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Hepatitis B Virus (HBV)-Specific Cytotoxic T-Cell Response in Humans: Production of Target Cells by Stable Expression of HBV-Encoded Proteins in Immortalized Human B-Cell Lines†

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To analyze the hepatitis B virus (HBV)-specific cytotoxic T-cell (CTL) response during acute and chronic viral hepatitis, target cells that express HBV-encoded antigens in the context of the appropriate HLA restriction element must be available for each subject studied. Since HBV is not infectious for human cells in vitro, such target cells must be produced by DNA-mediated gene transfer into cultured human primary cells or cell lines. For this purpose, we have developed a panel of Epstein-Barr virus-based episomal expression vectors containing each of the HBV open reading frames under the transcriptional control of the simian virus 40 early promoter. Transfection of Epstein-Barr virus-immortalized B-cell lines with this panel of recombinants consistently leads to stable expression of the HBV envelope, nucleocapsid, and polymerase proteins. The HBV X gene product is transiently expressed following transfection, but stable expression of this protein cannot be maintained on a long-term basis. To assess the suitability of this system for the identification of HBV-specific CTL in humans, a panel of EBO-HBV transfectants of defined HLA haplotype was used to monitor the HBV-specific CTL response in a patient with acute viral hepatitis type B. Transfectants that stably express the HBV nucleocapsid (core) antigen were found to serve as excellent targets for the detection of HLA class I-restricted CTL that recognize endogenously synthesized HBV core antigen in this patient; they were also successfully used to stimulate the specific expansion of these CTL in vitro.

Hepatitis B virus (HBV) is a hepatotropic DNA virus that infects more than 250 million people worldwide and causes acute and chronic liver disease and hepatocellular carcinoma (4). Circumstantial clinical evidence and data from a number of in vitro and animal model systems (2, 22) suggest that HBV is probably not directly cytopathic, at least when HBV replication and gene expression are normally regulated. Rather, it is assumed that HBV-associated liver disease is caused by a cytolytic immune response against HBV-encoded antigens expressed by the infected hepatocyte.

Definitive proof of this hypothesis requires (i) the presence of sufficient numbers of specific cytotoxic T cells (CTL) in the peripheral blood to be detectable and (ii) the existence of a sensitive and specific cytotoxicity assay. The assay must involve the presentation of endogenously synthesized and processed viral antigen to the CTL antigen receptor by an autologous HLA class I molecule on the surface of another cell which is then killed.

Several years ago, Mondelli and colleagues (16, 17) reported the detection of HBV core antigen (HBcAg)-specific CTL in the peripheral blood of patients with acute and chronic HBV infection, using an autologous hepatocytotoxicity assay which measured the capacity of peripheral blood lymphocytes (PBL) from their patients to reduce the adherence of autologous, liver biopsy-derived hepatocytes to a plastic surface. These interesting results were limited by a number of factors, including uncertain correlation between loss of cytoadherence and cell death, lack of knowledge of the percentage of infected liver cells in the biopsy, variation in the number of infected cells from biopsy to biopsy, high spontaneous death rate of the target cells, inability to define the antigenic fine specificity and HLA restriction of the response, and inability to repeat experiments with the same target cells. Clearly, a more conventional and reproducible cytotoxicity assay system is needed if an HBV-specific CTL response is to be reliably detected and characterized in these patients, and if its role in viral clearance and the pathogenesis of liver cell injury is to be thoroughly examined.

Since HBV is not infectious in vitro, cytolytic target cells that express each of the HBV gene products in the context of a large assortment of defined HLA molecules must be generated by alternative high-efficiency gene transfer methods. Accordingly, over the past several years we have used a panel of well-characterized vaccinia virus (21) and retrovirus (15) vectors that direct the expression of the HBV envelope antigen and nucleocapsid antigen in several human cell types to generate ^{51}Cr-labelled target cells for this purpose. All studies thus far, using freshly isolated PBL from patients with acute and chronic HBV infection, have been negative (7). These results suggest that the HBV-specific CTL precursor frequency in the peripheral blood of patients with acute and chronic HBV infection is too low to be detected directly, and that the selective in vitro expansion of HBV-specific CTL by stimulation with stable, HLA-compatible transfectants may be necessary in order to detect them. The high-efficiency vaccinia virus and retrovirus-based recombinants that we have used thus far are not suitable for repetitive in vitro stimulation because they generate infectious virus that can affect the responder T-cell population.

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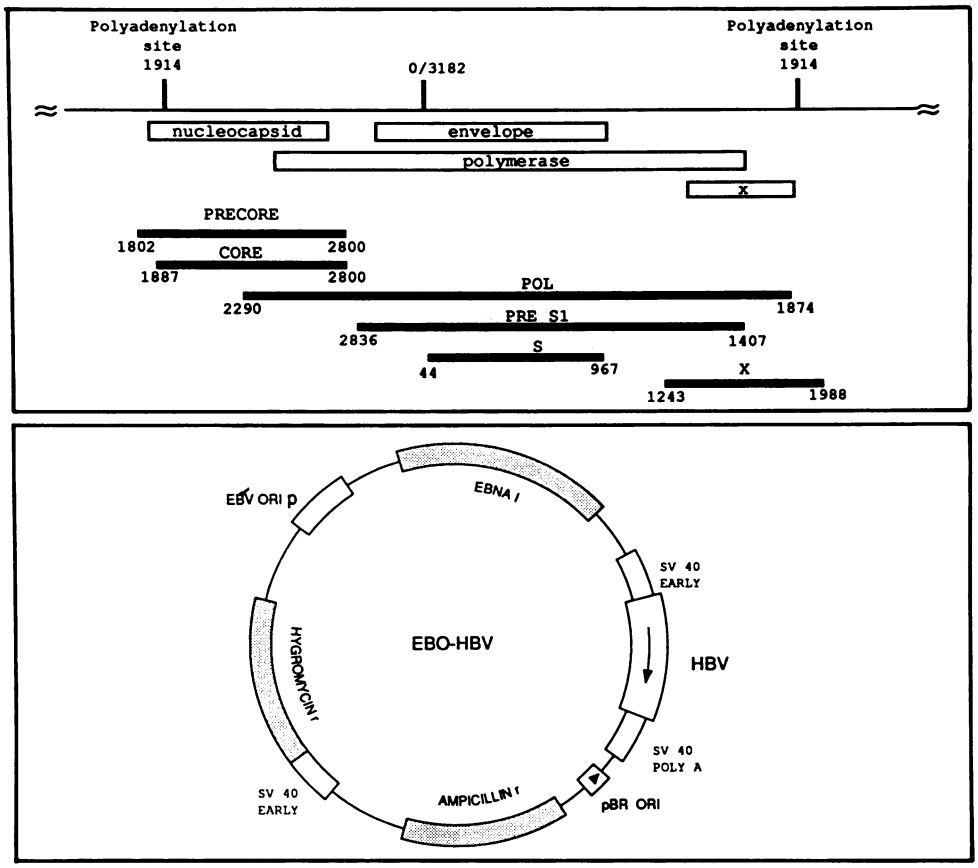

FIG. 1. Subcloning of the HBV ORF into the expression vector EBO-plpp. The different HBV ORFs are depicted in the open boxes under a virtual concatemer representing the HBV genome. The dark lines represents the fragments subcloned into EBO-plpp. The numbers correspond to the coordinates of each fragment; numbering is as in reference 10. The S fragment is within the pre-S1 fragment, which is itself part of the polymerase fragment.

Herein we report the production and characterization of a panel of noninfectious Epstein-Barr virus (EBV)-based expression vectors specifically intended to generate stimulator/target cells for this purpose. The recombinant plasmids contain all of the HBV open reading frames (ORFs), they replicate episomally in EBV-immortalized B-cell lines (BCL) after high-efficiency transfection, and they direct the stable expression of all of the viral gene products except the HBV transcriptional transactivating protein (the X gene product). A large library of HLA-defined BCL that express these antigens has been derived by transfection with these vectors and is being used to examine the HBV-specific CTL response in patients with acute and chronic HBV infection. Transfectants that stably express the HBV nucleocapsid region are specifically killed by CTL derived from the peripheral blood of patients with acute viral hepatitis type B in an HLA class I-restricted manner.

MATERIALS AND METHODS

Plasmid constructions. The EBO vector is an EBV-based vector designed for stable expression in eukaryotic cells (6). When used in human B cells, this vector replicates as an episome because of the EBV origin of replication and the EBNA I transactivator (Fig. 1). The sequences to be expressed are introduced into a polylinker between the simian virus 40 (SV40) early promoter and polyadenylation signal. The hygromycin resistance gene placed under the transcriptional control of a second SV40 early promoter allows positive selection of the transfected cells. The pBR322 origin of replication and ampicillin resistance gene allow the cloning and production process in bacteria.

The different HBV (ayw subtype) ORFs (10) were subcloned into the EBO-plpp vector by standard methods (13). Enzymes were used according to the manufacturer's recommendations. The coordinates of the HBV fragments (according to Galibert et al. [10]) inserted into EBO-plpp are as follows: precore (1803 to 2804), core (1890 to 2804), polymerase (2290 to 1874), pre-S1 (2839 to 1990), S (47 to 971), and X (1243 to 1988) (Fig. 1). Generation of the different envelope and nucleocapsid fragments has been previously reported (15). The X and polymerase fragments were generously provided by A. McLachlan and M. Riggs. The HBV ORFs were subcloned into the *SalI* site of M13mp18 and subsequently transferred into the EBO-plpp vector after a double digestion with *Hin*dIII and *Kpn*I. Because the HBV envelope region is completely contained within the HBV polymerase ORF, the EBO-Pol construct also has the potential to express the envelope proteins.

Cell culture and plasmid transfection. PBL from normal individuals or HBV-infected patients whose HLA haplotypes had been determined by standard microcytotoxicity analysis (23) using reagents purchased from One Lambda (Los Angeles, Calif.) were immortalized by infection with

EBV as described previously (8) except that cyclosporine (2 μg/ml) was added to the culture medium for 1 week after infection. The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 10 μg of gentamicin sulfate per ml, 100 μM nonessential amino acids, 1 mM sodium pyruvate, and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO_2 atmosphere. Plasmid transfections were done by electroporation essentially as described previously (14). Briefly, 10^7 cells were washed once and resuspended in 250 μl of electroporation buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose); 10 μg of plasmid DNA was added, and the cells were electroporated at room temperature in a 0.4-cm-wide cuvette with a Bio-Rad Electroporator set at 210 V and 960 μF. Selection was applied after 24 h with 250 μg of hygromycin (Sigma) per ml.

DNA and RNA analysis. DNA extraction and Southern blot analysis were performed according to standard methods (13). RNA extraction was performed by the acid pH-phenol method (13). RNA was separated on 1% agarose gels in the presence of formaldehyde. Nylon membranes (Hybond; Amersham) were used for both DNA and RNA transfer.

Immunoblotting. Cell lysates were prepared by three cycles of freezing and thawing in 60 mM Tris-Cl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–0.7 M 2-mercaptoethanol–0.1 mM phenylmethylsulfonyl fluoride–10% glycerol. Polyacrylamide gel electrophoresis was performed as described by Laemmli (12). Protein concentration was determined by the copper sulfate-bicinchoninic acid method (Pierce, Rockford, Ill.). For immunoblotting, the proteins were transferred from acrylamide gels by electroblotting onto nitrocellulose membranes (BA 85; 0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.). The membranes were blocked in phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.3% Tween 20 for 2 h at room temperature. Envelope-specific proteins were detected by probing the membranes with a rabbit antipeptide antiserum specific for HBV surface antigen (HBsAg) (anti-p49a) (11), incubating them with ^{125}I-labeled protein A (Amersham), and subjecting them to autoradiography as described previously (9). Nucleocapsid and polymerase products were detected by probing the membranes with rabbit anticore or antipolymerase antiserum (kindly provided by Robert Lanford and Heinz Schaller, respectively), incubating them with ^{125}I-labeled protein A, and subjecting them to autoradiography.

RT activity gel assay. Detection of reverse transcriptase (RT) activity was performed with slight adaptations of a method described elsewhere (3). In brief, 3×10^6 cells were centrifuged and washed once with ice-cold PBS. Cells were resuspended in 100 μl of 50 mM Tris-Cl (pH 6.8)–140 mM NaCl–0.5% Nonidet P40–1 mM phenylmethylsulfonyl fluoride–0.1% aprotinin and lysed by addition of 20 μl of 65 mM Tris-Cl (pH 6.8)–2 mM EDTA–20% glycerol–200 mM 2-mercaptoethanol–4% SDS. DNA was sheared with an insulin-type syringe (28.5-gauge needle), and an additional 20 μl of glycerol was added before loading. Electrophoresis was carried out in a 7.5% acrylamide gel containing 0.02% SDS and 10 μg of oligo$(dG)_{16}$ annealed to 100 μg of poly$(C)_{800-1500}$ per ml at 70 V overnight at 4°C. After protein renaturation by extensive washes in ice-cold 50 mM Tris-Cl (pH 7.5), in situ reverse transcription was carried out for 20 h at 37°C in 50 ml of 70 mM KCl–10 mM Tris-Cl (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol–0.5 mCi of [α-^{32}P]dGTP (800 Ci/mmol). Unincorporated radioactivity was removed by extensive washes in ice-cold 5% trichloroacetic acid plus 1% sodium PP_i. The gels were dried before autoradiography.

Immunofluorescence. The intracellular location of the envelope and nucleocapsid antigens was examined by immunofluorescence performed on air-dried, acetone-fixed cytospin preparations (for nuclear and cytoplasmic localization) and on thrice-washed viable cells (for surface staining) as previously described (15). Nucleocapsid antigens were detected with rabbit anticore antibody (Dako, Carpinteria, Calif.) followed by a fluorescein-conjugated goat anti-rabbit antibody (Pel-Freez, Rogers, Ark.). Envelope antigens were detected with a fluorescein-conjugated human anti-HBsAg antibody as previously described (15).

Antigen assays. HBsAg was measured by solid-phase radioimmunoassay (RIA) (AUSRIA II; Abbott Laboratories, Chicago, Ill.), with the manufacturer's HBsAg-positive control (20 ng/ml) as a standard. Nucleocapsid products were measured by a commercial solid-phase RIA that detects both HBcAg and HBV e antigen (HBeAg) (HBe [rDNA]; Abbott Laboratories). Purified recombinant core protein produced in *Escherichia coli* and purchased from Biogen (Geneva, Switzerland) was used as a standard.

CTL analysis. PBL were isolated by Ficoll-Hypaque (ρ = 1.077 g/ml) (Sigma) sedimentation from two patients (patients 1 and 2) during the acute phase of type B viral hepatitis (HBsAg positive; alanine aminotransferase > 2,000 IU/liter). Lymphocytes were analyzed for cytolytic function either immediately (at an effector-to-target cell ratio of 100) or following 1 to 2 weeks of stimulation in vitro either with irradiated (7,000 rads) EBO-HBV transfectants at a stimulator-to-effector cell ratio of 1:10 or with a pool of five synthetic peptides (10 μg/ml) corresponding to residues 11 to 27, 91 to 110, 111 to 126, 147 to 160, and 162 to 176 of HBcAg (ayw subtype [10]) plus recombinant HBcAg (Biogen) at 1 μg/ml. Peptide-stimulated cells were restimulated weekly with irradiated EBO-core transfectants as described above. Cytolytic capacity was assessed in a 4-h ^{51}Cr release assay as previously described (18), using chromium-labelled EBV B-cell targets that previously had been either incubated overnight at 37°C with synthetic peptides (10 μg/ml) or transfected with the EBO-core or EBO–pre-S1 expression vectors and stably selected for hygromycin resistance. All experiments were performed in triplicate. Results were expressed as percent specific lysis according to the following formula: percent specific lysis = (experimental release − spontaneous release)/(total release − spontaneous release), where experimental release is the mean counts per minute released by labelled targets in the presence of effector cells, total release is the mean counts per minute released in the presence of 0.5% Triton X-100, and spontaneous release is the mean counts per minute released in the presence of medium alone. In all instances, the spontaneous release was less than 25% of the total.

RESULTS

Transfection efficiency. Transfection experiments were carried out on 10^7 exponentially growing cells by electroporation. The electroporation conditions were designed to kill 80 to 90% of the starting cells. Because the BCL are growing in suspension, it is not possible to directly assess the number of effectively transfected cells. However, by establishing the growth curve of the transfected cell line after selection with hygromycin, it is possible to estimate the number of initially transfected cells by extrapolating this curve to the time point of transfection. Typical results reveal

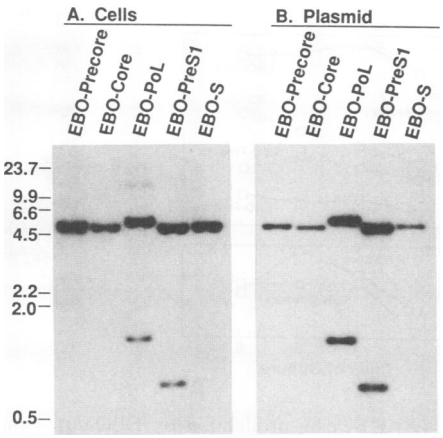

FIG. 2. Plasmid stability determined by Southern blot analysis of the EBO plasmids in transfected cells. Shown is *Eco*RI digestion of 20 μg of DNA from transfected cells (A) or 200 ng of plasmid DNA (B) probed with HBV DNA. All cells had been maintained in culture for several months before this analysis. Panel A was exposed for 2 days; panel B was exposed for 4 h. Sizes in kilobases are shown at the left.

a transfection efficiency of between 2 and 3% of the initial cell population. Routinely, electroporation of 10^7 cells with 10 μg of plasmid DNA yields 2×10^5 to 3×10^5 transfected cells available for selection, expansion, and further study (not shown). This level of efficiency permits the establishment of hygromycin-resistant, viral antigen-positive (see below) stable transfectants suitable for analysis within 2 to 3 weeks of initial transfection in most cases.

Episomal status and stability of transfected plasmid DNA. Because it contains the EBV origin of replication, the EBO plasmid has the capacity to be maintained as an episome in human B cells (14). The state and stability of the transfected vectors were assessed for each of the different EBO-HBV constructs after several months of culture except for EBO-X, which could not be stably expressed (see below). Both total cellular DNA from each transfected cell line and the corresponding starting plasmid DNA used to transfect each line were digested with *Eco*RI and analyzed by Southern hybridization using an HBV-specific probe.

Cellular DNA prepared from the transfected B cells (Fig. 2A) displays exactly the same hybridization pattern as does the input plasmid DNA (Fig. 2B). This finding demonstrates the absence of gross rearrangement of the vector within the cells, even after long-term culture, and it demonstrates that the transfected plasmid is maintained indefinitely as an episome in stably transfected cell lines.

To confirm the episomal status of the transfected DNA, bacteria were transformed with total DNA from cells stably transfected with the EBO-precore and EBO-core expression vectors or with the original EBO-precore and EBO-core plasmids. Minipreps derived from individual ampicillin-resistant colonies were compared by agarose gel electrophoresis after digestion with *Eco*RI. In each case, ampicillin-resistant bacterial colonies containing structurally intact plasmid DNA were easily established after transformation with cellular DNA from transfectants.

Approximately 10 copies of the episome are present per cell, as illustrated in Fig. 3 for EBO-S-transfected cells. In separate studies, the number of copies per cell line was shown to be independent of the particular HBV fragment

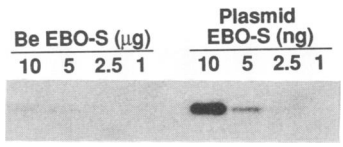

FIG. 3. Copy number. Shown is a Southern blot analysis of *Eco*RI-digested total cellular DNA form EBO-S-transfected Be cells (Be EBO-S) and *Eco*RI-digested EBO-S plasmid DNA probed with HBV DNA. The amount of loaded DNA is indicated above each lane.

carried by the vector, independent of the transfection experiment, and independent of the B-cell donor (not shown). These qualities, and the independence of expression of the HBV inserts from integration site influences in this episomal vector, contributed to the consistent expression level achieved with the different vectors in independent cell lines (see below).

HBV gene expression by transfected B cells. (i) RNA analysis. Northern (RNA) analysis of total RNA from cells stably transfected with each of the different EBO-HBV constructs, except EBO-X (see below), hybridized with an HBV-specific DNA probe reveals a transcript of the expected size in each case (Fig. 4). Additionally, the EBO-Pol transfectants display a less abundant smaller transcript that is probably initiated by the endogenous S gene promoter contained within the polymerase DNA fragment.

Unlike the nucleocapsid, envelope, and polymerase constructs, stable expression of X mRNA was not achieved following transfection of the EBO-X construct even though EBO-X-transfected cells can be selected with hygromycin, and despite the fact that Southern analysis of stable transfectants reveals the presence of the EBO-X plasmid as an episome in the cells without gross rearrangement (not shown). Interestingly, transient expression of a heterogeneous X-specific transcript is always observed during the first week after transfection with EBO-X, but expression is rapidly lost during the selection process (Fig. 5). No specific protein can be observed by Western immunoblotting of EBO-X-transfected cells (not shown).

Although it is possible that our failure to derive a stable EBO-X transfectant is due to some poorly understood incompatibility between the X gene product and the regulatory elements in the vector, it is also possible that this system affords some insight into the biological consequences of

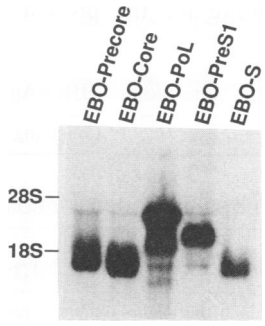

FIG. 4. Northern blot analysis. Total RNA (20 μg) prepared from cells stably transfected with the different EBO constructs was separated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane. The blot was probed with HBV DNA.

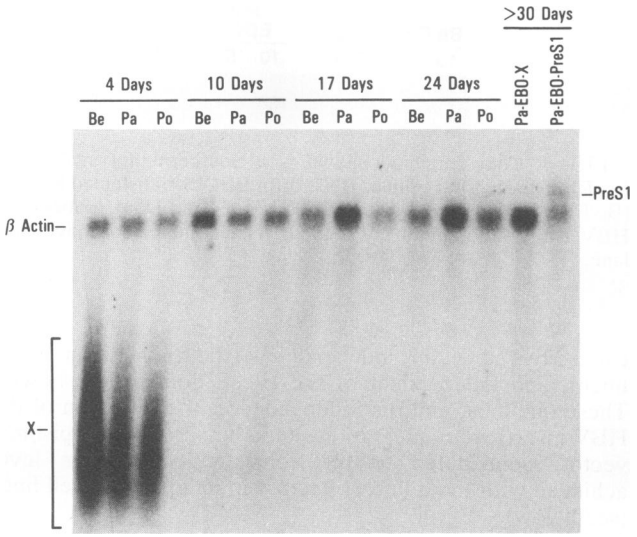

FIG. 5. Northern blot analysis of EBO-X-transfected cells hybridized with HBV and β-actin probes. Three EBV-immortalized BCL (Be, Pa, and Po) were transfected with EBO-X plasmid DNA, and total RNA was prepared at the indicated times after transfection. The X transcript appears as a highly heterogeneous population ranging from 0.3 to 0.9 kb. The amount of synthesized transcript decreases rapidly with time after transfection to reach undetectable levels by Northern blot analysis after 10 days posttransfection. This extinction of the transcription is not a peculiarity of these cell lines, as illustrated by the maintenance of the large envelope mRNA transcription in the Pa cell line more than 30 days after transfection with EBO–pre-S1 plasmid.

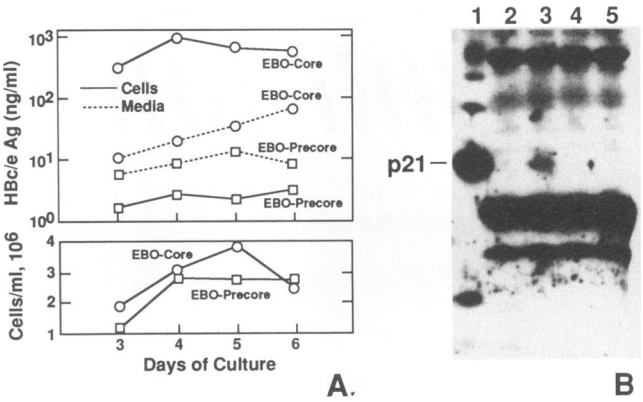

FIG. 6. (A) HBc/eAg production by EBO-core- and EBO-precore-transfected cells. On day 0, 6×10^5 cells in 3 ml of fresh culture medium were seeded in each well of a six-well culture plate. At the indicated days, cells from one well were separated from the culture medium by centrifugation and lysed in 1 ml of PBS by three cycles of freezing and thawing. The amount of intracellular and secreted HBc/eAg was quantified by RIA as described in Materials and Methods. The lower panel illustrates the growth of the cultures during that period. (B) Western blot analysis of HBV nucleocapsid products in cell lysates from EBO-precore (lane 2)- and EBO-core (lane 3)-transfected B cells. EBO-Pol (lane 4) and EBO-leu2 (lane 5) (14) were used as controls; lane 1 was loaded with 1 μg of recombinant core protein. Proteins were separated by electrophoresis in an SDS–15% polyacrylamide gel and transferred onto nitrocellulose. The blot was probed with rabbit anti-HBc/eAg antibody and ^{125}I-protein A.

unregulated X gene expression in the cell. The molecular basis for this interesting observation is under investigation.

(ii) Protein analysis of nucleocapsid constructs. EBO-core- and EBO-precore-transfected cells produce HBc/eAg which is readily detectable in the corresponding cell lysates and culture supernatants. As shown in Table 1, quantitative solid-phase RIA reveals that HBc/eAg is preferentially retained within the cell by EBO-core transfectants but is preferentially secreted by EBO-precore transfectants, in accord with the known behavior of these two related proteins (19). However, despite similar levels of mRNA detected in EBO-core- and EBO-precore-transfected cells (Fig. 4), the amount of HBc/eAg detected in EBO-precore-transfected cell lysate or culture supernatant (Fig. 6A) is significantly lower than with EBO-core transfectants, suggesting either a different translation efficiency of those two mRNAs or a shorter half-life for the protein products of EBO-precore. Western blot analysis of transfected cell lysates was performed to determine the molecular sizes of these proteins. The cell lysate from EBO-core transfectants displays an immunoreactive protein of 21 kDa (Fig. 6B, lane 3). Importantly, immunofluorescence analysis (Fig. 7) demonstrated that HBcAg was readily detectable in the cytoplasm, within the nucleus, and at the cell surface membrane in EBO-core-transfected cells, as it is in naturally infected hepatocytes in vivo. As expected, the nascent HBc/eAg product of the EBO-precore transfectants (predicted size, 24 kDa) cannot be detected intracellularly by Western blot (Fig. 6B, lane 2), presumably because it is rapidly secreted and does not accumulate to detectable levels within the cell (Fig. 6A). These data suggest that, like the EBO-core transfectants, the protein products of the EBO-precore transfectants follow the intracellular trafficking pathway of the native proteins produced in vivo by the naturally infected cell.

TABLE 1. Expression of HBc/eAg and HBsAg

Prepn	Concn (ng/ml)[a]				
	HBc/eAg		HBsAg		
	EBO-precore	EBO-core	EBO-Pol	EBO–pre-S1	EBO-S
Cell lysate[b]	0.1	102	0.01	0	3
Culture medium[c]	8.4	3	1.1	0.05	3.3

[a] Values are averages of the results obtained from three to eight different cell lines.
[b] One milliliter of cell lysate corresponds to 10^6 cells.
[c] Cells were seeded at a density of 2×10^5 cells per ml and grown for 1 week. The cells reached their maximum density of ca. 2×10^6 cells per ml after 3 days in culture.

(iii) Protein analysis of envelope constructs. The EBO–pre-S1 and EBO-S vectors were designed to direct the synthesis of the nonsecretable, water-insoluble, membrane-bound large envelope polypeptide and the rapidly secretable, water-soluble major envelope polypeptide, respectively. As expected, immunoreactive HBsAg is readily detected in the cell lysate and culture supernatant from EBO-S-transfected cells (Table 1). Also as expected, soluble HBsAg is detected only minimally or not at all in the lysates and supernatants derived from EBO–pre-S1-transfected cells (Table 1), in keeping with its behavior in other systems (9). Nonetheless, the large envelope polypeptide is produced by EBO–pre-S1 transfectants, as shown by Western blot and immunofluorescence analysis. Western blot analysis of the products of the EBO-S transfectants reveals the presence of two bands at 24 and 27 kDa, corresponding to the known 24-kDa major

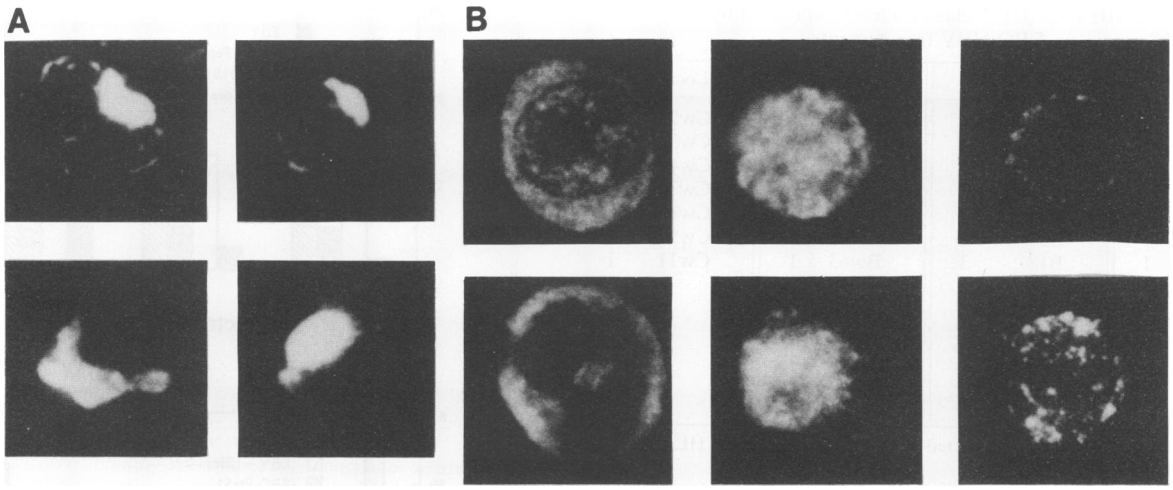

FIG. 7. Immunofluorescence analysis of EBO–pre-S1 (A)- and EBO-core (B)-transfected cells. Immunofluorescence was performed on fixed cells except for the right-hand photographs of panel B to reveal surface staining. EBO–pre-S1-transfected cells were stained with a fluorescein-conjugated anti-HBsAg antibody, and EBO-core-transfected cells were stained with a rabbit anti-HBeAg antibody and revealed with a fluorescein-conjugated goat anti-rabbit antibody.

envelope protein and its 27-kDa glycosylated derivative (Fig. 8, lane 3). Similarly, the EBO–pre-S1 transfectants produced products corresponding to the 39-kDa large envelope protein and its 42-kDa glycosylated derivative (Fig. 8, lane 1). Not surprisingly, immunofluorescence analysis of the EBO–pre-S1-transfected cells (Fig. 7) revealed the typical dense, perinuclear, cytoplasmic HBsAg inclusions that are characteristic of this protein, which is known to accumulate in the endoplasmic reticulum of the cell (9). The foregoing data indicate that like the EBO-core and EBO-precore transfectants, B cells transfected by the EBO-S and EBO–pre-S1 constructs synthesize HBV envelope proteins that follow trafficking pathways that are the same as or similar to those seen in naturally infected cells in vivo.

(iv) Protein analysis of polymerase construct. Because the HBV polymerase ORF contains the entire HBV envelope ORF and its corresponding promoters, the EBO-Pol construct has the potential to express the HBV envelope proteins as well as the viral polymerase. Indeed, low levels of HBsAg are detected in the supernatant of EBO-Pol-transfected cells by RIA (Table 1). Despite several attempts, we have not been able to detect the polymerase protein by Western blot analysis in EBO-Pol-transfected cells. To detect the polymerase protein, we used an RT activity gel assay based on a system previously described by Bavand et al. (3). Using this assay system, we have detected RT activity migrating as a ca. 75-kDa band in cell extracts from EBO-Pol transfectants (Fig. 9, lane 1). The specificity of the system was demonstrated by the failure to detect similar activity in cells transfected with EBO–pre-S1 or EBO-core (Fig. 9, lanes 2 and 3) even after prolonged exposure (not shown).

Preparation of a library of HLA-typed, EBO-HBV transfectants. Having demonstrated the capacity of the EBO–pre-S1, -S, -precore, -core, and -Pol constructs to generate stable, long-term BCL that express the corresponding HBV gene products, we produced a library of stable transfectants representing a wide variety of HLA haplotypes for subse-

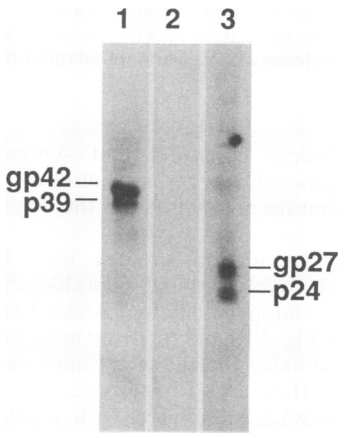

FIG. 8. Western blot analysis of HBV envelope products in cell lysate. Total protein (100 μg) from cells transfected with EBO–pre-S1 (lane 1), EBO-core (lane 2), and EBO-S (lane 3) was separated by electrophoresis in an SDS–10% polyacrylamide gel and transferred onto nitrocellulose. HBV envelope proteins were revealed by incubation with anti-HBsAg antibody and ^{125}I-protein A.

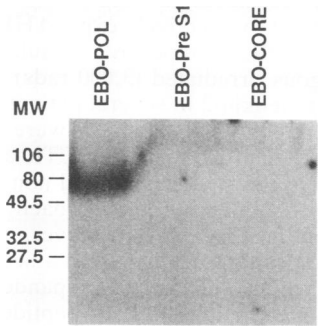

FIG. 9. RT gel activity assay. The assay was controlled for variable protein loading by staining of the gel with Coomassie blue, which confirmed that an equivalent amount of cellular protein was loaded in each lane. Molecular weights (MW) are shown in thousands.

TABLE 2. HLA class I alleles expressed by EBO-HBV transfectants

Locus	n^a	Locus	n	Locus	n	Locus	n
A1	6	B6	8	B51	3	Cw2	3
A2	16	B7	4	Bw52	2	Cw3	4
A3	9	B8	6	B56	1	Cw4	11
A11	6	B13	1	Bw58	1	Cw7	5
Aw23	2	B14	2	Bw60	1	Cw8	2
Aw24	7	B17	1	Bw62	2	Cw9	1
A25	1	B18	3	Bw63	1	Cw11	1
A26	2	B27	3	Bw65	2		
A29	1	B35	10	Bw67	1		
A31	4	Bw41	1	Bw73	1		
A33	1	B44	6	Bw75	1		
Aw69	1	B45	1				
Aw74	1	B46	1				

[a] Number of EBO-HBV-transfected cell lines that express the HLA class I antigen shown.

quent use as CTL targets. To date, a total of 37 BCL have been stably transfected with each of the EBO-HBV vectors. The EBO-S, -precore and -core transfectants have been shown to express stable levels of HBsAg or HBc/eAg by RIA for up to 1 year (not shown). The HLA class I alleles coexpressed by the library of transfectants, and therefore potentially able to present endogenously synthesized and processed HBV antigens to class I-restricted CTL as intended, are listed in Table 2. The broad spectrum of HLA restriction elements contained within this panel should cover the haplotypes of most patients who will be screened for HBV-specific CTL in the future and should also permit identification of the restriction element used by a given patient for each epitope recognized.

Cytotoxicity analysis. This panel of transfectants was used to detect the presence of HBV-specific CTL in the peripheral blood in several patients with acute and chronic viral hepatitis type B. Surprisingly, we were not able to detect HBV-specific CTL of any antigenic specificity in freshly isolated lymphocytes from the peripheral blood of patients with acute or chronic HBV infection in a standard ^{51}Cr release assay, even using autologous EBO-HBV transfectants as target cells (not shown). These findings suggest either that there is no HBV-specific CTL response in this disease or that the number of CTL in the circulation is below the detection limit of the assay and that in vitro stimulation and expansion of the CTL population may be required for detection. Accordingly, the same transfectants were irradiated (12,000 rads) and used to attempt to selectively expand HBV-specific CTL from autologous PBL by repetitive stimulation in the presence of autologous, irradiated (3,000 rads) antigen-presenting cells and interleukin-2 in several patients with acute and chronic hepatitis. HBV-specific CTL were not obtained by this strategy, although EBV-specific CTL capable of killing autologous EBO-HBV-transfected and nontransfected BCL were frequently obtained by this approach (not shown).

As illustrated in Fig. 10A, however, we successfully detected HBV-specific CTL by using a sequential stimulation strategy in which CTL, initially expanded by stimulation with a panel of overlapping synthetic peptides corresponding to the core protein of HBV, were restimulated by the corresponding HLA-matched EBO-core transfectant as we have recently reported (5). In this experiment, we observed the induction of a CTL population capable of killing not only peptide-pulsed target cells but also target cells transfected

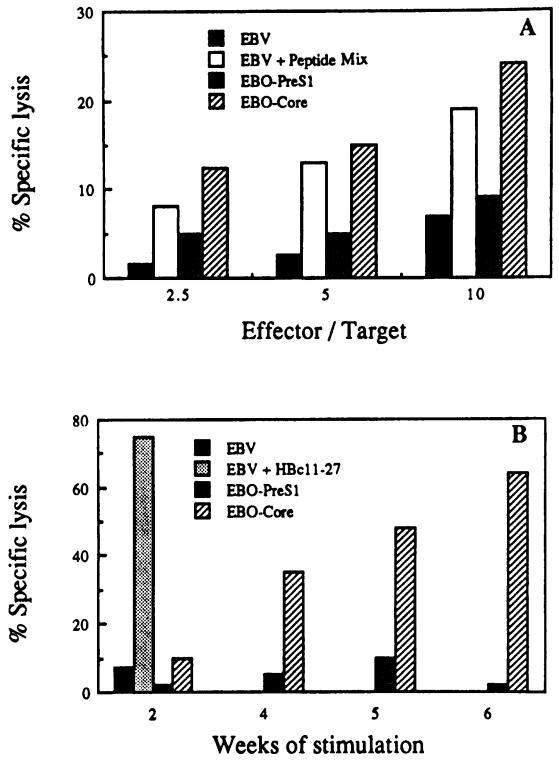

FIG. 10. (A) Peripheral blood mononuclear cells from a patient with acute viral hepatitis (patient 1) were stimulated with a mixture of overlapping HBcAg peptides and then tested for the ability to lyse ^{51}Cr-labelled HLA-A2-matched EBV-transformed B cells (EBV), B cells pulsed with one of the component peptides (EBV+HBc_{11-27}), or EBO–pre-S1 and EBO-core transfectants derived from the same BCL at the indicated effector-to-target cell ratio in a 4-h chromium release assay as described in Materials and Methods. (B) Selective expansion of CTL able to recognize endogenously synthesized HBcAg. Following 2 weeks of stimulation of PBL from patient 2 with the synthetic core sequence HBc_{11-27} and recombinant HBcAg, the derivative peptide-responsive CTL line was further restimulated weekly with the irradiated (7,000 rads) EBO-core transfectant at a stimulator-to-effector cell ratio of 1:10 and then tested for the ability to lyse ^{51}Cr-labelled HLA-A2-matched EBV-transformed B cells (EBV), B cells pulsed with one of the component peptides (EBV+HBc_{11-27}), or EBO–pre-S1 and EBO-core transfectants derived from the same BCL at an effector-to-target cell ratio of 20:1 in a 4-h chromium release assay. Data are adapted from reference 5.

with the EBO-core construct that synthesize the CTL epitope endogenously, whereas autologous EBO–pre-S1 transfectants that do not synthesize the cognate antigen are not killed.

The peptide stimulation strategy used herein has the potential to selectively expand peptide-specific CTL with low affinity for endogenously synthesized antigen. Indeed, as illustrated in Fig. 10B and reported in detail elsewhere (5), peripheral blood mononuclear cells from a patient with acute viral hepatitis (HLA class I haplotype: A2, A11, B44, B62, Cw5, DR4, DRw12) stimulated with a pool of synthetic peptides corresponding to the HBV nucleocapsid protein (HBcAg) very efficiently lysed an HLA-A2-matched EBV BCL pulsed with one of the peptides used in the stimulatory mixture (corresponding to amino residues 11 to 27 of HBcAg), while the BCL itself was not lysed. At the same time, however, an EBO-core-transfected, stably selected

derivative of the same BCL was only minimally lysed relative to a control EBO–pre-S1 transfectant. Restimulation of this short-term, peptide-specific CTL line with the EBO-core transfectant, however, preferentially expanded a CTL population whose lytic potential for the corresponding endogenously synthesized antigen was greatly increased. This strategy thus permits the identification and selective expansion of high-affinity HBV-specific CTL that unequivocally recognize endogenously synthesized HBV antigens in the peripheral blood of patients during HBV infection.

In additional experiments described elsewhere (5, 20), the HBcAg (residues 11 to 27)-specific effector cells that mediate this activity have been shown to be HLA class I-restricted, CD8-positive T cells that are readily detectable in the peripheral blood of patients with acute viral hepatitis but are not detectable in the peripheral blood of patients with chronic hepatitis type B and normal control subjects. Altogether, these results suggest that the peptide fragment derived from the intracellular processing of core antigen expressed by the EBO transfectants is closely related or identical to the antigen peptide fragment that is generated in vivo within infected cells and is responsible for the priming of core-specific CTL in vivo.

DISCUSSION

This study was prompted by our desire to examine the CTL response to HBV-encoded antigens in patients with viral hepatitis type B. Despite the likelihood that this response plays a major role in viral clearance and liver cell injury in this disease, little is known of its characteristics because the production of cytolytic target cells has been hampered by the restricted host range of HBV and because it is not infectious in vitro. The recombinant episomal vectors that direct expression of HBV gene products were developed specifically for this purpose.

If one is to study a physiologically relevant CTL response specific for a given viral antigen in an outbred population such as humans, one must have the capacity to present endogenously synthesized viral antigen to the CTL T-cell receptors of each subject studied in the context of the HLA restriction element(s) utilized by that patient for that antigen. Accordingly, it was important to demonstrate that the HBV proteins expressed by the EBO-HBV transfectants traverse intracellular processing pathways similar to those used by their natural counterparts in infected cells. Indeed, both envelope proteins are normally glycosylated in the EBO transfectants; the major envelope and the precore proteins are normally secreted, and the large envelope and the core proteins are retained in the appropriate compartments within the cell. Thus, the subcellular localization and the maturation, at least of the envelope and nucleocapsid proteins, are the same in the transfectants as in infected hepatocytes. It is reasonable to anticipate, therefore, that the transfected HBV proteins also follow natural intracellular antigen processing and presentation pathways and therefore deliver the requisite stimulatory signals to amplify and detect a limited HBV-specific CTL population in the peripheral blood of infected patients.

Indeed, our successful use of these EBO-HBV transfectants to identify HBV CTL that recognize endogenously synthesized HBV antigen in the peripheral blood of patients with acute hepatitis graphically demonstrates the merits of this expression vector and experimental approach to this heretofore elusive question. The EBO expression vector appears to be particularly well suited to this objective because it replicates as an episome at moderate levels in transfected long-term BCL, thereby permitting stable expression of the individual HBV-encoded antigens needed for selective expansion of HBV-specific CTL precursors that appear to be present at very low frequency in the peripheral blood of infected patients.

The failure to detect HBV-specific CTL either in freshly isolated PBL or even after in vitro stimulation by autologous EBO-HBV transfectants strongly suggests that the circulating HBV-specific CTL precursor population is exceedingly low in this disease, possibly because these cells are efficiently sequestered within the liver. The successful identification of CTL that can efficiently lyse EBO-core transfectants following a round of PBL stimulation with HBV core-specific synthetic peptides in vitro is compatible with recent data (1) suggesting that a higher density of antigenic determinants is needed for CTL sensitization than for recognition and lysis by CTL. If the requisite determinant density for primary CTL expansion is not present at the EBO-HBV-transfected cell surface, perhaps introduction of a stronger, B-cell-specific promoter to drive HBV gene expression into the EBO vector in place of the SV40 early promoter will solve this problem and further enhance the value of this system.

Nonetheless, we have demonstrated that the EBO-HBV transfectants function very well for secondary in vitro expansion of CTL and as cytolytic targets for CTL that recognize endogenously synthesized antigen. Therefore, although the determinant density needed for the primary in vitro expansion of HBV-specific CTL may not be present at the EBO-HBV-transfected cell surface, the transfectants function extremely well for the selective expansion of CTL that recognize endogenously synthesized antigen from within the initial pool of peptide-specific CTL. This is critically important because it is not possible to determine in advance the peptide structure that is naturally expressed as a result of antigen processing and presentation by infected cells in vivo. Therefore, secondary stimulation of peptide-specific CTL by the transfectants serves to focus the responding CTL population on the naturally expressed epitope contained within the original stimulatory peptide. This should permit, ultimately, the determination of the fine specificity of the corresponding CTL and the identification of the naturally processed peptide antigen produced in vivo. Additionally, this panel of stable EBO-HBV transfectants of defined HLA haplotype will permit the isolation of naturally processed HBV antigenic determinants from the binding groove of any HLA molecule shown to function as a restriction element for HBV-specific CTL.

Collectively, these properties should facilitate the identification of candidate CTL epitopes for the construction of T-cell-based vaccines and immunotherapeutic strategies for termination of persistent HBV infection. Finally, since the current panel of EBO-HBV expression vectors has permitted the first definitive identification of an HLA class I-restricted CTL response to a precisely mapped epitope within the HBV nucleocapsid antigen (5, 20), the data suggest that this system will facilitate a detailed examination of the role of the HBV-specific CTL response in the pathogenesis of viral clearance and liver cell injury in this disease.

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