Hepatitis B Virus (HBV)-Specific Cytotoxic T-Cell (CTL) Response in Humans: Characterization of HLA Class II-Restricted CTLs That Recognize Endogenously Synthesized HBV Envelope Antigens†

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In this study, we show that CD4⁺, hepatitis B virus (HBV) envelope-specific T-cell clones produced by stimulation with a particulate antigen preparation are able to recognize and kill not only autologous antigen-presenting cells incubated with exogenous HBV envelope antigens but also autologous HLA class II-positive cells expressing endogenously synthesized HBV envelope antigens following infection with recombinant vaccinia viruses or transfection with recombinant Epstein-Barr virus expression vectors. Experiments with lysosomotropic agents and brefeldin A suggest that the endosomal compartment is likely involved in the processing of endogenously synthesized viral proteins for recognition by CD4⁺ T cells. Our study indicates that HBV envelope-specific, HLA class II-restricted CD4⁺ cytotoxic T lymphocytes can potentially participate in the immune clearance of HBV-infected cells and the pathogenesis of hepatocellular injury in hepatitis B.

Little is known about the immune mechanisms responsible for liver cell damage during acute and chronic hepatitis B virus (HBV) infection. By inference from studies of other viral infections, a class I-restricted cytotoxic T-cell (CTL) response against viral peptides expressed on the liver cell membrane is believed to play a major role in the clearance of HBV-infected cells (13). Moreover, the observation that liver cells express HLA class II determinants on their surface following HBV infection (42) suggests that HLA class II-restricted CD4⁺ cells could also potentially participate in the clearance of infected hepatocytes. However, for this to occur, HLA class II-restricted T cells must efficiently recognize endogenously synthesized antigens and must express cytolytic activity against infected target cells.

Several lines of experimental evidence show that CD4⁺, HLA class II-restricted T lymphocytes can recognize exogenous antigens after internalization and intracellular processing by the antigen-presenting cells (APC). On the other hand, CD8⁺, HLA class I-restricted T cells are generally activated by antigens which are endogenously synthesized by the APC. As a general rule, exogenous proteins entering the APC by endocytosis are processed within the acidic endosomal compartment and associate with HLA class II molecules, whereas endogenously synthesized proteins are degraded through a nonendosomal pathway and preferentially interact with HLA class I determinants (21, 27, 28, 40). However, recent data from several authors indicate that the two intracellular pathways of protein processing may not be completely separated. Activation of CD8⁺, HLA class I-restricted T cells by exogenous antigens has been reported (6, 24, 32, 35, 46), and recognition by class II-restricted CTLs of endogenously synthesized antigens within virus-infected or

-transfected cells has also been described (22–24, 29, 39, 44). However, the importance of this pathway of HLA class II-restricted T-cell activation with respect to the immunopathogenesis of viral infections is still largely unknown, and the intracellular mechanisms leading to association between endogenous peptides and HLA class II molecules remain to be clarified. In addition, a cytotoxic activity of HBV-specific, HLA class II-restricted T cells against endogenous antigen has never been demonstrated, even though the lysis of APC prepulsed with exogenous antigen by HBV envelope-specific, CD4⁺ T cells has been reported (5, 10). In this study, we have used a panel of recombinant vectors capable of inducing endogenous synthesis of HBV envelope antigens to test whether HLA class II-restricted, HBV envelope-specific T cells can exhibit cytolytic activity against autologous target cells expressing endogenous viral peptides in association with HLA class II molecules and to investigate the intracellular pathways of exogenous and endogenous antigen processing for HLA class II-restricted T-cell activation.

Using a plasma-derived HBV envelope antigen preparation containing the S, pre-S2, and pre-S1 region-encoded determinants, purified from pooled HBV surface antigen-positive sera of ad and ay subtypes (19), we previously produced several antigen-specific T-cell clones from two HBV vaccine recipients immunized with a plasma-derived vaccine preparation (Hevac-B; Pasteur Institute) (18). As expected, all clones were CD4⁺, HLA class II restricted (Fig. 1A and B) and displayed proliferative response to HBV envelope antigens presented by the exogenous antigen processing pathway. Additionally, most of the clones expressed HLA class II-restricted cytotoxicity against autologous targets consisting of Epstein-Barr virus (EBV)-transformed B (EBV-B) cells preincubated with exogenous HBV envelope antigens (Fig. 1C). Although such cells might play a regulatory role in the HBV-specific immune response by destroy-

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FIG. 1. HLA class II restriction of antigen recognition by HBV envelope-specific, CD4⁺ T-cell clones. (A) Effect of anti-HLA class I and class II monoclonal antibodies on the antigen-stimulated proliferative response of a representative S-specific T-cell clone (E4). Results are expressed as percent inhibition of proliferation. The following monoclonal antibodies were used: D1-12 (immunoglobulin G2a [IgG2a]), specific for DR molecules; BT3/4 (IgG1), recognizing DQ molecules; B7/21 (IgG2a), specific for DP antigens; and W6/32 (IgG2a), anti-HLA-A,B,C specific. The specificity of these anti-HLA antibodies (1, 2, 8, 15, 16, 43) and the experimental conditions used (17) have been described in detail elsewhere. (B to D) Antigen presentation by allogeneic APC/target cells. Autologous and allogeneic mitomycin-treated EBV-B cells were cultured overnight with recombinant large HBV envelope protein (S+pre-S2+pre-S1) (1 μ g/ml) or with medium alone (B). After incubation, EBV-B cells were washed and added to 5×10^4 effector T cells from the representative T-cell clone E4. [³H]thymidine incorporation was measured after 3 days of culture. For the HLA restriction of the cytotoxic response, autologous and allogeneic EBV-B cell targets were either incubated overnight in the presence or absence of S+pre-S2+pre-S1 (C) or infected with V₇₃ recombinant (rec. V73) or wild-type (Vwr) vaccinia virus (D). Target cells were ⁵¹Cr labeled and cocultured for 4 h with effector T-cell clones. Results are expressed as ⁵¹Cr released by EBV-B cell targets (see also legend to Fig. 2). HLA class II molecules shared by allogeneic EBV-B cell lines with the T-cell donor are indicated. EBV-B cell lines were completely HLA class I mismatched. The T-cell donor was HLA-DR1,DR2,DQw1,DPw2,DPw4 positive. Identical HLA restriction was observed with T-cell clones E16 and A4 (DPw4 restricted).

ing APC (i.e., B cells and macrophages) that internalize and process exogenous antigen, they would not contribute to the clearance of infected hepatocytes unless they recognized endogenously synthesized antigen presented in the context of the appropriate HLA class II restriction element.

The availability of recombinant vectors capable of inducing efficient expression of HBV envelope gene products in autologous cells (11, 12, 34) made it possible to investigate this question. This issue is relevant to the immune pathogenesis of hepatitis B in humans, since HLA class II determinants are expressed on liver cells that synthesize endogenous HBV antigens during HBV infection (42). Three distinct approaches were followed to address this issue. First, a recombinant vaccinia virus (V₇₃; constructed as described by Cheng et al. [12]) encoding the large envelope polypeptide (S+pre-S2+pre-S1) was used to induce the endogenous synthesis of HBV envelope antigens in autologous EBV-B cell targets. This vector was used to avoid sensitization of target cells by release and reuptake of newly synthesized antigen, since the large HBV envelope polypep-

tide is not secreted by infected cells and remains entrapped in the endoplasmic reticulum (ER) (12). As shown in Fig. 2, all T-cell clones tested were able to recognize and kill not only EBV-B cells preincubated with exogenous HBV envelope proteins but also target cells synthesizing and processing endogenous HBV envelope antigens following infection with V₇₃ recombinant vaccinia virus. Lytic activity was generally significant at effector-to-target ratios as low as 2:1 or 5:1 (data not shown). Recognition of endogenously synthesized HBV envelope antigens showed the same pattern of HLA class II restriction observed with exogenous antigen (Fig. 1C and D).

Since the viability of V₇₃-infected target cells after overnight incubation was reduced, thus raising the possibility that cellular fragments with membrane-associated HBV envelope antigens could be released by dead cells and taken up by the APC, we used a second recombinant system to stably transfect the large envelope protein gene into autologous EBV-B cells. For these experiments, the HBV envelope and core open reading frames were inserted into the EBV-based

FIG. 2. Recognition of autologous EBV-B cells expressing either exogenous or endogenously synthesized HBV envelope antigens by CD4⁺, HLA class II-restricted, HBV envelope-specific cytotoxic T-cell clones (derived from vaccine recipients E and A). Target cells were autologous EBV-B cells (i) pulsed with (■) or without (□) exogenous large HBV envelope protein (S+pre-S2+pre-S1), (ii) infected with V₇₃ recombinant (□) or Vwr wild-type (▨) vaccinia virus, or (iii) transfected with EBO-pre-S1 (▩) or EBO-core (▧) plasmid vectors. Effector HBV envelope-specific T-cell clones (10⁵ per well) were incubated with ⁵¹Cr-labeled autologous target cells (5 × 10³ per well) at an effector/target ratio of 20:1 for 4 h at 37°C in round-bottom microtiter plates in RPMI containing 10% fetal calf serum. Before ⁵¹Cr labeling, target EBV-B cells were (i) incubated overnight in the presence or absence of 5 μg of exogenous S+pre-S2+pre-S1 per ml; (ii) infected for 1 h at room temperature with V₇₃ recombinant or Vwr wild-type vaccinia virus (multiplicity of infection of 100), washed, and recultured for a further 12 to 14 h to allow expression of virus-encoded gene products; or (iii) stably transfected with EBO plasmid vectors. The percentage of ⁵¹Cr release was calculated as follows: (cpm sample – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release) × 100. Spontaneous release was defined as the ⁵¹Cr released from target cells in the absence of effector T cells; maximum release was defined as the ⁵¹Cr released from target cells in the presence of 0.1 N HCl.

plasmid vector EBO-pLPP (9), which is maintained as an episome when transfected into EBV-transformed human B-cell lines. The HBV inserts are under the transcriptional control of the simian virus 40 early promoter and polyadenylation signal. The EBO-transfected cells are selected with hygromycin (250 μg/ml; Sigma Chemical Co., St. Louis, Mo.), a selection marker carried by the plasmid. EBO-pre-S1 and EBO-core encode the large envelope polypeptide and the nucleocapsid antigen of HBV (ayw subtype), respectively. Stable expression of HBV antigens was obtained following electroporation of EBV-B cells in the presence of each one of the plasmids carrying a specific HBV DNA sequence. The construction and characterization of these vectors will be reported elsewhere (21a). EBO transfection of EBV-B cells does not alter their viability and growth capacity; in addition, EBO-pre-S1-transfected cells do not secrete large envelope proteins which are not detectable by radioimmunoassay in the culture supernatants even after 100-fold concentration (data not shown). EBO-pre-S1-transfected autologous EBV-B cells expressing the HBV large envelope gene products were efficiently killed by CD4⁺, HBV envelope-specific CTLs, providing additional evidence that HLA class II molecules can present endogenous antigens to CD4⁺ T cells (Fig. 2).

FIG. 3. Evidence that autologous EBV-B cells cannot be sensitized by coculture with EBO-pre-S1-transfected B cells for recognition by CD4⁺, HBV envelope-specific T-cell clones. ⁵¹Cr-labeled autologous EBV-B cells were incubated overnight with an equal number of unlabeled EBO-pre-S1-transfected B cells (■); in parallel, ⁵¹Cr-labeled EBO-pre-S1-transfected B cells were incubated overnight in the absence of autologous EBV-B cells (□) or in the presence of an equal number of unlabeled EBO-pre-S1-transfected B cells (▩) as a control for a potential cold target inhibition. After washing, each target cell combination was added to the effector T-cell clones at an effector/target ratio of 20:1 in round-bottom well plates, and chromium release was measured after 4 h of incubation. Results identical to those illustrated were obtained by coculturing labeled EBV-B cells with higher numbers of unlabeled EBO-pre-S1-transfected B cells (1:5 and 1:10 ratios) and coculturing labeled EBO-pre-S1 transfectants with higher numbers of unlabeled EBO-pre-S1-transfected B cells (1:5 and 1:10). Spontaneous release of the different target populations was generally between 20 and 30% of the total release and never exceeded 35%.

Finally, the same conclusion was also suggested by coculture experiments between EBV-B cells and EBO-pre-S1-transfected B cells. Figure 3 shows that labeled EBV-B cells, cocultured in the presence of unlabeled EBO-pre-S1-transfected cells, are not killed by the CD4⁺, HBV envelope-specific CTLs, showing that the amount of antigen released by transfected cells, if any, is not sufficient to render EBV-B cells lysable by CD4⁺ T-cell clones. These results are also compatible with the possibility that EBO-pre-S1 transfectants do indeed transfer antigen to the labeled EBV-B cell targets but that they also block the killing of these targets by cold target inhibition. To address this hypothesis, the killing of labeled EBO-pre-S1 transfectants was tested in the presence and absence of unlabeled EBO-pre-S1 transfectants (Fig. 3). No cold target inhibition was observed in this experiment even at a cold target-to-labeled target ratio of 10:1, presumably because the experiment was done under conditions of effector cell excess. These results effectively eliminate the possibility that unlabeled EBO-pre-S1 transfectants were blocking the killing of labeled EBV-B cell targets and therefore also eliminate the possibility that shedding and reuptake of endogenously synthesized antigen is responsible for the HLA class II-restricted killing of the target cells in this study.

In contrast with our results for vaccinia virus vectors, it has been reported that target cells infected with a recombinant vaccinia virus carrying the influenza virus hemagglutinin gene are not recognized by class II-restricted CTLs (37), leading to the conclusion that only exogenous antigens can

be presented in the context of HLA class II molecules. Our results, however, are in agreement with data recently reported on the recognition of target cells infected with recombinant vaccinia virus expressing the influenza virus matrix protein (23, 29) and the recognition of endogenously produced immunoglobulin light chain (44) by class II-restricted T cells.

Since we have shown that HBV envelope-specific CD4⁺ T-cell clones can recognize both exogenous and endogenously synthesized large envelope polypeptides, we next investigated the intracellular pathways involved in the processing of the two forms of antigen. Thus, we assessed the effect of lysosomotropic agents (NH₄Cl and chloroquine) and brefeldin A (BFA) on the presentation of exogenous and endogenous HBV envelope protein antigens to the CD4⁺ CTLs. Chloroquine and NH₄Cl are weak bases that accumulate in acidic vesicles, raising their pH and therefore affecting the activity of the enzymes involved in the endosomal processing pathway (41). The presence of NH₄Cl or chloroquine during the overnight pulse of EBV-B cells with exogenous HBV envelope proteins or during the time of infection and expression of V₇₃ recombinant vaccinia virus abrogated recognition and killing of target cells (Fig. 4A and B). This effect is not due to inhibition of infection because the titers of vaccinia virus and the amounts of HBV envelope antigens produced by cells infected in the presence or absence of lysosomotropic agents were not different (data not shown). Therefore, these results suggest that both exogenous and endogenously synthesized antigens must reach an acidic compartment for efficient presentation.

We then tested the effect of BFA on antigen presentation to HLA class II-restricted, CD4⁺ T cells. BFA is a fungal metabolite originally described as capable of selectively interfering with antigen processing and presentation to HLA class I-restricted T lymphocytes (30, 45) by causing disassembly of the Golgi complex, with consequent accumulation of newly synthesized proteins in the ER (20, 25).

BFA had a profound and selective inhibitory effect on presentation of endogenously synthesized HBV envelope antigens, whereas no effect was observed on processing and presentation of exogenous large envelope proteins (Fig. 4C) which do not follow the ER-Golgi pathway to reach the cell membrane, as recently shown also for influenza A virus matrix protein (29). In contrast to particulate antigens, the presentation to class II-restricted T cells of soluble exogenous antigens is, however, inhibited by BFA (3, 36).

Fluorescence-activated cell sorting analysis of BFA-treated and untreated target cells did not show significant differences in HLA class II (or class I) expression (data not shown). However, since BFA blocks the egress of newly synthesized proteins from the ER, it is likely that its inhibition of endogenous HBV antigen presentation may depend on the effect on both newly synthesized HLA class II molecules and newly synthesized HBV envelope antigens.

In conclusion, our data demonstrate that endogenous HBV envelope antigens can be presented to CD4⁺ CTLs in association with HLA class II molecules and suggest that antigen processing or peptide-HLA association could take place in an acidic cellular compartment. Jin et al. have shown that the class II-restricted proliferative T-cell response to endogenous and exogenous HBV envelope antigens is blocked by chloroquine treatment of the APC (24). This finding is compatible with our conclusion that the endosomal compartment is involved in processing of both forms of antigen. Access to the endosomes could be related to the specific nature of the HBV envelope antigens which,

FIG. 4. Effect of lysosomotropic agents (NH₄Cl [A] and chloroquine [B]) and BFA (C) on presentation of exogenous and endogenously synthesized HBV envelope antigens to CD4⁺, HBV envelope-specific T-cell clones. Autologous EBV-B cells were incubated overnight with 5 μ g of HBV envelope antigens per ml in the presence or absence of 1 mM NH₄Cl, 5 μ g of BFA (Sandoz Ltd., Basel, Switzerland) per ml, or 0.4 μ M chloroquine (Sigma). Target cells infected with V₇₃ recombinant vaccinia virus were treated with 1 mM NH₄Cl or 5 μ g of BFA per ml during the time of infection and antigen expression; the chloroquine concentration was 4 μ M during infection and then 0.4 μ M during the expression period. All drugs were removed by washing the target cells before addition to the effector T-cell clones in round-bottom well plates at an effector/target ratio of 20:1 for a 4-h cytotoxicity assay (similar results were obtained when BFA was present also during the 4 h of cytotoxicity). Each drug was previously tested at different concentrations (NH₄Cl, 50, 20, 10, and 1 mM; chloroquine, 40, 4, and 0.4 μ M; BFA, 0.5 and 1 to 5 μ g/ml) to identify the best experimental conditions.

as typical membrane-associated proteins, could be internalized into the endosomal compartment by recycling from the cell membrane. Alternatively, activation of CD4⁺ CTLs through an endogenous pathway could be a peculiar feature of the HBV large envelope proteins (encoded by the recombinant vectors used in this study) which have been shown to specifically accumulate within the ER (14). Recent data indicate that this intracellular compartment can represent a processing site for HLA class II-restricted T-cell activation

by endogenous antigen (44). If this is the case, the inhibitory effect of chloroquine and NH_4Cl on endogenous antigen presentation would suggest that the processed antigen is transported to the endosomes in order to bind HLA class II molecules and that inhibition reflects a lack of HLA class II-invariant chain dissociation which is known to take place inside the endosomes and to be sensitive to lysosomotropic agents (7, 31, 38).

The distinct possibility that HLA class II molecules associate in a pre-Golgi compartment with antigenic peptides derived from processing of endogenous antigen has recently been suggested by the observation that presentation of endogenous influenza A matrix protein to class II-restricted T cells is chloroquine insensitive and BFA sensitive (29). Similarly, presentation of measles virus antigens by class II HLA molecules was insensitive to treatment with chloroquine (33). These apparently conflicting findings probably reflect distinct intracellular pathways of antigen trafficking for different viral proteins and may be related to distinct routing signals contained in protein antigens of different nature (soluble versus particulate, glycosylated versus non-glycosylated, secretory versus membrane bound, etc.).

Finally, our results demonstrating recognition of endogenous HBV envelope antigens by cytotoxic CD4^+ , class II-restricted T cells imply that HBV vaccination can induce envelope-specific CD4^+ T cells potentially able to participate in the clearance of virus-infected cells in the event of subsequent infection. The demonstration that liver cells express HLA class II molecules following HBV infection provides additional support for this possibility (42). Moreover, the observation of Barnaba et al. (5) demonstrating the presence of class II-restricted, HBV envelope-specific CTLs in the intrahepatic lymphoid infiltrate in a patient with chronic active hepatitis B suggests that this mechanism may also be induced as a consequence of HBV infection as well as following immunization.

The lytic activity of this T-cell subset could be especially relevant during the early stages of HBV infection, when HBV envelope antigens have been shown to be strongly expressed on the liver cell membrane (4, 26).

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