there is heterogeneity in the biological response to HTLV-I infection which is, in part, dependent on the infecting strain of virus.

Infection by human T-lymphotropic virus type I (HTLV-I) is primarily associated with 2 diverse disease syndromes, adult T-cell leukemia/lymphoma (ATL) and a chronic progressive myelopathy [HTLV-I-associated myelopathy or tropical spastic paraparesis (HAM/TSP)] (Hinuma *et al.*, 1981; Gessain *et al.*, 1985). The pathogenesis of HTLV-I infection and disease is poorly understood, in part, because of the long latent period between exposure and disease manifestation. The virus is transmitted in a cell-associated manner, and major routes of transmission involve sexual spread, mother-to-child transmission, and parenteral injection of contaminated blood (Iwahara *et al.*, 1990; Kajiyama *et al.*, 1986; Okochi *et al.*, 1984). Proposed mechanisms of HTLV-I tumor induction include a multi-step model in which HTLV-I induces cellular proliferation (*e.g.*, *tax*-mediated transactivation), with subsequent "secondary" signals producing cellular transformation (Fujii *et al.*, 1988). The pathogenic mechanisms involved in the generation of HAM/TSP are equally unclear; the lesions appear primarily to be immunologically mediated, potentially driven by cytotoxic T-lymphocytes within nervous tissue (Moore *et al.*, 1989). Proposed determinants of lymphoproliferative *vs.* neurodegenerative disease manifestations of HTLV-I infection include host factors (*e.g.*, certain major histocompatibility complex antigen haplotypes may determine disease susceptibility), environmental influences (*e.g.*, carcinogens), or viral factors (*e.g.*, strain differences) (Osame *et al.*, 1989; Yanagihara *et al.*, 1991; Yoshida *et al.*, 1989).

To date, a few structural differences between ATL and HAM/TSP-derived HTLV-I isolates have been defined; however, this may reflect the relatively few HTLV nucleotide sequences that have been completely analyzed. Molecular heterogeneity exists in the *tax* and *env* regions of HTLV-I isolates (Daenke *et al.*, 1990; Ratner *et al.*, 1985). HTLV-I isolates derived from the Caribbean may have greater similarity in their nucleotide sequences compared with Japanese isolates, suggesting geographic clustering of specific HTLV-I types (Kaplan *et al.*, 1991; Malik *et al.*, 1988). The description of HTLV-I variant strains from the United States, Africa, and Papua-New-Guinea suggests that subtypes of HTLV-I infect geographically isolated populations (De *et al.*, 1991; Ratner *et al.*, 1985; Yangihara *et al.*, 1991).

Animal models have provided important information concerning oral and sexual transmission of HTLV-I (Iwahara *et al.*, 1990; Miyoshi *et al.*, 1983), infectivity of blood products containing HTLV-I-infected cells (Kotani *et al.*, 1986), and potential vaccines against the viral infection (Shida *et al.*, 1987). We have shown the usefulness of the rabbit model of HTLV-I infection in determining the sequential antibody responses to the virus infection and detection of infected tissues, using polymerase chain reaction (PCR) technology (Cockerell *et al.*, 1990). In the present study we used this animal model to test for potential differences in the anti-viral responses of rabbits to infection by unique HTLV-I strains derived from both ATL and HAM/TSP patients. Our data suggest that viral strain differences may determine the infectivity of HTLV-I *in vivo* and provide a basis for improved utilization of the rabbit model to assess the influence of co-factors of infection in a biological system.

MATERIAL AND METHODS

Cell lines

Established HTLV-I-infected cell lines MT-2 (Miyoshi *et al.*, 1981), HuT 102 (Gazdar *et al.*, 1980) and Ra-1 (Miyoshi *et al.*, 1983) were maintained in complete RPMI 1640 medium with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin, and 2 mM glutamine, at 37°C in a 7% CO_2 atmosphere incubator. Peripheral blood mononuclear cells (PBMC) or cerebrospinal fluid (CSF) cell-derived cultures were established from patients with a clinical diagnosis of ATL (Kuefler and Bunn, 1986) or HAM/TSP (Osame *et al.*, 1986); the clinical and virologic data for each patient are summarized in Table I. Serum and CSF specimens from each patient were tested for antibodies against HTLV-I in an immunoblot (IB) assay (Hartley *et al.*, 1990). Patient PBMC-derived cell lines were established after Ficoll/diatrizoate separation (Bionetics, Charleston, SC) of heparinized blood samples, followed by stimulation with 1% phytohemagglutinin (PHA-P, Difco, Detroit, MI) and culture at a density of 2×10^6 cells/ml in complete medium supplemented with a purified source of human interleukin 2 (IL-2) (10% of total medium) (Advanced Biotechnologies, Silver Spring, MD). After 72 hr of culture,

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Received: June 10, 1991 and in revised form July 26, 1991.

TABLE I – HTLV-I CELL LINES USED FOR RABBIT INOCULATIONS

HTLV-I-infected patient and cell line data							
Patient	Origin	Disease	Age/sex	Ethnic group	Risk	Cell line[1]	Reference
No. 1	US	HAM/TSP	62/M	White	Transf.	PBMC/CSF	Kaplan *et al.*, 1991
No. 2	US	HAM/TSP	49/M	Black	ND[2]	PBMC/CSF	McKendall *et al.*, 1991
No. 3	US	ATL	43/F	Black	Family	PMBC	Ratner *et al.*, 1990

HTLV-I established cell lines			
Cell line	Origin	Disease	Reference
HuT 102	Caribbean	ATL	Gazdar *et al.*, 1980
MT-2	Japan	ATL	Miyoshi *et al.*, 1981
Ra-1	Japan	ATL	Miyoshi *et al.*, 1983

[1]All patient sera and CSF were positive for HTLV-I antibodies as described (Hartley *et al.*, 1990) and all patient cell lines were positive by PCR using HTLV-I gag and pol primers as described (Lairmore *et al.*, 1990).–[2]ND = not determined.

patient PBMCs were split 1:2 and subcultured both as primary cultures and at equal ratios (each 1×10^6 cells/ml) with a common source of mitogen-stimulated normal donor PBMC; these PBMC were predetermined to be negative both for HTLV-I proviral DNA by PCR and for HTLV-I virus antigen production by cell culture. CSF-derived cultures were established by concentrating patient CSF cells (approximately 1×10^4 cells from 5 to 10 ml of patient CSF) by centrifugation followed by co-culture with normal donor PBMC (1×10^6) in a single well of a 24-well plate (2 cm^2) (Costar, Cambridge, MA). Patient cell cultures (both PBMC- and CSF-derived) were subsequently expanded and maintained in 75-cm^2 flasks in complete RPMI 1640 medium (with 10% IL-2) and tested for the presence of soluble HTLV-I antigen from cell culture supernatants (below) and for the presence of HTLV-I nucleotide sequences by PCR.

Animal inoculation procedures

Weanling (6- to 8-week-old) New Zealand white rabbits were obtained from a colony of the Centers For Disease Control and a commercial rabbitry (Western Oregon Rabbit Co., Philomath, OR). Rabbits were inoculated either intravenously (i.v.) (5.0×10^5 to 1.6×10^8 cells via lateral ear vein) or intracisternally (i.c.) (1×10^5 cells via foramen magnum) with lethally irradiated (5,500 to 10,000 rads) HTLV-I-infected or control cells (Table II). To compare the relative infectivity of HTLV-I cell lines, we equilibrated the inoculum between groups based on the amount of HTLV-I *gag* p19 protein in cell culture supernatants by antigen-capture assay at the time of inoculation; each rabbit was injected with 0.60–0.64 pg HTLV-I p19 per 10^7 cells inoculated (Table II). Comparison of viral proteins from cellular lysate preparations from our HTLV-I cell lines by IB correlated directly with the amount of released viral antigen (data not shown). Aliquots of all cellular inocula were maintained in culture to verify the lethality of the irradiation procedure (viable cell concentrations were determined by Trypan-blue exclusion).

Clinical and hematologic analysis

Complete hematologic analysis was performed by automated cell counting (Coulter, Hialeah, FL); values obtained included total erythrocyte, platelet and leukocyte counts. Wright-Giemsa-stained blood films were used to determine differential enumeration of leukocytes and erythrocyte morphology. Body weights were monitored and rabbits were regularly evaluated for any overt clinical signs of disease. Rabbits were killed for necropsy at post-inoculation (p.i.) intervals of 4, 12 or 24 weeks (1 to 2 rabbits per interval). Gross and histologic examinations of *post-mortem* tissues were assayed for any pathologic alterations induced by the inoculation of HTLV-I.

TABLE II – HTLV-I INOCULA: ORIGIN, ANTIGEN PRODUCTION, ROUTE, AND GROUP SIZES

HTLV-I-inoculated rabbits						
Inocula[1]	Disease origin	HTLV-I p19 Ag/cell (pg)	Number of cells inoculated per rabbit	Ag/cell inoculated ($\times 10^7$)	Route[3]	Number of rabbits
Pat. 1	HAM/TSP	nd	1.0×10^6	ND	i.c.	5
Pat. 1	HAM/TSP	0.16	4.0×10^7	0.64	i.v.	4
Pat. 2	HAM/TSP	0.06	1.0×10^8	0.60	i.v.	4
Pat. 3	ATL	0.04	1.6×10^8	0.64	i.v.	4
HuT 102	ATL	0.62	1.0×10^7	0.62	i.v.	4
MT-2	ATL	0.12	5.0×10^7	0.60	i.v.	4
Ra-1	ATL	ND	5.0×10^6	ND	i.c.	4

Control-inoculated rabbits			
Inocula[2]	Number of cells inoculated per rabbit	Route	Number of rabbits
Human PBMC	10^7	IV	2
Rabbit PBMC	10^7	IC	2
HuT 78	10^7	IC	2
HuT 78	10^7	IV	2

[1]HTLV-I cell line origin and inoculation procedure as described in Methods. Pat # = patient number.–[2]Controls: PBMC = mitogen stimulated cells of HTLV-I negative donor (human and rabbit). HuT 78 = HTLV-I negative, transformed human T cell line. [3]I.v., intravenous; i.c., intracisternal.

Serologic tests

IB was performed to detect the presence of anti-HTLV-I antibodies as described by Hartley *et al.* (1990). Briefly, HTLV-I antigen was obtained from a commercial source (Hillcrest, Cypress, CA). Antigen (10 μg/cm of gel width) was suspended in equal volumes of sample buffer, heated and electrophoresed on 10% polyacrylamide gels with 3% stacking gels. Proteins resolved by electrophoresis were electrophoretically transferred to nitrocellulose sheets, blocked, and cut into 3-mm strips. Individual strips were incubated overnight at room temperature with 1:100 dilutions of serum or 1:10 dilutions of CSF. Antibody banding patterns were detected by using biotinylated goat anti-rabbit immunoglobulin G followed by incubation with avidin-biotin-horseradish peroxidase conjugate (Vector, Burlingame, CA) and visualized with diaminobenzidine-nickel chloride-hydrogen peroxide (Sigma, St. Louis, MO). A serum or CSF sample was considered positive for HTLV-I antibody according to published US Public Health Service guidelines (reactivity to both HTLV-I gag p24 and env 46 [external envelope] or env 61/68 [envelope precursor] (Anderson *et al.*, 1988). Specific antigen bands were verified by demonstration of reactivity to murine monoclonal antibodies against HTLV-I p19, p24 and gp46 (Palker *et al.*, 1989).

Detection of HTLV-I in PBMC and tissue cultures

HTLV-I was detected by co-culture methods as described by Lairmore *et al.* (1990). Briefly, Ficoll/diatrizoate-separated rabbit PBMC or single-cell suspensions of lymphohematopoietic tissues were stimulated with mitogen (concanavalin A, 3 μg/ml, Sigma, or 1% PHA-P) and co-cultured with equal numbers of mitogen-stimulated human PBMC from an HTLV-I PCR-confirmed negative donor. Cultures were maintained in complete RPMI 1640 medium supplemented with 10% IL-2. Culture supernatants were monitored at approximately weekly intervals for the presence of HTLV antigens by antigen-capture assays (HTLV-I p19, Cellular Products, Buffalo; NY, Papsidero *et al.*, 1990; and HTLV-I/II p24, Coulter; Lairmore *et al.*, 1990). Resultant absorbance values of both tests were compared with known standard curves of viral core antigens performed in the same assay.

PCR assay

Genomic DNA extracted from rabbit PBMC or tissues was examined for the presence of HTLV-I nucleotide sequences by PCR (Ou *et al.*, 1988). Oligonucleotide primer pairs from the *gag* and *pol* genes of HTLV-I (De and Srinivasan, 1989) were used to amplify 1 μg of DNA (equivalent to approximately 150,000 cells) for each PCR amplification. The amplification consisted of 34 repetitive 3-step cycles, with the following conditions: 25°C to 95°C, and then 2-min periods of incubation at 95°C, 55°C, and 72°C per cycle in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The amplified products were separated in 1.8% agarose gels and probed by Southern hybridization, using *gag*- and *pol*-specific, ^{32}P-labelled oligonucleotide probes for HTLV-I (De and Srinivasan, 1989). Genomic DNA from MT-2 cells (HTLV-I-positive cell line) and either HuT 78 (uninfected T-cell line) cells or normal PBMCs (rabbit and human) were used as positive and negative controls, respectively. The sensitivity of our PCR procedure for HTLV-I (detection of one copy of HTLV-I DNA in 10^5 cells) was determined in each PCR trial by serial dilution of MT-2 cells with negative HuT 78 control cells.

RESULTS

Serologic and clinicopathologic responses of inoculated rabbits

Of the 37 rabbits used in the study, 29 were inoculated with HTLV-I-infected cells (16 received cells derived from ATL patients and 13 received cells derived from HAM/TSP patients), and 8 rabbits were used as controls (inoculated with HTLV-I-negative cells) (Table II). Sera were tested for immunoreactivity to HTLV-I by IB at 4, 8, 12 and 24 weeks p.i. HTLV-I-inoculated rabbits that developed antibody reactivity did so as early as 4 weeks p.i. and remained persistently seroreactive, frequently with increased intensity through the 24 weeks of the study. Table III shows the HTLV-I-inoculated groups arranged in decreasing order of seroreactivity; 2 of the 4 HTLV-1 inocula resulted in 75% or greater seropositivity among inoculated rabbits. HuT 102 strain (administered i.v.) and patient 1 strain (administered i.c.) were the most immunogenic according to this criterion (Table III). In contrast, only 2 of 4 rabbits inoculated with patient 2 strain were considered seropositive, while none of 4 MT-2-strain-inoculated rabbits were seropositive (Table III). None of 8 rabbits inoculated with HTLV-I-negative cell lines seroconverted to HTLV-I-specific viral proteins. Among seropositive rabbits, the intensity of IB bands measured at sequential intervals correlated with the inoculum; those inocula that elicited the greatest percentage of seropositive animals also produced the most intense virus-specific bands (Fig. 1). No clinical, hematologic or pathologic alterations were detected among the inoculated rabbits during the course of the 24-week study (data not shown).

TABLE III RANKED ANTI-HTLV-I ANTIBODY RESPONSES BY IMMUNOBLOT ASSAY[1]

Inocula	Route	HTLV-I Protein (number positive/number tested)				% Seropositive (p24 + gp46/68)
		p19	p24	p53	gp46 or 68	
Pat. 1	i.c.	5/5	5/5	5/5	5/5	100%
HuT 102	i.v.	3/4	4/4	3/4	4/4	100%
Pat. 3	i.v.	4/4	4/4	3/4	3/4	75%
Ra-1	i.c.	4/4	4/4	4/4	3/4	75%
Pat. 1[2]	i.v.	2/3	3/3	2/3	2/3	68%
Pat. 2	i.v.	1/4	3/4	1/4	2/4	50%
MT-2	i.v.	1/4	0/4	0/4	0/4	0
Controls	i.c./i.v.	8/0	0/8	0/8	0/8	0

[1]Immunoblot assay as described in "Methods"; US Public Health Service criteria for HTLV-I seropositivity (Anderson *et al.*, 1988).–[2]Only 3 rabbits of group 6 were available for immunoblot assay.

Detection of HTLV-I antigen in cell-culture supernatants

To determine the extent of virus replication among inoculated rabbits, PBMC and tissue suspension cultures were monitored for HTLV-I by antigen-capture assay. In a comparison of inoculated groups, the percentage of positive cultures was dependent on viral strain and correlated with those groups seropositive for HTLV-I (Fig. 2). Rabbits inoculated with patient 3 strain contained the broadest tissue distribution of virus (4 of 4 PBMC, 4 of 4 spleen, 3 of 4 mesenteric lymph node, and 2 of 4 bone marrow), whereas the MT-2-strain-inoculated group had the fewest positive cultures (only 1 of 4 PBMC, 0 of 4 spleen, 0 of 4 mesenteric lymph node and 1 of 4 bone marrow) (Fig. 2). When all groups were compared, the greatest number of virus-positive cultures were obtained from PBMC, followed by spleen, mesenteric lymph nodes and bone marrow (Fig. 2). Similar tissue cultures from control animals were negative when tested by antigen-capture assay.

To estimate and compare the quantity of viral proteins produced from the different groups, PBMC cultures of all inoculated groups (except Ra-1 strain rabbits) established at 4 weeks p.i. were tested for HTLV-I p19 by the antigen-capture assay. The PBMC cultures of rabbits inoculated with patient 3 strain contained the greatest amount of HTLV-I p19 antigen in culture supernatants, followed by groups inoculated with strains derived from patient No. 1, HuT 102 cultures, patient 2, and MT-2 cultures (Fig. 3).

Detection of HTLV proviral sequences by PCR

The extent and distribution of tissues containing HTLV-I DNA sequences were determined by the PCR technique among all HTLV-I-inoculated groups (except Ra-1) by using genomic DNA from PBMC at 4 weeks p.i, as well as other lympho-hematopoietic tissues at 4, 12 or 24 weeks p.i. Like our serologic and virus culture data, the PCR results suggested differences among experimental animals, depending on the HTLV-I strain inoculated. Rabbits inoculated with patient 1, patient 3, and HuT 102 strains contained the greatest number of PCR-positive samples, while groups inoculated with patient No. 2 and MT-2 strains had the least number of PCR-positive samples (Table IV, Fig. 4). In all groups, PBMC produced the greatest percentage of positive PCR tests compared with other tissues (Table IV). All control animals examined by PCR were negative (Table IV).

DISCUSSION

We have shown that the response of rabbits to HTLV-I infection is, in part, dependent on the viral strain used for inoculation. Our HTLV-I cell lines could be classified as highly, moderately, or poorly infectious in rabbits. The serologic responses to different HTLV-I strains, while being strain-dependent, were consistent with anti-HTLV-I antibody

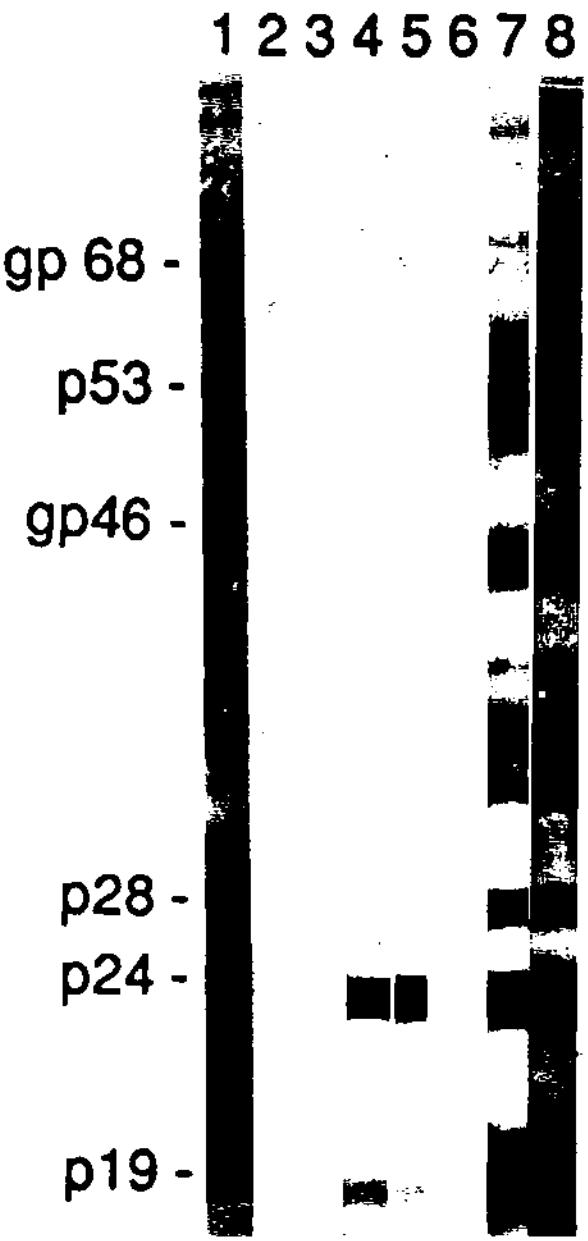

FIGURE 1 – HTLV-I immunoblot assay. Positive control serum from ATL patient (lane 1) and negative control from normal human donor (lane 2). Serum immunoreactivity of a rabbit inoculated with Ra-1 cell line (lanes 3, 4 and 5) compared to immunoreactivity of a rabbit inoculated with patient No. 1 strain. Lanes 3 and 6 are strips incubated with serum obtained prior to inoculation, lanes 3 and 7 with serum 4 weeks post inoculation and lanes 5 and 8 with serum 12 weeks post inoculation. Note more intense response of the rabbit inoculated with patient 1 strain.

responses of infected humans (Hartley *et al.*, 1990). Using US Public Health Service criteria for seropositivity to HTLV-I, we found that the HuT 102 strain was the most immunogenic inoculum, followed by patient 3, patient 1, and Ra-1 strains. Patient 2 strain was moderately immunogenic, while the MT-2 strain failed to elicit any significant antibody responses. The failure of MT-2 cells to infect or elicit antibody responses in our study is in contrast to published reports in which other MT-2 strains were highly infectious for rabbits (Miyoshi *et al.*, 1983). Our MT-2 cell line was originally obtained from Dr. I. Miyoshi (Kochi Medical School, Kochi, Japan) and maintains

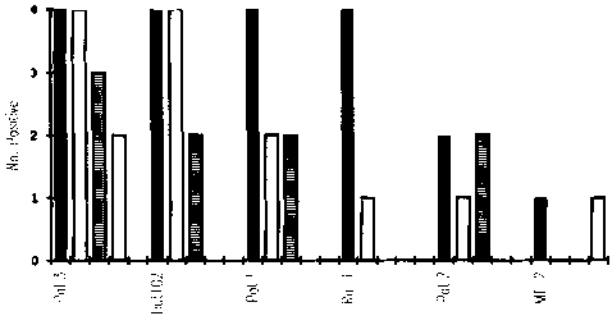

FIGURE 2 – HTLV-I p19 antigen capture assay of cell culture supernatants taken at 21 days of culture. Each rabbit group (n = 4) was necropsied and PBMC or tissue suspensions cultured as in "Methods". Columns show equal number of positive cultures for each tissue tested. Filled columns = PBMC; light gray (hatched) columns = spleen; striped columns = mesenteric lymph node; dark gray (hatched) columns = bone marrow. Note greater number of positive cultures and wider tissue distribution of HTLV-I p19 from tissues derived from rabbits inoculated with patient 1, patient 3 and HuT 102 strains.

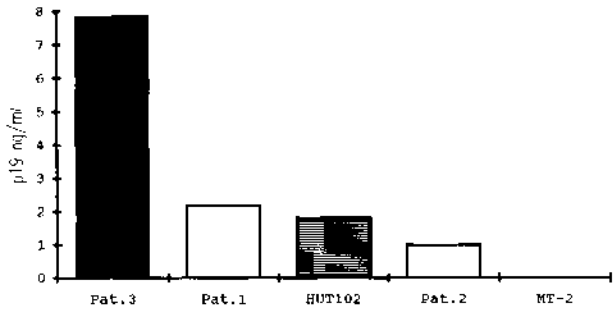

FIGURE 3 – HTLV-I p19 antigen capture assay from PBMC cultures obtained from rabbits 4 weeks p.i. (mean of 4 rabbits per group). Supernatants taken at 14 days of culture. Note the relatively greater quantity of viral antigen produced from PBMC cultures of rabbits inoculated with patient 3 strain.

an identical protein profile in immunoblots and a cell-surface phenotype similar to that of the original MT-2 cell line. Our MT-2 cells, while producing high quantities of viral gene products and mature budding virus particles (Cockerell *et al.*, 1990; Hartley *et al.*, 1990), may have acquired mutations during repeated passage in culture that reduced or eliminated their infectivity.

The serologic responses observed in our study were not due to the variable number of HTLV-I-infected cells inoculated. For example, rabbits inoculated with the HuT 102 strain received 5- to 20-fold less cells than groups inoculated with MT-2 or patient 3 strains, yet HuT 102-inoculated rabbits were more frequently seropositive. The direct correlation between the detection of HTLV-I by antigen-capture assay and PCR from infected rabbits and the strength of the anti-HTLV-I serologic response suggests that the antibody response to HTLV-I infection may depend on the ability of the infecting viral strain to replicate efficiently in the host. One implication of these results is that studies utilizing this animal model of HTLV-I infection to assess the humoral immune response to potential vaccines must use an HTLV-I challenge strain capable of eliciting a complete pattern of anti-HTLV-I antibodies after infection. By analogy, the pattern and quality of the anti-HTLV-I antibody response in an infected human may also be, in part, viral-strain-dependent; the identification of HTLV-II-infected individuals with "indeterminate" serologic patterns to HTLV-I antigens suggests that this occurs when HTLV-I

TABLE IV – RANKED HTLV-I PCR RESULTS FROM RABBIT PBMC[1]

Inocula	HTLV-I p24 gene PCR[2] Number positive/number tested	% positive
HuT 102	4/4	100%
Pat. 3	3/4	75%
Pat. 1	2/4	50%
Pat. 2	1/4	25%
MT-2	0/4	0

[1]PBMC obtained 4 weeks p.i. [2]PCR using p24 (gag 49/51 primer pair) as described (De and Srinivasan, 1989).

antibody testing is performed in certain at-risk human populations (Khabbaz *et al.*, 1991).

Results of virus detection by antigen-capture and PCR methods clearly indicated the relative replication efficiency of the HTLV-I strains in inoculated rabbits. The infectious nature of our viral strains did not correlate with the copy number of HTLV-I provirus inoculated; the highly infectious patient 3 inoculum contains only 1 proviral copy of HTLV-I per cell, while the MT-2 cell line contains at least 3 copies of HTLV-I provirus per cell (estimated by Southern blot assay; data not shown). We have reported that the nucleotide sequences of partial clones derived from strains of patients 1 and 3 are genetically distinguishable from both MT-2 and HuT 102 strains in the 5′ domain of the envelope gene (De *et al.*, 1991; Kaplan *et al.*, 1991). These data suggest that the replicative capacities of HTLV-I strains in the host are due to nucleotide sequence differences.

We infected our rabbits by using cell-associated HTLV-I strains to mimic the natural method of viral transmission in humans (Okochi *et al.*, 1984). Our viral inocula were equilibrated based upon viral antigen production on a per cell basis and we have determined that viral antigen in culture supernatants directly correlates with the quantity of intracellular viral antigens produced by our cellular inocula (data not shown). The ability of the inocula to infect rabbits did depend upon the IL-2-dependent state of the cell producing our strains (*e.g.*, HuT 102 IL-2-independent; patient 3 IL-2-dependent), lymphocyte subset of the inocula (*e.g.*, HuT 102 $CD4^+$; patient 3 $CD8^+$), or length of time each inoculum was maintained in culture (*e.g.*, HuT 102 high passage; patient 3 strain in culture less than 4 months).

Rabbits inoculated with the highly infectious HTLV-I strains contained the greatest tissue distribution of HTLV-I. Among all infected animals, virus detection was most successful when PBMC samples were tested. Among the lympho-hematopoietic tissues examined (other than PBMC), the spleen was the best source of HTLV-I for antigen production and proviral DNA; these results may be due, in part, to PBMC in this tissue. However, we found a low percentage of HTLV-I-inoculated rabbits with positive bone-marrow samples, a tissue that would be expected to contain a large quantity of blood. Our study was limited to the detection of viral antigen expressed from cultured tissues or amplification of proviral DNA sequences in infected cells. Further virus titration and mRNA expression studies from tissues are needed to clarify the *in vivo* state of HTLV-I replication in infected animals.

The rabbit model was used here to test the effects of infection by various HTLV-I strains in a uniform population of animals. The success of HTLV-I infection in these rabbits did not correlate with the source of our isolates; HTLV-I strains derived from ATL patients appeared to replicate as efficiently as those derived from HAM/TSP patients. These results may suggest that disease expression in humans, while dependent on strain-associated replication efficiency of HTLV-I, may also be influenced by co-factors (*e.g.*, host major histocompatibility haplotypes) (Miyai *et al.*, 1987; Osame *et al.*, 1989). The absence of disease in our HTLV-I-inoculated rabbits parallels

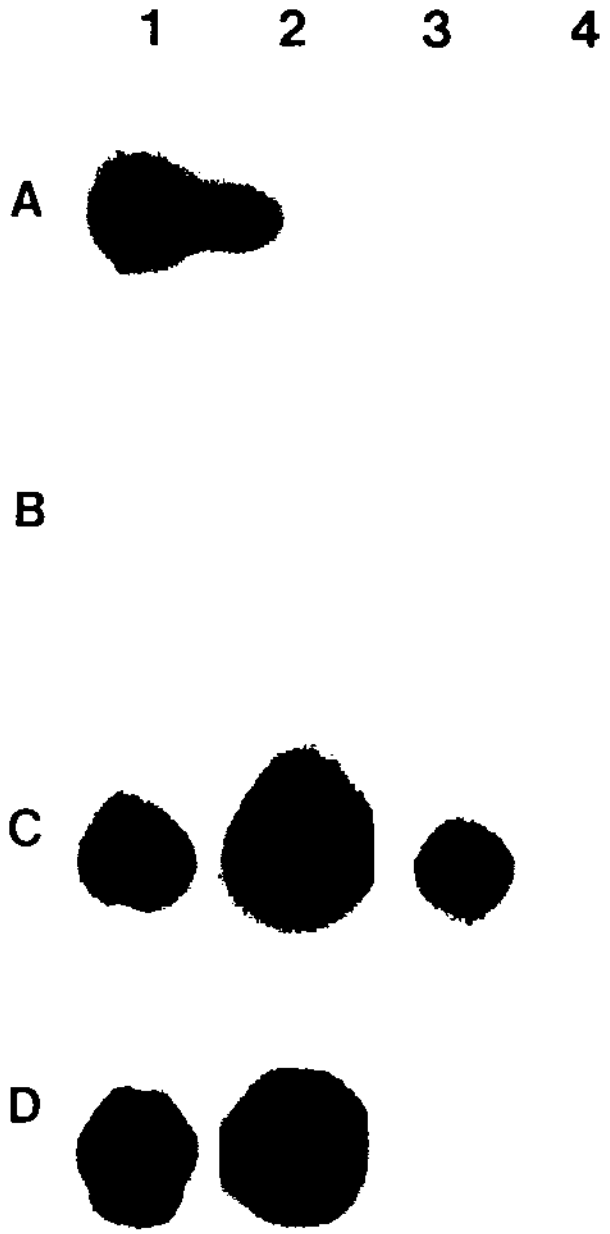

FIGURE 4 – HTLV-I polymerase chain reaction using gag p24 primers (De and Srinivasen, 1989) to amplify genomic DNA from PBMC taken from rabbits 4 weeks p.i. Panel A: MT-2-positive control (lane 1 diluted 10^4, lane 2 diluted 10^5) and negative controls (HuT 78 lane 3 and normal human PBMC lane 4). Panel B: PBMC from rabbits inoculated with negative control cells HuT78 (lanes 1 and 2) or normal PBMC of rabbits (lanes 3 and 4). Panel C: PBMC from rabbits inoculated with patient 3 strain (each rabbit separate lane 1–4). Panel D: PBMC from rabbits inoculated with patient 1 strain (each rabbit separate lane 1–4).

the human infection in which 96 to 99% of HTLV-I-infected persons remain asymptomatic (Blattner, 1989). The absence of disease among our inoculated rabbits may be due, in part, to the limited duration of our study (total of 24 weeks), or the absence of the necessary secondary co-factors that allow for the development of lymphoproliferative disease. However, even in the absence of disease, the course of HTLV-I infection, including viral tropisms and expression in tissues, can be investigated in this animal model.

Our results suggest there are differences among HTLV-I strains in their ability to replicate, infect different tissues, and elicit anti-viral antibody responses in an infected host. We have identified nucleotide differences among our viral strains in the 5′ domain of the envelope gene (De *et al.*, 1991; Kaplan *et al.*, 1991). Clarification of the pathogenesis of HTLV-I infection and disease may depend on the development of full-length infectious molecular clones of HTLV-I; to date, such clones have not been reported. Our identification of HTLV-I strains that replicate efficiently *in vivo* makes these viruses attractive starting material for the development of such reagents. Finally, the demonstration of the biological heterogeneity of HTLV-I infection in rabbits suggests that similar differences among HTLV-I strains in humans may determine transmission patterns and influence disease expression in persons infected with HTLV-I.

ACKNOWLEDGEMENTS

The authors thank Dr. G. Toedter, Coulter Immunology, for HTLV-I/II antigen capture assays. The research was supported, in part, by grants from the American Cancer Society (IRG-16-30) and the National Cancer Institute (RO1CA40714-04).

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Delineation of an Immunodominant and Human T-Cell Lymphotropic Virus (HTLV)-Specific Epitope Within the HTLV-I Transmembrane Glycoprotein

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Antibody reactivity to the transmembrane region of human T-cell lymphotropic virus type I (HTLV-I) envelope, gp21, is observed in virtually all individuals infected with HTLV-I or HTLV-II. Recombinant proteins encoding selected portions of gp21 are described and used to define two immunogenic regions. The first epitope (designated GD21-I) contains amino acids 361 to 404 of the HTLV-I envelope and reacted with all of 54 sera from HTLV-I– and HTLV-II–infected individuals. The second epitope (designated BA21) expresses amino acids 397 to 430 of the HTLV-I envelope and was recognized by 33 of 54 HTLV antisera. To determine the specificity of GD21-I and BA21, sera from 17 HTLV-negative individuals with nonspecific reactivity to p21E were tested. None of these sera reacted with GD21-I, but 16 of 17 sera reacted with BA21. With virtually complete reactivity to sera from HTLV-infected individuals and no reactivity to sera from p21E-reactive uninfected individuals, GD21-I will be useful in immunoassays for the detection of HTLV infection.

HUMAN T-CELL LYMPHOTROPIC virus type I (HTLV-I) and HTLV-II are human retroviruses that have the ability to transform human cells in vitro. Infection of target cells is mediated through interaction of the viral *env* glycoproteins with an as yet undefined cellular receptor. HTLV-I causes adult T-cell leukemia and HTLV-I–associated myelopathy (HAM)[1] and has been associated with several other human diseases, including polymyositis, arthropathy, and cutaneous disorders.[2-4] HTLV-II has been reported in patients with a rare T-cell variant of hairy cell leukemia[5] and has recently been associated with a neurologic disorder similar to HTLV-I–associated myelopathy,[6,7] although neither association is firm. Both HTLV-I and HTLV-II can be transmitted parenterally, by sexual contact with an infected partner, and from mother to child, via breast feeding.[1]

The *env* gene of HTLV is expressed as a single precursor, gp61, that is processed into two smaller proteins gp21 and gp46. The larger protein gp46 is expressed on the surface of both virions and infected cells and is associated with gp21, which spans the cellular/viral membrane. The *env* proteins contain multiple immunogenic epitopes to which HTLV-infected individuals develop an antibody response.[8-13] Recombinant proteins expressing the majority of HTLV-I gp21 are broadly immunoreactive with sera from HTLV-I– and HTLV-II–infected individuals.[9,14-16] One of these recombinant proteins, p21E, which expresses amino acids 306 to 439 of the HTLV *env* gene,[14] has been incorporated into several commercially available assays and has greatly facilitated the detection of *env* specific antibodies in HTLV-I/II–infected individuals.[17-19] However, several reports have indicated that antibodies to the p21E recombinant protein are also found in sera from some uninfected individuals.[18-20] These sera are negative for anti–HTLV-I antibodies by radioimmunoprecipitation assay (RIPA), and peripheral blood mononuclear cells (PBMCs) from these individuals are negative for the presence of HTLV-specific nucleic acid by polymerase chain reaction (PCR) analysis using HTLV-I– and HTLV-II–specific oligonucleotide primers and probes.[18,20] It is not known whether the immunogenic regions of HTLV-I gp21 recognized by infected and uninfected individuals are the same or can be differentiated.

To further define the immunodominant epitopes within HTLV-I gp21, we isolated anti-gp21 antibodies by Epstein-Barr virus (EBV) activation of B-cell lines derived from an HTLV-I–infected individual suffering from HAM. The highly specific anti-gp21 antibodies obtained were used to screen a series of recombinant proteins expressing selected portions of HTLV-I gp21. Recombinant proteins found to be immunoreactive with either the anti-gp21 oligoclonal antibodies and/or HTLV antisera were purified, and the immunoreactivity of the purified proteins was determined with a panel of sera from HTLV-I– and HTLV-II–infected individuals. Evidence for the presence of two highly immunoreactive epitopes within the sequences of the p21E recombinant protein is presented. The specificity of these two epitopes was then evaluated with a panel of sera derived from p21E-reactive but HTLV-negative individuals. One of the two epitopes, designated GD21-I, did not react with any of p21E reactive but HTLV-negative antisera and is thus highly HTLV specific.

MATERIALS AND METHODS

Antisera. The antisera used in these analyses included a well-characterized panel of sera from 26 HTLV-I– and 28 HTLV-II–infected individuals.[13,21] All of the HTLV-I and HTLV-II sera had antibody profiles meeting standard criteria for HTLV infection (antibodies to p24 *gag* and gp46 and/or gp61 *env* proteins). In addition, the sera were typed as being HTLV-I infected both by virtue of their positive reactivity towards the recombinant HTLV-I antigen MTA1 and by PCR using HTLV-I–specific oligonucleotide primers and probes[18,21] or as HTLV-II infected by their reactivity towards the recombinant HTLV-II antigen K55 and by PCR using HTLV-II–specific primers and probes.[18,21] HTLV-negative sera were derived from HTLV enzyme immunoassay (EIA)-negative blood donors to the Stanford University Blood Bank. The anti-sj26 sera used was

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Submitted January 9, 1995; accepted March 30, 1995.

Supported in part by Public Health Service Grants No. DA60596 and HL33811.

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0006-4971/95/8604-0031$3.00/0

produced by inoculating New Zealand White rabbits with approximately 500 μg of purified glutathione-S-transferase produced by bacteria expressing nonrecombinant pGEX-1 plasmid.[22]

This study also uses a panel of 17 sera that are reactive with the p21E protein from individuals with no other evidence for HTLV infection. The sera were identified in routine blood screening with HTLV-I EIA (Abbott Laboratories, Chicago, IL) and were found to have antibodies to the p21E recombinant protein (Cambridge Biotech, Rockville, MA, and/or Genelabs Diagnostics, Singapore Science Park, Singapore). Some of these sera also had *gag* protein reactivity. (The reactivity of these sera to viral *gag* antigens is presented in Table 2 below.) Reactivity of the p21E reactive sera to gp46 *env* protein was determined using a modified Western blot (HTLV Blot 2.3; Genelabs Diagnostics) that includes the recombinant proteins MTA-1 and K55 derived from the central portion of HTLV-I and HTLV-II gp46, respectively.[21] Additionally 10 of the 17 sera were tested for the presence of anti-*env* antibodies by RIPA as described.[18] Finally, 14 of the 17 sera were tested for the presence of HTLV nucleic acids by PCR using the primer pairs SK 110/111 and SK 43/44, which amplify both HTLV-I and HTLV-II nucleic acids, and the primer pairs SK54/55 and SK 58/59, which are specific for HTLV-I and HTLV-II nucleic acids, respectively.[23] PCR reaction and subsequent hybridizations with HTLV-I– and HTLV-II–specific probes were performed as described.[18,23] PCR analysis of the other three p21E reactive sera used was prohibited by the insufficient volume of the sample.

Production of gp21-specific antibodies. Peripheral B cells were isolated from an HTLV-I–infected individual who was suffering from HAM. T cells were removed by rosetting with sheep red blood cells and the resulting enriched preparation of B cells was seeded at 10^4 cells/well in 96-well microtiter plates (Corning Costar Corp, Cambridge, MA) and activated with EBV as described.[24,25] Cultures exhibiting specific anti–HTLV-I IgG activity were identified using an indirect immunofluorescence assay in which supernatants from actively dividing cultures were incubated with the HTLV-I infected cell line MT-2.[26] Two of the activated B-cell cultures secreted antibodies to gp21 by Western blot analysis (HTLVblot 2.3; Genelabs Diagnostics). Tissue culture supernants from these two cell lines, 5G4 and 6E9, were used in this study. Additional supernatants from these activated B cells were produced and used in subsequent studies.

Construction of recombinant clones. Oligonucleotide primers whose DNA sequences were based on the sequence reported for the ATK strain of HTLV-I[27] were designed and synthesized on an automated synthesizer (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. All of the primers contained either a *Bam*HI, *Nco* I, and/or *Eco*RI site located at their 5′ ends to facilitate cloning of the amplified DNA fragments as an in-frame insertion into a modified version of the vector pGEX-2[22] (obtained from Pharmacia, Piscataway, NJ) that contains an *Nco* I site just 5′ to the existing *Bam*HI and *Eco*RI sites. The locations of the 5′ and 3′ ends of the HTLV DNA sequences of the various HTLV-I gp21 recombinant antigens were selected on the basis of the hydrophilicity profiles of HTLV-I gp21 as determined by the program Antigen[28] within the software package PC-Gene (Intelligenetics, Mountain View, CA).

PCR was performed according to the manufacturer's instructions (Perkin-Elmer/Cetus, Norwalk, CT), and all PCR reactions contained 2 ng of the HTLV-I clone sp65 MT-2 (generously provided by Dr F. Wong-Staal, Department of Medicine, University of California, San Diego, CA) as template and 1.0 μmol/L of the appropriate oligonucleotide primers. PCR amplification was performed for 25 cycles of template denaturation (1 minute at 94°C), primer annealing (2 minutes at 50°C), and primer extension (2 minutes at 72°C). Amplified DNA fragments were purified, digested for 2 hours with the appropriate restriction enzymes, and then ligated into similarly digested modified pGEX-2. The recombinant plasmids were then used to transform *Escherichia coli* strain JM101 (Epicurean Coli; Stratagene, La Jolla, CA). Plasmid-containing bacteria were screened for protein production by Western blot analysis of crude lysates prepared from 2-mL cultures of the transformed *E coli* as described.[13] The resulting Western blots were then incubated overnight at room temperature with HTLV-infected or control antisera diluted 1/100 or with tissue culture supernatants from the cell lines 6E9 and 5G4 diluted 1/2 in BLOTTO (10 mmol/L Tris-HCl, pH 7.4, 5% nonfat dry milk, 2.5% normal goat sera, and 0.5% Tween-20). The Western blots were washed, and bound human IgG was detected as described previously.[13,21]

Purification of recombinant proteins. Purification of recombinant fusion protein was performed essentially as described.[13,22] Briefly, a 10-mL overnight culture of bacteria containing the recombinant plasmid of interest was diluted 1/100 into flasks containing 500 mL of NZYDT media[29] with 100 μg/mL ampicillin. Expression of fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 0.2 mmol/L) to log-phase cultures. The cultures were grown for an additional 3 to 4 hours at 37°C, at which point the bacteria were pelleted by centrifugation at 5,000*g* for 10 minutes. The cells were resuspended in 20 mL of cold phosphate-buffered saline (PBS) and were lysed by several cycles of freezing and thawing. After lysis, proteins were solubilized by the addition of Triton X-100 (Sigma, St Louis, MO) to 1.0%, DNAse I to 1 μg/mL, and aprotinin to 1.0%. After incubation for 5 minutes at 25°C, insoluble cellular debris was pelleted by centrifugation 2 times at 10,000*g* for 10 minutes, and the supernatants were reserved. Aliquots from both the pellet and supernatant fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[30] to determine if the recombinant proteins were solubilized by the above procedure.

Although the majority of GD21-I was insoluble, sufficient quantities for further studies of both the GD21-I and BA21 recombinant proteins were present in the soluble fraction. The supernatants were then passed through a column containing 0.8 mL of glutathione agarose (Pharmacia, Piscataway, NJ) that was pretreated as recommended by the manufacturer. The column was washed with 10 mL of MTBS (150 mmol/L NaCl, 16 mmol/L Na_2HPO_4, 4 mmol/L NaH_2PO_4; pH 7.3)[22] plus 1% Triton and 1% Aprotinin, followed by a 5-mL wash with MTBS alone. Bound proteins were eluted with buffer containing 5 mmol/L glutathione in 50 mmol/L Tris, pH 8.0, and 10 1-mL fractions were collected. The location of the peak of eluted protein was determined by measuring the absorbance at 280 nm of the fractions and by SDS-PAGE analysis of aliquots of the fractions. For both of the recombinant proteins a 1 L culture resulted in the purification of 1 to 2 mg of fusion protein at a purity of approximately 70%. Fractions containing significant amounts of protein were pooled, and aliquots of this pool were frozen at −70°C for subsequent analysis.

Serologic analysis of purified recombinant peptides. Aliquots of the purified GD21-I and BA21 proteins were separated under reducing conditions on a 11.5% polyacrylamide gel. The resolved proteins were electroblotted onto a nitrocellulose membrane, blocked with BLOTTO, air-dried, and cut into 2-mm wide strips. The strips were rehydrated in TTBS buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.5, 0.2% Tween-20) and incubated overnight with sera diluted 1:50 in BLOTTO. The strips were washed three times with TTBS and incubated for 1 hour with goat antihuman IgG conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA). After washing four times with TBS, bound antibody was detected by incubating the strips in a substrate solution containing NBT and BCIP in 100 mmol/L Tris-HCl buffer, pH 9.5, 50 mmol/L $MgCl_2$. Color development was continued until a uniform background developed on the strip and was halted by rinsing the strips two times with deionized water.

RESULTS

Peripheral B cells from an HTLV-infected individual with HAM were isolated and activated by infection with EBV. Two cell lines, 5G4 and 6E9, were isolated that reacted specifically with Western blotted p21E recombinant protein (Fig 1). No immunoreactivity to any other HTLV protein was detected. Although the two cell lines were originally derived from the EBV activation of 10^7 B cells, they produced antibodies that were highly specific for reactivity to the p21E region of HTLV-I gp21.

The p21E-specific antibodies produced by the 6E9 and 5G4 cell lines were then used to determine the immunogenic sequence(s) within gp21. Eight oligonucleotide primers (Table 1) were synthesized and used to amplify seven DNA

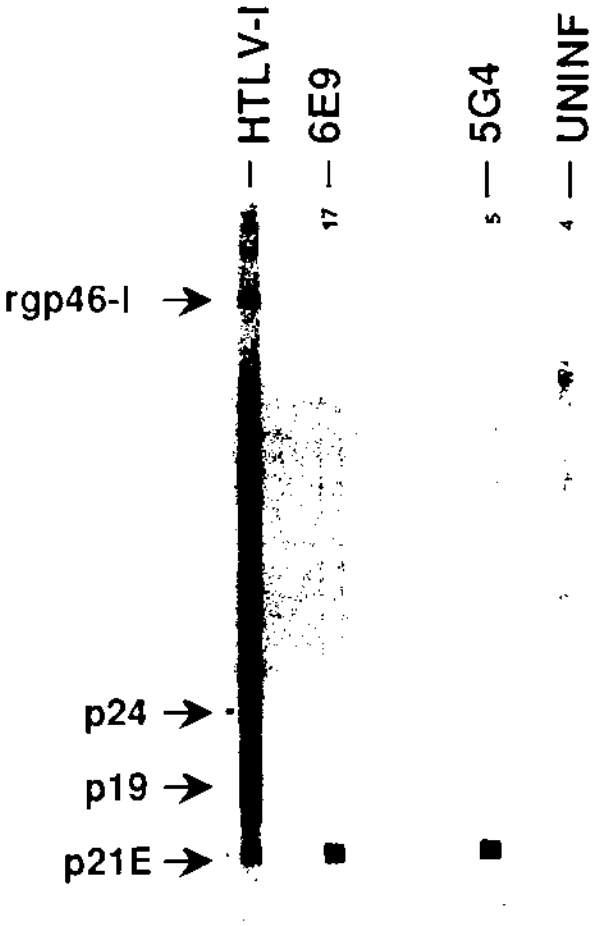


Fig 1. Reactivity of tissue culture supernatants from the 5G4 and 6E9 cell lines. Western blots containing HTLV-I viral lysate and the recombinant HTLV-I *env* antigens p21E and MTA-1[12] were incubated with the indicated sera or tissue culture supernatants. HTLV-I, sera from an HTLV-I infected individual diluted 1/100; 6E9 and 5G4, tissue culture supernatants diluted 1/2 obtained from two cell lines derived from the EBV activation of peripheral B cells from an HTLV-I–infected individual; UNINF, sera from a HTLV-negative individual diluted 1/100. The migration of the HTLV-I *gag* proteins p19 and p24 as well as the migration of the p21E and MTA-1 (denoted as rgp46-I) recombinant *env* antigens are indicated.

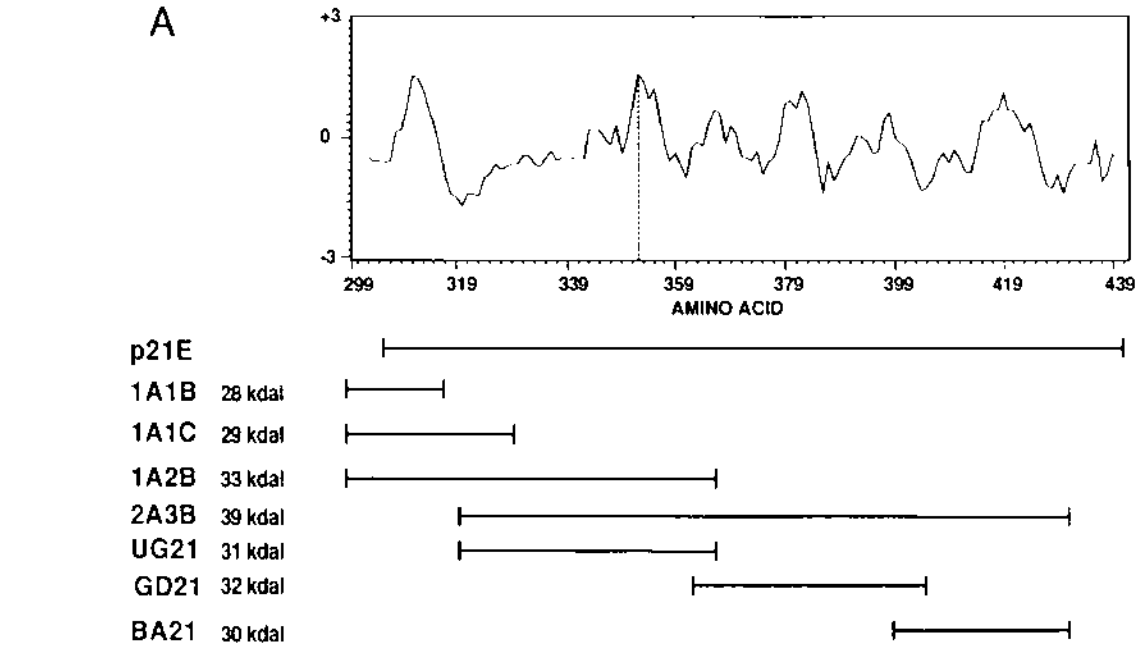
Table 1. PCR Primers Used in the Synthesis of the gp21 Recombinant Proteins

Forward primers		
	6076	
1A	5′ TCC GAA TTC TCC ATG GGT TCC TTG TCA CCT GTT CCC ACC	
	6135	
2A	5′ TCC GAA TTC GGA TCC TGG CTT GTC TCC GCC CTG GCC	
	6261	
MR1	5′ GC GAA TTC GGA TCC ATA GTC AAA AAC CAC AAA AAT C	
	6369	
3A	5′ TCC GAA TTC ACT AGT GGA TCC CAA GAA CAG TGC CGT TTT CCG	
Reverse primers		
	6126	
1B	5′ ACC ACT AGT ACC ACC ACC ACC GAA TTC CAC CGG TAC CGC TCG GCG GGA	
	6164	
1C	5′ ACC ACT AGT ACC ACC ACC ACC GAA TTC GGC TGC CAT GGC CAG CGC GGA	
	6275	
2B	5′ TGG GAA TTC GTG GTT TTT GAC TAT TGC TTG	
	6391	
MR1	5′ CGC GAA TTC GGA AAA CGG CAC TGT TC	
	6466	
3B	5′ TGG GAA TTC GTT AAG GCC CCA GCC AGT CAG	

Primers used in the construction of the recombinant proteins 2A3B, 1A1B, 1A2B, and 1A1C are indicated by their names (eg, recombinant protein 2A3B was synthesized using forward primer 2A and reverse primer 3B). The primers used for the construction of UG21, GD21-I, and BA21 are 2A and 2B. MR1 and MR1 and 3A and 3B, respectively. The dot in the sequence of the primer indicates the beginning of HTLV-I homologous sequences, and the number above the primer indicates the first nucleotide of HTLV-I homologous sequences. The restriction sites used in the cloning of the PCR products are underlined.

fragments that expressed selected portions of HTLV-I gp21 (Fig 2A). The start and stopping points of the various recombinant clones were designed to express the four major peaks of hydrophilicity present in the p21E region of gp21 either alone or in combination (Fig 2A). All of the inserts were successfully expressed as recombinant fusion proteins and four of the recombinant clones 2A3B, UG21, GD21-I, and BA21 were also DNA sequenced by the dideoxy termination procedure.[13] The sequence of the DNA inserts obtained confirmed that the four recombinant proteins were expressing the desired regions of HTLV-I gp21.

The immunogenicity of supernatants from the 5G4 and 6E9 cell lines was assessed by Western blot analysis of whole cell lysates of bacteria expressing six of the gp21 recombinant proteins (Fig 2). The expression of all six of the recombinant proteins tested was verified by Coomassie Blue protein staining (data not shown). None of the four constructs expressing all or part of amino acids 299 to 365 of the HTLV *env* proteins reacted with tissue culture supernatants from either of the anti-p21E activated B cells tested (Fig 2B). In addition, neither of the two p21E antibodies reacted with the recombinant protein BA21, which expressed amino acids 397 to 430 of the HTLV-I *env* protein. However, tissue culture supernatants from both the 5G4 and 6E9 cell lines reacted strongly with the 2A3B recombinant protein, which expresses amino acids 319 to 430 of the HTLV *env* gene. Both the full-length protein, which has a molecular weight of 39 kD, and several breakdown products derived from proteolytic cleavage of 2A3B were immunoreactive.

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                    10        20        30        40
GD21-I    IVKNHKNLLK IAQYAAQNRR GLDLLFWEQG GLCKALQEQC RFPN

BA21      QEQCRFPNIT NSHVSILQER PPLENRVLTG WGLN
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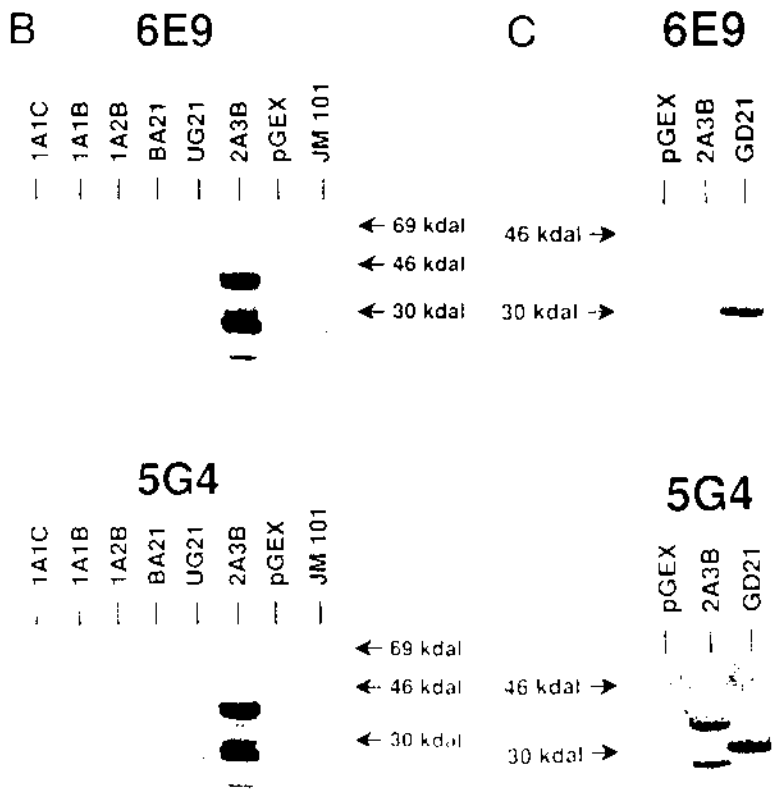

Fig 2. (A) Hydrophilicty profile of the p21E region of HTLV-I *env* gene calculated by the method of Hopp and Woods[28] indicating the sequences of gp21 expressed by the recombinant proteins used in this study. The calculated sizes of the recombinant fusion proteins, including both the insert and the glutathione-S-transferase portion, are given after their names. The sequence used in this analysis is from the ATK strain of HTLV-I,[27] and the amino acids are numbered from the initial methionine of the HTLV-I *env* precursor protein. The dotted line indicates the location of the highest peak of hydrophilicity in this region, and the scale of the y axis is in arbitrary units, with regions of higher charge density being indicated by increasingly positive numbers. The amino acid sequences of the inserts of the recombinant proteins GD21-I and BA21 are also given. (B) Western blot analysis of 1/2 diluted tissue culture supernatant from the 6E9 or 5G4 cell lines against whole cell lysates derived from bacteria expressing the indicated recombinant proteins. Bacterial lysates were prepared and Western blots were performed as described in the Materials and Methods. The lane marked JM101 contains whole cell lysate derived from untransformed *E coli* and the lane marked pGEX contains whole cell lysate derived from *E coli* transformed with nonrecombinant modified pGEX-2. The migration of molecular weight markers is indicated at left. (C) Western blot analysis of 1/2 diluted tissue culture supernatants from the 6E9 or 5G4 cell lines against whole cell lysates of bacteria expressing the 2A3B and GD21-I recombinant proteins. Lanes are marked as for (B).

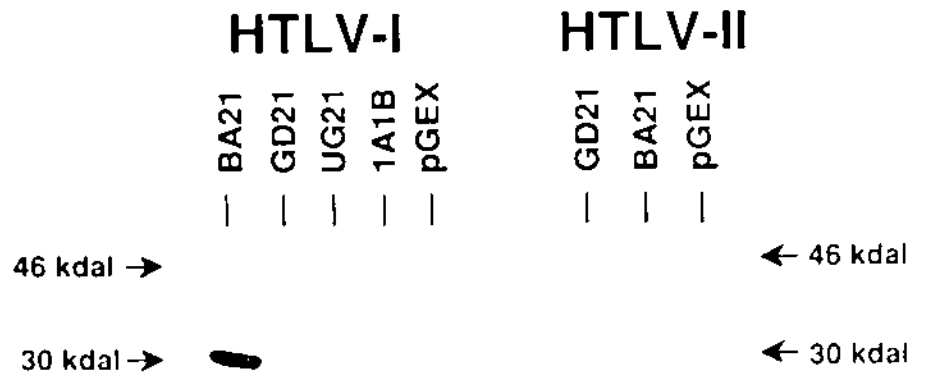

Fig 3 Western blot analysis of whole cell lysates derived from bacteria expressing the indicated recombinant proteins against sera from HTLV-infected individuals. The blots labeled HTLV-I, HTLV-II or UNINF were incubated with sera from individuals infected with the indicated virus or an uninfected individual. All sera were diluted 1/100 in BLOTTO. The lane marked JM101 contains whole cell lysate derived from untransformed *E coli* and the lane marked pGEX contains whole cell lysate derived from *E coli* transformed with nonrecombinant modified pGEX-2. The migration of molecular weight markers is indicated at left.

These data suggested that an immunodominant epitope was located between amino acids 365 and 397 of the HTLV I *env* gene in the sequences of the 2A3B recombinant protein that were not shared with the UG21 or BA21 recombinant proteins. Therefore, a recombinant protein, GD21-I, that expressed amino acids 361 to 403 of the HTLV-I *env* gene was constructed and the immunoreactivity of GD21-I with tissue culture supernatants from the 6H9 and 5G4 cell lines was determined (Fig 2C). As expected, both anti-p21E antibodies reacted with the smaller GD21-I recombinant protein.

To confirm that the results obtained with the anti-p21E antibodies would also be true for polyclonal sera, the reactivity of the recombinant proteins against sera from 5 HTLV-I and 5 HTLV-II infected individuals was also determined. Whole cell lysates from bacteria expressing several of the recombinant proteins were fractionated by SDS-PAGE and Western blotted. Representative Western blots obtained are presented in Fig 3. All HTLV-I and HTLV-II infected sera tested reacted with the recombinant protein GD21-I. Serum from 1 HTLV-I and 1 HTLV-II infected individual tested also reacted with the BA21 recombinant protein. This finding suggested that a second epitope might exist within p21E. Additionally, serum from 1 of 5 of the HTLV infected individuals also reacted with recombinant protein 2A3B and any of the other recombinant proteins expressing amino acids 209 to 365 of the HTLV *env* protein (data not shown). No immunoreactivity to the recombinant proteins was observed when sera from an uninfected individual was tested.

To determine the overall reactivity of GD21-I and BA21 to HTLV infected sera, the recombinant proteins were purified via glutathione agarose affinity chromatography and Western blots were prepared as described. The resulting blots were then cut into 2 mm strips and incubated with panels of sera from HTLV-III infected individuals, p24E reactive but HTLV negative individuals, and HTLV-I negative blood donors. Representative Western blots obtained are presented in Fig 4. The immunoreactive band(s) in several sera migrating below GD21-I was not reactive with either the 6H9 and 5G4 supernatants or antisera directed against the sp26 portion of the GD21-I recombinant protein and represents a contaminant of the purification. The sera had reactivity to full length GD21-I observed in three of the control

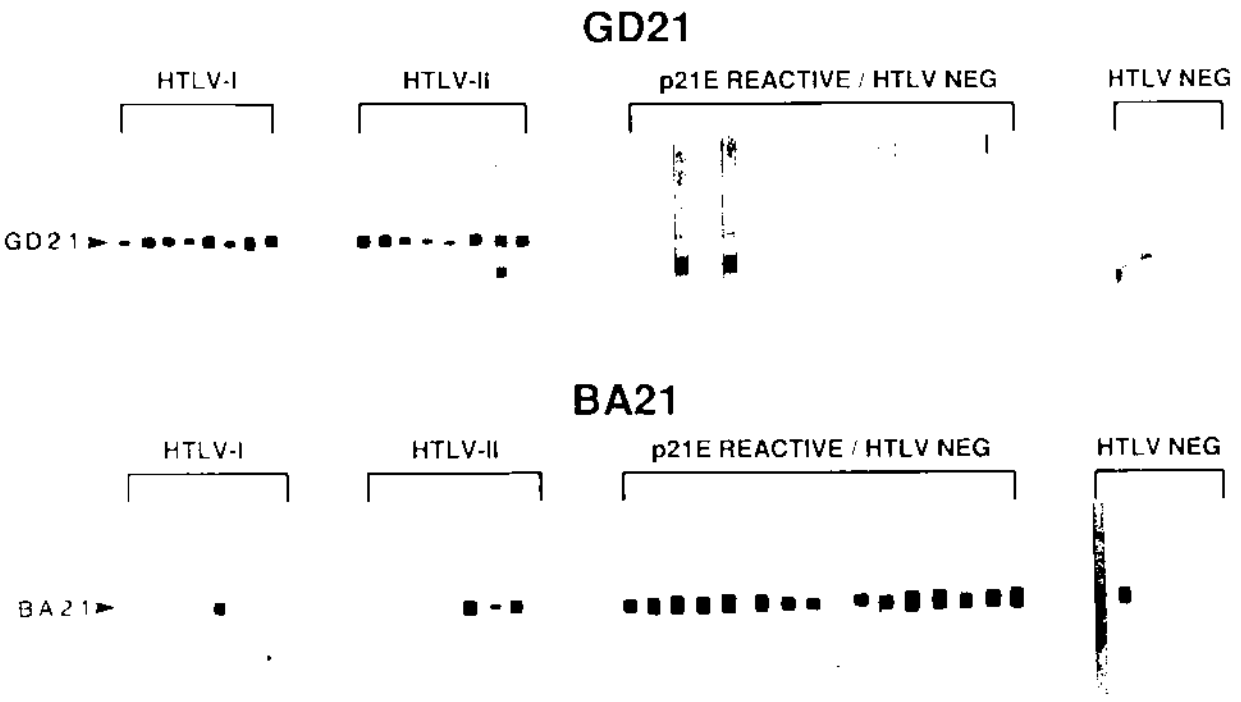

Fig 4. Western blot analysis of purified GD21-I and BA21 recombinant protein against a representative panel of sera diluted 1/100 in BLOTTO. The 8 strips marked HTLV-I were incubated with sera from HTLV-I–infected individuals; the 8 strips marked HTLV-II were incubated with sera from HTLV-II–infected individuals; the 16 strips marked p21E reactive/HTLV negative were incubated with sera from p21E-reactive/HTLV-negative individuals; and the 6 strips labeled HTLV NEG were incubated with sea from uninfected individuals. The migration of full-length GD21-I and BA21 recombinant proteins is indicated at left. The 30-kD molecular weight marker comigrated with the full-length GD21-I and BA21 proteins. GD21-I and BA21 Western blot strips were prepared and sera were incubated as described in the Materials and Methods.

sera (Fig 4) was not reproducible in two additional experiments, and the sera were determined to be GD21-I negative.

The results from the entire panel of sera are presented in Table 2. We tested sera from 54 individuals who were positive for infection with HTLV-I or HTLV-II by both serologic criteria and whose sera were positive when tested by PCR with HTLV-specific primers and probes. Overall, sera from 26 of 26 HTLV-I–infected individuals and 28 of 28 HTLV-II–infected individuals reacted strongly with the recombinant protein GD21-I. The BA21 protein was reactive with 14 of 26 HTLV-I–infected and 19 of 28 HTLV-II–infected individuals, although in some cases the reactivity observed was relatively weak (Fig 4).

To assess the specificity of the epitopes, the GD21-I and BA21 recombinant proteins were tested against a panel of 24 HTLV-negative sera. This panel included sera from 17 HTLV indeterminate individuals, including 8 that were reactive with p21E and 9 that reacted with p21E and an HTLV *gag* protein (Table 2). PCR amplification of 14 of the 17 HTLV indeterminate sera with HTLV-specific primers and probes was negative. Of these sera, only the 3 that are reactive to both p21E and p24 might be classified as HTLV infected on the basis of p24 plus *env* reactivity; however, these sera were neither reactive with the HTLV-specific gp46 proteins MTA1 and K55 nor positive for HTLV infection when tested by PCR. None of the 24 control sera, including the 17 p21E-reactive HTLV-negative sera, reacted with GD21-I recombinant protein. In contrast, a total of 17 sera, including 16 of the p21E-reactive HTLV indeterminate sera reacted with the BA21 protein (Table 2). Thus, the GD21-I recombinant protein expresses an epitope that is both highly sensitive and highly specific for sera from individuals who can be shown to be HTLV infected by both serologic and PCR-based criteria.

DISCUSSION

Immunogenic epitopes within the surface glycoprotein gp46 and the transmembrane glycoprotein gp21 have been shown to elicit strong antibody responses from HTLV-I– and HTLV-II–infected individuals.[2-7] Several reports have confirmed that recombinant proteins that express the majority of the sequence of HTLV-I gp21 are broadly reactive with sera from HTLV-I– and HTLV-II–infected individuals.[3,4,6] In addition, work by Manns et al[3] has indicated that antibodies to the gp21, detected using the recombinant protein p21E,[3] are generally among the first to appear during seroconversion to HTLV-I. The broad reactivity of gp21 recombinant proteins coupled with the difficulty of establishing reactivity to native Western blotted *env* proteins[3,5,7] has led to the incorporation of the recombinant protein p21E into HTLV screening and confirmatory assays and has reduced the need to perform more cumbersome confirmatory tests, such as RIPA.[3,7,8] However, it has also been established that some individuals who are not HTLV-I or HTLV-II infected

Table 2. Reactivity of Purified GD21-I and BA21 Recombinant Proteins With HTLV-I, HTLV-II, and P21E Indeterminate Sera

	N	p21E Recombinant Proteins		HI/HII PCR-*
		GD21-I	BA21	
HTLV-positive sera seroreactivity				
p21E, p24, and MTA-1 (HTLV-I infected)	26	26	14	26
p21E, p24, and K55 (HTLV-II infected)	28	28	19	28
Totals (HTLV infected)	54	54	33	54
HTLV indeterminate sera seroreactivity†				
p21E only	8	0	8	0/5
p21E and p19	6	0	5	0/6
p21E and p24	2	0	2	0/2
p21E, p24, and p19	1	0	1	0/1
None	7	0	1	0/7
Totals	24	0	17	0/21

* PCR analysis of HTLV-infected and indeterminate sera was performed as described in the Materials and Methods. Unless otherwise indicated, the total number of samples tested is indicated in the column labeled N.

† None of the HTLV indeterminate sera were reactive with the HTLV-I– and HTLV-II–specific gp46 recombinant proteins MTA-1 or K55. Additionally, 10 of the p21E-reactive sera, including all that were coreactive with p24, were negative for evidence for HTLV infection when tested by RIPA.

also possess an antibody response to the p21E protein.[18,19] In one study, antibodies to p21E were observed in 0.6% of uninfected blood donors.[19] Subsequent RIPA testing of these p21E-reactive sera confirmed that they do not react with native gp61 *env* protein.[19] PCR testing of HTLV indeterminate p21E-reactive individuals with HTLV-specific primers and probes has also indicated that these individuals are HTLV negative.[18,20] The cross-reactivity of the p21E recombinant protein with sera from uninfected individuals limits its utility in the diagnosis of HTLV infection.[19]

It is therefore important to further define the immunogenic regions recognized by HTLV-I– and HTLV-II–infected sera within the 134 amino acid sequence of the p21E protein. To accomplish this, we used highly specific preparations of anti-p21E antibodies from EBV-activated B cells of an HTLV-I–infected individual and determined the reactivity of these antibodies towards a series of recombinant proteins that expressed selected portions of gp21. This approach is very analogous to that used previously to isolate the HTLV-I–specific epitope contained within the gp46 recombinant protein MTA-1.[10,21] Antibodies produced by two anti-p21E EBV-activated B-cell lines were specifically reactive with a recombinant protein, GD21-I, that expressed amino acids 361 to 404 of the HTLV-I *env* gene. Analysis of a panel of 54 sera from HTLV-I– and HTLV-II–infected individuals indicated that 100% of the sera reacted with the GD21-I protein. A second highly immunoreactive domain, recognized by approximately 60% of HTLV antisera tested, was contained within the recombinant protein BA21 that expressed amino acids 397 to 430 of the HTLV-I *env* gene. Only minor amounts of reactivity to recombinant proteins expressing amino acids 299 to 365 of the HTLV *env* gene were observed after limited testing. Thus, we show that two immunogenic regions are contained within the sequences of p21E.

Our results contrast with those of several previous studies that failed to delineate an immunodominant epitope within HTLV-I gp21.[8,11,12] These studies all used synthetic peptides encoding sequences derived from selected regions of HTLV-I gp21. In a very thorough analysis by Horal et al,[11] the entire coding sequence of the HTLV-I *env* gene was synthesized as a series of overlapping synthetic peptides of approximately 22 amino acids in length. The results obtained indicated that there were multiple immunogenic epitopes present within the sequences of p21E; however, none of the peptides was universally reactive with HTLV-I– and HTLV-II–infected sera. We believe that the use of human monoclonal antibodies permits a more definitive analysis of epitopes.[10,13] In this effort the oligoclonal human antibody preparations possessed highly specific antibodies to gp21, which allowed for the isolation of GD21-I and permitted a more definitive characterization of the immondominant epitope of HTLV gp21.

This study is also the first to delineate the location of the epitope(s) recognized by individuals who are reactive with HTLV-I p21E but are negative for HTLV nucleic acids by PCR. Along with reacting with approximately 60% of truly HTLV-infected individuals, the BA21 recombinant protein reacted with 16 of 17 sera from HTLV indeterminate p21E-reactive individuals. None of these HTLV indeterminate p21E-reactive sera recognized GD21-I. The localization of the cross-reactive epitope within p21E to a 34 amino acid sequence should facilitate research directed at identifying what causes immunoreactivity to HTLV-I gp21 in uninfected individuals.

Thus, the immunodominant epitope recognized by truly HTLV-infected individuals and the epitope recognized by p21E-reactive but HTLV PCR-negative individuals are distinct. The lack of immunoreactivity towards this sequence among HTLV indeterminate individuals indicates that the GD21-I protein holds great promise as being the basis of improved HTLV confirmatory and screening assays possessing an increased specificity over currently used tests. More specific serologic assays for HTLV infection would greatly benefit accurate testing and counseling of volunteer blood donors as well as epidemiologic studies of HTLV-I and HTLV-II infection.

ACKNOWLEDGMENT

The authors thank Martin Ruda and James J. Lipka for performing some of the PCR analyses of HTLV-infected sera. Additionally, the authors are indebted to Cameron Hoover for DNA sequencing of the GD21-I and other recombinant proteins. Special thanks are appropriate as well to James J Lipka for his part in the collection and organization of the sera used in these studies.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 8, Number 4, 1992
Mary Ann Liebert, Inc., Publishers

Development of a Monoclonal Antibody-Based p24 Capsid Antigen Detection Assay for HTLV-I, HTLV-II, and STLV-I Infection

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ABSTRACT

A monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay (ELISA) was developed and employed to detect p24 capsid antigen from human T-cell lymphotropic viruses type I and II (HTLV-I, HTLV-II), simian T-cell lymphotropic virus type I (STLV-I) -infected cell lines, and from mononuclear cell cocultures of HTLV-infected humans and STLV-I infected monkeys. A monoclonal antibody specific for HTLV p24 and p53 capsid antigens was coated onto 96-well microtiter plates to capture HTLV/STLV antigen. Captured antigen was then detected by the addition of a polyclonal, biotinylated human anti-HTLV-I antibody, and color developed with tetramethyl benzidine/H_2O_2 substrate. As little as 15 pg/ml of HTLV-I p24 antigen could be detected in this assay. Culture supernatants from HTLV-I-infected cell lines (HUT-102, MT-2, C5/MJ), HTLV-II-infected cell lines (Mo-T, Mo-B, PanG 12.1, NRA) and STLV-I-infected cell lines (Matsu, NEPC M39) were all positive in the assay. In addition, p24 was detected from peripheral blood mononuclear cell (PBMC) cocultures of 8 of 8 (100%) HTLV-I diseased patients, 14 of 20 (70%) HTLV-I and HTLV-II-infected, asymptomatic persons, and 8 of 8 (100%) STLV-I-infected, asymptomatic monkeys. Culture supernatants of cells infected with human immunodeficiency virus type (HIV-1), simian immunodeficiency virus (SIV), *Chlamydia trachomatis*, cytomegalovirus (CMV), herpes simplex I and II (HSV), feline leukemia virus (FELV), bovine leukemia virus (BLV), and bovine immunodeficiency virus (BIV) were all negative. Similarly, normal human peripheral blood mononuclear cells and uninfected, transformed human T cells, were also negative in the assay. The antigen assay is a sensitive and specific method for the detection of HTLV-I, HTLV-II, and STLV-I p24 capsid antigen and can replace reverse transcriptase assays in the confirmation of tissue cultures for these retroviruses.

INTRODUCTION

INFECTION BY HUMAN T-LYMPHOTROPIC VIRUSES (HTLV) has recently emerged as an important public health problem. Human T-lymphotropic virus type I (HTLV-I) is considered the etiologic agent for two primary disease syndromes, adult T-cell leukemia/lymphoma (ATL) and a chronic, degenerative myelopathy, HTLV-I-associated myelopathy, also referred to as tropical spastic paraparesis (HAM/TSP).[1] In addition, HTLV-I infection has tentatively been associated with certain forms of polymyositis and polyarthritis.[2,3] While these later reports require further epidemiologic and laboratory confirmation,

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these studies suggest an even broader spectrum of HTLV-I-associated disease. In contrast, HTLV type II (HTLV-II) has not been definitively associated with a particular disease syndrome. However, HTLV-II infection is common among intravenous (IV) drug users.[4] Among normal blood donors, a high percentage of those seropositive for HTLV have been demonstrated to have HTLV-II infection.[5] Interestingly, certain American Indian populations have been reported to have HTLV-II infection with no apparent incidence of HTLV-associated disease.[6] A closely related virus, simian T-cell leukemia virus-type I (STLV-I)[7] is suspected of being associated with a lymphoproliferative disorder in nonhuman primates[8] and STLV-I infection among nonhuman primates provides a valuable animal model for examining the pathogenesis of HTLV-I associated disease.[9]

HTLV I, II, and STLV have similar genome organization and similar protein profiles. Indeed, none of the serologic assays can distinguish between these virus types. These observations led us to believe that the antibodies to common epitopes should be able to detect all three viruses. We describe here the production and development of a monoclonal antibody to p24 and p53 capsid antigen which reacts with a common epitope of HTLV-I, II, and STLV-I. The monoclonal antibody was used to develop a sensitive and efficient antigen capture assay. The capture assay was capable of detecting capsid antigen in culture supernatants from a majority of infected individuals, and all HTLV-I, II-, and STLV-I infected cell lines. The ability to monitor cultures for the presence of HTLV p24/p53 antigen will be useful in studies concerning the transmission, epidemology, and treatment of diseases associated with HTLV.

MATERIAL AND METHODS

Cell lines and viral antigen production

Cells were grown in RPMI-1640 (Flow Laboratories) containing 10 to 15% bovine calf serum (Hyclone, Logan, UT). The cell lines were: HUT-102 (HTLV-I),[10] MT-2 (HTLV-I),[11] C5/MJ (HTLV-I),[12] PanG 12.1 (HTLV-II),[6] NRA (HTLV-II),[13] Matsu (Isolated by J. Blakeslee, from STLV-I$^+$ Japanese macaque), NEPC M39 (STLV-I, gift of Dr. Muthiah Daniels, New England Primate Center), HUT-78,[14] MOLT-4,[15] 8E5/LAV,[16] $SIV_{mac}251$/HUT-78,[17] MoT (HTLV-II),[18] MoB (HTLV-II),[19] FELV (Gardner strain, provided by Dr. Pradip Roy-burman, University of Southern California), fetal lamb kidney cells (FLK) infected with bovine leukemia virus (BLV),[20] and primary bovine embryonic lung cells infected with bovine immunodeficiency-like virus (BIV).[21] All cell lines were used to produce cell-free culture media, which were obtained by centrifugation at 500 × g. Cytomegalovirus and herpes simplex 1 and 2 were obtained from the American Type Culture Collection (Rockville, MD).

Viral antigens for mouse immunizations were obtained from HUT-102 supernatant by differential centrifugation at 50,000 × g for 3 hours. Antigen which was used to quantitate the capture assay sensitivity was isolated by affinity purification of HUT-102 culture media. Culture media was lysed with 0.1% Triton X-100, then passed over an affinity column of murine monoclonal antibodies specific for HTLV capsid proteins linked to Sepharose-4B (Pharmacia). The monoclonal antibody used for the affinity purification (GE 157) was developed by the authors. After washing to baseline with 0.1 M phosphate-buffered saline (PBS), the bound antigen was eluted with 3 M sodium thiocyanate. The eluted antigen was dialysed against PBS, and the total protein quantitated by Lowry assay.[22] Purified proteins were diluted in lysis buffer (0.1% SDS/0.5% Triton X-100 in PBS) containing dithiothrietol, and heated at 100°C for 5 min. The proteins were separated on a 12.5% SDS-PAGE slab gel,[23] then electrophoretically transferred onto nitrocellulose membranes.[24] The nitrocellulose was stained after transfer for protein content with 0.1% amido black. Immunoreactive proteins were detected with polyclonal human anti-HTLV-I antibody. Bound antibody was detected with ^{125}I-goat anti-human immunoglobulins (New England Nuclear, Boston, MA) and visualized by autoradiography. When stained for either protein content or immunoreactivity, the affinity purified protein showed two bands, at 24,000 and 53,000 daltons.

Affinity purified polyclonal anti-HTLV antibody

Human sera positive for antibodies to HTLV-I were obtained from TSP patients undergoing treatment (provided by Dr. William Sherematta, University of Miami, Miami, FL). Sera were screened by Western blot for reactivity against HTLV-I p24. The Western blots used HUT-102 cells as a source of antigen, which were disrupted in lysis buffer (0.1% SDS/0.5% Triton X-100 in PBS), reduced in buffer containing dithiothreitol at 100°C for 5 min, and the proteins separated on a 12.5% SDS-PAGE slab gel and electrophoresed as above. The nitrocellulose strips were incubated with the positive human sera samples. Bound antibodies were detected with ^{125}I-goat anti-human immunoglobulins (New England Nuclear, Boston, MA) and visualized by autoradiography. Those sera found to be positive were further screened by ELISA against detergent disrupted HTLV-I coated onto microtiter plates. The sera which gave the highest titer were purified by protein A-Sepharose (Pharmacia LKB-Piscataway, NJ), and conjugated to biotin (Pierce Chemical Co., Rockford, IL).

Monoclonal antibody development

Female BALB/c mice were immunized with 20 μg of detergent disrupted (0.1% sodium dodecyl sulfate) HUT-102 viral antigens. The antigen was suspended in Freund's complete adjuvant and injected interperitoneally. After three subsequent immunizations of 20 μg of disrupted antigen in phosphate-buffered saline (PBS; 100 mM phosphate/0.5 M NaCl; pH 7.1), splenocytes from one mouse were fused with SP2/0-Ag14 myeloma cells, using polyethylene glycol 1500 to effect fusion.[25] After selection with hypoxanthine, aminopterin, and thymidine, culture supernatants were screened by ELISA against disrupted HTLV-I antigen coated onto 96-well polystyrene plates by absorbtion in bicarbonate buffer (0.1 M, pH 9.6). Positive cultures were cloned in agarose and then further screened by Western blot assay. The monoclonal antibodies reacting with the p24 capsid antigen were further expanded.

Characterization of monoclonal antibodies

HUT-102 (HTLV-I), MoB (HTLV-II), MoT (HTLV-II), and Matsu (STLV-I) cells were disrupted in lysis buffer, reduced in

sample buffer containing dithiothrietol at 100°C for 5 min, and the proteins electrophoresed and Western blotted as above. The blots were then used to test the ELISA$^+$ culture supernatants.

Development of p24 capture assay

Monoclonal antibodies specific for HTLV capsid antigens were purified by protein A chromatography. The purified antibodies were coated at a concentration of 2 μg/ml onto 96-well polystyrene microtiter plates (Nunc, Denmark) by adsorbtion in 0.1 M Tris-HCl, pH 8.0. Nonspecific binding was prevented by blocking with 5% bovine serum albumin in 0.1 M Tris-HCl. Culture supernatants from cell cultures, 200 μl and 20 μl of 0.2% Tween-20/0.5% Triton X-100 were added, and the plate was incubated for 2 hours at 37°C. This was followed by three washes in 0.1% Tween-20 in PBS. Biotinylated human anti-HTLV-I antibodies, 200 μl at 1.0 μg/ml were then added to the wells. After incubation at 37°C for 1 hour, the wells were washed three times, streptavidin-peroxidase (0.01 μg/ml) was added, and incubated for 30 min at room temperature. After again washing three times, a 0.04 M tetramethylbenzidine/ 0.03% H_2O_2 substrate solution was added to develop color. The reaction was stopped with 50 μl of 1 M H_2SO_4, and the optical density read at 450 nm.

Viral cultures from seropositive individuals

Peripheral blood mononuclear cell (PBMC) cultures were produced from heparinized blood obtained from HTLV seropositive individuals or STLV-I seropositive monkeys. Cells were separated by Ficoll/Diatrizoate (LSM solution, Bionetics Laboratory Product, Charleston, SC) and stimulated with 1% phytohemagglutinin (PHA-P, Difco, Detroit, MI) and cultured at 2 × 10^6/ml in RPMI-1640 containing 15% fetal bovine serum and 10% interleukin 2 (Advanced Biotechnologies, Inc., Silver Springs, MD). After 72 h, 1 × 10^6 cells/ml were cocultured with an equal number of PHA-P-stimulated PBMC from HTLV seronegative donors. The culture supernatants were collected on day 14 postcoculture. Some of the culture supernatants were tested for reverse transcriptase activity for comparison purposes. Western blots for determining the seroreactivity of nonhuman primates to SIV were performed as previously described.[26]

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed as described,[6] using total genomic DNA isolated from PBMC.

RESULTS

Characterization of monoclonal antibodies

The production of hybridomas from the splenocytes of mice immunized with purified HTLV-I virus yielded a number of HTLV-I reactive monoclonal antibodies, as determined by ELISA reactivity on purified virus. The purpose of the monoclonal antibody production was to obtain clones which detect all strains of HTLV, as capsid proteins are more conserved than the envelope proteins of HTLV-I and HTLV-II. Western blot analysis was performed to identify those monoclonal antibodies

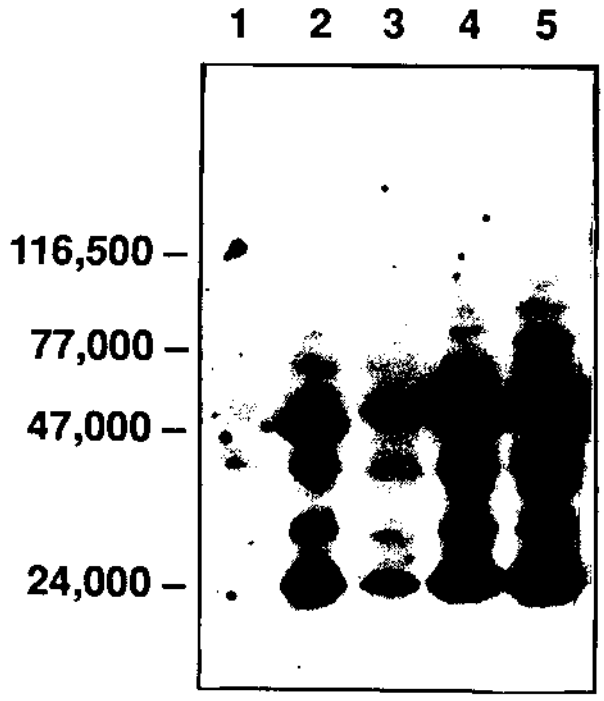

FIG. 1. Western blot of KC88 monoclonal antibody. Detergent lysed tissue culture cells were used as the source of antigen. Lanes 1 and 2: HUT-102(HTLV-I); Lane 3: MoB (HTLV-II); Lane 4: Matsu (STLV-I); Lane 5: MoT (HTLV-II). Lanes 2-5, strips stained with KC88; Lane 1 stained with nonrelevant mouse immunoglobulin. Molecular weights (× 10^3) indicated on the left margin.

reacting only with the capsid proteins. One of these antibodies, KC88, reacted strongly with the p24 and p53 capsid antigens of HTLV-I, HTLV-II and STLV-I by Western blot analysis (Fig. 1). Fainter bands at 33 and 42 kD were also evident.

Development of antigen capture assay

The KC88 antibody, when purified and coated onto 96-well microtiter plates, formed an effective capture phase for a monoclonal antibody-based antigen capture ELISA. Detection of the captured antigen was effected by utilizing purified, biotinylated human anti-HTLV-I antibodies. The human antibodies were shown by Western blot to react with the capsid proteins of HTLV-I, along with other viral components.

The sensitivity of the assay was established by the purification and quantitation of HTLV-I capsid antigen from HUT-102 culture supernatants by affinity chromatography. The quantitation of the assay was based upon the protein concentration of the purified antigen. Serial dilution of the quantitated antigen demonstrated that the assay had a sensitivity of 15 pg/ml (Fig. 2).

The interassay reproducibility of the antigen assay was examined by repeatedly assaying a stored HTLV-I$^+$ sample. Culture media conditioned by HUT-102 cells was aliquoted and refrigerated at 2–8°C. Samples were retrieved, serially diluted, and assayed over a 10-day period (10 separate plates, n = 47 for each dilution). The concentration of antigen in each dilution was

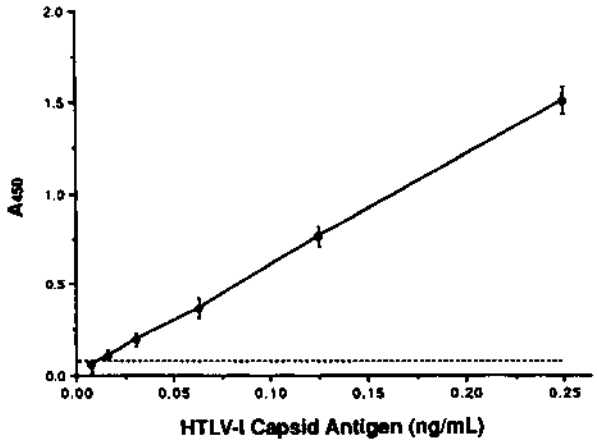

FIG. 2. Sensitivity of the HTLV-I, HTLV-II antigen capture assay. Affinity-purified HTLV-I core antigen was quantitated, serially diluted in RPMI-1640, 10% FBS, and assayed in triplicate. The cutoff (---) was three standard deviations above the negative control (RPMI-1640, 10% FBS without p24 antigen).

determined by calculation from a standard curve, run in each assay. The results are presented in Table 2. The low standard deviation and coefficient of variation indicates that the assay is capable of reliably measuring antigen from sample to sample.

TABLE 1. SPECIFICITY OF THE HTLV-I, II ANTIGEN CAPTURE ASSAY

Specimen	*ELISA reactivity*[a]
HTLV-I cell lines[b]	
HUT-102	>2.0
MT-2	>2.0
C5/MJ	>2.0
HTLV-II cell lines	
MoB	
MoT	>2.0
PanG 12.1	>2.0
NRA	>2.0
STLV-I cell lines	
Matsu	>2.0
NEPC M39	>2.0
Other retrovirus producing cell lines	
8E5 (HIV-I)	<0.05
HUT-78/SIV mac-251	<0.05
FELV	<0.05
BLV	<0.05
BIV	<0.05
Nonretrovirus	
HUT-78	<0.05
MOLT 4	<0.05
CMV	<0.05
HSV I, II	<0.05

[a]Optical density (A 450/570).

[b]Cell-free culture medium was obtained by centrifugation at 500 × g, then assayed in the capture ELISA. Cultures were assayed a minimum of two times.

TABLE 2. INTERASSAY VARIABILITY OF ANTIGEN CAPTURE

Dilution	*Mean ng/ml*[a]	*Standard deviation*	*Coefficient of variation*
1:10	0.126	0.008	6.50%
1:20	0.061	0.004	7.19%
1:40	0.030	0.003	8.87%

[a]Concentration of serially diluted HUT-102 medium was determined by reference to a standard curve.

n = 47 determinations over 10 assays.

Specificity of antigen capture for HTLV and STLV

The monoclonal antibody was employed as the capture phase to give specificity to the assay. The specificity of the capture assay was evaluated using supernatants of cell cultures infected with a variety of HTLV-related and unrelated viruses, and uninfected cells. The results (Table 1) showed that established cell lines infected with HTLV-I, HTLV-II, and STLV-I demonstrated significant levels of p24/p53 antigen in culture supernatants, while none of the uninfected cells or cells infected with other viruses had measurable levels of p24/p53 antigen.

The application of the antigen capture assay to identify HTLV-I and HTLV-II infection was examined with PBMC cocultures from seropositive and seronegative persons. PBMC cocultures from 8 of 8 (100%) persons with HTLV-I-diseases (ATL or HAM/TSP) were culture positive in the assay; seronegative control PBMC cultures were negative when tested in parallel cultures (Table 3). In addition, the assay successfully detected p24/p53 antigen in 8 of 12 (67%) HTLV-II-infected asymptomatic persons. Overall, 22/28 (79%) of cocultures from HTLV-I, II-infected individuals were positive. As is typical for HTLV cultures, supernatants of HTLV-infected cultures contained low reverse transcriptase activity and were difficult to distinguish from background control activity (data not shown).

To extend the utilization of the antigen assay, similar PBMC cocultures from STLV-infected nonhuman primates were tested for the presence of the p24/p53 antigen. Eight of eight (100%) infected monkey PBMC cultures were positively identified in the antigen assay, while 10 of 10 noninfected monkeys were negative (Table 3). The same nonhuman primates were also tested for SIV seropositivity by Western blot assay (data not shown). The only PBMC cultures positive for STLV-I antigen were those derived from primates positive for STLV-I antibody. In primates positive for both STLV-I and SIV antibodies, the HTLV-I antigen capture assay was able to detect the STLV-I antigen. Since the assay failed to detect bovine leukemia virus and other retrovirus positive cultures (Table 1), these data suggest that the antigen capture assay detected only the STLV-I antigen present in these PBMC cultures.

DISCUSSION

Using a monoclonal antibody (KC88) directed against a conserved epitope of p24/p53 capsid antigen, we have developed a highly sensitive and specific enzyme immunoassay for HTLV-I, HTLV-II, and STLV-I. The assay is capable of detecting capsid antigens, not only from chronically infected

TABLE 3. DETECTION OF p24 ANTIGEN IN COCULTURES FROM HUMAN OR MONKEY PBMC-INFECTED WITH HTLV-I, HTLV-II, OR STLV-I

	Polymerase chain reaction		*Antigen assay*	
Group	*Result*	*Total positive/ total tested*	*Total positive/ total tested*	*% Positive*
HTLV-I				
ATL & HAM/TSP	HTLV-I+	8/8	8/8	100
Asymptomatic	HTLV-I+	8/8	6/8	75
HTLV-II				
Asymptomatic	HTLV-II+	12/12	8/12	67
		Total:	22/28	79
STLV-I[a]				
Asymptomatic	STLV-I+	8/8[c]	8/8	100
Uninfected[b]				
Human PBMC	HTLV-I−	0/3	0/3	0
Monkey PBMC	STLV-I−	0/10	0/10	0

[a]Determined by Western blot on HTLV-I.
[b]Seronegative and PCR negative.
[c]Determined with HTLV-I-specific primer pairs, *pol* and *tax*.

T-cell lines, but also from culture supernatants derived from PBMC infected with these viruses. The sensitivity of the assay described here makes the assay a reasonable substitute for reverse transcriptase assays. Furthermore, the antigen capture assay does not require radionucleotides for the detection of virus and eliminates the need for radioactive waste disposal.

The KC88 antibody recognizes an epitope present on the capsid proteins of HTLV-I, HTLV-II, and STLV-I. The epitope thus apparently represents a conserved region in the p24/p53 antigen of these three viral types. With the emergence of HTLV-II as a prevalent virus in blood donors,[5] the ability of the antigen assay to detect both HTLV-I and HTLV-II allows a more complete examination of PBMC cultures from HTLV-infected populations. A previous study has described an HTLV-I antigen assay based on a monoclonal antibody to p19, which is specific for HTLV-I only and does not detect HTLV-II.[27] Type-specific peptide sequences have been mapped to the p19 capsid antigen.[28]

A monoclonal antibody to a common epitope of the p24 antigen has allowed the detection not only of HTLV-I, but also closely related HTLV-II and STLV-I. The antigen assay detected 100% of those with HTLV-I associated disease, while among asymptomatic persons, 75% of those with HTLV-I and 67% of those with HTLV-II were antigen positive. The reduced detection of antigen in the asymptomatic HTLV-I and HTLV-II seropositive cultures is likely due to the low number of virus-positive cells in these individuals. Alternatively, PBMC from these persons may carry replication-defective HTLV or are infected with a closely related, but distinct HTLV. However, these PBMC cultures were confirmed positive for the virus infection using HTLV-I- or HTLV-II-specific primers in the PCR assay. The success rate of HTLV detection from PBMC cultures in our study was comparable to previously published reports of other forms of antigen detection from seropositive, but asymptomatic persons' lymphocyte cultures.[14,10] However, the antigen-capture assay format offers the advantage of convenient quantitation of p24 capsid antigen by comparing values to those of standardized controls. Also, the microtiter format allows easy testing of multiple samples from cell culture supernatants to confirm the presence of HTLV-I, HTLV-II, or STLV-I. This feature is particularly useful when attempting to establish replication kinetics for these viruses.

Animal models have proven useful in providing information concerning the pathogenesis of retrovirus infections. STLV-I infection is a common infection among many primate species.[26] Naturally or experimentally STLV-I-infected macaques have been utilized to demonstrate the transmissibility of the virus infection[14] and have provided a relevant animal model in the development of HTLV vaccines.[9] Here we demonstrate the use of our antigen assay to successfully detect STLV-I infection in monkey PBMC cultures. These results are not unexpected, considering the 95% degree of nucleotide sequence homology between HTLV-I and STLV-I.[29]

The antigen detection system described here will be useful in laboratory investigations into the pathogenesis and treatment of HTLV-I-associated disease. The total viral load of HTLV-I-infected PBMC may reveal useful information about the replication competence of the virus and disease development. The ability to detect antigen from HTLV-II-infected PBMC can also be useful to study the epidemiology of HTLV-II infection, and to determine if the virus is associated with a disease syndrome.

ACKNOWLEDGMENTS

This research was supported, in part, by a grant from the American Cancer Society No. IRG-16-30, and The National Cancer Institute No. CA-40714.

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The EMBO Journal vol.4 no.6 pp.1455–1460, 1985

Isolation and characterization of a human T cell leukemia virus type II from a hemophilia-A patient with pancytopenia

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Communicated by H. Diggelmann

Human T cell leukemia virus (HTLV) type I has been isolated from the cultured T cells of several patients with adult T cell leukemia (ATL) and has been etiologically linked to ATL. However, HTLV-type II has been isolated only once, from the T cells of a patient with a T cell variant of hairy-cell leukemia. We report here the isolation of HTLV-II-related virus from the cultured T cells of a hemophilia-A patient with pancytopenia. The T cell line (CM) grows in the absence of T cell growth factor. Cord blood T cells were rapidly transformed when co-cultivated with irradiated CM cells. Heterologous competition radioimmunoassays using purified HTLV-I p24 showed the expression of HTLV-II_{MO}-related protein in these cells. Electron microscopy of the CM cells showed the presence of intracellular and extracellular type C viral particles. Comparison of the proviral genome in the CM cell line and the prototype HTLV-II_{MO}-containing cell line (MO) by molecular hybridization with probes specific for HTLV-II_{MO} indicated that restriction cleavage sites were identical. The fresh peripheral blood leukocytes of the patient contained two complete copies of the proviral genome, despite the lack of HTLV-II p24 expression. The virus from the cell line CM is designated as HTLV-II_{CM} to distinguish it from the original HTLV-II_{MO} isolate.

Key words: HTLV-II/hemophilia-A/p24 competition RIA/provirus in fresh cells

Introduction

Two different types of human T cell leukemia viruses (HTLV) have been reported. The first subtype, HTLV-I, was isolated from the T cells of several patients with leukemias and lymphomas of mature T cells (Poiesz *et al.*, 1980; Popovic *et al.*, 1983; Yoshida *et al.*, 1982). Seroepidemiologic surveys of patients with adult T cell leukemia in Japan and in the Caribbean suggest a causative relationship between HTLV-I and adult T cell leukemia (ATL) (Kalyanaraman *et al.*, 1982a; Gallo *et al.*, 1983; Hinuma *et al.*, 1981; Blattner *et al.*, 1982). The virus has also been shown to be exogenous to humans (Gallo *et al.*, 1982). More recently another subtype of this T-lymphotropic virus, HTLV-II, has been shown to be present in the cultured T cells of a patient with a T cell variant of hairy-cell leukemia (Kalyanaraman *et al.*, 1982b). The virus has been shown to be distinct from HTLV-I by immunologic and nucleic acid analyses (Kalyanaraman *et al.*, 1982b; Chen *et al.*, 1983a; Gelman *et al.*, 1984). However, unlike HTLV-I, HTLV-II has not been isolated from other patients with leukemia or lymphoma. During studies on HTLV-I in patients with hemophilia, we detected an HTLV-II-like virus in a T-cell line established from a patient with hemophilia and pancytopenia. The cell line (CM) releases HTLV-II in high titers. This report describes the biological and biochemical characterization of the cell line.

Results

Establishment and characterization of the cell line CM

The CM cell line was established from the T cells of a 39-year-old white man with hemophilia-A and pancytopenia who had been a recipient of large amounts of Factor VIII concentrate. His serum showed low antibody titer to HTLV-I p24 (titer $1 \geq 50$). Mononuclear cells were separated from the patient's (CM) blood and stimulated with phytohemogglutinin (PHA) in RPMI medium containing 20% fetal calf serum. After 3 days the cells were placed in medium containing lectin-free T cell growth factor. After 2–3 weeks in culture, the cells continued to grow in the absence of T cell growth factor (TCGF). Table I summarizes the phenotypic properties of the TCGF-independent CM cells. A high proportion of the cells reacted strongly with OKT-4 antibody, characteristic of the helper-inducer subset of T lymphocytes. The cells were negative for the suppressor-cytotoxic T lymphocyte phenotype OKT-8 and for surface immunoglobulins, and a high proportion (95%) expressed HLA-DR antigen and TCGF receptors. The properties of the CM cells described above indicate that it is a transformed human T-cell line capable of TCGF-independent proliferation, a property characteristic of several HTLV-I-transformed T cells (Salahuddin *et al.*, 1983; Popovic *et al.*, 1983).

Electron microscopy of CM and the transformed cord blood T cell lines shows type C retroviruses budding from plasma membranes, as well as mature extracellular virus particles (Figure 1A and B). The virus is morphologically similar to HTLV-I, but the nucleoid of the virus is sometimes seen in intimate contact with the inner layers of the viral membrane.

Table I. Characteristics of HTLV-II-producing CM cells and transformed cord blood T-cells

Characteristic	CM cells	Transformed cord blood T cells
HTLV-II p24 expression[a]	+	+
TCGF requirement	None	None
Virus particles by electron microscopy	+	+
HLA-DR (%)	95	84
Surface IgG (%)	2.5	1
OKT-3 (%)	9	87
OKT-4 (%)	92	71
OKT-8 (%)	2	41
OKT-10 (%)	25	73
TCGF receptors (TAC) (%)	93	96
Karyotype (sex chromosome)	XY	XX

[a]HTLV p24 expression was tested in a heterologous radioimmunoassay with ^{125}I-labeled HTLV-I p24 and a limiting dilution of antibody to HTLV-II_{MO}.

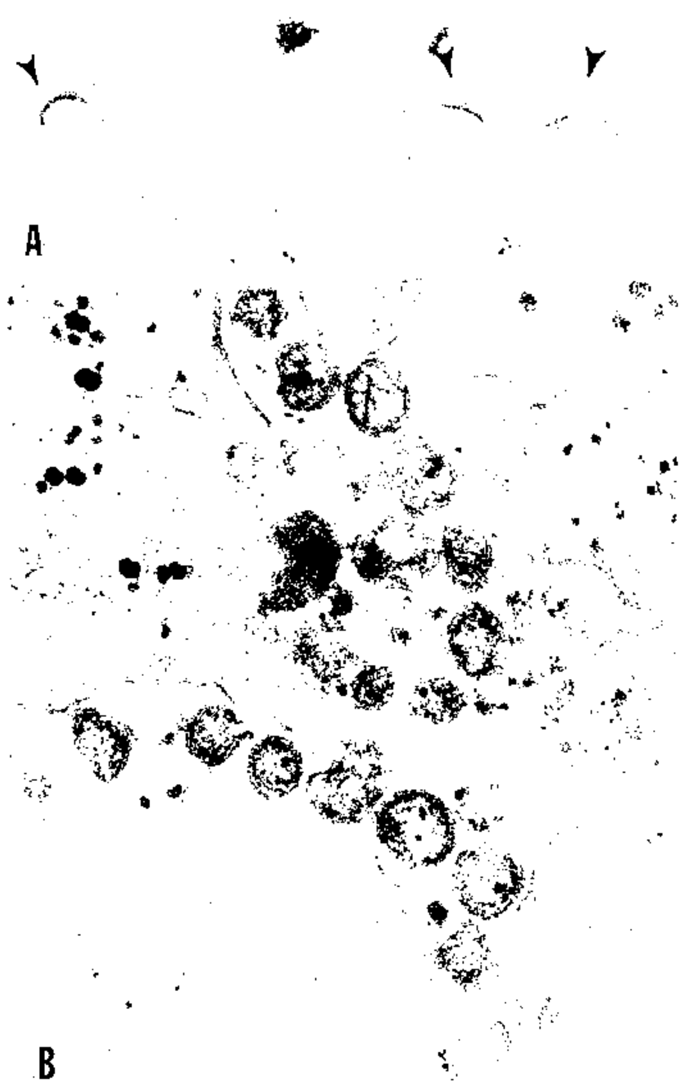

Fig. 1. Electron microscopy of HTLV-II-infected CM cells. **(A)** Immature type C particle budding from plasma membrane of CM cells by thin section electron microscopy (x 82 150). **(B)** Mature type C particles in extracellular space.

Characterization of viral proteins from the CM cell line

The CM cell line was further analyzed for the expression of HTLV proteins. Solubilized proteins of the CM cell line were analyzed by competitive radioimmunoassays for the HTLV core proteins described earlier (Kalyanaraman *et al.*, 1982b). Briefly, the major core protein p24 of HTLV-I was purified from the solubilized HTLV-I (released by MT-2 cells) by phosphocellulose and gel filtration chromatography (Kalyanaraman *et al.*, 1981). The purified HTLV-I p24 was labeled with ^{125}I and used to develop competition radioimmunoassays with limiting dilutions of rabbit antibodies to either HTLV-I_{MT-2} or HTLV-II_{MO}, the two subtypes of the HTLV family of viruses. The unlabeled cell extracts were used as competing antigens in these assays. The CM and the CM/CB proteins competed only partially in the assay specific for HTLV-I (Figure 2A). Further, the slopes of the curves were very different from that of the prototype HTLV-I (Figure 2A). However, competition curves of the CM proteins were indistinguishable from those of MT-2 cells when tested in the heterologous assay (Figure 2B) for HTLV p24. These results strongly suggest that the virus harbored by CM cells is more closely related to HTLV-II_{MO} rather than to HTLV-I.

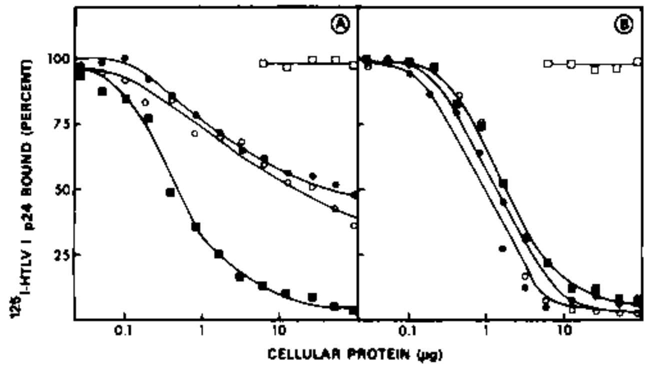

Fig. 2. Homologous and heterologous radioimmunoassay of HTLV p24. HTLV-I p24 was purified and labeled according to previously described methods (Kalyanaraman *et al.*, 1982b). The competition radioimmunoassays were done with ^{125}I-labeled HTLV-I p24 and a limiting dilution of either **(A)** rabbit antibody to HTLV-I virus or **(B)** rabbit anti-HTLV-II_{MO}. The competing unlabeled antigens used in the assay are MT-2 cells ■ – ■; CM cells ○ – ○; CM/CB cells ● – ●; and normal human T-cells □ – □.

The virus released by the CM cells was further analyzed for its relationship to HTLV-I and HTLV-II_{MO}. Virus was concentrated from cell-free supernatant by being pelleted at high speed (19 000 r.p.m. in a Beckman T-19* rotor for 2 h). Virus was then resuspended in TNE (10 mM Tris-H pH 7.5, 100 mM NaCl, and 1 mM EDTA) and banded in a 20 – 60% (w/w) sucrose gradient; the virus banded at a density of 1.16 to 1.18 and was used for further analysis. The SDS-polyacrylamide gel electrophoretic profile of the proteins of HTLV-I, HTLV-II_{MO} and HTLV-II_{CM} demonstrated that HTLV-II_{MO} and HTLV-II_{CM} have exactly the same group of major proteins (Figure 3). They contain, in addition to the core protein p24, two other lower mol. wt. proteins, p21 and p15. HTLV-I, however, had p24, p19 and p15. The p24 and p15 of HTLV-II_{CM} are similar to those of the corresponding proteins in HTLV-I. That the p19 equivalent of HTLV-I is p21 in HTLV-II was confirmed by labeling HTLV-I and II with [^{3}H]myristic acid. For this purpose the MT-2 cells and the CM cells were labeled with [^{3}H]myristic acid (^{50}MC/ml) overnight. The labeled viruses were purified by density banding in sucrose and analyzed by SDS-polyacrylamide gel electrophoresis. The label was incorporated only in the p19 of HTLV-I and p21 of HTLV-II, which proved that there are equivalent proteins in the two viruses (data not shown). The presence of myristic acid also shows that the proteins form the N-terminal part of the *gag* gene product, in a manner analogous to other retroviruses. To distinguish the virus released by the cell line CM from the original HTLV-II_{MO}, we refer to it as HTLV-II_{CM}. The p24 and p15 of HTLV-II_{CM} behave in the same way as those of HTLV-I during purification.

Relationship of the provirus in the CM cell line to HTLV-I and II

High mol. wt. DNA was extracted from the cell lines CM and CM-CB (CM virus-transformed cord blood T cells). Since the restriction enzyme *Eco*RI does not cleave HTLV-I genome internally, high mol. wt. CM DNA was digested using *Eco*RI and analyzed by Southern blot hybridization. Figure 4A demonstrates the lack of HTLV-I-related sequences in CM when HTLV-I *env px* containing DNA fragment was used as hybridization probe. In comparison, MT2 cellular DNA digested with *Eco*RI showed at least three bands with mol. wts. >9 kb.

To explore the relationship of CM virus to HTLV-II, high mol. wt. DNA was digested with *Hind*III, which does not cleave within the proviral genome of HTLV-II_{MO}. Two high mol. wt. bands

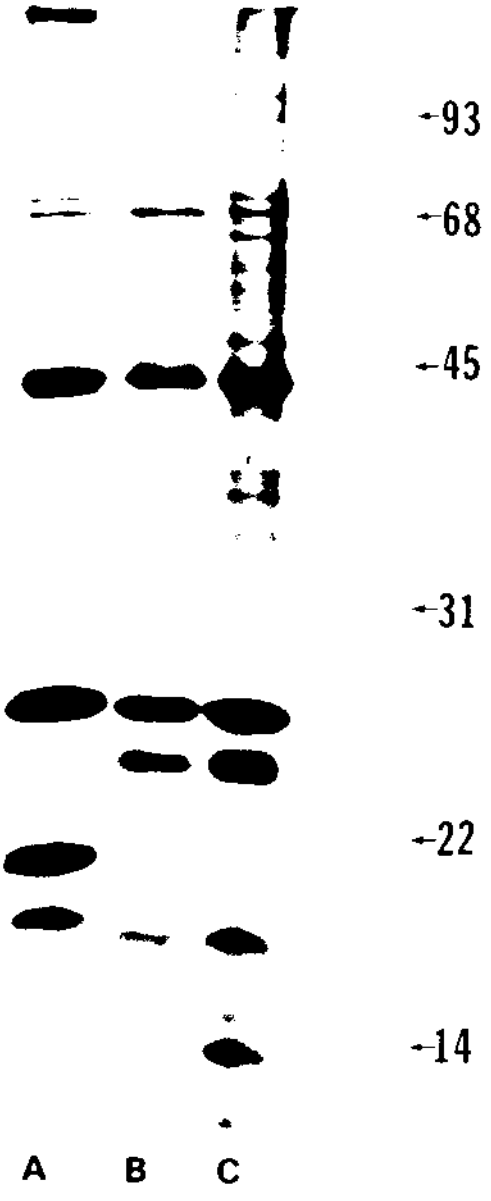

Fig. 3. SDS-polyacrylamide gel electrophoretic profile of **(A)** HTLV-I, **(B)** HTLV-II_{MO}, and **(C)** HTLV-II_{CM} proteins. The sucrose gradient banded viruses were solubilized in SDS and β-mercaptoethanol and separated in a 12% polyacrylamide gel. The proteins were stained with Coomassie R.250, destained, and photographed.

of ~10 and 12 kb were generated which hybridized to probes specific for the 5′ and 3′ ends of HTLV-II (Figure 4B and C). This result suggested the presence of two copies of the proviral genome related to HTLV-II in the cell line CM. The structural similarity of HTLV-II genome in the CM cell line was further analyzed with a battery of restriction enzymes known to release characteristic internal fragments of the genome. *Eco*RI, *Bam*HI, *Pst*I and *Sac*I produced 3.6, 2.6 and 3.6 kb fragments hybridizable to *gag-pol* (Chen *et al.*, 1983a,1983b) probe (p^E) as expected. Combination of enzymes such as *Bam*HI + *Bgl*II or *Bam*HI + *Sma*I also produced the predicted internal fragments of 2 and 2.1 kb, respectively. These results showed the conservation of cleavage sites of HTLV-II_{MO} in the 5′ end of HTLV-II_{CM} DNA. We then examined the CM proviral DNA at the 3′ end for homology to HTLV-II_{MO}. CM cellular DNA was digested with *Bam*HI and *Pst*I and analyzed with an HTLV-II-derived probe specific for the *env-px* region (p^B). *Bam*HI and *Pst*I produced 3.6 and 2.6 kb HTLV-II-related fragments (Figure 4C). *Bam*HI cleaves within the HTLV-II_{MO} LTRs and envelope region to produce two internal fragments of 4.6 and 3.6 kb. HTLV-II LTR-specific probe (pBE) detected two fragments of 3.6 and 4.6 kb as expected (Figure 4D), indicating a similar set of restriction enzyme cleavage sites in the 3′ end of both HTLV-II_{CM} and HTLV-II_{MO}. Figure 4 shows the restriction map of HTLV-II_{CM} derived from the molecular hybridization data.

To determine whether the HTLV-II proviral sequences were expressed in CM cells, we extracted cellular RNA from the cell line CM, poly(A)-selected, and analyzed by dot-blot hybridization with both the 5′ and 3′ HTLV-II probes. Both probes hybridized to the poly(A)-enriched cellular RNAs of CM (Figure 5), suggesting that the HTLV-II_{MO} genome is transcribed in the cell line CM.

Transformation of cord blood T cells by the HTLV-II_{CM} virus

We then tried to transmit the virus into human umbilical cord blood T cells and to establish a transformed human cord blood T cell line. The donor CM cells were initially irradiated (10 000 rads) and mixed with three times the number of 3-day-old PHA-stimulated human cord blood leukocytes. The cells were initially grown in 10% TCGF, but were subsequently selected for TCGF-independent cell growth. In this manner, we could easily establish human cord blood T cell lines consistently transformed by CM cells. The transformed cord blood cell line produced HTLV-II_{MO}-related p24 (Figure 2). The properties of one such transformed human cord blood T cell line are listed in Table I. The cells were of the T cell sub-type and were positive for OKT-4 phenotype. a high percentage of the cells also expressed OKT-8 cell surface marker. The CM cell-transformed cord blood T cells (CM/CB) also expressed HLA-DR antigen and TCGF receptors. The transformed cord blood T cells, as expected, had the karyotype of the recipient and not the donor CM cells. Thus, the CM cells exhibit the phenotypic properties of previously described HTLV-transformed cell lines (Popovic *et al.*, 1983) and are fully capable of transforming human T cells.

Presence of provirus in the uncultured CM cells

The infectious nature of virus produced by CM cell line and its expression in T cells *in vitro* prompted us to check for the presence of HTLV-II-related provirus and its expression in the patient's circulating blood cells. For this purpose, mononuclear cells of CM were separated from blood by the ficoll-hypaque technique. Digestion of the DNA from the uncultured cells with *Hind*III generated two copies of the HTLV-II proviral genome, which hybridized to the 5′ (*gag-pol*) and 3′ (*env-px*) specific probes of HTLV-II_{MO} (Figure 6A and B). The 5′ probe also detected 3.6-kb *Eco*RI, 4.6-kb *Bam*HI fragment and a 2.1-kb fragment when it was digested with both *Bam*HI and *Bgl*II enzymes. Another line of evidence for the presence of complete HTLV-II provirus in the fresh cells came from hybridization studies with HTLV-II_{MO} LTR-specific probe. As observed in the cultured CM cells, the LTR probe detected two fragments (3.6 and 4.6 kb) in DNA from peripheral blood cells after digestion with *Bam*HI. The expression of HTLV-II genome in the fresh uncultured cells of the patient was then tested by competition radioimmunoassay of the core proteins of HTLV-I and HTLV-II_{MO}. However, there was no expression of the major core protein of HTLV-II (p24) in these cells despite the presence of the integrated form of HTLV-II provirus.

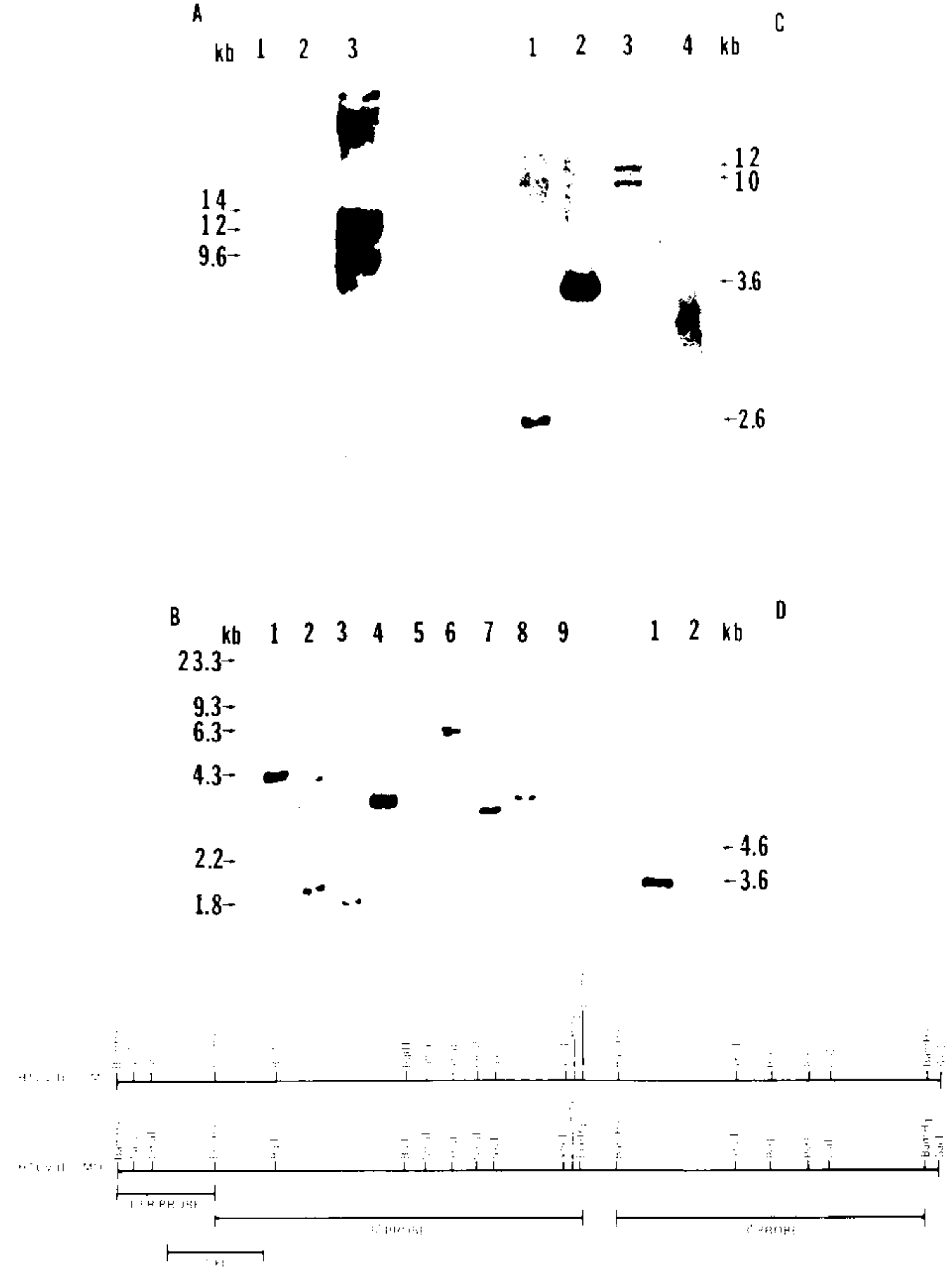

Fig. 4. Detection of HTLV-II proviral sequences in the genomic DNA from the cell line CM. **Panel A, lane 1:** Genomic DNA from CM digested with *Eco*RI; **lane 2:** genomic DNA from uninfected cord blood lymphocytes digested with *Eco*RI; **lane 3:** genomic DNA from MT-w digested with *Eco*RI and hybridized to HTLV-I (*env-px*) p^{HX}. **Panel B, lanes 1–7:** DNA from CM digested with **(1)** *Bam*HI, **(2)** *Bam*HI + *Bgl*II, **(3)** *Bam*HI + *Sma*I, **(4)** *Eco*RI, **(5)** *Hind*III, **(6)** *Pst*I and **(7)** *Sac*I. **Lane 8:** DNA from MO-*Eco*RI, **(lane 9)** uninfected normal human cord blood DNA-EcoRI. **Lanes 1–9** hybridized to the 5′ end of HTLV-II MO (p^{E}). **Panel C, lanes 1–3:** high mol. wt. DNA from CM digested with **(1)** *Pst*I, **(2)** *Bam*HI and **(3)** *Hind*III and hybridized to the 3′ end of HTLV-II MO (p^{B}). **Lane 4:** DNA from uninfected cells hybridized to the 3′ end of HTLV-II MO (p^{B}). **Panel D, lane 1:** DNA from CM digested with *Bam*HI; **lane 2:** DNA from uninfected normal human cord blood; *Bam*HI hybridized to LTR probe derived from HTLV-II-MO (p^{BE}).
Insert: shows the restriction endonuclease map of HTLV-II (MO) and the proviral genome of CM. The enzyme sites of CM were mapped from the Southern blots and compared with the cloned HTLV-II (MO). The probes derived from MO used in this study are shown.

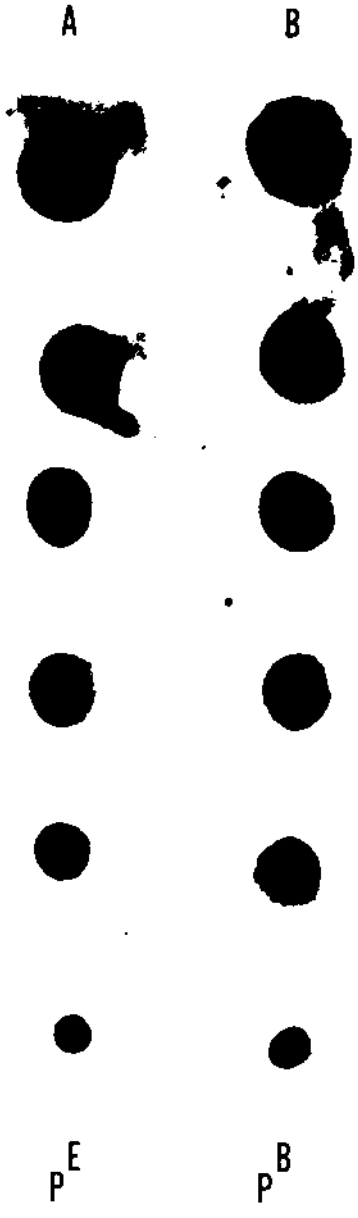

Fig. 5. Expression of HTLV-II proviral genome in the cell line CM. **Panel A** shows the results of dot-blot hybridization of the poly(A)-enriched cellular RNA with the *gag-pol* (p^E) probe of HTLV-II. **Panel B** shows the results of dot-blot hybridization of the poly(A)-enriched cellular RNA with the *env-px* (p^B) probe of HTLV-II. Total cellular RNA was poly(A)-selected, dissolved in H_2O, boiled and quick-chilled in ice, and loaded onto nitrocellulose (0.3, 0.06, 0.125, 0.25, 0.5 and 1 μg), air-dried at 80°C for 2 h, and hybridized. Filters were washed at room temperature 3 x 5 min, in 2 x SSC and 0.1% SDS for 3 x 15 min, in 0.1 × SSC and 0.1% SDS at 65°C. **Panels A** and **B**: poly(A) RNA from CM hybridized with *gag-pol* and *env-px* probes, respectively.

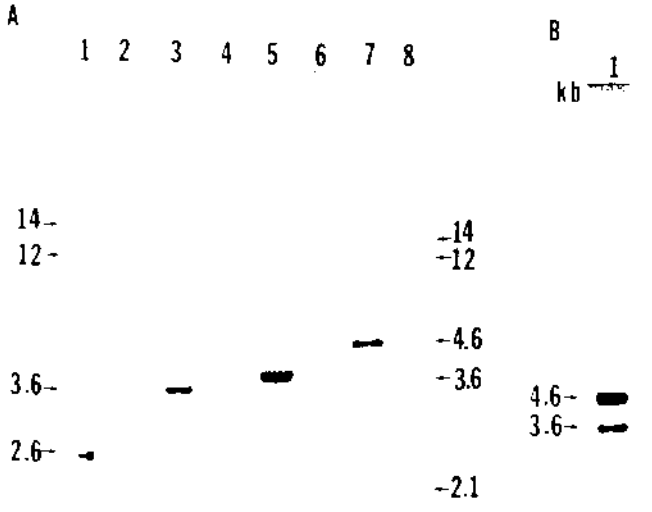

Fig. 6. Analysis of DNA from the fresh lymphocytes of the hemophilia-A patient. **Panel A** shows the hybridization results with 5′- and 3′-derived probe of HTLV-II_{MO}, and **Panel B** shows the results of hybridization with LTR-specific probe of HTLV-II_{MO}. High mol. wt. DNA from the lymphocytes was extracted and 5 μg of DNA was digested with **(1)** *Pst*I, **(2)** *Hind*III and **(3)** *Bam*HI and probed with the 3′ end of HTLV-II. **Lane 4**, *Hind*III; **lane 5**, *Sac*I; **lane 6**, *Bam*HI + *Bgl*II; **lane 7**, *Eco*RI, and **lane 8**, uninfected normal human cord blood DNA-*Eco*RI were probed with the 5′ end of HTLV-II_{MO}. **Lanes 1–3** and **4–8** are from two different gels. **Panel B** shows: **lane 1**, 5 μg of DNA from the fresh lymphocytes digested with *Bam*HI, and **lane 2**, 5 μg of uninfected normal human cord blood DNA digested with *Bam*HI and hybridized to LTR probe.

Discussion

Thus, we have established a continuous cell line from the cultured T cells of a hemophilia patient with pancytopenia. The cell line does not depend on TCGF for its growth and produces HTLV-II particles in high titers (from 10^8 to 10^9 particles/ml). The CM cells as well as the cord blood T cells are also very similar to other HTLV-I producers. The protein profile of HTLV-II_{MO} and HTLV-II_{CM} demonstrate their close relationship which is further substantiated by the fact that they compete similarly in the heterologous radioimmunoassay of HTLV p24. We have now purified the p24 of HTLV-II_{CM} to homogeneity and have developed homologous competition radioimmunoassay for HTLV-II_{CM} p24 (Kalyanaraman *et al.*, 1982b). Although HTLV-II_{MO} had a profile in this assay identical to that of HTLV-II_{CM}, HTLV-I competed only partially and with a different slope. Partial sequencing analysis carried out with the purified p24 and p15 of HTLV-II_{CM} further reveals that HTLV-II_{CM} is related to HTLV-I, but clearly different from it (Devare *et al.*, in preparation).

The results of the analysis of the proteins of the virus released by the CM cells are further strengthened by the analysis of the proviral genome in the CM cells. Hybridization with molecular probes specific for HTLV-I and HTLV-II viruses showed the presence of complete viral genome closely related to, if not identical to, HTLV-II in the cell line CM. On the basis of restriction enzyme analysis, we could not distinguish the proviral genomes present in the cell line CM described in this paper and the one in the cell line MO producing the prototype HTLV-II virus. However, of particular interest is the presence of two complete copies of the HTLV-II provirus in the circulating mononuclear cells of the patient. There was no expression of at least the major core protein p24 in these cells. A similar phenomenon has also been observed in adult T cell leukemia patients who harbored complete HTLV-I proviral genomes with no viral antigen expression (Reitz *et al.*, 1983). In the case of bovine leukemia virus, suppression of viral genome expression has been shown to be mediated by non-immunoglobulin protein factors in the plasma of infected animals (Gupta and Ferrer, 1982). The mechanism by which the plasma factors block the expression of the BLV genome is not known. Similar factor(s) may account for the lack of expression observed in fresh CM peripheral blood

cells. The patient CM has had pancytopenia since 1978 and has a history of anklyloses and traumatic hemorrhages requiring multiple transfusions with packed red cells and therapy with Factor VIII. Human T cell leukemia virus has been shown to be transmitted through blood in a number of studies (Miyoshi *et al.*, 1982; Saxinger and Gallo, 1982; Tedder *et al.*, 1984). Based on this, it is possible that the patient's disease may result from the acquisition of HTLV-II through blood or it may be an opportunistic infection.

HTLV-II, though markedly different from HTLV-I in its occurrence, seems to have similar biological properties *in vitro* as HTLV-I (Chen *et al.*, 1983a). The pattern of restriction enzyme cleavage sites of HTLV-II_{CM} is distinct, and molecular hybridization studies show further that the viral genome is not homologous to HTLV-I. Although HTLV-II has been found associated only with single cases of a relatively benign T-cell variant of hairy-cell leukemia and of hemophilia with pancytopenia, HTLV-I is usually found in association with highly malignant T cell leukemia and lymphoma (Poiesz *et al.*, 1980,1981; Popovic *et al.*, 1982,1983). Further studies involving a large number of patients are needed to verify the association of HTLV-II to any specific disease.

Materials and methods

Cells and viruses

The HTLV-I_{MT-2}-producing cell line was obtained from Dr. Hinuma of Kyoto University. The MO-B cells were received from Dr. Golde of UCLA. The HTLV-II_{MO} virus was subsequently transferred to normal human cord blood **T** cells by co-cultivation with irradiated MO-B cells. The cells were grown in RPMI-1640 medium containing 10% fetal calf serum. For large-scale virus purifications, the cells were grown in roller bottles in a final density of 1 – 2 x 10^6 cells/ml. The medium was clarified by low-speed centrifugation to remove the cells, then centrifuged for 3 h in a Beckmann type-14 rotor at 19 000 r.p.m. to pellet the virus. The virus was then resuspended in a small volume of TNE Buffer (Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) and pelleted through a cushion of 30% sucrose (w/w) in TNE. The virus was further purified if necessary by banding in a step gradient of 30 and 45% (w/w) sucrose. The virus banded on the 45% sucrose after centrifuging for 18 h at 27 000 r.p.m. in an SW-28 rotor.

Competition radioimmunoassays

The major core protein p24 of HTLV-I was purified from the sucrose gradient-banded HTLV-I_{MT-2} virus as described previously (Kalyanaraman *et al.*, 1981). The virus was solubilized in Triton X-100-containing buffer and freed of nucleic acids by passage through a column of DEAE cellulose. The unadsorbed protein fraction was then loaded on a column of phosphocellulose equilibrated with Buffer 1 (10 mM BES, 1 mM EDTA pH 6.5). The column was then eluted with a linear gradient of NaCl. The p24 was eluted from the column at a salt concentration of 200 – 300 mM NaCl. The fractions containing nearly homogeneous HTLV-I p24 were used for subsequent analysis. The HTLV-I p24 was labeled by the Chloramine-T method (Greenwood *et al.*, 1963). The labeled p24 was >95% immunoprecipitated by the rabbit antibody to HTLV-I. Radioimmunoassays were performed by the double antibody method described by Kalyanaraman *et al.*, 1981.

Serial 2-fold dilutions of antisera were incubated with the labeled antigen (~10 000 c.p.m.) for 2 h at 27°C and further incubated overnight at 4°C. These samples were in a buffered solution containing 200 mM NaCl, 20 mM Tris-hydrochloride (pH 7.5), 0.3% Triton X-100, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride in a final volume of 200 µl. A 30-fold excess of the appropriate second antibody was then added and further incubated for 2 h at 37°C followed by 1 h at 4°C. The incubation mixtures were diluted to 1 ml with the buffer and centrifuged at 8000 r.p.m. for 4 min, and the percentage radioactivity bound in the pellets was determined in an LKB Ultrogamma counter. In competition radioimmunoassays, unlabeled antigen was pre-incubated for 1 h at 37°C with an unlimiting dilution of the immune serum capable of precipitating 30% of the labeled antigen before addition of the labeled antigen. Further steps of the procedure were the same as described above for the standard radioimmune precipitations.

Hybridization techniques and derivation of HTLV-I and HTLV-II-specific probes

High mol. wt. DNA was extracted from different cells, digested with various restriction enzymes, size-fractionated on 0.8% agarose gel, transferred to nitrocellulose and hybridized to the respective nick-translated probes from MO and MT-2 using 50% formamide, 6 × SSC (SSC = 0.15 M NaCl, and 0.015 M sodium citrate pH7.0), 0.05 mM phosphate buffer pH 6.4, 5 x Denhardt's (0.02% of bovine serum albumin, polyvinylpyrrolidine and ficoll 4000), denatured salmon sperm DNA (200 µg/ml) and 10% dextran sulfate. The filters were washed in 0.1 x SSC – 0.1% SDS at 72°C, air dried, and exposed to Kodak XAR film for 48 – 72 h with intensifying screens.

The HTLV-I genomic clone used in these experiments was derived from a HTLV-I-producing cell line MT-2 (Yoshida *et al.*, 1982). A synthetic oligonucleotide comprising 30 bases from the HTLV-I long terminal repeat (LTR) region, based on the available sequence information (Seiki *et al.*, 1983), was used as probe to clone an integrated provirus from the MT-2 genomic library (unpublished data). An HTLV-I subgenomic clone containing *env* and *px* sequences (pHX-2.1 kb) was constructed and used in hybridization experiments. The characterization of the 5'-, and 3'- and LTR-specific probes of HTLV-II have been previously reported (Chen *et al.*, 1983a,1983b). Under normal stringency conditions (see Figure 4 legend), HTLV-I- and HTLV-II-specific probes hybridized only to the DNA from HTLV-I-producing MT-2 cells and HTLV-II-producing MO cells, respectively. Neither set of probes hybridized to DNA from uninfected human cord blood lymphocytes.

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Received on 23 January 1985; revised on 29 March 1985

Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8840–8844, November 1990
Medical Sciences

Isolation of human T-cell lymphotropic virus type 2 from Guaymi Indians in Panama

(retrovirus)

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Communicated by Robert H. Purcell, August 23, 1990

ABSTRACT **Human T-lymphotropic virus type I (HTLV-I) is associated with adult T-cell leukemia/lymphoma and with a chronic degenerative myelopathy. However, another major type of HTLV, HTLV-II, has been isolated only sporadically, and little is known of disease associations, transmission routes, and risk factors for HTLV-II infection. Recent studies indicate that a high percentage of certain groups of i.v. drug users and blood donors are infected with HTLV-II. Seroepidemiologic studies have found an elevated rate of seroreactivity to HTLV among Guaymi Indians from Bocas del Toro Province, Panama. To identify the cause of seroreactivity among this unique population we used HTLV-II-specific polymerase chain reaction techniques to detect HTLV genetic sequences from blood leukocytes of three seropositive Guaymi Indians. The HTLV-II primer-amplified polymerase chain reaction products from two of these subjects were partially sequenced and matched published HTLV-II nucleotide sequences in both p24 *gag* (94% of 107 bases) and *pol* (98% of 112 bases) regions. A CD4$^+$ T-lymphocyte line established from one of these same subjects produced HTLV-II-specific proteins when tested in antigen-capture and immunoblot assays, as well as mature HTLV particles. The demonstration of HTLV-II infection in this geographically and culturally isolated Central American Indian population without typical risk factors of HTLV infection suggests that HTLV-II infection is endemic in this population and provides an important clue to a potential natural reservoir for this virus.**

Human T-cell lymphotropic virus type I (HTLV-I) is associated with adult T-cell leukemia/lymphoma and with a chronic degenerative neurologic disease, HTLV-I-associated myelopathy/tropical spastic paraparesis (1). A second type of HTLV, HTLV-II, was initially isolated from a patient with hairy cell leukemia (2) but has subsequently been isolated only sporadically (3); detailed studies regarding disease associations, transmission routes, and risk factors for HTLV-II infection have not been reported. Recent studies using the polymerase chain reaction (PCR) technique have indicated that a high percentage of HTLV seroreactivity among i.v. drug users and blood donors in certain regions of the United States may be from HTLV-II (4, 5).

HTLV-I and HTLV-II are distinguished by restriction endonuclease cleavage sites, nucleotide sequence, major core protein size, and immunogenic properties (6–8). The two viruses appear to share ≈60% overall nucleotide sequence. Despite nucleotide differences, the two virus types have a number of similar biological properties, including an ability to transform lymphocytes, predominant CD4 lymphocyte tropism, and an ability to elicit cytokine production from transformed cell lines (9–11).

Recent population-based seroepidemiologic studies revealed that 8% of 337 Guaymi Indians residing in Bocas del Toro Province, Republic of Panama, had antibody against HTLV (12, 13). Antibody was found almost exclusively in subjects 15 yr old and older (16% seropositivity), there was no evidence for household clustering of infection, and neither hematologic nor neurologic diseases usually associated with HTLV infection were identified (13). Furthermore, serum specimens from these HTLV-seropositive persons demonstrated weak immunoreactivity to envelope antigens of HTLV-I compared with the seroreactivity of HTLV-I seropositive controls (13). The Guaymi are descendents of Indian groups who have lived in relative isolation since the arrival of the Spanish in the 16th century and are still largely unadmixed with other racial or ethnic groups (14). Traditionally, the Guaymi practice a subsistence economy, although in recent years some families have migrated to Changuinola on the Caribbean coast to work on banana plantations. The Guaymi do not practice i.v. drug use, tattooing, or scarification, and medical procedures requiring blood transfusion are rare. The unusually high prevalence of HTLV seroreactivity, atypical epidemiology, and weak antibody reactivity to HTLV-I envelope antigens suggested that variant HTLV strains might be endemic in this population. The present studies were initiated to identify the nature of the virus accounting for this seroreactivity.‖

MATERIALS AND METHODS

Guaymi Indian Subjects. Demographic, cultural, and HTLV serologic information regarding the Guaymi Indians have been reported (12–14). Briefly, the Guaymi subjects studied were individuals who migrated to Changuinola, Bocas del Toro Province, Panama to seek employment at a banana plantation. During the seroepidemiologic studies (12, 13) blood samples were collected (after obtaining oral informed consent from each subject or guardian), and peripheral blood mononuclear cells (PBMC) were cryopreserved after Ficoll separation. In addition, a study physician and staff carried out interviews, physical examinations, and clin-

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Abbreviations: HTLV, human T-lymphotropic virus; PCR, polymerase chain reaction; IL-2, interleukin 2; gag, group-specific antigen; env, envelope; pol, polymerase; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody.

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‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M38253 for *pol* and M38254 for *gag* p24).

Table 1. Summary of family relationships, HTLV antibody status, and PCR results

Family	Subject	Age, yr	Relation	HTLV antibody	PCR results					
					HTLV-I			HTLV-II		
					gag	pol	env	gag	pol	env
12	1*	26	Mother	+	–	–	–	+	+	–
12	3	30	Father	–	–	–	ND	–	–	ND
12	2	11	Son	–	–	–	ND	–	–	ND
12	6	4	Daughter	–	–	–	ND	–	–	ND
66	11	33	Mother	+	–	–	–	+	+	+
66	10	29	Father	ND	–	–	ND	–	+	–
66	14	6	Son	–	–	–	ND	–	+	–
66	15	8	Daughter	–	–	–	ND	–	–	ND
132	5	24	Mother	+	–	–	–	+	+	+
132	4	23	Father	–	–	–	ND	–	–	ND
132	7	5	Son	ND	–	–	ND	–	–	ND

A study physician and staff carried out interviews, examinations, and collection of specimens in the subject's home. Serum samples were tested for HTLV antibodies by using commercial enzyme immunoabsorbant assay (DuPont), according to the manufacturer's recommendations. Seropositivity was confirmed by immunoblot assays as described (15). ND, not determined.

*T-cell line (Fig. 5) was derived from subject 12.1.

ical evaluations. Family relationships, HTLV antibody status, and HTLV PCR (below) results are summarized in Table 1. Serum samples were tested for HTLV antibody by using commercial enzyme immunoabsorbant assay (DuPont) according to the manufacturer's recommendations. Seropositivity (reactivity to *gag* p24 and *env* gp46 or gp 61/68) was confirmed by immunoblot assays as described (8, 15). Due to extensive cross-reactivity among viral proteins, our serologic methods did not distinguish antibody reactivity to HTLV-I from reactivity to HTLV-II (8).

Polymerase Chain Reaction and Nucleotide Sequence. PCR was performed using total genomic DNA as described (16, 17). Primers were used to amplify 1 μg of total genomic DNA (equivalent to ≈150,000 PBMC) for each PCR amplification in 100-μl reaction volumes of 5 mM KCl/10 mM Tris, pH 8.5/10 mM $MgCl_2$/0.2 mM of each dNTP/each primer at 100 ng/μl/2 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin–Elmer/Cetus) (18). The amplification consisted of 34 repetitive three-step cycles under the following conditions: 25°–95°C and then 2-min periods of incubation at 95°C, 55°C, and 72°C per cycle in a thermal cycler (Perkin–Elmer/Cetus). PBMC DNA was amplified by using both HTLV-I- and HTLV-II-specific *gag* and *pol* primers (16, 19). Samples that were positive when amplified with either HTLV *gag* or *pol* primers were also PCR-amplified with *env* primers. HTLV-I primers were derived from *gag* sequence positions 1423–1444 in the sense strand and 1558–1537 in the antisense strand, from *pol* positions, 3015–3034 in the sense strand and 3154–3134 in the antisense strand, and from *env* positions 5627–5648 in the sense strand and 5792–5771 in the antisense strand. HTLV-II primers were derived from *gag* positions 1424–1445 in the sense strand and 1561–1540 in the antisense strand, from *pol* positions 2989–3010 in the sense strand and 3131–3110 in the antisense strand, and from *env* positions 5602–5620 in the sense strand and 5804–5787 in the antisense strand**. The amplified products were separated in 1.8% agarose gels and probe by Southern hybridization by using specific ^{32}P-labeled probes for HTLV-I—*gag* 1489–1513, *pol* 3050–3074, and *env* 5713–5737—and for HTLV-II—*gag* 1490–1514, *pol* 3025–3049, and *env* 5758–5779**. After blocking of blotted membranes, labeled probes were hybridized overnight at 42°C in prehybridization solution. Membranes were subsequently washed under high-stringency conditions [twice with 2% SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) at 56°C for 30 min, once with 1% SSC at 56°C for 15 min]. A sample was considered positive if amplified with primers from each of two separate viral regions. To obtain nucleotide sequence data the PCR products were isolated with 1.8% agarose, denatured by dimethylsulfoxide, and sequenced dideoxynucleotide chain termination with Sequenase version 2.0, according to the manufacturer's recommendations (United States Biochemical).

Cell-Surface Antigen Expression and HTLV Antigen Assays. Expression of CD4 (Leu 3a), CD8 (Leu 2), CD25 [interleukin 2 (IL-2) receptor], and IgG1 control was detected with fluorescein isothiocyanate-conjugated murine monoclonal antibodies (mAbs; Becton Dickinson) and then analyzed by fluorescence-activated analysis (FACS-scan, Becton Dickinson). Soluble HTLV antigen from subject 12-1 PBMC culture supernatant was determined by enzyme immunoabsorbant capture assays for HTLV-I p19 (Cellular Products) and for HTLV-I and -II p24 (Coulter). The assay specific for HTLV-I used polyvalent rabbit antiserum to HTLV-I coated onto microtiter plates to capture soluble HTLV-I antigen, which detects HTLV-I antigen by using a mAb specific for HTLV-I p19 (20). Bound mAb is detected with peroxidase-conjugated goat anti-mouse IgG, and color is developed with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. The antigen-capture assay, which recognized both HTLV-I and HTLV-II p24 core antigen, uses a murine mAb specific for p24 of HTLV-I and -II coated onto microwell strips to capture soluble HTLV-I/II p24 antigen. Bound HTLV antigen is recognized by biotinylated human antibodies to HTLV-I/II. Streptavidin-horseradish peroxidase is then complexed with biotin-linked antibodies, and color develops from the reaction of the peroxidase with hydrogen peroxide substrate in the presence of 3,3',5,5'-tetramethylbenzidine chromagen. Resultant absorbance values of both tests were detected and compared with known standard curves of viral core antigens in the same trial. Immunoblotting was used to detect HTLV antigens from cell culture lysates as described (15). Cellular lysates were prepared from Guaymi 12-1 cell line (10^8 cells per 5 ml of lysing buffer, ref. 15). HTLV-I (MT-2, ref. 21) and HTLV-II (Mo-T, ref. 22) lysate antigens were obtained from a commercial source (Hillcrest Biologicals, Cypress, CA). Viral antigens were suspended in sample buffer [Tris buffer at 0.1 mol/liter, pH 6.8, containing 0.5% SDS, bromophenol blue at 0.10 μg/ml, 20% (vol/vol) glycerol, and 10% (vol/vol) 2-mercaptoethanol, heated at 95°C for 4 min and electrophoresed in precast gradient gels (4–20%

**Nucleotide sequence positions were derived from The Los Alamos National Laboratory, Human Retroviruses and AIDS, 1989: HTLV-I, J02029; HTLV-II, M10060.

polyacrylamide, EmproTech, Bethesda, MD], and probed by using an avidin–biotin–peroxidase procedure as described (15).

Electron Microscopy. Ultrastructural examination for HTLV particles was done by using a PBMC suspension culture (from subject 12-1, Table 1) containing $\approx 1 \times 10^7$ cells, which was washed free of media and resuspended in 2.5% glutaraldehyde in phosphate buffer at pH 7.4 for 2 hr at 4°C. The cells were then pelleted, and the fixative solution was removed and replaced with phosphate buffer at pH 7.4. The pellets were postfixed by 1% osmium tetroxide and stained with uranium and lead acetate salts.

RESULTS

To identify HTLV we collected PBMC from three HTLV-seropositive Guaymi and eight of their seronegative family members. Genomic DNA was isolated from the PBMC samples and assayed by PCR to test for HTLV sequences. We used oligonucleotide primer pairs that would selectively amplify HTLV-I or HTLV-II nucleotide sequences. DNA from the three seropositive Guaymi were positive when using HTLV-II *gag* and *pol* sequence primers (Fig. 1). In addition, HTLV-II *env* primers consistently amplified two of the three PBMC samples from these seropositive subjects (Fig. 1). These same DNA samples were PCR negative when using HTLV-I-specific primers derived from HTLV-I *gag*, *pol*, and *env* sequences (Table 1).

Six of the eight PBMC DNA samples from family members were negative for HTLV-II amplification with both *gag* and *pol* primers, and two samples (from subjects 66-10 and 66-14, Table 1) were amplified with *pol* primers but were negative with *gag* primers. All eight family members were negative for HTLV-I PCR amplification when using conserved *gag* and *pol* primers.

To further characterize the identity of the PCR-amplified products, we directly sequenced the HTLV-II *gag* and *pol* PCR products from two of the seropositive persons (Fig. 2).

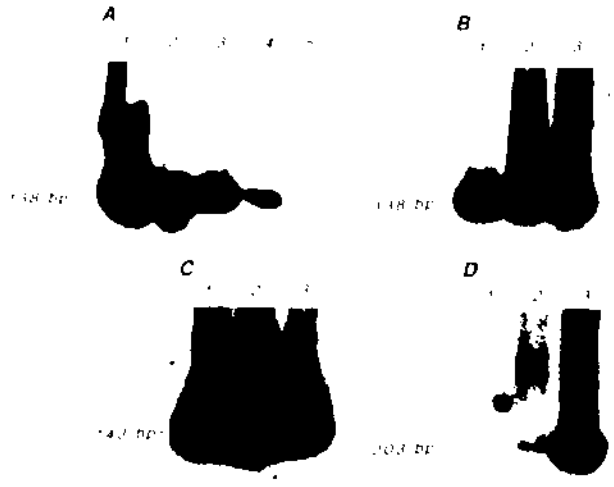

FIG. 1. Southern analysis of PCR-amplified products using ^{32}P-labeled oligonucleotide probes. The results with HTLV-I primers indicated no specific amplified products, whereas the HTLV-II primers amplified the specific products under the same conditions. (*A*) Amplification and probing for HTLV-II *gag* sequences. Serial 10-fold dilutions (undiluted–10^{-4}) of control HTLV-II cell line Mo-T (lanes 1–4) and normal donor PBMC (lane 5). (*B*) Guaymi PBMC samples 12-1 (lane 1), 66-11 (lane 2), and 132-5 (lane 3) amplified and probed for HTLV-II *gag* sequences. (*C*) Amplification and probing for HTLV-II *pol* sequences: Guaymi PBMC samples 12-1 (lane 1), 66-11 (lane 2), and 132-5 (lane 3). (*D*) Amplification and probing for HTLV-II *env* sequences: Guaymi PBMC samples 12-1 (lane 1), 66-11 (lane 2), and 132-5 (lane 3).

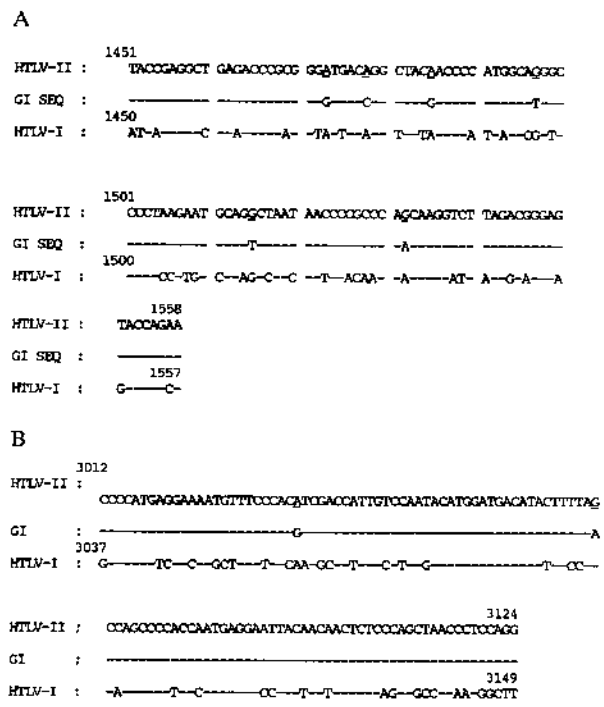

FIG. 2. Comparison of genomic sequence of HTLV Guaymi Indian (GI) PBMC PCR-amplified *gag* and *pol* products. (*A*) Nucleotide sequence alignment of genomic DNA product of *gag* region from PBMC sample of Guaymi subject 12-1, Table 1. Nucleotide differences are noted and compared with HTLV-II *gag* sequence (positions 1451–1558) and HTLV-I *gag* sequence (positions 1450–1557)**. (*B*) Nucleotide sequence alignment of genomic DNA product of *pol* region from PBMC sample from Guaymi subject 132-5 (GI), Table 1. Nucleotide differences are noted compared with HTLV-II *pol* (positions 3012–3124) and HTLV-I sequences (positions 3037–3149)**.

The DNA sequence of the *gag* p24 region (from subject 12-1, Table 1) matched the corresponding HTLV-II *gag* sequence in 101 (94%) of 107 nucleotide bases and only 66 (62%) of 107 HTLV-I *gag* nucleotide bases. The *pol* PCR product (from subject 132-5, Table 1) was sequenced and was identical to the corresponding HTLV-II *pol* sequence in 110 (98%) of 112 nucleotides and only 73 (65%) of 112 HTLV-I *pol* nucleotides.

To further define the HTLV-II infection in this population, we cultured Ficoll-separated PBMC from subject 12-1. We established a primary IL-2-dependent T-lymphocyte line, which at 14 weeks in culture had a surface phenotype characterized by $CD2^+$ (99%, T cell–sheep erythrocyte receptor), $CD3^+$ (99%, T-cell receptor complex), $CD4^+/CD8^-$ (80%, helper/inducer, T lymphocyte), $CD25^+$ (60%, IL-2 receptor) reactivity (Fig. 3). This primary cell line was maintained independent of normal donor feeder cells through 24 weeks, remained IL-2 dependent, but slowed in replicative capacity and was cryopreserved. The infectious capacity of the HTLV culture was established by cocultivation and infection of normal donor PBMC and by infection of rabbits with irradiated (5000 rads; 1 rad = 0.01 Gy) Guaymi PBMC 12-1 culture (23). Supernatants of both primary and cocultured cells were positive in an antigen-capture assay capable of detecting the major core antigen *gag* p24 of both HTLV-I and HTLV-II; these same supernatants were negative when using an HTLV-I-specific antigen-capture assay for HTLV-I *gag* p19 (Fig. 3).

HTLV proteins in cell culture lysates were identified by immunoblot analysis (Fig. 4). Polyvalent serum from an HTLV-I-infected patient and mAbs specific for either HTLV-I (*env* gp46) or recognizing both HTLV-I and

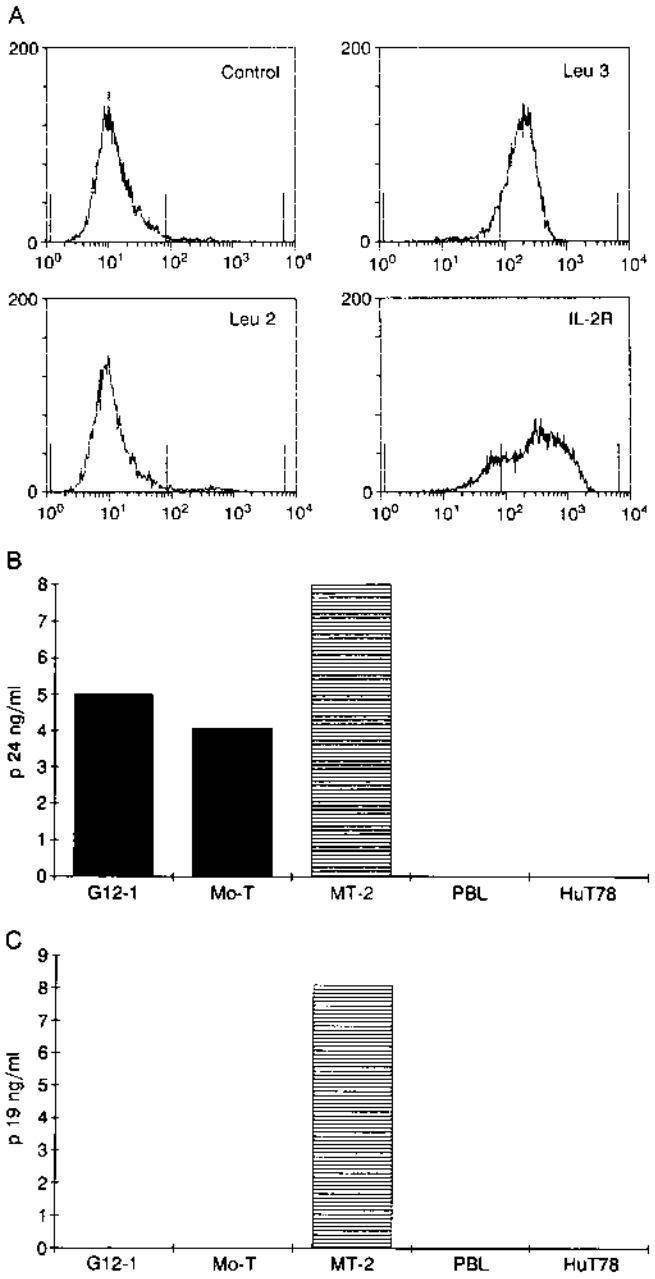

FIG. 3. Cell-surface antigen expression and HTLV antigen production of HTLV-II-producing cell line derived from Guaymi subject 12-1 of Table 1. Expression of CD4 (Leu 3a), CD8 (Leu 2), CD25 (IL-2 receptor), and IgG1 control was determined with fluorescein isothiocyanate-conjugated murine mAbs (Becton Dickinson) and then analyzed by fluorescence-activated analysis (FACSscan, Becton Dickinson). (*A*) Relative staining of cell-surface antigens is indicated for the primary PBMC culture established from Guaymi 12-1 at 14 weeks after culture initiation. (*B* and *C*) Comparison of viral antigen from Guaymi (G 12-1) PBMC culture supernatants versus known HTLV-I (MT-2) and HTLV-II (Mo-T) culture supernatants. Guaymi PBMC 12-1 culture supernatant antigen was detected by an antigen-capture assay that used a mAb to common epitopes of p24 of both HTLV-I and -II (*B*) and is not detected by HTLV-I p19-specific antigen-capture assay (*C*). Supernatants from HuT 78 (HTLV-negative cell line) and PBL (normal cultured peripheral blood lymphocyte) used for negative controls.

HTLV-II (*gag* p24) were used to demonstrate that the cell lysate reactivity was consistent with HTLV-II and not HTLV-I (Fig. 4). The lysates failed to react with mAbs directed against a specific epitope of HTLV-I *env* p46/gp65 (24). However, cell lysates did react with HTLV-I patient

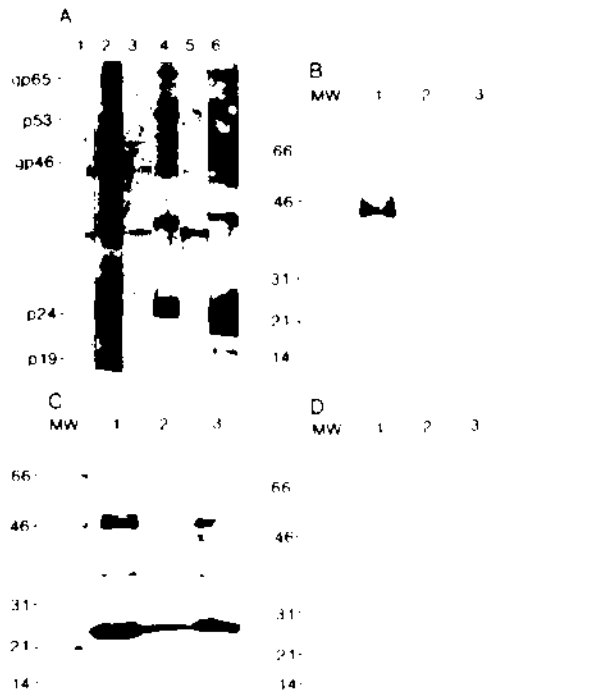

FIG. 4. Immunoblot cell lysate analysis of T-cell line from Guaymi subject 12-1. Reactivity to polyvalent and mAbs compared with HTLV-I and HTLV-II cell lysates. Guaymi 12-1 PBMC lysate reacted positive to polyclonal antiserum and mAb that recognize both HTLV-I and HTLV-II but failed to react to murine mAb specific for HTLV-I. (*A*) HTLV-I MT-2 lysate (lanes 1 and 2), Guaymi 12-1 lysate (lanes 3 and 4), and HTLV-II Mo-T lysate (lanes 5 and 6). Cell lysates reacted against polyvalent HTLV antiserum lanes (2, 4, and 6) or negative control serum (lanes 1, 3, and 5). (*B–D*) Lanes: MW, molecular weight markers; 1, HTLV-I MT-2 lysate; 2, Guaymi 12-1 lysate; 3, HTLV-II Mo-T lysate. (*B*) Cell lysates reacted with murine mAb to HTLV-I envelope gp46 and gp68 epitope (24). (*C*) Cell lysates reacted with murine mAb to HTLV-I and HTLV-II p24 and precursors (25). (*D*) Cell lysates reacted against normal mouse ascites.

serum that contained cross-reactive antibodies for HTLV-I and -II and with a mAb reactive to common epitopes shared in *gag* p24 of both HTLV-I and -II (25) (Fig. 4).

Ultrastructural analysis of the cell culture revealed 80- to 120-nm mature retroviral particles consistent with HTLV (Fig. 5). Mature intact viral particles were primarily adjacent to the surface of plasma membranes or between cell processes and were less frequently found budding from the plasmalemma (Fig. 5). Viral particles consisted of irregular nucleocapsids with fine granular core material surrounded by an envelope. Some viral particles assumed hexagonal to polyhedral shapes.

DISCUSSION

We report HTLV-II infection from a defined non-i.v.-drug-using population, a finding that has important implications for understanding the phylogeny of human retroviruses. HTLV-II genomic DNA was amplified by PCR in all three PBMC samples from seropositive Guaymi, each from separate family units; PCR products were sequenced and matched known HTLV-II nucleotide sequences in two conserved viral genomic regions (*gag* and *pol*). In addition, we isolated HTLV-II from cultured PBMC from one of these same subjects. These data together with the generally weak serologic reactivity to HTLV-I envelope antigens in the Guaymi population (13) suggest that our HTLV-II isolate is primarily

FIG. 5. The T-cell line derived from Guaymi subject 12-1 was used for ultrastructural analysis. Electron photomicrograph of HTLV particles from 12-1 cell line adjacent to cell membrane. (×26,000; bar = 200 nm.) (*Inset*) HTLV viral particles. (×48,000; bar = 100 nm.)

responsible for the HTLV seroreactivity in this Central American Indian population.

The Guaymi are descendents of Indian groups who have lived in relative isolation since the arrival of the Spanish in the 16th century and are still largely unadmixed with other racial or ethnic groups (14). Typical risk factors for HTLV infection could not be identified among the population. Guaymi Indians do not practice ritual scarification or tatooing; i.v. drug use does not occur, disposable needles and syringes are almost universally used in Panama by physicians and in all hospitals and health centers, and transfusion of blood is rare (12).

The mode of transmission of HTLV-II among the Guaymi Indian population was not investigated in our study. Mother–child transmission and sexual transmission may be important in maintaining endemicity of the virus infection in the population, but seropositivity is rare in Guaymi children, and familial clustering is inconsistently demonstrated (13). Our finding of HTLV-II *pol* PCR amplification among two of eight family members suggests that the virus infection may be latent in certain individuals as suggested (1) for HTLV-I infection. However, this PCR reactivity was demonstrated for only one set of PCR primers in a conserved region (*pol*), and we cannot exclude the possibility of nonspecific amplification of similar host or closely related viral sequences. The absence of known HTLV-associated disease, either adult T-cell leukemia or spastic paraparesis, may suggest that the HTLV-II infection is less pathogenic than HTLV-I or has evolved a more benign relationship in the population. However, to determine the role of HTLV-II in disease among the Guaymi, further monitoring of infected persons is necessary because of the long latent period between infection and disease characteristic of this group of retroviruses and because of the relatively small population studied.

It is possible that the HTLV-II isolate we have identified in this study may differ from previous HTLV-II isolates. In the United States, HTLV-II infection has recently been found to be more prevalent than previously believed in certain groups of i.v. drug users and normal blood donors (5). It will be important to identify the role of HTLV-II in human disease (if any) and to identify transmission routes, risk factors, and pathogenetic mechanisms for this human retrovirus. Further molecular characterization, including complete sequencing, may provide important clues for the origin of the Guaymi HTLV-II isolate and for comparative phylogenic studies of this family of retroviruses.

We thank Ms. Cynthia Goldsmith for electron microscopic examination of cell cultures; Drs. Rima Khabbaz, Dan Bednarik, Renu Lal, and Tom Folks for their discussions and constructive reviews of the manuscript; and Dr. Gary Toedter (Coulter Immunology) for supply of antigen capture assays. Our study was supported, in part, by the National Cancer Institute, National Institutes of Health, Contract NCI-CP-31015 with the Gorgas Memorial Institute.

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CURRENT MICROBIOLOGY Vol. 27 (1993), pp. 355–360

Current Microbiology

Isolation, Characterization, and Transmission of Human T-Lymphotropic Virus Types I and II in Culture

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Abstract. Highly sensitive coculture methods were developed both for isolation of human T-lymphotropic virus types I and II (HTLV-I and HTLV-II) from infected individuals and for productive infection of lymphoid cells. Mitogen-activated peripheral blood mononuclear cells (PBMC) from 13 HTLV-I- and 20 HTLV-II-positive specimens were cocultured with an equal number of mitogen-activated PBMC from HTLV-seronegative individuals, and culture supernatants were tested for the presence of soluble $p24^{gag}$ antigens at weekly intervals for 4 weeks. Eleven of 13 (85%) HTLV-I and 14 of 20 (70%) HTLV-II cultures were positive for p24 antigens. None of the 17 HTLV-seroindeterminate or six HTLV-seronegative specimens were positive for the presence of p24 antigen. The isolation rates for HTLV-I and HTLV-II by an alternative whole-blood lysis procedure were comparable to those obtained by standard PBMC cultures. Furthermore, cocultivation of PHA-stimulated PBMC from healthy donors with lethally irradiated HTLV-I- and HTLV-II-infected cell lines (SP and Mo-T, respectively) resulted in productive viral infection, as reflected by the appearance of $p24^{gag}$ antigens concomitant with specific genomic amplification of HTLV proviral DNA after 3 weeks of cocultivation. Thus, the coculti-vation technique provides a highly sensitive and specific procedure both for HTLV isolation and for infection of target cells.

Human T-lymphotropic virus type I (HTLV-I) is the etiologic agent of adult T-cell leukemia (ATL) and HTLV-I-associated neuropathy (HAM) [4, 18, 25]. HTLV-II, a closely related retrovirus, has not been associated with clinical disease [18], although both HTLV-I and HTLV-II have tropism for T-lymphocytes [24]. Antigenic analysis of HTLV-I has led to the development of both indirect and direct methods for identifying HTLV-I-infected persons [1]. Indirect serologic tests are based upon the fact that almost all HTLV-I-infected individuals develop antibodies specifically directed against HTLV-I proteins. Furthermore, because of the significant antigenic similarity between HTLV-I and HTLV-II, antibody assays for HTLV-I have commonly been used for detecting HTLV-II antibodies as well [4, 18].

In contrast, direct demonstration of HTLV-I/II in infected individuals is based on the detection of the viral genome or proteins in the body fluids. Although in situ hybridization [2] and immunofluorescence assays have been used to detect the presence of viral nucleic acid sequences and viral proteins [10], respectively, both of these procedures are time consuming and not suited for large epidemiologic studies. In addition, the detection of reverse transcriptase activity, a generic marker of retroviral infection [8], is highly insensitive for HTLV detection. Therefore, the presence of viral antigens, as measured by antigen capture assays, is considered to be the most sensitive and specific assay for detecting virus in infected lymphocyte cultures [10, 23, 26]. The two commercially available antigen capture assays for HTLV detection use a monoclonal antibody directed against either a type-specific epitope of $p19^{gag}$ of HTLV-I[23] or a common epitope of $p24^{gag}$ of both HTLV-I and HTLV-II [26]. The former assay specifically detects antigen in HTLV-I-infected cultures, whereas the latter detects antigens in both HTLV-I- and HTLV-II-infected cultures. However, optimal culture procedures and the kinetics of antigen expression are not well characterized.

In vitro infection with cell-free HTLVs has been difficult to demonstrate when compared with infec-

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tion involving other retroviruses, including human immunodeficiency virus-I [8]. Recent studies have identified the presence of newly synthesized HTLV-DNA after incubation with purified HTLV-I virions [7], but it is not known whether a replication-competent virus could be isolated by this procedure. Several investigators have utilized a cocultivation system using lethally irradiated, HTLV-I-infected donor cells with activated target cells, and infection is detected by the presence of viral antigens or syncytia production in the target cells [5, 10, 27]. However, neither the kinetics of acute infection nor infection with HTLV-II has thus far been tested.

In the present study, we describe optimal culture conditions for isolation of HTLVs from peripheral blood mononuclear cells (PBMC) as well as whole blood leukocytes from individuals infected with HTLV-I and HTLV-II. We have also developed procedures for successful transmission of HTLV-I and HTLV-II to the lymphocytes. Finally, we demonstrate the ability of the infected lymphocyte to express replication-competent virus, concomitant with soluble *gag* antigen released in the culture supernatants.

Materials and Methods

Culture techniques for HTLV isolation. PBMC were stimulated with 0.1% phytohemagglutinin (PHA-P; DIFCO Co., Detroit, Michigan) and cultured at a density of 2×10^6 cells/ml in RPMI-1640 with 15% fetal bovine serum (Gibco, Grand Island, New York) (C-RPMI) and 10% partially purified interleukin-2 (IL-2; Advanced Biotechnologies Inc., Silver Spring, Maryland). After 72 h of culture, the lymphocyte cultures were subcultured in 24-well plates (Costar) either as primary cultures or cocultured at equal ratios (each 2×10^6 cells/ml) with PHA-stimulated PBMC from an HTLV-seronegative donor. The culture supernatants were collected every 3 days, and soluble HTLV antigen production was determined by an antigen capture assay (Coulter Immunology, Hialeah, Florida) with monoclonal antibody to $p24^{gag}$ that recognizes *gag* epitopes common to both HTLV-I and HTLV-II [26]. In some experiments, primary cultures were maintained in the presence of the rIL-2, rIL-6, and recombinant tumor necrosis factor-α (TNFα; Genzyme Corp) or as a mixture of all three cytokines for 28 days in cultures (100 U/ml and 10 U/ml). The culture supernatants were assayed for soluble HTLV antigen as described above.

The whole-blood lysis procedure (Immunolyse, Coulter Immunology) was used according to the manufacturer's instructions with slight modifications. Briefly, 1-ml sample of heparinized blood was suspended in 9 ml of 1 : 10 diluted sterile ammonium chloride-based lysing solution to lyse the erythrocytes. After 2–3 min, cells were washed three times in RPMI and cultured as described above.

Electron microscopic analysis. The ultrastructural analysis for HTLV particles was performed on 21-day cultured PBMC. After two washes in 0.2 M sodium cacodylate buffer, cells were fixed in 2.5% buffered glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through a graded series of alcohols and propylene oxide, and embedded in Epon/Araldite epoxy resins. Sections were stained with uranyl acetate and lead citrate.

Productive HTLV infection. Long-term T-cell lines derived from an HTLV-I-infected patient (SP; [12]) and an HTLV-II-infected patient with hairy-cell leukemia (Mo-T) were maintained in C-RPMI. Acute infections were established by cocultivating an equal number of irradiated SP or Mo-T cell lines (6,000 and 10,000 rads) with PHA-stimulated normal PBMC. Culture supernatants were collected every third day to test for presence of viral antigens. Two million cells each were harvested from the irradiated cell line cocultivated with normal PBMC, the irradiated cell line alone, and the nonirradiated cell line cultured at the similar cell density at the indicated time intervals and extracted in polymerase chain reaction (PCR) lysis buffer at 6×10^6 cells/ml. Previous studies have demonstrated that the HTLV-I-infected cell line SP leads to productive infection in an experimental rabbit model system [12].

Polymerase chain reaction. The amplification and detection of HTLV sequences by PCR were performed on DNA specimens from HTLV-positive individuals and their cultured PBMC. Total genomic DNA was prepared from 6×10^6 cells in PCR lysis buffer (50 mM KCl; 10 mM Tris HCl, pH 8.3; 2.5 mM $MgCl_2$; 1 mg/ml gelatin; 0.45% NP-40; 0.45% Tween-20) supplemented with 60 μg/ml of proteinase K for 1–3 h at 56°C. Proteinase K was subsequently inactivated by heat at 95°C for 10 min. PCR was performed with minor modifications under the conditions described previously [15]. Briefly, 50 μl of cell lysate (approximately 2 μg DNA) and 200 ng of each primer were amplified for 35 cycles at 94°C for 1 min, 45°C for 1 min, and 72° for 30 s in a thermocycler (Perkin Elmer, Emeryville, California). The amplified products were analyzed by 1.2% agarose gel electrophoresis and Southern blot hybridization to ^{32}P end-labeled oligonucleotide probes. The amplification of the *pol* region with HTLV-I/II consensus primers (SK110/111) and type-specific probes (SK112 for HTLV-I and SK188 for HTLV-II) were used to test for the presence of the HTLV genome [9, 11]. The 5′–3′ sequences of the primer pairs and probes from the *pol* region, based on HTLV-I (GenBank accession no J02029) and HTLV-II (GenBank accession no M10060) sequences, were as follows: SK110 (*pol*, HTLV-$I^{4757-4778}$, HTLV-$II^{4735-4756}$)- CCC TAC AAT CCA ACC AGC TCA G; SK111 (*pol*, HTLV-$I^{4942-4919}$, HTLV-$I^{4920-4897}$)- GTG GTG AAG CTG CCA TCG GGT TTT; SK112 (*pol*, HTLV-$I^{4825-4840}$)- GTA CTT TAC TGA CAA ACC CGA CCT AC; SK188 (*pol*, HTLV-$II^{4880-4998}$)- TCA TGA ACC CCA GTG GTA A.

Reference HTLV antibody tests. Serum specimens were tested for HTLV-I/II antibodies with a commercial enzyme-immunoassay (Abbott, Chicago, Illinois), and repeatedly reactive specimens were further tested by Western blot (WB) with HuT-102 lysate (Cambridge Biotech, Rockville, Maryland), and by radioimmunoprecipitation (RIPA) with HTLV-I-infected MT-2 lysates [15]. Sera containing antibodies to at least two HTLV structural gene products (*gag* p24 and *env* gp46 and/or gp68, r21e), by WB alone or in combination with RIPA, were considered positive (HTLV-positive), whereas sera reacting with only *gag* gene products were considered indeterminate (HTLV-indeterminate). Sera exhibiting repeat EIA reactivity but no reactivity to any HTLV gene product were considered negative (HTLV-negative).

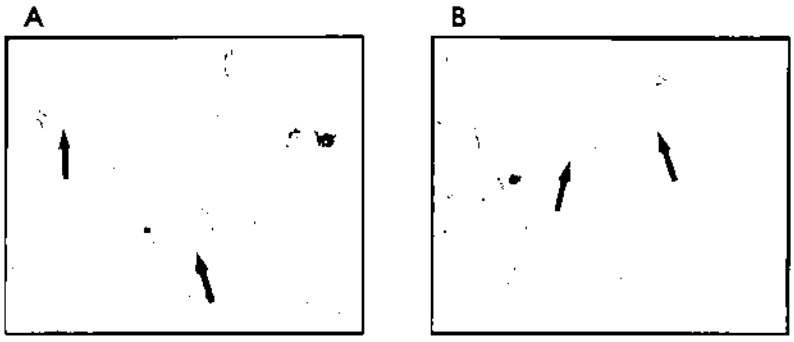

Fig. 1. Electron microscopic analysis of 21-day cocultured PBMC from two individuals (original magnification, 94,300). The arrows indicate the presence of mature viral particles resembling HTLVs in extracellular spaces.

Study population. Blood specimens were collected from the following individuals who were previously identified to be seropositive for antibodies to HTLV-I/II: three patients with HAM, 20 blood donors, and six injecting drug users from the United States, and four female prostitutes from Mexico. PCR analysis of PBMC demonstrated that all three HAM patients were infected with HTLV-I, 10 of 20 blood donors were infected with HTLV-I, and the other 10 blood donors were infected with HTLV-II. All six injecting drug users and all four prostitutes were infected with HTLV-II. For comparison, 17 blood donors with HTLV-indeterminate reactivity (*gag* antibodies only) and six blood donors seronegative for HTLV were included as controls. None of the seroindeterminate or seronegative specimens had evidence of HTLV genome by PCR analysis. All of the individuals in the present study were screened for antibodies to human immunodeficiency virus type 1 (HIV-1) and were found to be negative.

Results and Discussion

Virus isolation procedures for HTLVs. To optimize the culture techniques best suited for isolation of HTLVs, PBMC from 11 HTLV-infected individuals were either cultured with PHA alone (primary) or cocultured with PHA-stimulated normal PBMC (coculture). Whereas only 3 of the 11 (27%) primary cultures contained detectable levels of $p24^{gag}$ antigens, 9 of the cocultures (82%) had detectable levels of HTLV antigens by day 14 in cultures (data not shown), suggesting that the mixed lymphocyte reaction (MLR) provided by the cocultivation may be required for the isolation of these cell-associated T-lymphotropic viruses [16]. Electron microscopic analysis of the cultures revealed mature retroviral particles resembling HTLVs in extracellular spaces (Fig. 1). No viral antigens could be detected from the remaining two cultures. Of the three positive primary cultures, two were from patients with HAM, supporting an earlier finding in which HTLV-I antigens were detectable in approximately half of primary cultures from HAM patients, by an indirect immunofluorescence technique [21].

We further attempted to analyze parameters that could enhance sensitivity of isolations from primary cultures. The spontaneous release of IL-6 and TNFα in cultures derived from patients infected with HTLV-I and HTLV-II [13] together with the role of TNFα in mixed lymphocyte reaction (MLR) [19] prompted us to analyze the effect of exogenously added cytokines (rIL-2, rIL-6, and rTNFα) on primary culture isolations. In parallel, these same cultures were cocultured with PHA-stimulated normal donor PBMC. While $p24^{gag}$ antigen could be detected in five of six (83%) cocultured supernatants, none of the cytokines were capable of enhancing viral isolations alone or in combination with other cytokines (data not shown).

Although the precise mechanism leading to viral replication and production during cocultivation is not known, recent studies have demonstrated the requirement of intracellular adhesion molecule-1 (ICAM-1) in the initiation of MLR [22]. Indeed, soluble levels of sICAM-1 have recently been demonstrated in the serum from HTLV-I-infected patients, suggesting a role for this molecule in viral expression [17]. Of greater significance is the finding that expression of the cytokines alone is not sufficient for viral expression; instead, viral expression requires the effect of at least one additional signal provided by cocultivation of activated PBMC of normal donors. These results suggest that the MLR induced by cocultivation provides a cell-to-cell interaction or additional soluble product(s) necessary for HTLV-I/II antigen expression.

The cocultivation culture technique was next applied to specimens of PBMC infected with either

HTLV-I or HTLV-II. Of the 13 PCR-confirmed HTLV-I-infected PBMC, 11 (85%) cultures had detectable levels of $p24^{gag}$ antigens in the supernatants. Similarly, of the 20 PCR-confirmed HTLV-II-infected PBMC from individuals with various risk factors, 14 (70%) cultures had detectable levels of $p24^{gag}$ antigens. Our inability to detect virus from culture supernatants in 2 of 13 HTLV-I and 6 of 20 HTLV-II PCR-confirmed seropositive individuals presumably reflects the low frequency of HTLV-infected cells in the peripheral blood. We have not excluded the possiblity that virus-negative cultures may be the result of HTLV-infected individuals harboring replicative defective HTLVs. Moreover, the sensitivity of positive cultures in a clinical setting largely depends upon the sensitivity of soluble antigen detection, in this case of p24 antigen. It is possible that more sensitive PCR-based assay might enhance sensitivity of current detection systems. Indeed, PCR amplification of total RNA after reverse transcription has yielded a greater sensitivity of HTLV detection when compared with $p24^{gag}$ antigen production from the same cultures (unpublished observation). Taken together, our results indicate that virus culture alone should not be used to diagnose HTLV-infection.

The cocultivation technique for isolation of HTLV-I/II is similar to what has previously been used for isolation of HIV-1 [3, 6], although the kinetics of viral-specific antigen production is earlier (days 5–10) for HIV-1 when compared with that for HTLV-I/II (days 14–21). In addition, the efficiency of HIV-1 isolation appears to be higher than that for HTLV-I/II and could either be due to high viral load or the cell-free nature of the HIV-1. While the exact mechanism of human retrovirus isolation by co-cultivation remains to be determined, it is postulated that the mixed cultures of immunocompetent cells promote the presentation of a variety of stimuli, including viral antigens as well as heterologous HLA and cell surface markers that in turn trigger a complex cascade of immune modulators from the participating cells.

The specificity of the culture system for virus isolation was next examined in specimens derived from individuals with isolated *gag* antibodies to HTLV (HTLV-indeterminate). Analysis of culture supernatants from 17 HTLV-indeterminate specimens demonstrated no evidence of HTLV infection; in none of the 17 specimens was HTLV-genome present, as tested by PCR [15]. The seroindeterminate patterns are believed to result from antigenic mimicry of the *gag* proteins with other microbial and cellular proteins, including endogenous retroviral sequences [15]. Indeed, expression of human endogenous retroviral sequences in healthy individuals has been reported, providing further evidence that such antigens have the potential to yield cross-reactive antibodies [20].

Table 1. Virus isolations in PBMC or whole-blood cultures from HTLV-I/II-positive and HTLV-indeterminate specimens

Group	PBMC cultures		Whole blood cultures	
	#pos/ # tested	%	#pos/ #tested	%
PCR-confirmed HTLV-I:				
HAM	3/3	100	1/1	100
Asymptomatic	8/10	80	3/4	75
PCR-confirmed HTLV-II:				
Blood donors	7/10	70	4/7	57
Injecting drug users	4/6	67	3/4	75
Prostitutes	3/4	75	ND[a]	ND
HTLV-seroindeterminate:				
Blood donors	0/17	0	ND	ND

[a] ND, not done.

Recently, a whole-blood lysis procedure has been successfully used for phenotypic analysis of lymphocytes [14], as well as for PCR asssays to detect the presence of HTLV genome [9]. Because of the ease of the specimen preparation, we next used a whole-blood lysis procedure to obtain mononuclear cells for the isolation procedure. Similar to virus isolations from PBMC, 4 of 5 (80%) of HTLV-I- and 7 of 11 (64%) of HTLV-II-infected blood specimens had detectable levels of $p24^{gag}$ antigen (Table 1). Thus, the frequency of virus isolation by whole blood was comparable to that obtained by separated lymphocytes. Some of the antigen-positive cultures have now been maintained for over 2 years, with stable $p24^{gag}$ antigen production. The whole-blood procedure has several advantages over separated PBMC procedure in that less than 1 ml of blood is needed, and the time and reagents needed for lymphocyte separation are eliminated. More importantly, the whole-blood lysis method results in a preparative yield of all leukocytes. Therefore, if the virus is harbored in cells other than lymphocytes, the likelihood of isolation would increase. In addition, the procedure would also be suitable for pediatric cases when large amounts of blood cannot be drawn.

Productive infection with HTLVs. The in vitro infection with cell-free HTLV-I has been difficult to dem-

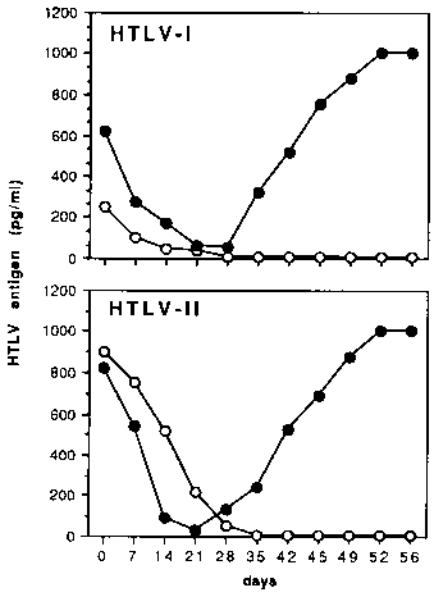

Fig. 2. Infection of normal PBMC with HTLV-I (SP cell line) or HTLV-II (Mo-T cell line). PHA-activated PBMC (2×10^6) were cocultured with equal numbers of lethally irradiated (6000 rad) SP (Panel A) or Mo-T (Panel B) cell lines. The culture supernatants were tested for presence of $p24^{gag}$ antigen in cocultured cells (closed circles) or irradiated cells alone (open circles). The results shown are representative data from one of eight experiments for HTLV-I and one of five experiments for HTLV-II.

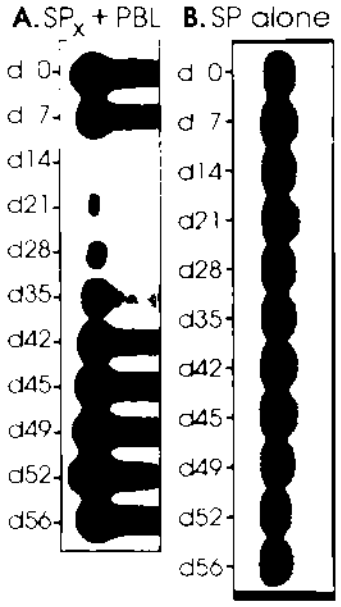

Fig. 3. Kinetic expression of HTLV-I *pol* gene sequences. Southern blot analysis of enzymatic amplified *pol* sequences during cocultivation of normal PBMC with irradiated SP (A) or nonirradiated SP cells (B) cultured for up to 56 days in a similar way as cocultures.

onstrate, because cell-free particles do not effectively infect target cells [5, 27]. We therefore developed a cocultivation procedure, using irradiated HTLV-I-infected (SP) or HTLV-II-infected (Mo-T) cell lines with PHA-activated normal PBMC; cultures were monitored for 56 days postcocultivation. The residual $p24^{gag}$ antigens from the irradiated input cell lines could be detected in the cultures for both HTLV-I (Fig. 2a) and HTLV-II (Fig. 2b) for up to 21 days, after which time a steady increase in the levels of $p24^{gag}$ antigen was observed. The presence of viral antigens during the initial phase of the cultures represents residual antigens released by the dying cells. Our repeated attempts to reduce the levels of residual $p24^{gag}$ antigens in the cultures by extensive washing after cocultivation or increasing the dose of lethal radiation (10,000 rad) were unsuccessful (data not shown).

The kinetic expression of cellular HTLV-genomic sequences (pol) was determined in parallel to supernatant $p24^{gag}$ antigen production. Although no detectable sequences could be amplified from the irradiated SP cell line after day 14 of culture alone, cocultivation with normal PBMC resulted in reappearance of strong *pol* hybridized products by day 28 (Fig. 3) and followed a similar kinetics as the antigen production in the culture supernatants (Fig. 2). The nonirradiated SP cell line demonstrated stable production, as evidenced by hybridized bands at all the time points (Fig. 3). These results demonstrate a slower kinetics of acute spreading HTLV infection than that seen for HIV-1, for which acute infection is followed by marked cytopathicity and can be observed by day 10 in most cultures [8].

In summary, we have demonstrated the presence of newly synthesized viral proteins and proviral HTLV-DNA in 28 days in culture for both HTLV-I and HTLV-II. These results corroborate previous reports that cell-to-cell contact is required for transmission of HTLV-I to target cells [5, 27]. The experimental system presented here would allow us to determine not only the cellular tropism for HTLVs, but also T-cell functional abnormalities after infection.

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Clinical and Diagnostic Virology 9 (1998) 17–23

Clinical and Diagnostic Virology

Molecular detection and isolation of human T-cell lymphotropic virus type I (HTLV-I) from patients with HAM/TSP in São Paulo, Brazil

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Received 16 April 1997; received in revised form 21 October 1997; accepted 28 October 1997

Abstract

Background: Infection with HTLV-I is etiologically linked with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). However some patients with chronic progressive paraparesis resembling HAM/TSP have been shown to be infected with HTLV-II.

Objective: To clarify the role of each of these human retroviruses in the etiology of HAM/TSP in São Paulo, Brazil.

Study design: A detailed serological and molecular analysis of HTLV-I/II infection was performed in a cohort of 19 patients with HAM/TSP attending a neurological clinic.

Results: Plasma samples analyzed for anti-HTLV-I/II antibodies using a Western blot assay, comprising HTLV-I ($rgp46^{I}$)- and HTLV-II ($rgp46^{II}$)-specific recombinant *env* epitopes, demonstrated reactivity to $rgp46^{I}$ and hence were typed as seropositive for HTLV-I. Presence of HTLV genomic sequences in peripheral blood mononuclear cells (PBMC) was sought after by PCR using consensus primers SK 110 and SK 111 for the *pol* region of HTLV proviral DNA followed by hybridization with type-specific probes SK 112 (HTLV-I) and SK 188 (HTLV-II). Southern blots from all individuals hybridized with SK 112 but not with SK 188, further confirming HTLV-I infection. Cocultivation of PBMC from eight of these patients with activated lymphocytes from normal individuals resulted in active viral production, detected as presence of soluble $p24^{gag}$ antigen in culture supernatants. Investigation of risk factors for HTLV-I infection in these individuals revealed that five out of 19 patients studied (26.3%) had received blood transfusions previous to disease onset.

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PII S0928-0197(97)10015-0

Conclusions: We demonstrate HTLV-I as the only viral type involved in the etiology of HAM/TSP in a cohort from São Paulo, Brazil, and emphasize that prevention measures, including widespread routine screening of blood donations for HTLV should be conducted in Brazil.

Keywords: HAM/TSP; HTLV-I; Diagnosis; PCR; Cell culture

1. Introduction

Tropical spastic paraparesis or HTLV-I-associated myelopathy (HAM/TSP) is a chronic neurological syndrome, etiologically linked to human T-cell lymphotropic virus type I (HTLV-I) and clinically characterized by progressive crural paraparesis with involvement of the pyramidal tract that may lead to severe disablement, urinary sphincter dysfunction, sexual impotence and mild sensory disturbances (Gessain et al., 1985; Osame et al., 1986). However, patients with a HAM/TSP-like syndrome have been reported to be infected with HTLV-II, a genetically related retrovirus that had not been previously associated with any human disease (Hjelle et al., 1992; Jacobson et al., 1993).

Both HTLV-I and HTLV-II are transmitted by transfusion of cell-component blood products or through sharing of blood-contaminated needles, through sexual intercourse and from infected mother to child, specially by protracted breast feeding. Infection with HTLV-I is endemic among non-related populations in southern Japan, the Caribbean area, Central and West Africa, Central and South America, Melanesian islands and some other regions of the world (Blattner, 1990), whereas high seroprevalence of HTLV-II infection is detected in Native Amerindians (Maloney et al., 1992) and in intravenous drug users from Europe and the USA (Zella et al., 1990; Khabbaz et al., 1992). High genetic homologies between both viral types is responsible for their serological cross-reactivity. However type-specific seroassays and molecular diagnostic techniques have been developed, which allow distinction between HTLV-I and HTLV-II infections (Lipka et al., 1992; Heneine et al., 1992).

HTLV-I/II infections have been shown to be endemic in Brazil, and 0.3% of asymptomatic blood donors are detected as seropositive for these retroviruses in routine screening in São Paulo (Matutes et al., 1994). Likewise, occurrence of TSP/HAM in Brazil has been confirmed by detection of HTLV-I antibodies in patients' serum or CSF in sporadic case reports (Castro et al., 1989; Takayanagui et al., 1991; Castro-Costa et al., 1995; Araujo and Andrada-Serpa, 1996) and HTLV-I isolates from Brazilian HAM/TSP patients have been sequenced (Liu et al., 1996). We have been following a cohort of Brazilian HAM/TSP patients at the Neurology outpatient clinic, Hospital das Clinicas, School of Medicine, University of São Paulo, in São Paulo, Brazil and collecting information on their clinical and epidemiological features since 1989. In the present study we have undertaken a serological and molecular confirmation of HTLV infection in these HAM/TSP patients. Our data indicate the presence of HTLV-I-specific antibodies in serum and HTLV-I proviral sequences in PBMC of all HAM/TSP patients and further demonstrate virus isolation from several patients.

2. Material and methods

2.1. Study population

Nineteen symptomatic adult patients with chronic progressive paraparesis seen at the outpatient clinic at the Department of Neurology, School of Medicine, University of São Paulo, from July to December 1993 were enrolled for the study. They presented progressive weakness and spasticity of lower limbs. After clinical and neurological examination, they had blood and CSF specimens collected for diagnostic investigation. Informed consent was obtained prior to clinical specimen collection. PBMC and plasma were separated using the Ficoll–Hypaque gradient method and cells cryopreserved.

2.2. HTLV antibody assays

Initial HTLV serological screening was carried out in São Paulo. All sera and CSF samples were tested in duplicate with commercially available enzyme immunoassay (EIA) kits (Ortho Diagnostics, Raritan, NJ, USA; Organon Teknika Corp., Durham, NC, USA; Embrabio, São Paulo, Brazil), and repeatedly reactive specimens were confirmed by a Western blot (WB) that incorporates a recombinant transmembrane protein antigen (r21e) to the whole virus lysate (Cambridge Biotech. Research Laboratories, Rockville, MD, USA). Samples were considered seropositive when reactivity was shown to both *gag* (p24) and *env* (r21e or gp46) proteins. Additional serological confirmation and discrimination between HTLV-I and HTLV-II were carried out at the Retrovirus Diseases Branch, Centers for Disease Control and Prevention in Atlanta, GA, USA. A modified Western blot, containing type-specific immunodominant epitopes of the external glycoprotein (rgp46^{I} and rgp46^{II}) and a recombinant transmembrane antigen (r21e) added to viral lysate (Diagnostic Biotechnology Ltd., Genelabs Diagnostics, Singapore), was used for simultaneous confirmation and discrimination between HTLV-I and HTLV-II infections. Serological confirmation was based upon reactivity to *gag* (p24) and *env* (rgp46^{I} or rgp46^{II} and r21e) proteins and discrimination was achieved by demonstration of reactivity to either rgp46^{I} (HTLV-I) or rgp46^{II} (HTLV-II).

2.3. HTLV PCR assays

Polymerase chain reaction (PCR) was used for the amplification of HTLV proviral sequences from PBMC of all patients, as previously described (Heneine et al., 1992). Briefly, cell lysates were obtained by proteinase K digestion and amplification of the proviral *pol* region was carried out using consensus primers (SK 110, 5′-CCC TAC AAT CCA ACC AGC TCA G-3′; and SK 111, 5′-GTG GTG AAG CTG CCA TCG GGT TTT-3′). Amplified DNA was transferred to nitrocellulose membranes and hybridization of Southern blots with specific ^{32}P-end-labeled oligonucleotides (SK 112, 5′-GTA CTT TAC TGA CAA ACC CGA CCT AC-3′; and SK 188, 5′-GAC CCG ATA ACG CGT CCA TCG-3′) enabled discrimination between HTLV-I (SK 112) and HTLV-II (SK 188).

2.4. Viral isolation

PBMC from eight patients were cocultivated with PHA-stimulated peripheral blood lymphocytes from a normal donor in RPMI-1640, with 15% fetal bovine serum and 10% partially purified interleukin-2 (IL-2), as previously described (Kitamura et al., 1993). Samples were chosen for coculture setup, according to cell viability in cryopreserved specimens. Culture supernatants were collected on days 7, 14, 21 and 28, and soluble HTLV p24^{gag} antigen was sought after by an antigen capture immunoassay, using a monoclonal antibody (Toedter et al., 1992).

3. Results

Serum and CSF specimens from all 19 patients enrolled in this study demonstrated HTLV-specific antibodies on both EIA- and r21e-spiked WB tests. Their ages ranged from 33 to 74 years (mean 48.5). Seven (36.8%) were male and 12 (63.2%) were female. Five were white, four were black, and 10 were of mixed black and white race. None was of Caribbean descent. Risk factors for HTLV infection included sexual partnership with a seropositive individual (one case) or with a sexually promiscuous partner (one case), sexual promiscuity (three cases), and past history of blood transfusion predating HAM/TSP onset (five cases). Demographic data, risk factors and clinical features of the studied population are summarized in Table 1. Patients were symptomatic for 10 months to 13 years (mean 75.5 months). Onset of disease was predominantly asymmetrical. Sphincter dysfunction and sensory involvement were seen in 17 of 19 (89.4%) patients.

Serological analysis of the 19 specimens on modified Western blot revealed that all reacted with *gag* (p19, p24) and *env* (r21e) proteins. All reacted with HTLV-I-specific recombinant

Table 1
Epidemiological and clinical features of 19 patients with chronic progressive paraparesis from São Paulo, Brazil

Patient	Age sex	Race	Risk factor	Clinical features		
				Duration of illness (months)	Onset	Severity of paraparesis
01	45 M	w	Unknown	84	S	I ·
02	62 F	w	Unknown	120	A	+ −
03	64 F	w-b	Unknown	48	A	+ − +
04	42 F	b	Blood transfusion	132	A	+ −
05	54 F	w-b	Blood transfusion	90	A	I ·
06	39 F	b	Unknown	72	A	+ − +
07	57 F	w	Blood transfusion	60	A	+ −
08	53 F	w-b	Sexual promiscuity	36	A	+ −
09	33 M	w-b	Unknown	36	S	I
10	33 F	b	Unknown	10	S	+ −
11	38 M	w-b	Sexual promiscuity	152	S	+ −
12	34 M	w	Sexual promiscuity	72	A	+
13	45 M	w-b	Unknown	144	A	I · I
14	43 F	w-b	Blood transfusion	90	A	+
15	35 F	w-b	Unknown	36	A	+ −
16	53 F	w-b	Seropositive spouse	36	A	+ − +
17	74 F	w	Blood transfusion	120	S	I ·
18	53 M	b	Unknown	72	A	+ −
19	65 M	w-b	Sexual promiscuity	24	A	+ − +

Race: w, white; b, black; w b, white black mixed race. Onset: A, asymmetrical; S, symmetrical. Severity of paraparesis: I, walks with no support; + +, walks with support; + + −, wheelchair restricted. All except patients 4 and 9 had sphincter dysfunction, and all except patients 1 and 11 also had sensory involvement.

protein, thus classifying them as HTLV-I infected. Two of the specimens also showed reactivity with HTLV-II-specific rgp46^{II}, attributed to serological cross-reactivity of *env* epitopes, since none of the specimens amplified HTLV-II-specific sequences (see below).

Presence of HTLV genomic sequences in PBMC was sought after by PCR. Amplification of the *pol* region with consensus primers followed by hybridization with HTLV-I-specific probe (SK 112) revealed HTLV-I proviral sequences in all 19 samples. None of the specimens, including two from patients who had demonstrated seroreactivity to HTLV-II-specific *env* protein rgp46^{II}, hybridized with HTLV-II-specific probe (SK 188) (see 12 representative samples in Fig. 1). These data confirm that all patients are infected with HTLV-I.

To further examine whether these individuals harbored replication-competent HTLV-I, viral isolation was attempted in eight HAM/TSP patients. Culture supernatants from all eight patients demonstrated presence of p24^{gag} antigen by day 7, with continuous viral antigen production through day 28. Four of these cultures have been maintained through day 56 with consistent viral antigen production.

4. Discussion

After the original description of HAM/TSP as a chronic myelopathy caused by HTLV-I in the Caribbean basin and the Southern islands of the Japanese archipelago (Gessain et al., 1985; Osame et al., 1986), similar cases have been reported from different parts of the world, including South America (Castro et al., 1989; Takayanagui et al., 1991; Dekaban et al., 1992; Castro-Costa et al., 1995; Araujo and Andrada-Serpa, 1996). In Brazil, HAM/TSP patients have been recognized through their clinical manifestations and through diagnostic confirmation, based on serological or molecular techniques. Asymptomatic HTLV-I/II

infection is also being currently identified in the country after routine screening of blood donors for HTLV was implemented in some areas, or as a result of seroepidemiological surveys carried out in high-risk groups for human retroviral infection (Cortes et al., 1989; Moreira et al., 1993). This shows that HTLV-I infection is prevalent in the country and that additional cases of HTLV-related illnesses may be further diagnosed as awareness of their occurrence and diagnosis increases among physicians.

Our study has been able to identify HTLV-I infection in 19 Brazilian patients with chronic progressive paraparesis, by serological evidence of type-specific reactivity to $rgp46^{I}$, which is unique to HTLV-I. In all cases, PCR amplification of *pol* sequences that hybridized with HTLV-I-specific probes only was shown. None of the *pol* fragments hybridized with HTLV-II-specific probes, therefore all studied Brazilian HAM/TSP cases were demonstrated as HTLV-I positive.

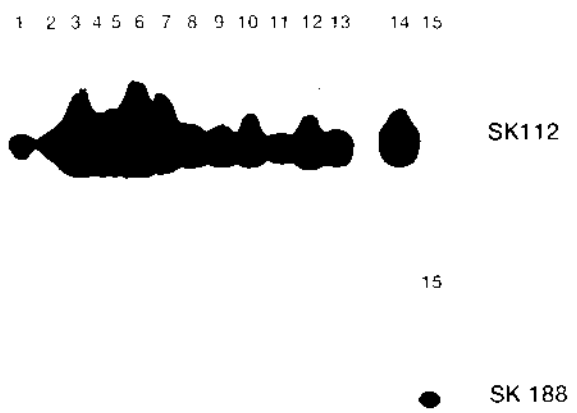

Fig. 1. Southern blot hybridization analysis of proviral DNA from peripheral blood mononuclear cells (PBMC) of HAM TSP patients. All DNA specimens from HAM TSP patients were amplified using consensus primers (SK 110 111) for HTLV-I II *pol* region and probed with HTLV-I-specific (SK 112) and HTLV-II-specific (SK 188) oligoprobes. Lanes 1–13 show representative specimens from HAM TSP patients. Positive controls for HTLV-I and HTLV-II are shown on lanes 14 and 15, respectively.

Two of the 19 sera specimens also showed reactivity to HTLV-II-specific *env* recombinant protein ($rgp46^{II}$) on Western blot assays. We believe this could reflect cross-reactivity of high-titered antibodies in HAM/TSP patients with HTLV-II protein. As there are several shared amino acids between $rgp46^{I}$ and $rgp46^{II}$, sera containing high-titered antibodies to HTLV could potentially cross-react with both of these recombinant antigens. In this regard, Western blot reactivity patterns alone should not be taken as reliable evidence for the diagnosis of dual HTLV-I/HTLV-II infections, and molecular techniques should always be used with this particular aim. Even though inadequate sensitivity of HTLV-II PCR could also be have been argued, this seems unlikely since molecular techniques using consensus primers for the amplification of *pol* sequences of proviral DNA have clearly been shown to possess high sensitivity and specificity (Heneine et al., 1992). Furthermore, this study also includes the report of viral isolation from peripheral blood mononuclear cells of HAM/TSP patients in Brazil. Replication-competent virus could be isolated from 100% of tested samples and continuous viral antigen production in culture supernatants was seen for nearly 2 months. The ease of isolation within 7 days in culture indicates high proviral load in these individuals. Long-term maintenance of in vitro co-cultures of PBMC may in fact lead to the establishment of infected cell lines as previously described (Andrada-Serpa et al., 1995).

Transfusion-transmitted HTLV-I has been reported to be an important cause of HAM/TSP in Japan (Osame et al., 1990), and the establishment of compulsory serological screening of HTLV-I-infected blood donors may reduce the incidence of the disease in that country. Likewise we have identified that five of our HAM/TSP patients had received blood transfusions prior to disease onset and did not have other risk factors for retroviral exposure. Even though biological samples from these patients, collected previously to transfusion, were unavailable and a look-back study could not be carried out in order to identify the HTLV-I serostatus of their blood donors, we may presume that transfusion-transmitted HTLV-I infection

may have caused HAM/TSP in these individuals. Therefore one should emphasize the need for wider serological screening of blood donors for HTLV-I infection in areas where seroprevalence of infection is significant, such as occurs in Brazil.

In conclusion, HTLV-I was shown to be the only viral type involved in the etiology of HAM/TSP in a cohort of HTLV-seropositive patients with chronic progressive paraparesis from São Paulo, Brazil. Recognition of the involved pathogen in this population was achieved by modified Western blot tests, comprising recombinant proteins representing immunodominant regions of envelope glycoprotein, by proviral DNA amplification, and by virus isolation from peripheral blood.

Acknowledgements

Aluisio A.C. Segurado was supported by a grant from CNPq/Secretary of Science and Technology, Brasilia, Brazil.

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Transformed T-lymphocytes infected by a novel isolate of human T cell leukemia virus type II

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Transformed T Lymphocytes Infected by a Novel Isolate of Human T Cell Leukemia Virus Type II

By T.L. Chorba, R. Brynes, V.S. Kalyanaraman, M. Telfer, R. Ramsey, A. Mawle, E.L. Palmer, A.T.L. Chen, P. Feorino, and B.L. Evatt

Human T cell leukemia virus type II (HTLV-II) has been isolated from a patient (Mo) with features of leukemic reticuloendotheliosis (LRE) and from a patient with acquired immunodeficiency syndrome (AIDS). We have obtained another isolate of HTLV-II from a patient (CM) with severe hemophilia A, pancytopenia, and a 14-year history of staphylococcal and candidal infections but no evidence of T cell leukemia/lymphoma, AIDS, or LRE. Fresh mononuclear cells and cultured lymphocytes from CM express retroviral antigens indistinguishable by molecular criteria from HTLV-II_{Mo}. Leukocyte cultures from CM yield hyperdiploid (48,XY,+2,+19) continuous lymphoid lines; human fetal cord blood lymphocytes (CBL) are transformed by cocultivation with these CM cell cultures but retain normal cytogenetic constitution. Electron microscopic examination of the CM cultures and transformed CBL reveals budding of extracellular viral particles, intracellular tubuloreticular structures, and viral particles contained within intracellular vesicles. CM cell cultures and the transformed CBL do not require exogenous interleukin 2, have T cell cytochemical features and mature T helper phenotypes, and exhibit minimal T helper and profound T suppressor activity on pokeweed mitogen-stimulated differentiation of normal B cells. These characteristics, which are similar to those observed with the first HTLV-II isolate, may represent properties of all HTLV-II–infected T cells.

HUMAN T CELL LEUKEMIA VIRUS (HTLV) was initially isolated from cases of adult T cell leukemia/lymphoma in the United States[1,2] and differs from all other known animal retroviruses in studies involving nucleic acid hybridization,[3] immunologic analyses of structural proteins[4,5] and reverse transcriptase,[6] and amino acid sequencing of structural proteins.[7] HTLV-II is a second subtype that is molecularly distinct from HTLV-I,[8,9] and from HTLV-III/lymphadenopathy virus (LAV), the subtype associated with acquired immunodeficiency syndrome (AIDS).[10] HTLV-II was originally obtained from a patient (Mo) with clinical and pathologic features of hairy cell leukemia/leukemic reticuloendotheliosis (LRE).[11,12] From the splenic tissue of patient Mo, a cell line (Mo-T) was derived that formed spontaneous rosettes with sheep red blood cells and was positive for tartrate-resistant acid phosphatase activity.[12] This T cell line is $OKT4^+$ and has been shown to be infected with HTLV-II.[13] Normal human peripheral blood lymphocytes (PBL) can be rapidly transformed in cocultivation with Mo-T cells by infection with HTLV-II.[14] The transformed cells bear a mature T helper cell phenotype and are constitutive lymphokine producers.[15,16] A second HTLV-II isolate has been reported from an AIDS patient with a history of intravenous drug abuse.[17]

We have investigated a patient (CM) with severe hemophilia A, pancytopenia, and a 14-year history of staphylococcal and candidal infections but no clinical or laboratory evidence of LRE. Serum from CM reacts in low titer with the major internal structural protein (HTLV-p24) of HTLV-I in an immunoprecipitation assay. Cultured CM lymphocytes yield continuous lymphoid lines with mature T helper phenotypes and profound suppressor activity on pokeweed mitogen (PWM)-induced normal B cell differentiation and do not require interleukin 2 (IL 2). Fresh CM mononuclear cells and cultured CM leukocytes express retroviral antigens indistinguishable from HTLV-II_{Mo} by molecular criteria. Normal human fetal cord blood lymphocytes (CBL) are rapidly transformed by cocultivation with CM leukocyte cultures. The transformed CBL have a mature T helper cell phenotype, exhibit suppressor activity on PWM-induced B cell differentiation, and are IL 2-independent. These characteristics are similar to those observed in Mo-T cells[14,15,18] and in the primary CM cell cultures and may represent properties of all HTLV-II–infected T cells. We present here the cytochemical, ultrastructural, cytogenetic, phenotypic, and functional characterization of these cell lines.

CASE HISTORY

A 38-year-old heterosexual man (CM) with severe hemophilia A (factor VIII:C < 1.0% activity) was hospitalized in August 1978 with pancytopenia and a staphylococcal leg abscess that resolved with appropriate antibiotic therapy. His medical history was significant for a chronic seizure disorder, multiple staphylococcal infections at venipuncture sites since 1970, staphylococcal septicemia in 1973, and multiple hemarthroses, ankyloses, and traumatic hemorrhages necessitating multiple transfusions with packed red cells and therapy with factor VIII concentrate. In January 1979, an arteriovenous graft was placed in the left thigh for convenient venous access. Pancytopenia was again noted and a bone marrow aspirate revealed normal cellularity. Bacterial cultures of bone marrow were negative. Multiple graft revisions were subsequently required, complicated by *Escherichia coli* and *Staphylococcus aureus* septicemia. Because of recurrent staphylococcal infections, he was placed on oral Keflex, which was continued until April 1983.

In March 1983, the patient was hospitalized with severe dysphagia. Endoscopy revealed candida esophagitis. A diagnosis of AIDS was considered, but the patient did not fit the strict Centers for Disease Control AIDS case definition because of his history of pancytopenia. His hemoglobin level was 8.8 g/dL; hematocrit, 28%; mean corpuscular volume, 69 fL; platelet count, 169,000/μL; and

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Submitted Jan 14, 1985; accepted June 17, 1985.

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0006-4971/85/6606-0023$00.00/0

leukocyte count, 2,800/μL (3% bands, 44% neutrophils, 35% lymphocytes, 8% monocytes, 8% eosinophils, and 2% basophils). His absolute T lymphocyte count was 922/μL (N = 800 to 1,800); absolute T helper cell count, 371/μL (468 to 1,433); and absolute T suppressor cell count, 538/μL (192 to 726), with a T helper-T suppressor ratio of 0.69 (0.9 to 3.3). Serum iron was 22 μg/dL (60 to 150); iron-binding capacity, 300 μg/dL (250 to 350); ferritin, 21 ng/mL (15 to 200); vitamin B_{12}, 115 pg/mL (200 to 900); and serum folate, 1.4 ng/mL (2 to 16). Skin tests for *Trichophyton* reactivity were positive. *Mycoplasma* antibody titer was 1:4, heterophile antibodies were negative, Epstein-Barr virus (EBV) antibody titer was 1:80, and cytomegalovirus antibody titer was 1:8. There was mild polyclonal immunoglobulin (Ig) evaluation. No hepatosplenomegaly was noted, and the rest of the physical examination was unremarkable. The candida esophagitis resolved with amphotericin B and ketoconazole.

In October 1983, CM was readmitted with candida esophagitis, which again resolved with amphotericin B. In January 1984, a routine hemogram revealed a hemoglobin of 15.3 g/dL; hematocrit, 46%; platelet count, 116,000/μL; and leukocyte count, 3,100/μL (8% bands, 52% neutrophils, 23% lymphocytes, 15% monocytes, 1% eosinophils, and 1% basophils). Lymphocytes had normal morphology and were negative for tartrate-resistant acid phosphatase activity. In November 1984, the arteriovenous graft was removed because of a pseudomonas infection. Since then the patient has remained well.

In 1979, the patient was noted to have IgG antibody to hepatitis A and to hepatitis B surface antigen. In 1980, hepatitis B core antibody was noted. In October 1983, his serum reacted in low titer with HTLV-I p24 in an immunoprecipitation assay, which prompted closer evaluation for retroviral infection. This serum specimen also reacted with HTLV-III/LAV p25 and p41 in a Western blot assay performed in May 1984. Family history was noncontributory.

MATERIALS AND METHODS

Cell cultures. Mononuclear cells were separated from CM's blood and stimulated with phytohemagglutinin (PHA) in RPMI 1640 (GIBCO, Chagrin Falls, Ohio*) + 20% heat-inactivated fetal calf serum (FCS). After three days, the cells were grown in medium with lectin-free IL 2. After three weeks, the cells were perpetuated without IL 2.

The ability of cultured CM lymphocytes, which produce HTLV-II, to transform CBL was assessed. Cultured CM cells were lethally irradiated (10,000 rad in ten minutes) and added in a ratio of 3:1 to three-day-old PHA-stimulated normal female CBL. This mixed culture was initially grown in 10% IL 2 but was subsequently selected for IL 2-independent growth.

Cytology, cytochemical studies, and terminal deoxynucleotidyl transferase determination. Cytocentrifuge preparations of cultured CM cells and transformed CBL were stained with Wright-Giemsa stain. Cytochemical reactions for α-naphthyl acetate esterase with and without fluoride inhibition, α-naphthyl butyrate esterase with and without fluoride inhibition,[19] acid phosphatase with and without tartrate inhibition,[20] myeloperoxidase,[21] chloroacetate esterase,[22] and the periodic-acid Schiff (PAS) reaction[23] were performed. Smears were stained for presence of terminal deoxynucleotidyl transferase (TdT) by an indirect immunofluorescence method (Bethesda Research Lab, Rockville, Md).[24]

Electron microscopy. Cell pellets from the CM cell line and from the transformed CBL were fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, postfixed in 1% osmium tetroxide, embedded in eponaraldite, and processed for transmission electron microscopy (EM).

Hybridization of cellular DNA with HTLV-I and -II specific probes. High-mol-wt DNA from cultured CM cells and from the transformed CBL cells was digested with different restriction enzymes, run in agarose gels, and transferred to nitrocellulose paper. The transferred DNA was hybridized with env-px specific probes of HTLV-I cloned DNA from MT-2 cells and 3'- and 5'-specific probes of HTLV-II_{Mo} (gift of Dr I.S.Y. Chen, UCLA).[8] The hybridizations were performed under stringent conditions in 50% formamide. High-mol-wt DNA was also digested with *Hind*III, a restriction enzyme that does not cleave within the proviral genome of HTLV-II_{Mo}.[8,25] Details of the hybridization studies and derivation of HTLV-I- and HTLV-II-specific probes are presented elsewhere.[26]

Cytogenetics. Cytogenetic evaluation of CM cell cultures and transformed CBL was performed by resuspending 1×10^7 cells in fresh RPMI 1640 + 20% FCS for 16 hours. Colcemid (10 μg/mL) was added during the final hour, followed by treatment with 0.07 mol/L KCl and final fixation in 3:1 methanol-acetic acid. Banded metaphase spreads were prepared by Giemsa-trypsin[27] and quinacrine fluorescence[28] techniques.

Phenotype characterization of cell cultures. Cell surface markers were analyzed by indirect immunofluorescence on a FACS IV (Becton Dickinson, Sunnyvale, Calif) as described.[29] The following murine monoclonal antibodies were used: α-T4, α-T8, α-T3, α-T9 (OKT4, OKT8, OKT3, OKT9) (Ortho Pharmaceutical Corp, Raritan, NJ); α-T11, α-Mo2, α-B1, α-B4 (Coulter Corp, Hialeah, Fla); α-Leu-8, α-Leu-11b, α-Ia (α-HLA-DR, nonpolymorphic) (Becton Dickinson); α-Tac (gift of Dr T.A. Waldmann, National Institutes of Health). All reagents were pretitered (regardless of manufacturer's suggested dilution) by using normal lymphocytes to determine optimal conditions for binding. Surface Ig-positive cells were detected with a fluoresceinated polyvalent goat antihuman Fab' reagent (Kallestad Laboratories, Chaska, Minn). Cytoplasmic Ig was evaluated by standard techniques on fixed, cytocentrifuged mononuclear cells with affinity-purified, fluorescein-conjugated goat antisera of known specificity.[30]

Functional assays. T helper and suppressor activity of CM and CBL lines were evaluated on PWM-driven Ig synthesis of normal B cells as described.[31]

For assay of helper activity, T and B cell populations from normal donors were isolated on Ficoll-Hypaque density gradient centrifugation of heparinized blood,[32] followed by E rosette sedimentation using sheep red blood cells treated with 2-aminoethylisothiouronium bromide hydrobromide (AET).[33] Normal T cells, cultured CM cells, and CBL cells were irradiated (3,300 rad), and graded numbers were mixed with 5×10^4 purified B cells. Cultures were stimulated with 1:200 vol/vol PWM (GIBCO) and cultured in round-bottomed microtiter plates for six days in a final volume of 0.2 mL. The culture medium was RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin. Ig production in the supernatants was measured using enzyme-linked immunosorbent assay (ELISA).[31]

Suppressor activity was assessed by coculturing 1×10^5 CM or CBL with 1×10^5 normal PBL in flat-bottomed microtiter plates. Cultures were stimulated with 1:200 PWM in a final volume of 0.2 mL. Purified allogeneic T cells and CEM cells (a pre-T cell line) were used as controls. Ig production was measured by ELISA.

RESULTS

Cytology, cytochemical studies, and terminal deoxynucleotidyl transferase. The CM line consisted of cells the size of large reactive lymphocytes and rare larger cells with

*Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the US Department of Health and Human Services.

Table 1. Cytochemical Profile of Fresh Peripheral Blood Lymphocytes From Patient CM, Cultured CM Lymphocytes, and CBLs Transformed in Coculture With Irradiated Cultured CM Lymphocytes

Stain	Blood Lymphocytes	CM	CBL
Periodic-acid Schiff	rare +	+ + +	−
Acid phosphatase without tartrate	+	+ + +	+ + +
Acid phosphatase with tartrate	−	+	+
Chloroacetate esterase	ND	−	−
α-Naphthyl acetate esterase (without fluoride)	−	+	−
α-Naphthyl acetate esterase (with fluoride)	−	−	−
α-Naphthyl butyrate esterase (without fluoride)	−	+ +	+
α-Naphthyl butyrate esterase (with fluoride)	−	+ +	+
Myeloperoxidase	−	−	−
Terminal deoxynucleotidyl transferase	−	−	−

−, negative; +, weak positivity; + +, moderate positivity; + + +, marked positivity; ND, not done.

folded nuclei. All were characterized by large amounts of cytoplasm. The cytochemical profile of CM and CBL cells is seen in Table 1. Many CM cells showed multiple blocks of PAS-positive material. They were weakly positive for tartrate-resistant acid phosphatase activity and demonstrated focal nonspecific esterase activity with α-naphthyl acetate and butyrate substrates. This reaction was resistant to fluoride inhibition with α-naphthyl butyrate but was completely inhibited when α-naphthyl acetate was used. TdT reactivity was not present. Cells from the CBL line were similar in size and appearance to the smaller CM cells.

Electron microscopy. On EM examination, the smaller CM cells had round to slightly irregular nuclei, often containing a rim of heterochromatin clumped at the nuclear envelope. Scattered mitochondria, strands of rough endoplasmic reticulum, free ribosomes, polyribosomes, glycogen deposits, and multivesicular bodies were identified throughout the cytoplasm. Larger cells contained prominently folded nuclei and large nucleoli (Fig 1). Their cytoplasm occasionally demonstrated tubuloreticular structures but was otherwise similar to that of smaller cells (Fig 1, inset).

EM examination of CM cell cultures and transformed CBL revealed budding of extracellular viral particles (Fig 2, inset) and viral particles contained within intracellular vesicles (Fig 2). The HTLV-II particles produced by CM cells have a denser core than do HTLV-I particles.

Hybridization of cellular DNA with HTLV-I– and HTLV-II–specific probes. Although there was no hybridization of genomic DNA from cultured CM cells with the HTLV-I probe, the CM cellular DNA hybridized strongly with both the 3'- and 5'-specific HTLV-II_{Mo} viral DNA. *Hind*III did not cleave the proviral genome in HTLV-II_{CM}. Similar results were observed with transformed CBL cells. Detailed analyses with extensive restriction maps demonstrating that the proviral genome of HTLV-II_{CM} and HTLV-II_{Mo} are identical are presented elsewhere.[26] Competition radioimmunoassays demonstrating that the virus harbored by these cell lines is more closely related to HTLV-II_{Mo} than to HTLV-I are also presented elsewhere.[26]

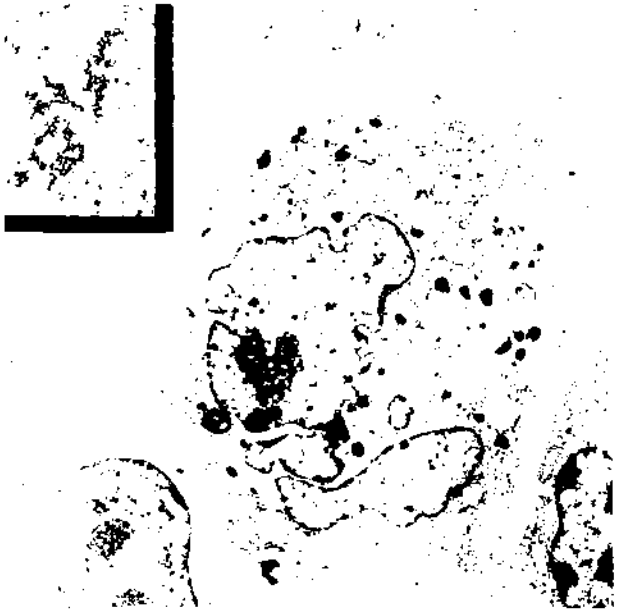

Fig 1. Larger cells of the CM line demonstrate prominently folded nuclei. Uranyl acetate, lead citrate. Original magnification ×4,850; current magnification ×4,185. Inset: Tubuloreticular structures seen in occasional large cells. Uranyl acetate, lead citrate. Original magnification ×16,700; current magnification ×15,030.

Fig 2. Numerous extracellular virus particles associated with the surface of a cultured CM line cell. Uranyl acetate, lead citrate. Original magnification ×36,300; current magnification ×32,670. Inset: Virus particles budding from the cell membrane. Uranyl acetate, lead citrate. Original magnification ×62,230; current magnification ×56,000.

Table 2. Phenotypic Characterization of Cultured CM Leukocytes and Transformed CBL

Cell Set	Putative Specificity	Cultures Positive (%) CM	CBL
T3	Pan T	7	86
T11	Pan T (E rosette receptor)	81	98
T4	T inducer cell set	90	70
T8	T suppressor/cytotoxic cell set	0	40
T10	Early hematopoietic stem cells and activated lymphocytes	23	72
T9	Proliferating cells (transferrin receptor)	44	48
Leu-8	T cell subsets	74	57
B1	B cell	0	0
Mo2	Monocyte/macrophage	0	0
Ia	B cells, monocytes, and activated T cells	93	14
Leu-11b	Fc receptor of NK cells, neutrophils, and basophils	0	1
B4	Immature and mature B cells (not plasma cells)	0	0
Tac	Activated T cells (IL 2 receptor)	91	95
Surface IgG	B cells	3	1
Cytoplasmic IgG	Plasma cells	0	0

Cytogenetics. Examination of 27 metaphases revealed long-term CM cell cultures to be of mosaic cell constitution presenting 11 (41%) metaphases of apparently normal male karyology (46,XY) and 16 (59%) metaphases of hyperdiploid constitution (48,XY,+2,+19). Short-term cultures of peripheral mononuclear cells from the patient were of normal constitution (46,XY). Normal female CBL cocultivated with irradiated CM cells were consistently transformed, grew continuously, and had normal female constitution (46,XX).

Phenotypes of cell cultures. Table 2 summarizes the phenotypic characterization of the cultured CM cells and the transformed CBL. The T cell phenotype of the cultured CM cells and the transformed CBL was determined by the presence of Tac-antigen (91% and 95%, respectively), T4 (90% and 70%), T11 (81% and 98%), and Leu-8 (74% and 57%).

Functional assays. Profound suppression was observed with 1:1 ratios of effector cells to PWM-stimulated PBL, both for CM and CBL lines (Table 3). No suppression was observed with fresh allogeneic cells. No suppression was observed with the CEM line, indicating that the suppression observed in CM and CBL assays was not due to culture overgrowth.

Typical results of the T helper assay are presented in Table 4. Both CM and CBL lines demonstrated some minimal helper activity.

No significant natural killer (NK) activity was observed by either CM or CBL lines against any of six NK targets (data not shown).

DISCUSSION

Although HTLV-II transmission to normal human PBL by cocultivation with lethally irradiated HTLV-II–infected cells has been demonstrated,[14,34–36] HTLV-II–infected cell lines have heretofore only produced infectious virus in low titers.[37] The establishment of continuous lymphoid cultures described here that do not require IL 2 and produce HTLV-II particles in high titer (from 10^8 to 10^9 particles per milliliter) will permit more detailed immunologic and molecular analysis of this retrovirus. Like cells transformed by HTLV-I[34–36] and HTLV-II_{Mo},[14] the HTLV-II_{CM}–transformed cells appear to have indefinite growth potential. Although subtle structural differences may exist between HTLV-II_{Mo} and HTLV-II_{CM}, retroviral antigens expressed by cells infected with HTLV-II_{CM} have been indistinguishable thus far from those expressed by cells infected with HTLV-II_{Mo}.[26]

Unlike patient Mo, patient CM has no evidence to date of malignancy. At the time of splenectomy of patient Mo, histologic features of classic LRE were noted, as was the presence of tartrate-resistant acid phosphatase activity in transformed T cell cultures derived from Mo spleen cells. Because HTLV-II_{Mo} is only one of two previously described isolates of HTLV-II, the evidence for causality in the HTLV-II–LRE association has been limited. B cell origin is characteristic of most cases of LRE, the vast majority of which have surface Ig.[6,38–40] The case of patient Mo may represent fortuitous HTLV-II infection with an unrelated malignancy[41] in the same fashion that EBV-positive cell lines are isolated from healthy normals. With only three HTLV-II isolates described in patients with different clinical presentations,[12,17] the role of HTLV-II in disease remains unclear.

Tubuloreticular structures are associated with elevated levels of interferon in viral infections and have been found in cells from patients with systemic lupus erythematosus, patients with AIDS, and persons belonging to AIDS risk groups[42–46]; they are primarily found in suppressor cells of patients with AIDS, but because they are also found in a variety of other tissues, their presence in T helper cells cannot be excluded.[42,46] We found tubuloreticular structures in cultured CM cells and CBL that expressed an inducer/

Table 3. Demonstration of T Suppressor Effect of Cell Lines on PWM Response of Fresh PBL (1:1 ratio)

Cells Added (10^4/culture)	Donor 1 −PWM	Donor 1 +PWM	Donor 1 Suppression (%)	Donor 2 −PWM	Donor 2 +PWM	Donor 2 Suppression (%)
PBL only	0.3	3.7	—	0.1	2.3	—
Fresh allogeneic T cells	0.4	>27.3	−640	0.1	9.7	−320
CM cells	0.2	0.1	97	0.1	0.1	96
CBL cells	0.1	0.1	97	0.1	0.1	96
CEM cells	0.2	>27.3	−640	0.1	3.1	−35

Results are expressed as μg/mL Ig secreted. All cultures were performed in triplicate.

Table 4. Demonstration of T Helper Effect on PWM Response of Fresh T-Depleted PBL

Number of T Cells Added	Autologous T Cells	Allogeneic T Cells	CM Cells	CBL Cells
B Cell Donor 1				
0 (B cells alone)	42	42	42	42
5×10^4 (1:1)	185,000	368,000	125	275
1×10^4 (1:5)	828	503	192	484
5×10^3 (1:10)	86	329	146	254
B Cell Donor 2				
0 (B cells alone)	110	110	110	110
5×10^4 (1:1)	187,000	359,000	167	778
1×10^4 (1:5)	939	1,600	1,600	31,000
5×10^3 (1:10)	234	481	376	4,400

Results expressed as ng/ml Ig secreted. Figures in parentheses denote T cell-B cell ratio. All cultures were performed in triplicate.

helper T cell antigen (OKT4) but suppressed Ig secretion by B cells in vitro. The same dichotomy between function and phenotypic expression has been noted in HTLV-I–infected cell lines.[47] Because HTLV-I and -II share certain regions of nucleic acid homology,[48,49] these regions may be responsible for the shared ability to transform primary T cells in vitro[36,50-52] and for a T cell tropism preferential for a subpopulation of T cells that expresses a mature helper/inducer phenotype but functions as suppressor cells. However, just as HTLV-I has been shown to be capable of infecting and transforming T cells expressing a suppressor phenotype and bone marrow cells devoid of phenotypic markers,[52] and as HTLV-II_{Mo} has been shown to be capable of infecting B cells,[14] HTLV-II_{CM}–transformed lymphoid cells expressing other than a helper/inducer phenotype will probably be eventually cultivated.

The cytochemical profile of CM cells and CBL cells is similar to that of primary Mo-T cells,[14] secondary cultures transformed by Mo-T cells,[53] and other T cell lines in which strong PAS positivity and nonspecific esterase activity are commonly seen.[54] Tartrate-resitant acid phosphatase activity is observed not only in LRE, but also in HTLV-I–associated leukemia/lymphoma as well as other lymphoid malignancies.[43] This reaction was weakly positive in both CM and CBL cell cultures; its absence in the fresh PBL of patient CM is consistent with the absence of clinical evidence to support malignancy.

Because chromosomal aberrations may be fundamental to a malignancy, and because the first isolate of HTLV-II was associated with LRE, we reviewed 3,844 published cases of chromosome aberrations in cancer.[55] Although the hyperdiploid constitution (48,XY,+2,+19) observed in long-term CM cell cultures has been observed in several reports of various lymphomas and leukemias,[55] no association of LRE with duplications at either chromosome 2 or 19 has been reported. These chromosomal changes were either induced by the artificial culture conditions or may reflect genetic instability that may be associated with retroviral infection. As these aberrations were not noted in the HTLV-II_{CM}–infected CBL, it appears that transformation of the primary CM cultures is the result of viral infection and not chromosomal alteration.

ACKNOWLEDGMENT

We acknowledge Drs C. Cabradilla, J.P. Getchell, J.M. Jason, L. Martin, J.S. McDougal, R. Narayanan, and J.K.A. Nicholson for their assistance; D. Basinger, R. Green, A.K. Harrison, D. Jackson, B.M. Jones, M.L. Martin, J. Scheppler-Campbell, and C. Sporborg for their valuable technical support; Dr I.S.Y. Chen for generously providing the 3'- and 5'-specific probes of HTLV-II_{Mo}; Dr T.A. Waldmann for generously providing the α-Tac monoclonal antibody; and Patsy Bellamy for the preparation of the manuscript.

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