concerned in the classification of cell types in this unit readily admit that the process is in part arbitrary. In addition, the slides have been read by about a dozen different pathologists. In this particular series there is no admixture of surgical cases with the autopsy cases. For about 20 years there has been great interest in the cell type of lung cancers in the mining population and therefore great interest in the accurate determination of cell type in all lung cancers. Since all tissue blocks and the slides from which the original diagnosis was made are extant it would be possible to review the data used in this preliminary analysis. It would also be possible to investigate the high prevalence of lung cancer in asbestos-exposed white miners (20%) compared with all other exposure groups in terms of their exposure, smoking habits, and possible selection bias.

Among black asbestos miners adenocarcinoma is not the dominant cell type (Table III). Large-cell carcinoma appears to be much commoner than in any other subgroup of black or white miners, except asbestos-exposed white miners. The small data set of coloured miners (which, in fact, contains a very high proportion of asbestos-exposed miners) supports the opinion expressed by Churg,2 that adenocarcinoma is among the least common cell types in this group (Table IV).

It is not strictly true to say, as did Churg,2 that 'virtually every review article or textbook' carries a categorical statement that 'adenocarcinoma is the most common type of lung cancer found in those with asbestos exposure'. For instance, Becklake3 wrote as follows: '... there is some evidence to suggest that the distribution of cell types is different from that seen in smokers without [asbestos] exposure, namely, a greater preponderance of adenocarcinoma. ... Not all series show this greater preponderance of adenocarcinoma, however, and the question remains open.'

In the light of this statement, and the opinion expressed by Churg, our preliminary analysis is further evidence against the hypothesis that adenocarcinoma is the commonest cell type in lung cancer among asbestos-exposed subjects.

The data on which this analysis is based has been collected by a number of pathologists working at the National Centre for Occupational Health. The importance of this carefully collected data set cannot be exaggerated.

REFERENCES


Isolation of a new human herpesvirus producing a lytic infection of helper (CD4) T-lymphocytes in peripheral blood lymphocyte cultures — another cause of acquired immunodeficiency?

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Summary

A new human helper (CD4) T-lymphotropic herpesvirus (HTLV) was first isolated in February 1985 from the cultured peripheral blood lymphocytes (PBL) of a patient with the acquired immunodeficiency syndrome, and subsequently from the PBL of 1 patient with hairy cell leukaemia and 2 patients with lymphoproliferative disease associated with human T-lymphotropic virus type I infection. The viruses could be serially subcultured in umbilical cord PBL cultures in which they infected helper (CD4) T-lymphocytes producing multinucleate giant cells with intranuclear inclusions followed by cell lysis. Electron microscopy of infected cultures revealed that the isolates were herpesviruses. Specific DNA probing showed that the 4 isolates were related to one another but were distinct from cytomegalovirus, Epstein-Barr virus, Herpesvirus hominis types 1 and 2, and varicella-zoster virus. HTLV lyases the same target cell as human immunodeficiency virus in PBL cultures suggesting that it may have a similar potential to cause acquired immune deficiency. The development of an unequivocally diagnostic serological test is a priority, so that the epidemiology and pathogenesis of HTLV infection can be studied.

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There are 5 well-characterised human herpesviruses: cytomegalovirus (CMV); Epstein-Barr virus (EBV); Herpesvirus hominis types 1 and 2 (HVH-1, HVH-2); and varicella-zoster virus (VZV). These are important pathogens which persist in a latent state in the body after the primary infection, but may become reactivated to produce clinical disease, especially under immunosuppressive conditions.

Recently the isolation in the USA of a newly recognised human herpesvirus tropic for B-lymphocytes was reported and named human B-lymphotropic herpesvirus (HBLV). The isolation of a new human helper (CD4) T-lymphotropic herpesvirus (HTHLV), which produced a lytic infection of helper T-lymphocytes in the peripheral blood lymphocyte (PBL) cultures of 4 patients, is reported.

**Patients**

The following brief details of the patients are provided without necessarily relating the virus infection to their clinical condition.

1. In February 1985 a 58-year-old Scandinavian heterosexual man, who had resided in Central Africa for 25 years, was admitted to hospital in the RSA. His illness had started 16 months previously and had progressed to the acquired immunodeficiency syndrome (AIDS) at the time of admission. He discharged himself 17 days later and was lost to follow-up. He was human immunodeficiency virus type 1 (HIV-1)-seropositive and HTLHV (strain H) was isolated from a peripheral blood sample taken during his hospitalisation.

2. An 83-year-old white heterosexual man was diagnosed in January 1982 as having hairy cell leukaemia. In November of 1983 he was HIV-seronegative.

3. A 23-year-old coloured heterosexual man was admitted to hospital in December 1982 with acute T-cell lymphoblastic lymphoma associated with human T-lymphotropic virus type 1 (HTLV-I) infection. He had first presented in 1970, at the age of 11 years, with splenomegaly and thrombocytosis and was followed up for 6 years, after which he defaulted. His illness ran a fluctuating course until his last admission in April 1986. HTLHV (strain K) was cultured from his PBL shortly before death. He was HIV-seronegative.

4. A 49-year-old heterosexual Japanese man was admitted to hospital with a pulmonary infection and died several weeks later in September 1987. He was HIV-seronegative. HTLV-I was cultured in PBL using methods previously described and HTLHV (strain E) was isolated from two peripheral blood samples taken 10 days apart shortly before his death.

**Materials and methods**

**PBL cultures**

PBL cultures were established and maintained as described previously. Briefly, PBLs were separated from heparinised blood by centrifugation on a Ficoll gradient and cultured in an atmosphere of 5% carbon dioxide in air in RPMI 1640 medium supplemented with 0.2% Na(HCO3)2, 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, antibiotics, 2% highly purified interleukin-2 and sometimes with 100 μg/ml phytohaemagglutinin (PHA). Medium was replaced at 1-3-day intervals.

**Reverse transcriptase assay**

The reverse transcriptase activity was assayed on the pellets of the supernatant fluids (SNFs) of infected and control uninfected PBL cultures as described previously.

**Electron microscopy**

Infected and uninfected control PBL cultures were prepared by the standard negative staining technique for electron microscopy. Cells from infected and control PBL cultures were prepared for ultrathin-section electron microscopy as previously described.

**Immunofluorescence assay (IFA)**

The indirect IFA method was carried out, as previously described, to identify viral antigens in infected cells by their reaction with sera of known specificity, or to detect specific viral antibodies in sera by their reaction with antigens in cells infected with known viruses.

The following viral antigens and antisera were used in IFAs:

1. HVH-1 — strain HVH-145/67 isolated from a child with fatal disseminated herpesvirus infection. This strain reacted specifically in IFA tests using the specific bivalent HVH-1/HVH-2 monoclonal antisera below.

2. CMV — strain CMV-499/87 was isolated from the urine of a renal transplant patient and reacted specifically in IFA tests with the specific CMV monoclonal antisera below.

3. EBV — EBV nuclear antigen (EBVNA); EBV capsid antigen (EBVCA), and EBV early antigen (EBVEA) supplied in antibody test kits (Zeus Scientific Inc., Raritan, NJ, USA).

4. VZV — strain VZV-3215/87 was isolated from a child with varicella and reacted specifically in IFA tests using the specific VZV monoclonal antisera below.

5. HTLHV — the 4 isolates reported in this article.

Mouse monoclonal antisera specific for CMV, EBV, HVH-1/HVH-2 (bivalent) or VZV were obtained from Whittaker MA Bioproducts (Walkersville, Md, USA).

FITC-labelled goat anti-mouse immunoglobulin (IgG + IgA + IgM [H + L]) obtained from Zymed Laboratories Inc., San Francisco, California, USA, was used as second antibody in IFA when murine sera were employed as first antibodies.

**Lymphocyte typing**

Lymphocytes were typed by the standard indirect IFA method. The following murine monoclonal antibodies of the BMA series (Behringwerke AG Diagnostica, Marburg, West Germany) directed against the human lymphocyte specific membrane antigens were used as the specific first antibodies — BMA 081 anti-suppressor/cytotoxic (CD8) T-lymphocytes, BMA 040 and BMA 041. Lot 7018 anti-helper (CD4) T-lymphocytes, and BMA 0130 anti-pan (CD19) B-lymphocytes.

**Umbilical cord PBL cultures depleted of helper (CD4) T-lymphocytes**

A centrifuged pellet (250 g for 10 minutes) of 4 x 10⁶ PBL was mixed with 160 μl of helper (CD4) T-lymphocyte monoclonal mouse IgM antibodies (BMA 041 Lot 7018 listed under lymphocyte typing) and 1 ml of a 1:3 dilution of freshly filtered rabbit serum (as the source of complement) followed by incubation at 4°C for 45 minutes. Control cells were treated with rabbit serum alone.

The cells were then washed in phosphate-buffered saline (PBS) containing 5% FCS (PBS/FCS) and typed for helper T-lymphocytes. An absence of staining of intact cells compared with the control cells confirmed that the helper (CD4) T-lymphocytes had been lysed.
Lymphocyte subset tropism of HTLHV isolates

The following experiments were done to determine the lymphocyte subset tropism of HTLHV:

1. Umbilical cord PBLs stimulated with PHA and cultured for 3-4 days were typed before inoculation with HTLHV.
2. Inoculated cultures were typed to identify the subset(s) of infected lymphocytes. Because of the distinctive giant-cell formation infected cells could readily be identified to evaluate their reaction with specific typing sera.
3. The inoculation of cord PBL cultures depleted of helper (CD4) T-lymphocytes.

The virus inoculum used in these experiments consisted of the cell-free SNFs of HTLHV-infected cord PBL cultures.

Freshly separated umbilical cord lymphocytes were typed to determine the content of helper (CD4) T-lymphocytes. Five aliquots of 4 x 10^6 cells were pelleted at 150 g for 10 minutes. Two aliquots were treated with CD4 antiserum and complement to lyse the helper (CD4) T-cells, and one of these was typed to confirm that the helper (CD4) T-cells had been lysed; the second was the test sample. The other 3 aliquots served as controls: 1 was treated with antiserum alone, 1 with complement alone and 1 was untreated. The cells in the test sample and the latter 3 controls were washed in PBS/FCS, resuspended in 0,5 ml HTLHV virus suspension and incubated at room temperature for 1 hour. The cells were then pelleted and resuspended in culture medium containing 1% PHA and incubated for a further 1½ hours at room temperature. The cells were then washed 3 times resuspended in culture medium, incubated at 57°C in an atmosphere of 5% CO₂ and examined daily for cytopathic effect (CPE).

4. The following cell lines were tested for susceptibility to infection with HTLHV. They were established in our laboratory and cultured as for umbilical cord PBL (unpublished data).

T-191 is a line of helper T-cells in which all the cells type specifically with BMA 040 CD4 but not with BMA 081 antiserum. It was established in our laboratory by transforming human fetal thymocytes with simian T-lymphotropic virus type 1.

T-448 is a line of suppressor/cytotoxic T-lymphocytes typing with BMA 081 but not with BMA 040 antiserum. It was established by transforming human umbilical cord PBL with HTLV-I isolated in our laboratory. B-lymphocyte lines immortalised by infection with EBV were established from renal transplant patients.

HTLHV DNA Southern blot analysis and probing with specific herpesvirus DNA probes

The following specific herpesvirus probes were used:

1. CMV Hind III-S, -T, -U and -V DNA fragments cloned in pAT153.
2. EBV Bam I-W DNA fragment cloned in pACYC184.
3. HHV-2 Eco RI-G DNA fragment cloned in pSP65.
4. VZV Hind III-A DNA fragment cloned in pBR322.
5. An HTLV strain M Hind III 9.5 kb fragment cloned in pBR328 (unpublished data).

Cellular DNA was extracted from virus-infected cells by standard methods. Each of the specific probes was used on aliquots of DNA which were digested with the corresponding endonuclease (according to the method recommended by the suppliers, Boehringer Mannheim GmbH-Biochemica, Mannheim, West Germany) used in the preparation of the probe. The digest fragments were separated by gel electrophoresis and transferred to Hybond N membranes (Amerham International, Bucks, U.K.) by Southern blotting. The membrane-bound DNA was tested by standard methods for hybridisation with the specific herpesvirus probes under low- and/or high-stringency washing conditions. Low-stringency conditions involved washing twice at room temperature for 15 minutes in double-strength SSC buffer (0.15M NaCl, 0.015M trisodium citrate, pH 7.2) containing 0.1% sodium dodecyl sulphate [2 x SSC/SDS buffer], followed by a third wash at 65°C for 1 hour in the same buffer and a fourth wash at 65°C for 1 hour in SSC/SDS buffer. For high-stringency conditions the third wash was in SSC/SDS buffer and the fourth in 0.1 x SSC/SDS buffer.

Results

The results with all 4 isolates were similar.

Virus isolation

The patients' PBL cultures developed a CPE which was characterised by the formation of multinucleated giant cells illustrated in an unstained preparation. Three distinct cell lines were infected by HTLHV-M. A: The CPE is characterised by the formation of multinucleated giant cells illustrated in an unstained preparation (arrows) (x 75). B: Intranuclear inclusions in a stained preparation (x 400).

Infected PBL cultures were tested for reverse transcriptase activity at the time of maximal CPE and found to be negative.
Fig. 2. Electron micrographs of cord PBL cultures infected with HTLV. A, B and C: Ultrathin sections of infected cells — herpesvirus capsids in the nucleus in (A), and enveloped capsids in cytoplasmic cisternae (arrows) in (B) and extracellularly in (C). D: Negative stain of pelleted SNF showing a herpesvirus capsid (black arrow) and enveloped particles (white arrow).

Fig. 3. Southern blot endonuclease analysis and probing of DNA extracted from cord PBL cultures infected with HTLV. A: Ethidium bromide staining of HindIII digests — lanes 1 and 2 strain H; lanes 3 and 4 strain M; lane 5 lambda HindIII digest markers; lane 6 VZV probe DNA control. B: Filter with the DNA transferred from the gel in A by Southern blotting and probed with the specific VZV DNA probe (hybridisation under low-stringency conditions occurred only with the VZV probe control DNA in lane 6). C: The filter used in B was reprobed with the specific HTLV DNA probe — no hybridisation occurred with the VZV probe control DNA in lane 6 under low-stringency conditions; hybridisation did occur with the HTLV DNAs in lanes 1 - 4 under high-stringency conditions.

Electron microscopy

Electron microscopy using the negative staining technique revealed herpesviruses in infected cultures.

Ultrathin-section electron microscopy of infected cultured PBLs confirmed replicative infection with a herpesvirus. The cell nucleus contained viral capsids and membrane-bound virions were present in the cytoplasmic cisternae and also extracellularly.

IFAs

IFAs on HTLV-infected cells using specific monoclonal antisera to CMV, EBV, HVH-1 and HVH-2 and VZV were negative, indicating the HTLV was distinct from these other human herpesviruses.

All 4 patients had serum antibodies to the known human herpesviruses using IFA with virus-infected cells as antigen. The HTLV serum antibody titres were the same using cells infected with strains H or M as antigen, namely 320, 20, 1280 and 1280 for patients 1 - 4 from whom strains H, M, K and E were isolated respectively.
Specific DNA probing of viral DNA

Probing of DNA extracted from cells infected with each of the 4 HTLHV isolates showed no hybridisation with the DNA probes specific for CMV, EBV, HVH-1 and HVH-2 or VZV even under low-stringency conditions, but did hybridise with the HTLHV-specific DNA probe under high-stringency conditions.

These results showed that the isolates were genetically related to one another but distinct from the other human herpesviruses.

Helper (CD4) T-lymphocyte tropism of HTLHV

Umbilical cord PBL stimulated with PHA and cultured for 3 days were typed before inoculation with HTLHV. The content of B(CD19)-lymphocytes was 3 - 4% of helper (CD4) T-lymphocytes 46 - 49% and of suppressor (CD8) T-lymphocytes 43 - 53%.

The fact that B-lymphocytes comprised only 3 - 4% of the culture and that cell counts on the PBL cultures when CPE was maximal showed that 40 - 50% of the cells were infected indicated that the HTLHV strains infected T-lymphocytes. Furthermore, the cytopathic cells were identified as helper T-lymphocytes by their specific reaction with monoclonal CD4 antiserum.

Cord PBL cultures depleted of helper (CD4) T-lymphocytes and inoculated with virus showed no CPE for 4 days at which time maximum CPE had developed in control cultures. The scant cytopathic cells which emerged in the test cultures after 4 days were identified as helper T-lymphocytes. These cells had presumably escaped lysis initially when the cultures were depleted of their helper T-cell content by lysis with specific mouse monoclonal IgM CD4 antiserum and rabbit complement.

Further confirmation of the helper T-cell tropism of HTLHV was obtained from the inoculation of cell lines. Cultures of the T-191 CD4 T-lymphocytic line could be totally infected and these infected cells still reacted specifically with CD4 monoclonal antiserum. Furthermore, cultures of the T-448 CD8 T-lymphocytic line and of B-cell lines could not be infected with HTLHV.

Discussion

HTLHV is a distinct, newly recognised human herpesvirus which has in common with HIV the unique property of producing a cytoplastic infection of the helper (CD4) T-lymphocytes in the PBL cultures of infected patients. The possibility that HTLHV may spread in similar ways to HIV and have a similar effect in causing acquired immunosuppression must be considered. It is necessary that the epidemiology of HTLHV infection and its association with disease be established and it must be determined whether HTLHV is cleared from the body after primary infection, or whether persistent or latent infection, or incorporation of viral genomic DNA into the genome of infected cells occurs.

To obtain the necessary epidemiological information a specific diagnostic test for serum antibodies is required in addition to the available techniques of virus isolation and nucleic acid probing. The only serological test available at present is IFA, the results of which are difficult to evaluate because of probable cross-reactions with the other human herpesviruses. This problem has been overcome in the other human herpesviruses by cloning the viral genomes and identifying unique gene products; these, and the monoclonal antibodies prepared against them, are used in specific diagnostic serological tests. The same approach will be necessary in order to study the prevalence of infection with HTLHV and its role as a pathogen.

Since the first isolation of this new herpesvirus in our laboratory in February 1985 from a patient with AIDS only 3 further isolations have been made - from the PBLs of 1 patient with hairy cell leukaemia and 2 patients with lymphoproliferative disease associated with HTLV-I infection. No HTLHV was isolated from the PBL cultures of a further 117 people of whom 60 were HIV-1 seropositive, 29 had lymphoproliferative disease and 9 were HTLV-I seropositive.

If specific serological studies should confirm that this low rate of virus isolation is a true index of the prevalence of infection in the community it would favour the view that HTLHV is an emergent pathogen.

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REFERENCES