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Interaction of HIV-1 and HHV-6

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Introduction

The infection of a seronegative host with HIV is the begin of a viral infection which leads after a long incubation period to a chronic disease process as a consequence of a virus-induced immune suppression. So far, the destruction of a lymphocyte subpopulation essential in mounting an immune response has been identified as an important pathogenetic mechanism in the development of the disease. However, beside HIV as the etiological agent for AIDS other viruses have been implicated as co-factors in the activation of a latent HIV infection. It has been observed that several DNA viruses transactivate HIV (13, 23, 25, 29). One of these is the human herpesvirus type 6 (HHV-6). This virus infects like HIV peripheral blood lymphocytes (1, 2, 3, 4, 16, 20) and therefore its isolation from AIDS patients has led to the hypothesis that a HHV-6 infection may indeed interact with HIV and influences the course of AIDS.

In the present study we have started to evaluate the presence of HHV-6 antibodies in a population of AIDS and control patients in a urban and non-urban area of Tanzania, Africa. From Dar-es-Salaam and from Bukoba serum specimens were available for testing. Moreover, virus isolations were attempted from lymphocytes from AIDS and control patients to search for HIV as well as HHV-6. The data obtained indicate that HHV-6 is indeed transactivating HIV infection in lymphocytes but is also widely distributed in persons not infected by HIV. Since many AIDS patients in our test groups do not reveal a HHV-6 infection it is unlikely that HHV-6 plays a pathogenic role in the development of AIDS.

Materials and Methods

Collection and origin of samples

Serum samples were collected in a rural hospital in Kagera, Tanzania. 330 sera were collected from adults, ranging in age from 15 to 62 years (mean 26 years), 47 % male, and from 100 children ranging in age from 8 to 13 years (mean 10.5 years). Additional serum samples were drawn from patients of the Internal Medicine wards at the Muhimbili Medical Center (MMC), Dar-es-Salaam, Tanzania. 123 sera were collected from adults, ranging in age from 18 to 60 years (mean 30 years), 60 % male. During this visit to Dar-es-Salaam we also took peripheral blood for isolation of lymphocytes. Specimen were obtained from 100 subjects and were subsequently frozen in liquid nitrogen. Furthermore we received 42 sera from Ocean Road Hospital, Dar-es-Salaam, Tanzania. The patients' age ranged from 17 to 78 years (mean 35 years), 40 % male.

Serological test for HIV

Antibodies to HIV-1 or -2 were determined by enzyme linked immunosorbent assay (ELISA): one with whole viral lysate of HIV-1 (HTLV III[b]) on nitrocellulose membrane (Serion, Virion, Würzburg, FRG), and the second (PEIA: peptide ELISA, Biochrom, Berlin, FRG) using microtiter plates coated with synthetic peptides of HIV-1 (p17, p24, p31 and gp41) or of HIV-2 (p26 and gp36). Specimens that were positive on the initial test were subjected to Western blot analysis (WB). WB were considered positive when p24 or gp41 were observed along with other bands indicative for HIV infection. Only specimens confirmed in this manner were considered true positives.

Isolation of HIV and HHV-6 from PBL

Peripheral blood was drawn with anticoagulant. The patients PBL were separated from the buffer layer on a ficoll gradient and frozen in liquid nitrogen. Fresh normal lymphocytes depleted of $CD8^+$ cells (Dynabeads HLA class 1, Dynal, Oslo, Norway) were stimulated with 0.1 % PHA-P (Difco, Detroit, USA) for 72 hours then cocultivated with unstimulated CD8 depleted patient's lymphocytes. The cultures were screened for HIV-1 or HHV-6 by indirect immunofluorescence (IFA) or p24 antigen ELISA for HIV-1 (DuPont, Bad Homburg, FRG) beginning on day three of cocultivation.

Culture conditions

Cells were cultured in RPMI 1640 (Biochrom, Berlin, FRG) supplemented for cell lines with 10 % fetal calf serum (Biochrom, Berlin, FRG) 5 mM glutamine (Biochrom, Berlin, FRG), 10 μg/ml gentamycine (Merck, Darmstadt, FRG). For primary cultures 20 U/ml interleukin 2 (ICN, Eschwege, FRG) and 500 U/ml

anti leukocyte interferon (Renner, Dannstadt, FRG) were added. During the first three days of cocultivation $2 \mu g/ml$ polybrene (Aldrich, Steinheim, FRG) was added.

Cell lines

MT-4 cells (8, 20) were kindly provided by B. Zorr, Berlin, FRG. Raji cells (10), BjAB cells (19) and P3HR1 cells (10) were kindly provided by A. Pohl, Würzburg, FRG.

Cytofluorographic analysis (FACS)

For two colour staining 1.5×10^6 lymphoid cells were fixed with 3.5 % formaldehyde (FA) for 5 min. and subsequently treated for 30 min. at 4 °C with human serum positive for HHV-6 and goat anti human IgG Fab fragment phycoerythrein labeled (PE) (Dianova, Hamburg, FRG) for 30 min. at 4 °C. For intracellular staining we pretreated the cells at room temperature (RT) with 3.5 % FA for 25 min., with 0.25 % Triton X 100 (Sigma, St. Louis, USA) for 15 min. and blocked with 10 % goat serum for another 15 min. We stained for HIV-1 with monoclonal antibody p24 1:50 (Dianova, Hamburg, FRG) and goat anti mouse IgG fluorescein isothiocyanate (FITC) labeled for 60 min. at RT each. The fluorescence intensity was analysed by a FACSCAN flow cytometer (Becton and Dickenson, Heidelberg, FRG). Dead cells and debrids were excluded from analysis by selective gating based on low angle light scatter.

Electron microscopy (EM)

Single or double infected MT-4 cells were sedimented at 200 g for 5 min. and the pellet incubated with 1.25 % glutaraldehyde in 0.1 M cacodylate-HCL buffer, 0.1 M sucrose, and 2 mM $CaCl_2$, pH 7.2 for 1 h, postfixed with 1 % OsO_4 in the same buffer for 2.5 h and processed for transmission electron microscopy as previously described (3).

Transfection and chloramphenicol acetyltransferase (CAT-) assay

3×10^7 cells were transfected with $10 \mu g$ indicator plasmid (pHIV-1 CAT) and either $10 \mu g$ tat expression plasmid (pCV-1) or $10 \mu g$ pBR 322 by the DEAE-dextran method (27). Cells were harvested 36 h after transfection and processed as described (7). CAT-assays were performed with equal amounts of protein as determined with a commercial protein assay (Biorad, München, FRG), 0.12 μCi ^{14}C-chloramphenicol and

0.8 mM acetyl-CoA. Assays were incubated at 37 °C for 4 h. Acetylated chloramphenicol was separated from unacetylated by ascending thin-layer chromatography, and quantification was done by cutting out the spots from the chromatography plate and liquid scintillation counting.

Herpesvirus serology

HHV-6: The patients sera were titered for HHV-6 IgG antibodies using MT-4 cells infected with HHV-6 (Tanzanian isolate). 80 % infected cells were mixed 1:5 with uninfected MT-4 cells. Infected cells were harvested for testing, when about 50 % were infected and transformed to blasts (within 5 days). After rinsing of the cells, cells were dried, fixed in acetone for 5 min. and stored at –20 °C. Uninfected MT-4 cells were used as negative control. Incubation time with sera and conjugate (FITC labeled goat anti human IgG Fab fragment 1:50, Dianova, Hamburg, FRG) was 1 h at 37 °C. The slides were finally washed three times and mounted for UV-IFA. IFA was regarded positive with a titer of 1:30 and above.

EBV: IFA was used to detect IgG antibodies to EBV capsid antigen (EBV-VCA), to EBV nuclear antigen (EBNA) and EBV early antigen (EA). Antibodies to EBV-VCA were determined on acetone fixed cell smears with P3HR1 cells that were incubated with test sera 1:10 and then with FITC conjugated rabbit antibodies to human IgG 1:20 (Dakopatts, Hamburg, FRG) both for 1 h at 37 °C. For EBV-EA testing we induced Raji cells with 10 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, USA) for two days. Cells and slides were treated following our standard protocol for EBV-VCA IFA. The preparations were finally counterstained in Evans blue. Each test was run with positive and negative control sera. In testing for EBNA antibodies we used methanol-fixed Raji cells. BjAB cells were used as negative control. Preparations were successively overlaid with test sera, 1:10, serum from an individual lacking antibodies to EBV, 1:10, source of complement, and finally, FITC labeled rabbit antibodies to C3c globulins (Dakopatts, Hamburg, FRG), 1:20, each for 30 min. at 37 °C.

HSV-1, CMV, VZV: ELISA test kits were used for testing patients sera, 1:40, for IgG antibodies (Behring, Mannheim, FRG).

Results

During our attempts to isolate retrovirus from Tanzanian PBL we recovered also HHV-6 (no. T9, no.
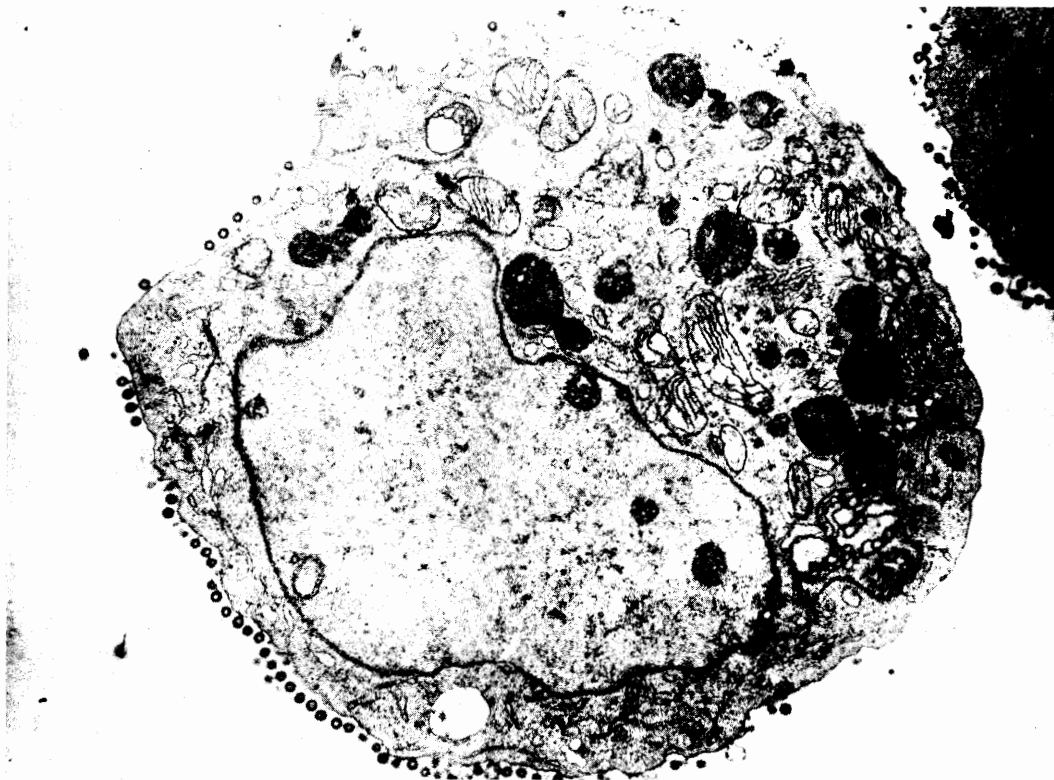
Fig. 1: Electron micrograph of an MT-4 cell infected with HHV-6 (T9). Magnification 5600 x

T87) from 2 HIV negative patients. Between days 7 and 12 of cocultivation, large refractile cells appeared and became the predominant cell type. Throughout the passage series, reverse transcriptase was not detected in cultures with extensive CPE. However, EM revealed the characteristic structure of a herpesvirus (Fig. 1).

Southern blotting of restriction enzyme digests of DNA from cultures of infected ,cells demonstrated hybridisation with HHV-6 probes, obtained from B. Honess (probe pHD 5). The infection was easily passaged on primary PBL cultures. Out of a large number of different cell lines only Jurkat, CEM, HSB-2, and MT-4 cells were susceptible to our HHV-6 isolates.

Double infection experiments

To investigate a possible interaction of HIV-1 and HHV-6 we established a double infection model based on MT-4 cells (Fig. 2). To achieve this we subcloned MT-4 cells (MT-4-T5) permanently infected with HIV-1 (Tanzanian isolate no. T5). This cell clone did no longer show any CPE for HIV-1 and produced $10^3 - 10^4$ infectious particles/ml supernatant. Growth pattern and viability did not differ from uninfected MT-4 cells. For double infection experiments we superinfected the MT-4-T5 clone with cell free HHV-6 virus (T9, T87) at a multiplicity of infection of 0.1 $TCID_{50}$/cell. The CPE for HHV-6 on the superinfected population and on single infected MT-4 cells occurred within 8 to 9 days. Cells infected only with HHV-6 died within 14 to 16 days p.i. Double infected populations died three days earlier. A marked increase in p24 antigen in supernatant of MT4-T5 was detected beginning at day 6 p.i. In contrast to the single infected cell clone we found a two to threefold increase of p24 in the supernatant of double infected cell cultures. To investigate the course of double infection over time we performed FACS analysis (Fig. 3). Single infected MT-4 cells on day 13 p.i. expressed
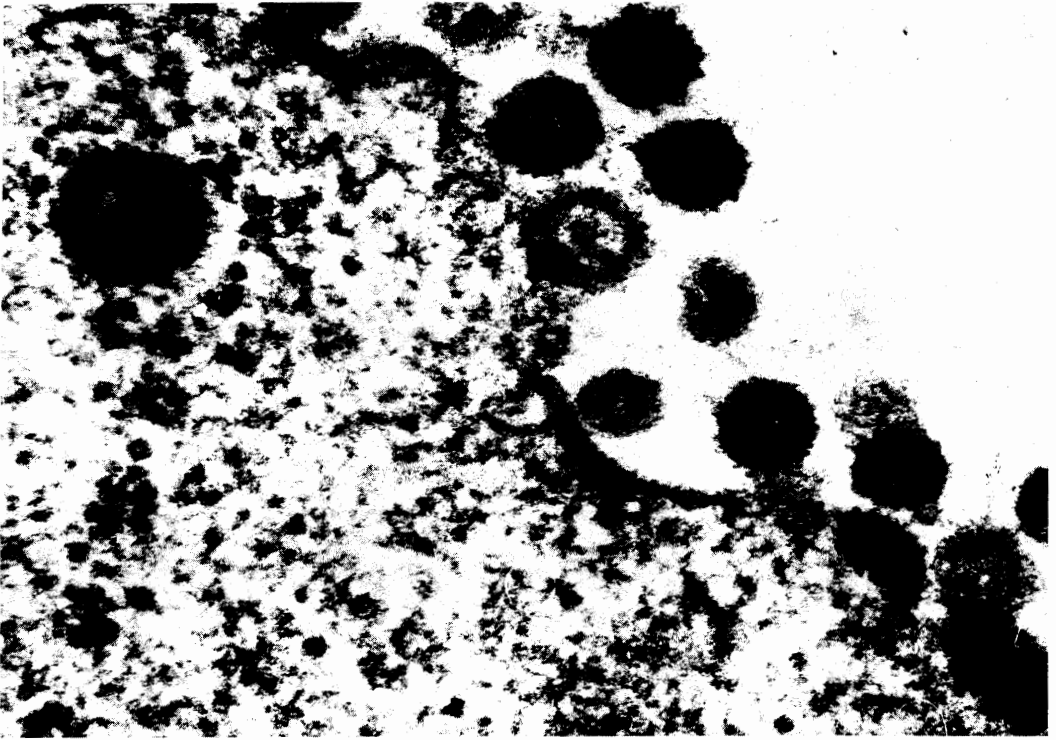
Fig. 2: Electron micrograph of double infected MT-4 cells (HIV-1 and HHV-6). Arrow heads indicate budding HIV particles. Seen intracellularly is the characteristic structures of a herpesvirus (asterisk). Magnification 31 500 x

nearly 100 % HHV-6 proteins at a mean of fluorescence (log PE) of 400. Double infected cells (MT-4-T5 + HHV-6, strain T9) at day 13 expressed HHV-6 proteins at a similar percentage but the fluorescence intensity (mean log PE) for HHV-6 was dramatically less when compared to single infected population (400 versus 127). Additionally the percentage of HIV-1 positive cells decreased over the course of double infection from 80 % in the beginning to 34 % on day 13.

As shown in Fig. 4 HHV-6 is able to activate transcription from the HIV-1 LTR in trans. HHV-6 infected MT-4 cells and uninfected MT-4, MT-2 and H9 cells were transfected with indicator plasmid pHIV-1 cat together with tat expression plasmid pCV-1 or with non-specific DNA (pBR322) to keep the DNA amount constant. In uninfected cells, transactivation was only observed by cotransfection of pHIV-1 cat and pCV-1 (compare lanes 1 and 2, 4 and 5, 7 and 8). In contrast, in HHV-6 infected cells, transcription

from the HIV-1 LTR is transactivated in the absence of pCV-1 (compare lanes 7, 10 and 13). The extent of transactivation is correlated to the percentage of HHV-6 infected cells as judged by immunofluorescence (lane 10: 5 % HHV-6 infected cells, lane 13: <1 % HHV-6 infected cells).

Serological evidence of Herpesviruses and HIV-1

To ascertain whether there was any correlation between the antibody prevalence of HIV-1 and herpesviruses we screened 493 sera of adult patients and 100 sera of school children from three Tanzanian hospitals for IgG antibodies to HIV-1, HSV-1, CMV, VZV, EBV and HHV-6 (Table 1). 49 % of the adults showed antibodies to HIV-1 confirmed by Western blotting. All children were HIV-1 seronegative. Of the 593 patients tested for HHV-6 antibodies, 46 % of the adults were seropositive (i.e. 227) and 37 % of the children (i.e. 37) when 1:30 was chosen as discrimina-
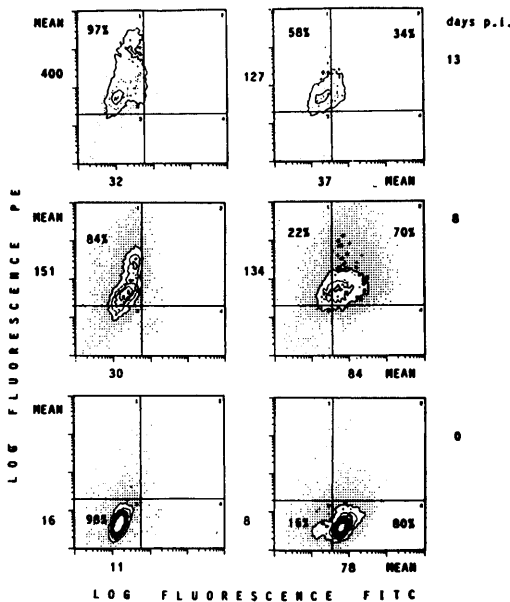
Fig. 3: FACSCAN analysis: Expression of HIV-1 and HHV-6 on MT-4 cells. Two colour immunofluorescence analysis (dot plot) of HHV-6 infected MT-4 cells (lane A) and MT-4-T5 (subclone permanently infected with HIV-1, lane B) at different times p.i. In this case the y axis indicates the level of positive HHV-6 expression (logPE) whereas the x axis shows HIV-1 expression (logFITC). Cell percentages are indicated in the quadrants.

tive IgG titer in the IFA. In regard to the titer there was no difference between adults and children. The 330 sera collected in a rural hospital in Kagera had a prevalence of 38 %, in sera from Muhimbili Medical Center we found 50 % of 123 sera and in sera from Ocean Road Hospital, 100 % of 42 sera positive for HHV-6. A difference in the rate of seropositivity against HHV-6 was found when men and women were compared. 41 % of the HHV-6 negative group (i.e. 83) and 62 % of the HHV-6 positive patients with a titer above 1:480 (i.e. 21) were women. We did not find any correlation between HHV-6 positivity or titer and HIV-1. 45 % of HIV-1 negative and 48 % of HIV-1 positive patients were HHV-6 positive. 51 % of the HHV-6 positive sera with a titer below 1:100 and 45 % of the sera with a titer above 1:480 were HIV-1 seropositive. Of 493 sera we obtained, 298 patients had well defined diseases (AIDS related complex, AIDS, tuberculosis, malaria and sexually transmitted diseases). We first tried to correlate the occurrence of antibodies against HIV-1 with the presence of anti-

bodies against EBV antigens such as EBNA, EA and VCA (indicative for respective an old, fresh or reactivated infection) and with the antibody titer against HHV-6. With respect to the serological markers no correlation was found between EBV and HIV nor between HHV-6 and HIV nor between EBV and HIV-1 and HHV-6. Further no correlation was seen between the above mentioned diseases (ARC, AIDS, etc.) and herpesvirus-, especially HHV-6 infection. The same sera were tested for antibody to other herpesviruses but antibody to HHV-6 did not correlate with the presence of antibody to HSV-1, CMV, VZV, and EBV, indicating that a positive anti HHV-6 IFA was specific and no correlation to other herpesviruses was observed.

Discussion

During our attempts to isolate retrovirus from Tanzanian PBL we cocultivated patients lymphocytes with HHV-6 and HIV negative donor PBL. Interestingly we isolated HHV-6 from two HIV seronegative patients. In contrast to other authors (23) we could not isolate HHV-6 from PBL of HIV seropositive patients. This may be due to the totally different growth pattern of both viruses and to the high isolation frequency for HIV-1 using our cocultivation method (>90 %). HIV-1 replicates in vitro much more rapidly than HHV-6. Normally we detect HIV-1 in primary cocultivation beginning on day 3 with maximum on day 8 whereas the detection of our HHV-6 strains began at day 10. In accordance with published results of other authors (18) we can show that our HHV-6 isolates from Tanzania are able to activate the transcription of HIV-1 in trans (Fig. 4). Our kinetical study of double infected MT-4 cells indicate: 1. HHV-6 leads to an enhancement of HIV-1 replication in early stages of double infection; 2. in later stages of double infection HHV-6 replication prevails over HIV-1 replication; 3. fluorescence intensity for HHV-6 expression in double infected populations does not reach the level of single infected MT-4 cells; 4. double infected cells are dying more rapidly than single infected cells.

Since HHV-6 is capable of increasing HIV-1 replication, infection by HHV-6 will consequently increase the cytopathic effect on coinfected $CD4^+$ cells in vitro. Because replication of HHV-6 itself is cytopathic for the same cells a synergistic effect may take place and progressively increase cell death. In this fashion HHV-6 infection or reactivation in immunosuppressed patients, for example AIDS patients, may exacerbate or modify the disease.

CAT-ASSAY

A   B   A   B   A   B   A   B   A   B

A
pHIV1 CAT

B
pHIV1CAT
pCV1

1   2   3   4   5   6   7   8   9   10
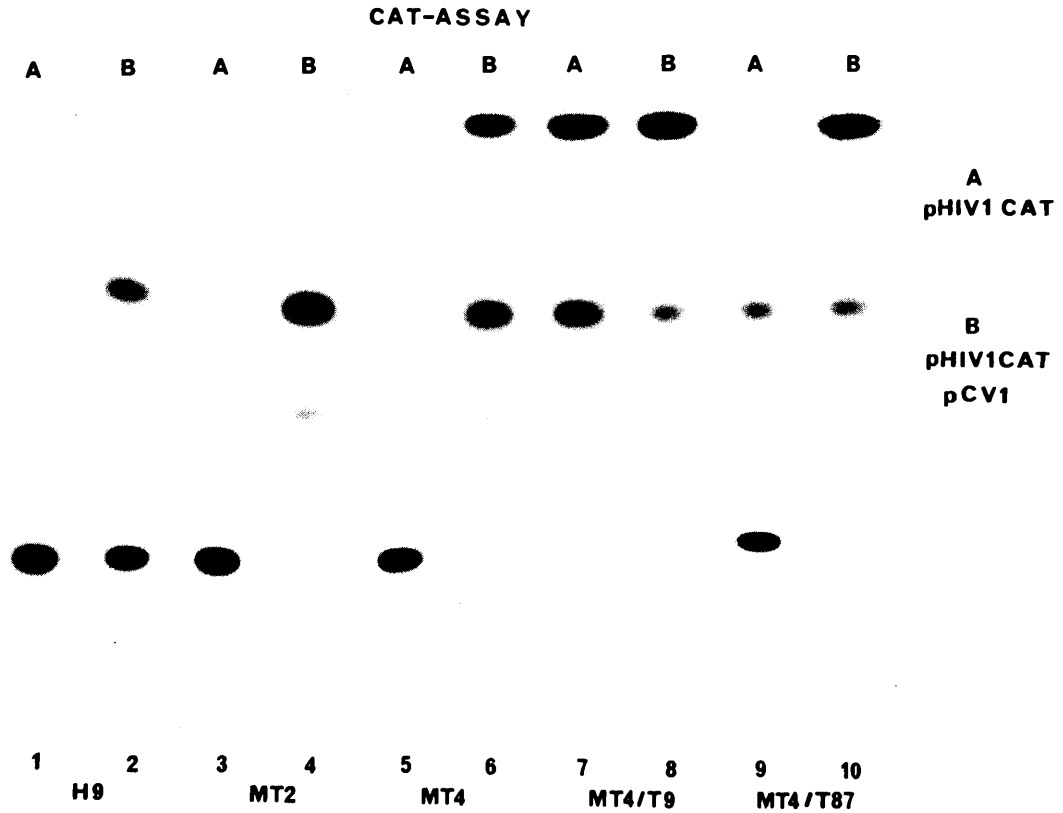H9   MT2   MT4   MT4/T9   MT4/T87

Fig. 4: CAT assay: HHV-6 infected MT-4 cells and uninfected MT-4, MT-2 and H9 cells were transfected with indicator plasmid pHIV-1cat together with tat expression plasmid pCV-1 or with non-specific DNA. In uninfected cells transactivation was only observed by cotransfection of pHIV-1 cat and pCV-1 (compare lanes 1 and 2, 4 and 5, 7 and 8). In HHV-6 infected cells transactivation of the HIV-1 LTR is also observed in cells, transfected only with pHIV-1 cat (compare lanes 7, 10 and 13).
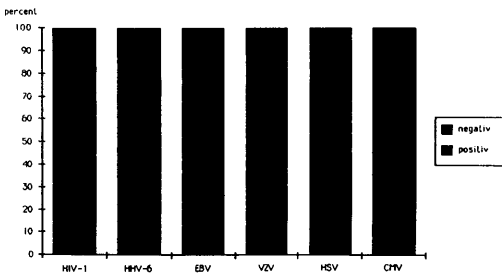
percent

Table 1: Seroprevalence for antibody against HIV-1, HHV-6, EBV, VZV, HSV-1, CMV (n = 493)

Many serological studies found prevalences for antibody against HHV-6 ranging from 20 to 98 % depending on the investigated populations and the discrimi-

nating titers (16, 17, 23, 24). With our method and a discriminating titer of 1:30 we found a seroprevalence for HHV-6 of 46 % in adults and 37 % in children. Significantly higher seroprevalences have been reported (5, 14, 15, 24) which compare better to our data if we could accept in IFA titer of 1:10 as positive. But in IFA we observed for African sera very high percentage of non specific reactions (approximately 25 %) on HHV-6 infected PBL, cord blood and MT-4 cells when using a discriminating titer of 1:10. In contrast to other studies the prevalence of HHV-6 in persons infected with HIV-1 or having AIDS syndrome was not significantly higher than in HIV-1 negative patients (Table 1). Ablashi et al. (1988) found a seroprevalence for HHV-6 of 26 % in a control group versus 70 % in a group of HIV infected patients. It is of

importance to state the differing transmission routes of HIV when African and American or European populations are compared. In Africa, HIV-1 infection has already entered the general population whereas in American or Europe the typical risk groups are still homosexuals, hemophiliacs and i.v. drug abusers. This may be one explanation for our findings.

Acknowledgement

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