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CD4-Independent, Productive Human Immunodeficiency Virus Type 1 Infection of Hepatoma Cell Lines In Vitro

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Five hepatoma cell lines, including CZHC/8571, PLC/PRF/5, Hep3B, HepG2, and HUH7, were inoculated with three diverse isolates of human immunodeficiency virus type 1 (HIV-1). Productive infection was noted in all hepatoma cell lines, and expression of viral p24 antigen lasted for over 3 months, but its level decreased in proportion to the number of viable cells. HIV-1 antigens were also found in the cells by immunohistochemical staining and radioimmunoprecipitation assay, as were viral RNA by in situ hybridization and HIV-1-like particles by electron microscopy. Virus yield assays were also positive on supernatant fluids collected from hepatoma cultures inoculated with HIV-1. Despite their susceptibility to infection, all five hepatoma cell lines were negative for CD4 by immunofluorescence and for CD4 mRNA by slot-blot hybridization. In addition, HIV-1 infection of hepatoma cell lines was not blocked by anti-CD4 monoclonal antibody or soluble CD4. Together, these findings clearly demonstrate that all five hepatoma cell lines were susceptible to productive infection by HIV-1 in vitro via a CD4-independent mechanism.

Human immunodeficiency virus type 1 (HIV-1) is tropic and cytopathic for human T lymphocytes bearing the CD4 receptor (18, 29). Macrophages, which also express CD4 on the cell surface, have also been shown to play a major role in the propagation and pathogenesis of HIV-1 infection (10, 15, 26, 30). However, many human cell lines which were not known to be CD4 positive have recently been found to be susceptible to HIV-1 infection in vitro. These include cells derived from colorectal carcinoma (1), rhabdomyosarcoma (6), bone marrow CD34$^+$ precursor cells (9), glioma (4–7, 13), and neuroblastoma (23). Several reports have also shown that HIV-1 is capable of infecting certain nonlymphoid tissues or cells in vivo, including colon, rectum, duodenum, cervix, retina, brain, and megakaryocytes (25, 27, 28, 35, 37). These in vitro and in vivo findings suggest that HIV-1 tropism may not be limited to CD4-positive cells.

Hepatic abnormalities associated with the acquired immunodeficiency syndrome (AIDS) include endothelialitis, hepatocellular necrosis, and granulomatosis (22, 31). To address whether HIV-1 can directly involve hepatocytes, we studied the susceptibility of hepatoma cell lines to infection in vitro by multiple strains of HIV-1.

MATERIALS AND METHODS

Cells and viruses. Five hepatoma cell lines were used in these studies, including three hepatocellular carcinoma (HCC) cell lines, PLC/PRF/5 (2), Hep3B (19), and CZHC/8571 (3), which contain the integrated gene of the hepatitis B surface antigen (HBsAg) and constitutively produce HBsAg into the culture medium. Another HCC cell line, HUH7 (24), and one hepatoblastoma cell line, HepG2 (19), did not produce HBsAg. These cell lines were cultured in Dulbecco modified Eagle minimal essential medium supplemented with heat-inactivated (56°C, 45 min) 15% fetal calf serum. Cell-free stocks of three strains of HIV-1, including HTLV-IIIB (29), HTLV-IIIRF (36), and HIV-AL (16), were harvested from infected H9 cultures. The infectious titers of these viral stocks were determined by serial dilutions on H9 cells and then adjusted to 5×10^5 50% tissue-culture infective doses ($TCID_{50}$) per ml.

Infection of hepatoma cell lines by HIV-1. Cells (5×10^6) were inoculated at multiplicities of infection ranging from 0.001 to 1 $TCID_{50}$/cell and then maintained in 25-cm^2 flasks. Each flask was washed four to six times with 1× Hanks solution 24 h after infection. The production of p24 antigen in the culture supernatant fluids was monitored once a week by an antigen capture enzyme immunoassay (Abbott Laboratories, North Chicago, Ill.). Virus yield assays were performed by harvesting cell-free supernatant fluids from select hepatoma cell cultures and inoculating onto the CD4$^+$ lymphoblastoid cell lines HPB-ALL and Molt-4 maintained in RPMI 1640 medium containing 10% fetal calf serum. The recovery of infectious HIV-1 was again determined by expression of HIV-1 p24 antigen in the culture supernatant once a week.

Light and electron microscopy. Hepatoma cell lines with and without inoculation of HIV-1 were serially monitored for morphologic changes by reverse-phase light microscopy. In addition, electron microscopy was performed with standard techniques to demonstrate HIV-1 infection in the CZHC/8571 HCC cells, as well as to document any associated cellular changes.

Immunohistochemical assay. Immunoperoxidase staining for HIV-1 antigens in hepatoma cells was performed by a previously described method with biotin-labeled antibodies (12). Cells were fixed onto the slides 1, 3, and 7 days after inoculation of HIV-1, using 5% acetic acid mixed in 95% methanol for 20 min. Slides were next incubated with 0.5% hydrogen peroxide for 30 min at room temperature and then washed with 0.05 M Tris hydrochloride for 20 min. After incubation with normal goat serum (Vector Laboratories, Inc., Burlington, Calif.) for 20 min at 37°C, the slides were incubated with mouse monoclonal antibodies to HIV-1 p24 or gp120 (NEN/Dupont, Billerica, Mass.) overnight at room temperature. After two washes, the slides were incubated

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TABLE 1. Infection of five hepatoma cell lines by HIV-1

Characteristic	PLC/PRF/5	Hep3B	CZHC/8571	HUH7	HepG2
Expression of HIV-1 antigens					
Supernatant p24	+	+	+	+	+
Cellular p24[a]	+	+	+	+	+
Cellular viral proteins[b]	NT[c]	NT	+	NT	NT
Supernatant reverse transcriptase activity	NT	NT	+	NT	NT
Expression of HIV-1 particles	NT	NT	+	NT	NT
Recovery of infectious HIV-1	+	+	+	+	+
Expression of CD4 protein by indirect immunofluorescence	−	−	−	−	−
Expression of CD4 mRNA	−	−	−	−	−

[a] Determined by immunoperoxidase method.
[b] Determined by radioimmunoprecipitation method.
[c] NT, Not tested.

with biotin-labeled goat anti-mouse immunoglobulin antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) at 37°C for 2 h. The slides were again washed before treatment with horseradish peroxidase-streptavidin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Reactions were subsequently developed with 3-amino-9-ethyl carbamazole, and cells were counterstained with hematoxylin. Appropriate positive and negative controls were included with each assay.

In situ hybridization. The hepatoma cells, with or without HIV-1 inoculation, were fixed onto poly-L-lysine-coated slides and then hydrated with 10 mM Tris–1 mM EDTA–1 mM dithiothreitol for 10 min, followed by addition of 0.2 N HCl for 20 min at room temperature. The cells on slides were subsequently permeabilized with proteinase K (1 μg/ml, 30 min, 37°C) and immersed in 5% paraformaldehyde for 10 min at room temperature. After several washes, the slides were immersed in 0.5 ml of acetic anhydride in 0.1 M triethanalamine for 10 min at room temperature, again followed by washes. The slides were prehybridized with 10 mM Tris–1 mM EDTA–5× Denhardt solution (0.2% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin) at 37°C for 30 min. After being washed, the slides were sequentially treated with 95% formamide in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 15 min and 0.1× SSC at 0°C for 5 min, followed by dehydration in ethanol. The slides were then covered by hybridization mixture (50% formamide, 10% dextran sulfate, 10 mM Tris, 1 mM EDTA, 1× Denhardt solution, 0.6 M NaCl, 1 mg of sperm DNA per ml) and 10^6 dpm of ^{35}S-labeled HIV RNA

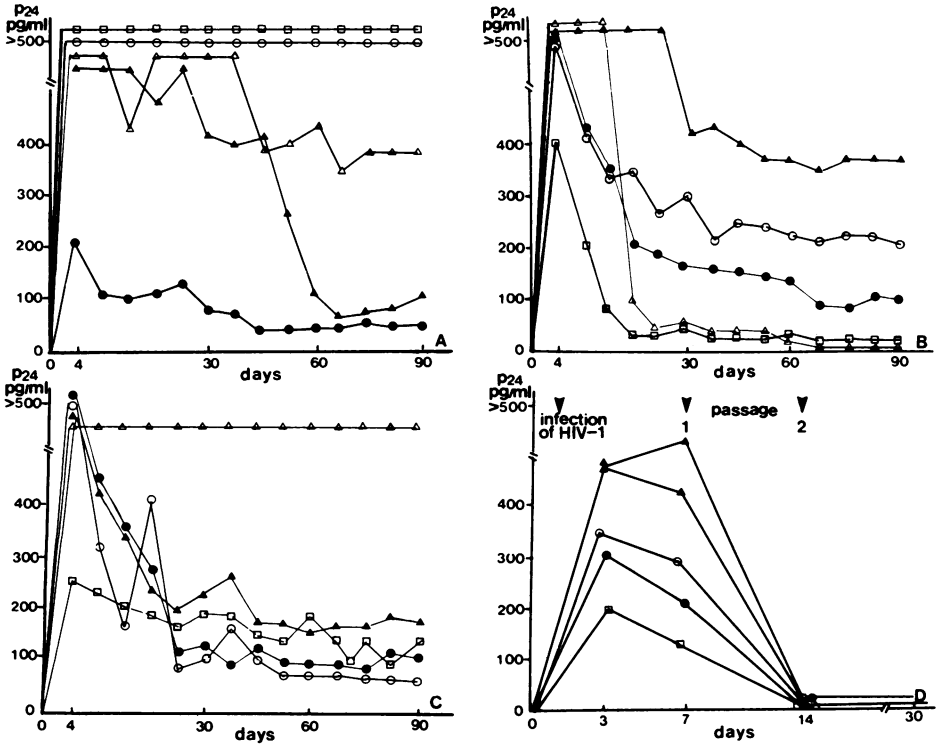

FIG. 1. Persistent HIV-1 infection of five hepatoma cell lines, PLC/PRF/5 (□), Hep3B (○), CZHC/8571 (△), HepG2 (▲), and HUH7 (●). (A, B, C) Monitoring of the HIV-1 p24 antigen level in the supernatant of cells inoculated with HTLV-IIIB (A), HIV-AL (B), and HTLV-IIIRF (C). (D) All five hepatoma cell lines were split 1:2 and refed with fresh medium 7 and 14 days after inoculation with HIV-1. p24 antigen was undetectable following the second passage.

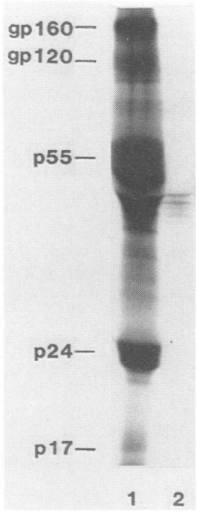

FIG. 2. Demonstration of HIV-1 envelope and core proteins in CZHC/8571 cells by radioimmunoprecipitation with a pooled human anti-HIV-1 immunoglobulin preparation (lane 1). Lane 2, Radioimmunoprecipitation with a normal human serum.

probe (NEN/Dupont) (37) per 10 μl at room temperature for 24 to 48 h. Subsequently, the cover slips were floated off in 2× SSC, and the slides were washed in 2× SSC twice at room temperature and at 55°C for 1 h. Following washes with 50% formamide–0.6 M NaCl–10 mM Tris–1 mM EDTA at room temperature overnight, the slides were treated with RNase A at 20 μg/ml in RNase buffer for 30 min at room temperature. After final washes, the cells on slides were dehydrated with ethanol, then coated with NTB2 emulsion (Kodak), and exposed for 3 to 7 days at room temperature, followed by development with Kodak D-19 and counterstaining with hematoxylin and eosin.

Radioimmunoprecipitation assay. A radioimmunonoprecipitation assay was used to demonstrate HIV-1 antigens in the hepatoma cells inoculated with virus. Three days after inoculation with HIV-1, CZHC/8571 HCC cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine (NEN/Dupont) overnight. A lysate of the cells was subjected to immunoprecipitation by pooled human anti-HIV-1 antibodies linked to staphylococcal protein A-Sepharose, and the precipitated proteins were then subjected to polyacrylamide gel electrophoresis as described previously (17), followed by autoradiography.

Reverse transcriptase assay. Supernatant fluids were collected from CZHC/8571 cultures 1, 2, 4, 7, 10, and 14 days after HIV-1 inoculation. Reverse transcriptase activity was assayed by a method previously described (14).

Detection of proviral DNA by the PCR method. CZHC/8571 cells inoculated with HIV-1 were serially passaged every 7 days. DNA was extracted from the cells at each passage and then subjected to polymerase chain reaction (PCR) with *gag* primers (SK38 and SK39) by the method of Kwok et al. (20). The amplified products were subsequently subjected to hybridization with a ^{32}P-labeled probe (SK19) to detect the HIV-1-specific DNA sequence.

Detection of CD4 on hepatoma cells by immunofluorescence. Hepatoma cells were grown in eight-well culture-chamber slides (Lab-Tek; Miles Scientific) and subsequently fixed in acetone. Anti-CD4 monoclonal antibodies Leu3A (Becton Dickinson), OKT4, and OKT4A (Ortho Diagnostics) at dilutions of 1:10, 1:25, or 1:50 were then added for 30 min at 37°C. The cells were washed three times in phosphate-buffered saline before the addition of goat anti-mouse immunoglobulin antibody conjugated to fluorescein isothiocyanate for another 30 min at 37°C. After three more washes, the slides were air dried, mounted, and examined under a UV microscope. Appropriate negative and positive controls for the immunofluorescence assay were included.

Detection of CD4 mRNA in hepatoma cells by slot-blot hybridization. RNA preparations extracted by the guanidinium-hot phenol method for CD4$^+$ T cells (HPB-ALL, 2×10^6), rat neuroblastoma cells (B35), and the five hepatoma cell lines were each spotted onto a nitrocellulose membrane with the Minifold II Slot Blot System (Schleicher & Schuell, Keene, N.H.). After being baked at 80°C for 1 h under vacuum, the blot was soaked for 5 h at 42°C in 10 ml of 5× SSC–1× Denhardt buffer–50% deionized formamide–0.1% sodium dodecyl sulfate prior to hybridization. A 1.8-kilobase fragment from *Xba*I-*Xho*I digestion of the CDM7:CD4 plasmid (gift of D. Camerini and B. Seed, Massachusetts General Hospital) was used as the probe. This fragment, containing the entire CD4 coding sequence, was labeled with 50 μCi of [α-^{32}P]dCTP (NEN/Dupont; specific activity, 3,000 Ci/mmol) and a random-priming DNA-labeling kit (Boehringer Mannheim Biochemicals). Hybridization was then carried out with the CD4 probe (2×10^7 cpm/blot) in buffer containing 10% dextran sulfate, 50 μg of denatured salmon sperm DNA per ml, and 50 μg of denatured yeast tRNA per ml for 36 h at 42°C. The blot was subsequently washed, air dried, and subjected to autoradiography.

HIV-1 blocking experiment in hepatoma cells. Recombinant soluble CD4 (30 μg; Biogen, Inc., Cambridge, Mass.) (8) was preincubated with the HTLV-IIIRF isolate (10^4 $TCID_{50}$) in 1 ml for 60 min at 37°C before inoculation onto T cells (HPB-ALL and CEM; 5×10^5) and the hepatoma cells (5×10^5). In addition, Leu3A (30 μg) and a control mouse monoclonal antibody were preincubated with T cells and hepatoma cells in 1 ml for 60 min at 37°C. These cells were then challenged with HTLV-IIIRF (10^4 $TCID_{50}$). After 24 h, all cultures were washed six times and replenished with medium. Each of the last washes was negative for detectable p24 antigen. On days 4 and 7 of the experiment, supernatant fluids were examined for HIV-1 expression by a p24 antigen capture assay (Abbott Laboratories). Four such experiments were performed.

RESULTS

Productive infection of hepatoma cells by HIV-1. Each of the five hepatoma cell lines was productively infected by three strains of HIV-1 at multiplicities of infection of 0.001 to 1.0 (Table 1). Figure 1 shows the significant levels of p24 antigen expression in supernatant fluids of hepatoma cultures inoculated with HTLV-IIIB (Fig. 1A), HIV-1 AL (Fig. 1B), and HTLV-IIIRF (Fig. 1C). This supernatant antigen expression was persistent, although the levels generally decreased with time with increasing cell mortality. Morphologically, the infected hepatoma cells exhibited cellular swelling and increased vacuolization. There was no evidence of cell-cell fusion. When infected hepatoma cells were serially passaged, HIV-1 p24 antigen and infectious virus were no longer detectable after the second passage (Fig. 1D). However, proviral DNA was still present until the fourth passage as determined by the PCR method (data not shown), suggesting that HIV-1 infection in the hepatoma cells may have become latent. Nevertheless, after the fourth passage, there was no evidence of HIV-1 infection by any method employed.

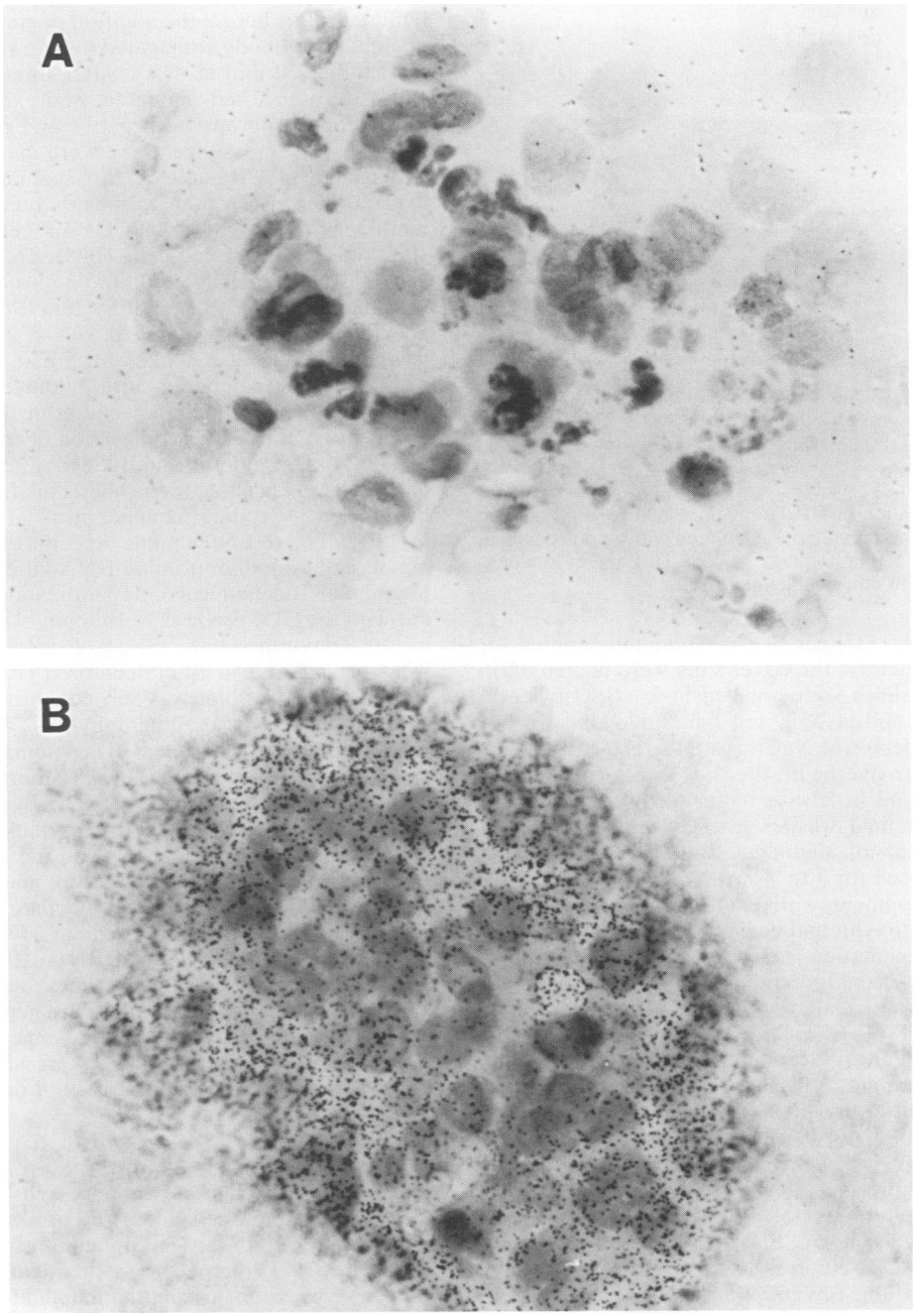

FIG. 3. In situ hybridization of uninfected (A) and HIV-1-infected (B) CZHC/8571 cells. Magnification, ×1,600.

HIV-1 antigen production was also detected within the hepatoma cells inoculated with virus. As summarized in Table 1, intracellular p24 antigen was detected in each of the hepatoma cell lines 3 days after inoculation with HIV-1 by the immunoperoxidase method with a specific mouse monoclonal antibody. Approximately 20 to 50% of the inoculated hepatoma cells expressed p24 protein, whereas no such antigen was found in uninoculated hepatoma cells. In addition, in one hepatoma cell line (CZHC/8571), viral antigen production was examined by radioimmunoprecipitation 3 days after exposure to HIV-1. As shown in Fig. 2, synthesis of several viral proteins (gp160, gp120, p55, p24, and p17) was clearly documented.

Supernatant particulate reverse transcriptase activity was also detected in a CZHC/8571 culture after inoculation with HIV-1 (Table 1), with values ranging from 1×10^3 to 7.7×10^4 cpm/ml, while the negative control values were less than 10^3 cpm/ml. The peak reverse transcriptase activity was, however, 10- to 100-fold less than that typically found for T-cell lines chronically infected with HIV-1.

The susceptibility of the hepatoma cells to productive HIV-1 infection was also documented by in situ hybridiza-

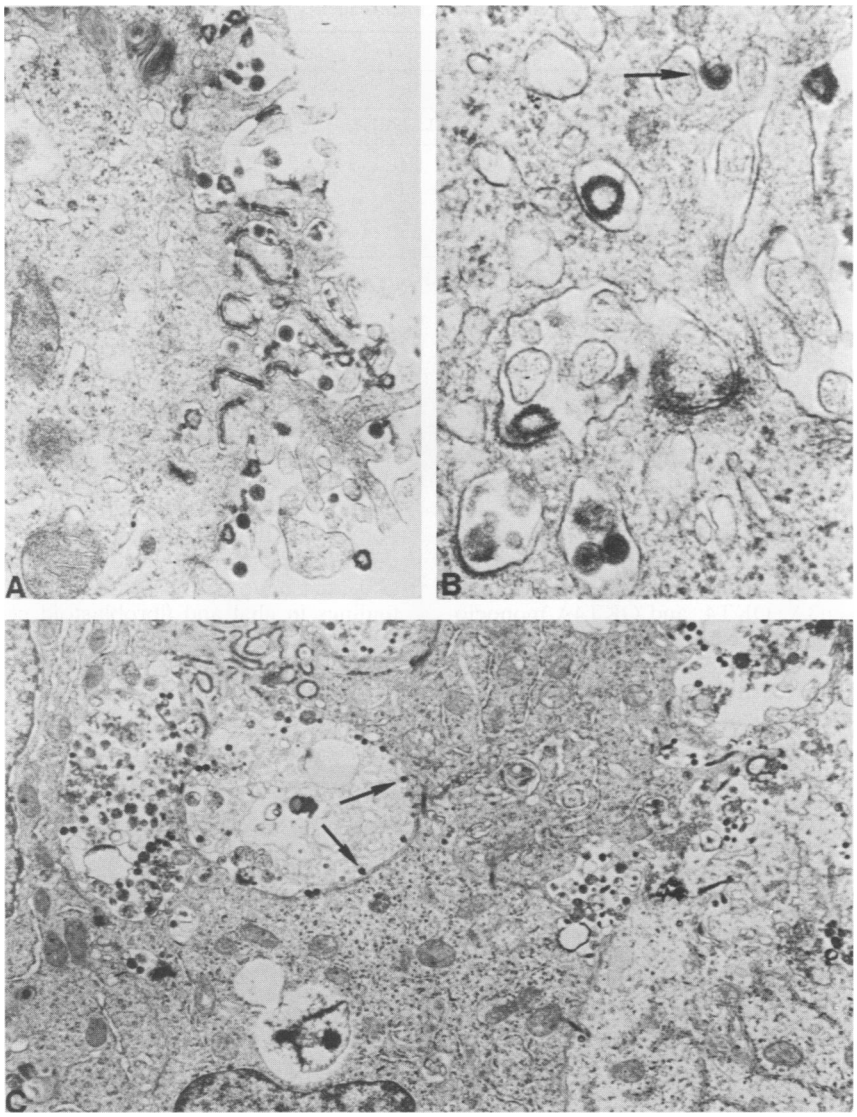

FIG. 4. Electron microscopy of HIV-1-inoculated hepatoma cells. (A) At 6 h after inoculation, numerous particles are adherent to the plasma membrane and within membrane invaginations. Magnification, ×30,000. (B) At 24 h, budding or fusion of virus is seen (arrow). Magnification, ×60,000. (C) At 7 days, many cells show vacuoles containing virus particles (small arrows), and cytolysis is often apparent. Magnification, ×11,000.

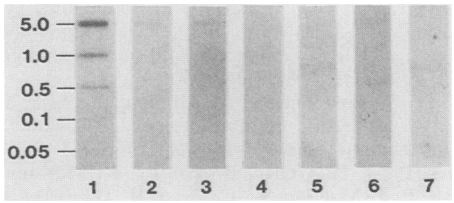

FIG. 5. Lack of CD4 mRNA in hepatoma cells by RNA slot-blot hybridization. Lane 1, $CD4^+$ HPB-ALL T cells (positive control); lane 2, rat neuroblastoma cells (negative control); lane 3, CZHC/8571; lane 4, HepG2; lane 5, Hep3B, lane 6, HUH7; lane 7, PLC/PRF/5. The amount of RNA (in micrograms) placed in each slot is shown at the left.

tion experiments. Three days after exposure to HIV-1, each of the hepatoma cells expressed viral mRNA as detected by in situ hybridization (Table 1). One example (CZHC/8571) is shown in Fig. 3.

HIV-1 infection of the CZHC/8571 hepatoma cell line was confirmed by electron microscopic studies. Six hours following exposure to virus, numerous HIV-1-like particles adhered to the plasma membrane of the cells, and some appeared to be in the process of being interiorized (Fig. 4A). At 24 h, there were profiles which could represent fusion or budding as the envelope of the virus appeared to be in continuity with the plasma membrane (Fig. 4B). On day 7, numerous HIV-1-like particles (110 to 140 nm) were observed within cytoplasmic vacuoles, as well as in the extracellular medium (Fig. 4C).

Virus yield assays were performed on supernatant fluids collected from hepatoma cultures 3 days after initial HIV-1

TABLE 2. Inhibition of HIV-1 expression by blocking reagents in T cells and five hepatoma cell lines

Reagent[a]	% Inhibition						
	T cells		Hepatoma cells				
	HPB-ALL	CEM-SS	PLC/PRF/5	Hep3B	CZHC/8571	HUH7	HepG2
Medium	0	ND[b]	0	0	0	0	0
Control MAB (30 μg/ml)	13	0	0	0	12	0	0
Leu3A (30 μg/ml)	97	95	0	0	20	0	0
Soluble CD4 (30 μg/ml)	100	100	0	15	10	0	0

[a] MAB, Monoclonal antibody.
[b] ND, Not determined.

inoculation. As summarized in Table 1, infectious HIV-1 was recovered, by using T cells, from each of the hepatoma cultures previously inoculated with virus. Together, the findings described in Table 1 clearly demonstrate that all five hepatoma cell lines were susceptible to productive infection by HIV-1 in vitro.

HIV-1 infection of hepatoma cells is CD4 independent. Despite their susceptibility to HIV-1 infection, CD4 protein was not detectable in the hepatoma cells by indirect immunofluorescence with Leu3A, OKT4, and OKT4A monoclonal antibodies (Table 1) or by flow cytometry (data not shown). In addition, CD4 mRNA could not be identified in RNA preparations of any of the hepatoma cell lines, as analyzed by slot-blot hybridization with a CD4-specific probe (Fig. 5, Table 1). Therefore, within the limits of the sensitivities of the techniques employed, it appears that CD4 is not expressed in these cells.

Since the possibility existed that CD4 present at subdetectable levels may still permit HIV-1 entry, a blockade of infection was attempted with anti-CD4 monoclonal antibodies and soluble CD4. Mean values from four blocking experiments are summarized in Table 2. Both Leu3A and soluble CD4 showed nearly complete (95 to 100%) inhibition of HIV-1 infection of T cells, as expected, but they failed to block virus infection of the hepatoma cells (0 to 20% inhibition). These findings strongly support the conclusion that HIV-1 infection of hepatoma cells is CD4 independent.

DISCUSSION

We have shown that hepatoma cells can be productively and persistently infected by HIV-1 with resultant viral antigen expression intracellularly and in the culture supernatant. In addition, HIV-1 mRNA and virions were demonstrated within the inoculated hepatoma cells by in situ hybridization and electron microscopy, respectively. The recovery of infectious virus from the inoculated cells confirmed the susceptibility of hepatoma cells to HIV-1 infection. These findings suggest that careful in situ studies should be performed on liver tissue obtained from patients with AIDS to determine whether HIV-1 can directly contribute to the occasional hepatic abnormalities seen in AIDS.

Three of the HIV-1 susceptible hepatoma cell lines (PLC/PRF/5, Hep3B, and CZHC/8571) harbor the hepatitis B virus genome and constitutively release HBsAg. Given that these cells can also be chronically infected by HIV-1, they thus represent a unique system to investigate possible HIV-1 and hepatitis B virus interactions at the cellular level. This is particularly important given that molecular interactions between the two viruses have already been shown (11, 32, 34) and that patients with AIDS are commonly coinfected with hepatitis B virus (21).

Despite their susceptibility to HIV-1, the hepatoma cells had no detectable CD4 or CD4 mRNA. Furthermore, HIV-1 infection in these cells could not be blocked by anti-CD4 monoclonal antibody or soluble CD4. These observations suggest that the hepatoma infection by HIV-1 is independent of CD4 and add to the growing evidence that there is a non-CD4 pathway by which the virus can enter cells, such as those of glial (4–7, 13), rhabdomyosarcoma (6), fibroblastoid (33), and neuronal (23) origin. In contrast to the reported findings in glial and fibroblastoid cells, HIV-1 infection of hepatoma cells is productive and does not require cocultivation to demonstrate the presence of the virus. Therefore, these cells are particularly useful for studies to define an alternative receptor for HIV-1.

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