Transferrin Receptor Mediates Uptake and Presentation of Hepatitis B Envelope Antigen by T Lymphocytes

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Summary

Human activated T lymphocytes expressing class II molecules are able to present only complex antigens that bind to their own surface receptors, and thus can be captured, internalized, and processed through the class II major histocompatibility complex processing pathway. We have used the antigen-presenting T cell system to identify the viral receptor used by hepatitis B virus (HBV) to enter cells, as well as the sequence of HB envelope antigen (HBenvAg) involved in this interaction. Results show that both $CD4^+$ and $CD8^+$ T clones can process and present HBenvAg to class II–restricted cytotoxic T lymphocytes and that the CD71 transferrin receptor (TfR) is involved in efficient HBenvAg uptake by T cells. Moreover, we provide evidence that the HBenvAg sequence interacting with the T cell surface is contained within the pre-S2 region. Since TfR is also expressed on hepatocytes, it might represent a portal of cellular entry for HBV infection. This system of antigen presentation by T cells may serve as a model to study both lymphocyte receptors used by lymphocytotropic viruses and viral proteins critical to bind them.

$CD4^+$ T lymphocytes recognize exogenous antigens as peptides bound to class II MHC molecules expressed on the surface of APC, generally represented by macrophages, dendritic cells, and B cells (1, 2). APC internalize these antigens, process them into endosomal compartments, and then express on their membrane short peptides in association with class II antigens (3).

Unlike mouse T cells, human activated T lymphocytes express class II molecules and have been shown to efficiently present only denatured antigens or peptides not requiring processing (4–7). In contrast, they usually fail to present complex antigens that need processing unless they capture antigens by their membrane molecules (8), as in the case of HIV gp120, which binds monovalently to the CD4 receptor (9, 10). In this manner, T cells can efficiently internalize antigens, process them through a class II processing pathway, and present their fragments to class II–restricted T lymphocytes, as specific B cells do via their Ig receptors (11–14).

In this study, we demonstrate that both $CD4^+$ and $CD8^+$ T cell clones can process and efficiently present hepatitis B envelope antigen (HBenvAg)[1] to class II–restricted CTL, which in turn kill the presenting T cells, and that the CD71 transferrin receptor (TfR) is involved in the uptake of HBenvAg by T cells. Moreover, we have identified the HBenvAg region involved in this interaction.

Materials and Methods

Reagents. Plasma-derived (native) HBenvAg (Sorin, Saluggia, Italy), containing all three (pre-S1, pre-S2, S) hepatitis B virus (HBV) envelope proteins, was purified from patients with chronic HBV infection as described (15). This antigen preparation contains high levels of pre-S1, pre-S2, and S reactivity as shown by solid phase ELISA (15). Recombinant (r) particles containing the entire surface protein of HBV (pre-S1, pre-S2, S) expressed in yeast (16) were a gift from P. J. Kniskern (Merck Sharp and Dohme Research Laboratories, West Point, PA). A deleted rHBenvAg form containing only the 12–52 sequence of the pre-S1 domain, the 133–145 sequence of the pre-S2 domain, and the entire S domain (S,L antigen [17]), a recombinant protein displaying antigenic determinants en-

A part of this work has appeared in abstract form (*J. Cell. Biochem.* 15:241*a* [Abstr.] 1991).

[1] *Abbreviations used in this paper:* HBenvAg, hepatitis B envelope antigen; HBV, hepatitis B virus; i, irradiated; TfR, transferrin receptor; TT, tetanus toxoid.

coded by pre-S2 and S regions (pre-S2, S), and a recombinant protein containing only the S region (S), all expressed in yeast (17), were kindly donated by P. Hauser and J. Petre (SmithKline Biologicals, Rixensart, Belgium). Another r(pre-S2, S) protein expressed in CHO cells (18) was provided by P. Adamowicz (Pasteur Vaccines, Marnes-La Coquette, France). Purified native tetanus toxoid (TT) was obtained from Institut Merieux (Marcy-L'Etoile, France). The synthetic peptide analogues of pre-S1, pre-S2, and S regions were purchased from American Peptide Company, Inc. (Santa Clara, CA). Soluble Tf was purchased from Calbiochem-Behring Corp. (San Diego, CA).

OKT11 (anti-CD2), OKT3 (anti-CD3), OKT4A (anti-CD4), OKT8 (anti-CD8), OKT1 (anti-CD5), OKT26a (anti-CD25), OKT10 (anti-CD38), and OKT9 (anti-CD71) mAbs were purchased from Ortho Pharmaceutical (Raritan, NJ). Anti-Leu-M5 (anti-CD18/LFA-1 β chain) and anti-Leu-19 (anti-CD56) mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA); anti-CD54 (anti-ICAM-1) mAb was from Immunotech (Marseille, France); anti-CD58 (anti-LFA-3; TS 2/9 clone) was obtained from T. A. Springer (Harvard Medical School, Boston, MA). The anti-CD71 mAbs (42/6, B3/25, T56/14, 43/31) were kindly donated by I. S. Trowbridge (Salk Institute, San Diego, CA). These anti-CD71 mAbs recognize different epitopes of the TfR; i.e., 42/6 and 43/31 mAbs inhibit Tf binding to its receptor, whereas the B3/25 and T56/14 mAbs, as well as OKT9, interact with a TfR epitope not involved in Tf binding (19, 20). The W6/32 mAb to monomorphic determinants of class I Ag was purchased from Cappel Laboratories (Malvern, PA). The Q5/13 mAb to framework determinants of HLA-DR was kindly provided by P. Giacomini (Regina Elena Cancer Institute, Rome, Italy). Anti-HLA-DQ and anti-HLA-DP were purchased from Becton Dickinson & Co. The purchased anti-MHC molecule mAbs were used after dialysis.

Antigen-specific T Cell Clones. Antigen-specific T cell clones were derived from PBL of two HB vaccine (HEVAC-B; Pasteur Institut, Paris, France) recipients, whose HLA-DR typings were DR5 and DR2,3, respectively. Briefly, PBL were plated (10^5 per well) in 96-well flat-bottomed plates (Falcon Labware, Oxnard, CA) in the presence of 10 μg/ml HBenvAg, and 20 U/ml rIL-2 (Hoffmann-La Roche, Basel, Switzerland) were added after 5 d. After an additional 5–6 d, growing cultures were expanded with IL-2, and a 15-d cycle of restimulation with antigen plus autologous irradiated (i)PBL was used as APC. Cultures chosen for their capacity to proliferate in response to HBenvAg in the presence of autologous iAPC were cloned by limiting dilution at 0.3 cells per well with 1 μg/ml PHA (Wellcome, Dartford, UK), rIL-2, and allogeneic iAPC, as described (14, 15). Table 1 shows antigen specificity and HLA restriction of the selected T clones. Random T clones, obtained by cloning autologous or allogeneic PBL with PHA and HLA-incompatible feeder cells, or EBV-transformed B (EBV-B) cells were used as APC.

Cytotoxicity Assay. ^{51}Cr-labeled EBV-B cells or T clones (target cells) were pulsed at 37°C (4 h) with increasing concentrations of entire HBenvAg (native or recombinant) or synthetic peptides. In specific cytotoxicity inhibition experiments, ^{51}Cr-labeled target cells were incubated with 2 μg/ml of various mAbs for 1 h at 37°C, before or after pulsing with either antigen or peptide. In some experiments, labeled target cells were treated with increasing concentrations of Tf for 2 h at 37°C, washed, and pulsed with HBenvAg or TT for another 2 h at 37°C. Viability of target cells before and after incubations with mAbs or Tf was 100%.

Target cells were washed, and cultured (5×10^3) for 4 h with antigen-specific T clones, at a 10:1 E/T ratio into 96-well round-bottomed plates in triplicate. Plates were then centrifuged, cell-free supernatants were collected from each well, and ^{51}Cr release was evaluated in a gamma counter. Percent specific lysis is expressed as the mean of triplicate determinations and is calculated as follows: 100× (experimental release − spontaneous release)/(maximal release − spontaneous release).

^{125}I-Tf, ^{125}I-HBenvAg, and Competitive Binding Assays. Purified human Tf (>99%) was conjugated with ^{125}I by the solid-phase lactoperoxidase system (New England Nuclear, Boston, MA). The binding reactions were performed in 12 × 75-mm polypropylene tubes in RPMI 1640 containing 0.2% BSA (Fraction V; Sigma Chemical Co., St. Louis, MO). Cell concentration was 5×10^6 cells/ml; labeled Tf, 500 ng/ml; and unlabeled Tf at different concentrations (0–500 μg/ml). In some experiments, unlabeled Tf was replaced by different molecular constructs of HBenvAg added at a final concentration ranging from 0 to 200 μg/ml. The cells were incubated for 120 min at 4°C, and unbound ligand was removed by passaging cells through a density cushion, as described (21). After incubation, 200-μl aliquots of the cell suspension, layered over 150 μl of a mixture of dibutyl phthalate (Merck, Darmstadt, Germany) up to a final density of 1.025 in 400-μl plastic microfuge tubes, were centrifuged in a microfuge (Jouan Inc., Winchester, VA) (10,000 *g*, 2 min). The resulting supernatant and most of the phthalate oil cushion were aspirated. The tips of the vials containing

Table 1. *MHC Class II Restriction of Antigen-specific Cytotoxic CD4$^+$ T Clones*

	Percent specific lysis		
	B41	VB26	VB27
mAbs			
None	100.0	78.5	63.7
Anti-DR	2.1	0.2	−0.1
Anti-DQ	88.4	75.3	62.9
Anti-DP	90.2	64.1	76.8
Anti-ABC	100.0	78.6	60.3
Target cells			
Allog. EBV-B (DR1)	1.6	−0.3	0.4
(DR2)	2.6	61.3	52.3
(DR3)	0.3	0.0	0.4
(DR4)	−0.1	0.1	0.7
(DR5)	110.0	0.2	−0.5
(DR6)	−0.3	−0.1	−0.2
(DR7)	−0.5	2.1	0.0
(DR8)	ND	0.0	0.6
(DR9)	ND	2.2	0.0

^{51}Cr-labeled autologous target EBV-B cells pulsed or not with 50 μg/ml HBenvAg at 37°C (4 h) were washed and then incubated with the respective cloned T cells (4 h), used as effector cells at an E/T ratio of 10:1, in the presence or absence of anti-MHC mAb. Percent specific lysis is expressed as the mean of triplicate determinations. ^{51}Cr-labeled allogeneic target EBV-B cells pulsed with antigen at 37°C (4 h) were washed and then incubated with cloned T cells (4 h) at an E/T ratio of 10:1 for the CTL assay. Percent specific lysis is expressed as the mean of triplicate determinations.

the cell pellet were severed with a scalpel and transferred into plastic vials, and the radioactivity was measured in a gamma counter. ^{125}I-HBenvAg binding was performed under similar experimental conditions, except for labeled ligand concentration (1.25 μg/ml). After incubation, cells were processed for ^{125}I-Tf binding assay.

Results

T Lymphocytes Process and Present HBenvAg. Fig. 1 shows that random CD4$^+$ and CD8$^+$ T clones, used as APC, were able to present HBenvAg to class II–restricted specific cytotoxic T clones. The specific CTL used in these experiments were a DR5-restricted T clone (B41) and two DR2-restricted T clones (VB26 and VB27) recognizing the synthetic 1–21 peptide (MGGWSSKPRKGMGTNLSVPNP) of the pre-S1 region, the 120–130 (MQWNSTAFHQT) of pre-S2 region, and the 193–207 (FFLLTRILTIPQSLD) of S region, respectively (Table 2). Lysis of presenting T cells was observed with a significantly lower concentration of antigen than that needed to induce a comparable level of EBV-B killing (Fig. 1). T cell clones, selected for presenting HBenvAg, were isolated and stimulated with PHA using allogeneic iPBL as feeders, which lacked the appropriate class II molecules, to exclude the presence of HLA-restricted conventional APC. These CD4$^+$ and CD8$^+$ clones were cytotoxic in a lectin-dependent cell-mediated cytotoxicity (LDCC) assay (data not shown). In some experiments, the HBenvAg-specific CD4$^+$ T clone, used as source of APC, was able to present HBenvAg to itself (data not shown). In this case, to exclude the presence of autologous conventional APC, the T clone was restimulated with PHA using allogeneic iPBL as feeders as described above. HBenvAg presentation by T cells required processing since cold pulsing resulted in a drastic inhibition of their APC capacity (Table 3). This ability was restored after at least 60 min of incubation at 37°C, except for T cells incubated in the presence of the lysosomotropic agent chloroquine (100 μM) (Sigma Chemical Co.), which inhibits the class II endosomal processing pathway (22) (Table 3). In contrast, recognition of 1-21 peptide, which presumably directly binds class II molecules without processing (23), was not affected by either cold or chloroquine treatments.

mAb to TfR Inhibits HBenvAg Capture and Presentation by T Cells. To define the putative receptor used by HBenvAg to interact with T cells, we attempted to inhibit specific lysis of presenting T lymphocytes by treatment with various randomly selected mAbs to lymphocyte surface molecules. Target cells were incubated with each mAb for 1 h at 37°C either before or after antigen pulsing. We assumed that the blocking of the HBenvAg uptake by T lymphocytes should occur only when the mAb to the presumed HBenvAg receptor was added before antigen pulsing. The B41 T clone was used as effector.

Results show that only the anti-CD71/TfR mAb (OKT9) was able to inhibit HBenvAg capture by T cells: indeed, only this mAb blocked specific lysis when added before, but not after, antigen pulsing (Table 4). Moreover, OKT9 also blocked, to a lesser degree, the specific lysis of EBV-B cells when added before HBenvAg pulsing (Table 5). In contrast, anti-TfR mAb did not inhibit either presentation of 1–21 peptide by T or EBV-B cells (Table 4 and 5), or the presentation of an unrelated antigen, the TT, by EBV-B cells to a TT-specific T line (Table 5).

An inhibitory effect was also obtained with adhesion molecule mAbs (anti-CD2, anti-ICAM-1, anti-LFA-1, anti-LFA-3) (24) (Tables 4 and 5). These mAbs showed an inhibitory effect both before and after HBenvAg pulsing of APC. They also

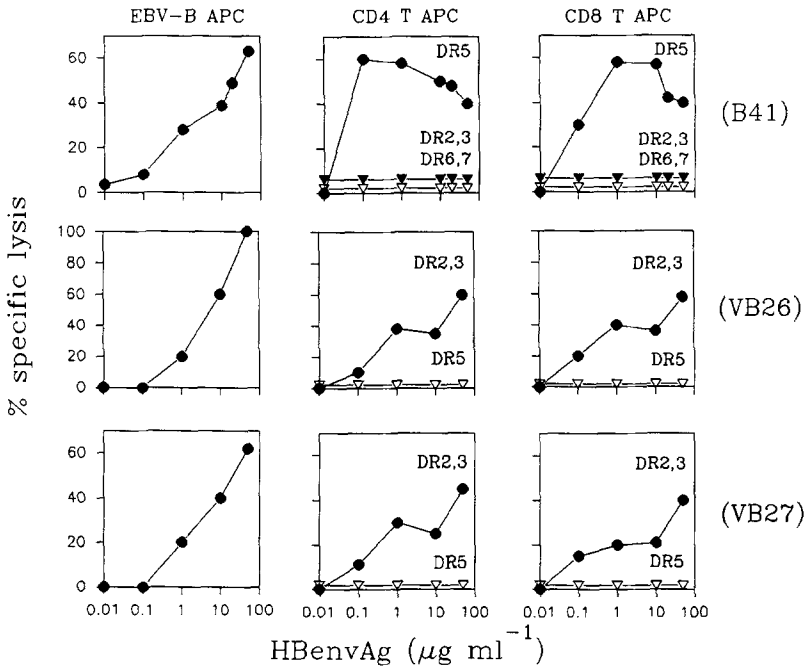


Figure 1. Cytotoxic activity of class II–restricted T cell clones against EBV-B or T cells presenting HBenvAg. Autologous or HLA-unrestricted target T clones were used for each experiment. ^{51}Cr-labeled target cells pulsed with increasing concentrations of entire HBenvAg at 37°C (4 h) were washed and incubated (4 h) with cloned T cells (indicated in parenthesis) at an E/T ratio of 10:1 for CTL assay. Percent specific lysis is expressed as mean of triplicate determinations.

Table 2. *Fine Specificity of Cytotoxic CD4+ T Clones*

	Percent specific lysis		
	B41	VB26	VB27
Recombinant antigens			
r(pre-S1, pre-S2, S)	100.0	70.3	82.0
r(pre-S2,S)	0.0	82.2	61.3
r(S)	0.2	0.3	72.4
Synthetic peptides			
Pre-S1 peptide pool	94.6	−0.3	0.2
1-21 pre-S1	101.2		
21-32 pre-S1	2.8		
32-53 pre-S1	−0.2		
74-94 pre-S1	0.0		
95-119 pre-S1	0.6		
pre-S2 peptide pool	0.0	82.2	0.2
120-130 pre-S2		76.4	
130-140 pre-S2		1.2	
141-174 pre-S2		2.1	
S peptide pool	0.5	1.9	83.2
172-184 pre-S2/S		0.3	0.0
193-207 S			81.3
289-304 S			0.0
374-389 S			0.2

^{51}Cr-labeled autologous target EBV-B cells pulsed with 50 μg/ml antigen or 10 μg/ml peptide at 37°C (4 h) were washed and then incubated with cloned T cells (4 h) at an E/T ratio of 10:1 for the CTL assay. Each peptide pool consisted of a mixture of single peptides (5 μg/ml) of pre-S1, pre-S2, and S proteins, respectively. The pre-S2/S peptide included the 172–175 sequence of pre-S2 protein and the 176–184 of S protein. Percent specific lysis is expressed as the mean of triplicate determinations.

inhibited presentation of the 1–21 peptide and the unrelated TT antigen.

Soluble Tf Blocks HBenvAg Uptake and Presentation by T Lymphocytes. To further clarify the role of TfR in HBenvAg uptake by T cells, we tested the effect of soluble Tf on specific lysis of presenting T and EBV-B cells. Incubation of APC with Tf for 2 h before antigen pulsing resulted in a drastic inhibition of their specific lysis. In control experiments, Tf did not block specific lysis of TT-pulsed EBV-B cells by the TT-specific T line (Fig. 2).

The HBenvAg Sequence Interacting with the T Cell Surface Is Contained in the Pre-S2 Region. To identify the viral envelope sequence relevant for virus/T cell interaction, we tested the ability of both T and EBV-B cells to present different HBV envelope antigen constructs. The VB27 T clone recognizing the 193–207 of S region, present in all tested proteins, was used as effector.

Table 3. *Processing Requirement for HBenvAg Presentation by T Cells*

CD4+ T cells	Time at 37°C		^{51}Cr release
	min		*%*
Unpulsed	0		0
HBenvAg pulsed	15		0
	30		5
	60		45
	120		100
	240		100
	240	+ chloroquine	10
	0	+ 1-21 peptide	50
	240	+ chloroquine + 1-21 peptide	49

^{51}Cr-labeled target CD4+ T clone was pulsed with 1 μg/ml rHBenvAg on ice (2 h), washed, and then cultured after different incubation times at 37°C, with the autologous B41 T clone at an E/T ratio of 10:1. In some experiments, prepulsed target cells were incubated at 37°C with 100 μM chloroquine (Sigma Chemical Co.) in the presence or absence of 1-21 peptide. Percent specific lysis is expressed as mean values from triplicate determinations. A representative experiment is presented. Equivalent results were obtained using a ^{51}Cr-labeled target CD8+ T clone (data not shown).

EBV-B cells presented all HBenvAg preparations tested with the same efficiency. T cells presented the entire recombinant or native (pre-S1, pre-S2, S) and the r(pre-S2, S) proteins with high efficiency (Fig. 3); they presented, to a lesser degree, the r(12–21 pre-S1, 133–145 pre-S2, S) S,L protein, and, not at all, the r(S) protein. These experiments were performed several times with equivalent results.

CD4+ CTL-mediated Lysis Does Not Induce Nonspecific Bystander Lysis. In this study, the antigen-specific CD4+ T clones killed T cells presenting HBenvAg. To examine whether they killed by a contact-dependent mechanism and not aspecifically by production of soluble factors (25), bystander lysis experiments were performed.

When unlabeled (cold) HBenvAg-pulsed target T cells were mixed with ^{51}Cr-labeled (hot) unpulsed T cells, class II–restricted CTL did not lyse the bystander targets (Table 6). In contrast, when pulsed T cells were added as hot targets to cold unpulsed T cells, strong lysis of the former was observed, thus suggesting that they killed by direct T cell/T cell contact and did not induce bystander lysis.

Activated T Lymphocytes Possess High Affinity Receptors for HBenvAg. Experiments were performed to evaluate the possible presence of high affinity membrane receptors for HBenvAg on T cells. Thus, T cells were incubated in the presence of a fixed amount of ^{125}I-HBenvAg (1.25 μg/ml S, L construct) and increasing amounts of cold HBenvAg (0–200 μg/ml). Scatchard plot of binding data shows that T cells possess high affinity receptors for HBenvAg (K_D 1.3 × 10^{-7} M) (Fig. 4). The binding of ^{125}IHBenvS,L Ag to T cells was inhibited by an excess concentration of some cold HBenv con-

Table 4. *Effect on the Antigen-specific Lysis by Treatment of Target T Cells with Different mAbs to Lymphocyte Surface Molecules*

mAb	Percent specific lysis of target T cells treated with mAbs		
	Before Ag pulsing	After Ag pulsing	After 1-21 peptide pulsing
Anti-CD3	100.0	80.5	98.6
Anti-CD4	71.2	66.7	76.3
Anti-CD8	92.8	72.1	100.0
Anti-CD5	88.6	94.3	ND
Anti-CD25	91.0	ND	99.4
Anti-CD38	78.3	ND	ND
Anti-CD56	84.7	73.2	ND
Anti-CD71	14.7	80.5	100.3
Anti-CD18 (LFA-1β chain)	0.0	ND	ND
Anti-CD54 (ICAM-1)	35.8	8.9	8.3
Anti-CD2	0.0	6.4	3.2
Anti-CD58 (LFA-3)	0.0	11.7	14.3
Anti-class I (A, B, C)	68.7	86.0	64.5

A ^{51}Cr-labeled target CD4$^+$ T clone was incubated with various mAbs either before or after entire HBenvAg pulsing as described in Materials and Methods. Target T cells were washed and cultured with the autologous B41 T clone at an E/T ratio of 10:1 for CTL assay. Cytotoxicity of B41 T clone against HBenvAg-pulsed target T cells without mAb treatment was 87%. Values expressing a percent inhibition >50% are underlined. Percent specific lysis is expressed as mean of triplicate determinations. Results represent the mean values of four experiments. Equivalent results were obtained using a ^{51}Cr-labeled target CD8$^+$ T clone (data not shown).

structs, such as native HBenvAg, or S,L, or r(pre-S2, S), but not by the form containing only the S protein (data not shown). Furthermore, using ^{125}I-HBenvAg, the level of nonspecific binding (i.e., the binding observed when the cells were incubated with labeled antigen and a large excess of cold antigen) is high and corresponds to 40–50% of the total binding.

Native form HBenvAg and the recombinant constructs are represented by a polymer exhibiting >250-kD formed by disulfur bridges (26). In light of this, we evaluated whether the antigen was still able to bind to cells after monomerisation by reducing agents such as DTT. The results clearly show that reduced HBenvAg loses its capacity to bind to cells (data not shown).

Table 5. *Effect on Antigen-specific Lysis by Treatment of Target EBV-B Cells with Different mAbs to Lymphocyte Surface Molecules*

mAb	Percent specific lysis of target EBV-B cells treated with mAbs				
	Before HBenvAg pulsing	After HBenvAg pulsing	After 1-21pep. pulsing	Before TT pulsing	After TT pulsing
Anti-CD3	88.3	67.1	ND	ND	ND
Anti-CD71	31.0	70.4	68.9	44.5	51.2
Anti-CD2	76.2	ND	64.1	35.9	40.0
Anti-CD58	28.3	17.7	24.2	10.6	8.3
Anti-CD18	37.1	16.2	36.5	8.8	8.2
Anti-CD54	19.8	7.4	0.8	1.3	4.0

^{51}CR-labeled EBV-B cells were incubated with various mAbs either before or after antigen pulsing as described in Materials and Methods. Target EBV-B cells were washed and cultured with the autologous B41 T clone for CTL assay. Cytotoxicities of the B41 T clone and the TT-specific T line against the respective antigen-pulsed target EBV-B cells without mAbs treatment were 79% and 40%, respectively. Values expressing percent inhibition >50% are underlined. Percent specific lysis is expressed as mean of triplicate determinations. The results represent the mean values of four experiments.

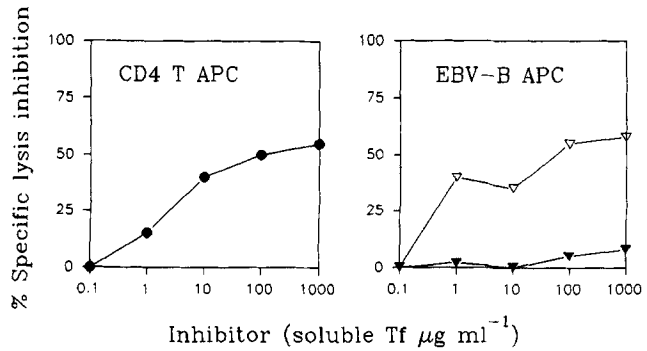
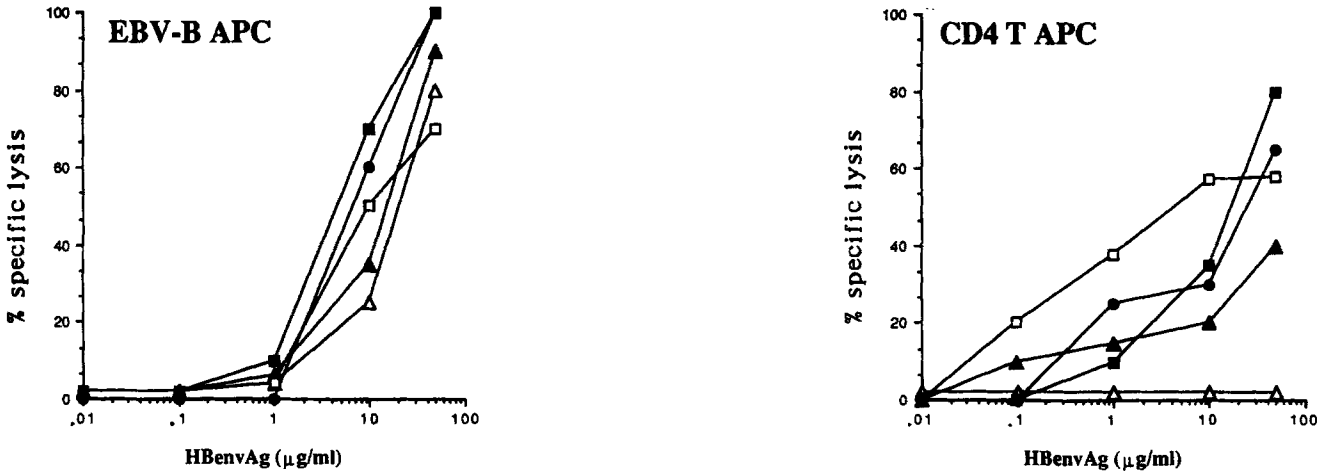


Figure 2. Dose-related inhibition induced by soluble Tf (inhibitor) on the lysis of CD4+ T cells by HBenvAg-specific CTL (●), and EBV-B cells by either HBenvAg-specific CTL (▽), or by TT-specific CTL (▼). ^{51}Cr-labeled target cells were treated with the indicated concentrations of Tf for 2 h at 37°C, washed, and pulsed with HBenvAg (10 μg/ml) for another 2 h. Target cells were then washed and cultured with the autologous B41 T clone for CTL assay. Percent specific lysis is expressed as mean of triplicate determinations. Results are representative of four experiments. Percent inhibition was calculated as follows: 100× [percent specific release (C) − percent specific release (T)]/[percent specific release (C)].

Table 6. *Lysis of Target T Cells by CD4+ CTL Is Not Mediated by Bystander Lysis*

	Percent specific lysis	
Clone	HBenvAg-pulsed cold T cells + unpulsed hot T cells	HBenvAg-pulsed hot T cells + unpulsed cold T cells
B41	−0.1	88.4
VB26	0.0	56.6
VB27	−0.5	62.0

Target cells were pulsed or not with 50 μg/ml HBenvAg for 4 h, washed, and used as ^{51}Cr-labeled (hot) or unlabeled (cold) targets. Experiments were performed in triplicate wells containing 5 × 10^3 cold targets. Effector cells were used at an E/T ratio of 10:1. Percent specific lysis is expressed as mean of triplicate determinations.



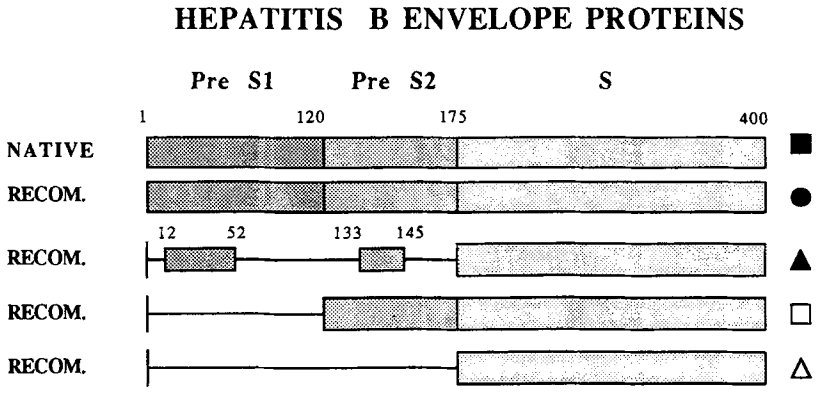


Figure 3. Capacity of EBV-B and T cells to present antigen preparations with different compositions in relation to HBV envelope. ^{51}Cr-labeled target cells pulsed with increasing concentrations of the indicated antigen preparations at 37°C (4 h) were washed and then incubated with the B27 T clone (4 h) at an E/T ratio of 10:1 for CTL assay. Percent specific lysis is expressed as mean of triplicate determinations. The results are representative of four separate experiments.

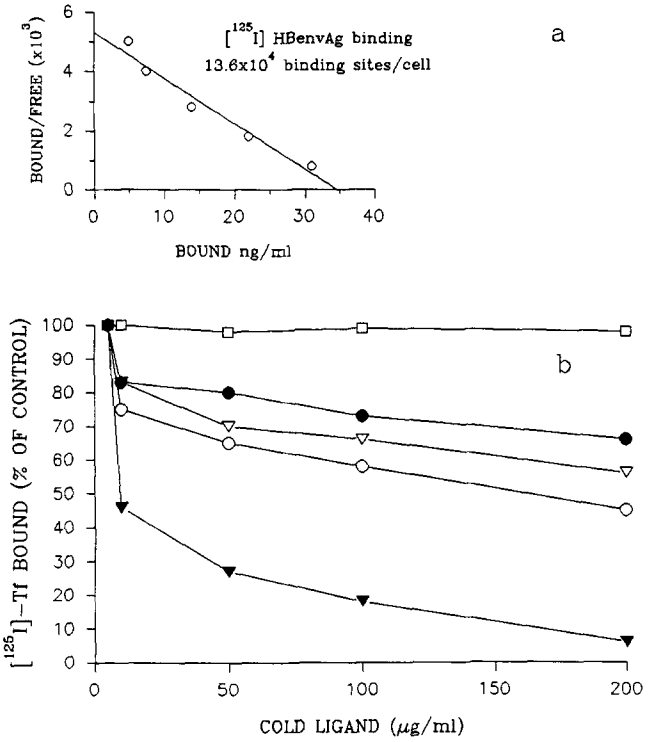


Figure 4. (*a*) Scatchard plot of ^{125}I-HBenvAg (S,L construct) binding to human activated T cells (CD4$^+$ nonspecific T cell clone). T cells were incubated in the presence of a fixed amount of ^{125}I-HBenvAg (1.25 μg/ml) alone or in the presence of increasing amounts of unlabeled HBenvAg (0–400 μg/ml). (*b*) Capacity of different HBenvAg constructs to inhibit ^{125}I-Tf binding to T cells. T cells were incubated in the presence of ^{125}I-Tf (500 ng/ml), in the absence or presence of increasing concentrations of HBenvAg constructs: plasma-derived native HBenvAg (▽); rHBenvAg only containing the S region (□); rHBenvAg containing the 12–52 pre-S1 sequence, 133–145 pre-S2 sequence, and the entire S domain (S,L antigen) (●); rHBenvAg composed by the entire pre-S2 and S domains (○); soluble Tf (▼).

The Binding of HBenvAg to Cells Is Inhibited by Some mAbs to TfR. Experiments were performed to evaluate whether ^{125}I-HBenvAg is able to interact with TfR. Cells were incubated with a panel of mAbs to human TfRs (42/6, B3/25, T9, T56/14, and 43/31), washed to remove excess unbound mAb, and then incubated for 120 min at 4°C in the presence of ^{125}I-HBenvAg. These experiments showed that: (*a*) on T cells, 43/31 anti-CD71 mAb produced a strong inhibition of ^{125}I-HBenvAg binding, whereas the other anti-CD71 mAbs induced only a slight inhibitory effect; and (*b*) on EBV-B cells, 43/31 mAb elicited a marked inhibition of HBenvAg binding, whereas only a moderate decrease of HBenvAg binding was observed with the remaining anti-CD71 mAbs (Fig. 5). Although OKT9 anti-TfR mAb had little effect in binding inhibition experiments performed at 4°C, it down-regulated surface TfRs when incubated at 37°C with a subsequent decrease of both ^{125}I-Tf and ^{125}I-HBenvAg binding (after a 60-min preincubation of T cells with OKT9, the binding of Tf and HBenvAg decreased by 60 and 50%, respectively).

The Binding of Tf to Its Receptor Is Partially Inhibited by HBenvAg. Competitive binding experiments were carried out to directly evaluate the ability of HBenvAg to interact with TfR. Recombinant HBenvAg S,L construct competed with radiolabeled Tf for TfR binding sites on both T and EBV-B cells, though to a significantly lesser extent than Tf (Fig. 5). Interestingly, rHBenvAg S,L construct exhibited a greater capacity to inhibit the binding of labeled Tf on T lymphocytes as compared with EBV-B cells (0.25 and 2.5 mg/ml of HBenvAg S,L construct was required to inhibit 50% of labeled Tf binding on T and EBV-B cells, respectively). This finding may explain why T cells present HBenvAg more efficiently than EBV-B lymphocytes.

Additional studies using different HBenvAg constructs provided results in line with those obtained on the capacity of T cells to present different forms of HBenvAg. Indeed, entire native HBenvAg and r(pre-S2, S) protein were able to inhibit Tf binding to TfR to a higher degree than rHBenvAg S,L; the S protein alone had no significant effect (Fig. 4).

Discussion

Human activated T lymphocytes express class II MHC molecules, but they usually fail to function as APC in presenting soluble antigens. Since T cells are able to present denatured antigens or peptides (4–7), it has been hypothesized that the defect was at the level of antigen processing, or alternatively at the level of antigen internalization due to the inability of T cells to take up antigens aspecifically (27). This last possibility has recently been supported by studies indicating that these cells are unable to present conventional antigens but internalize, process, and present antigens that bind to their membrane receptors with high efficiency (27). These studies show that T cells need receptor-mediated interactions with antigens to effectively capture and present them.

In light of these results, we evaluated whether the system of antigen presentation by T cells could be used to identify receptors used by lymphocytotropic viruses to enter T cells (28). In particular, the possibility was considered that HBV, demonstrated to infect T lymphocytes (28, 29), is captured by T cells via a receptor-mediated mechanism to be subsequently processed and presented to specific T lymphocytes.

Our study demonstrates that both CD4$^+$ and CD8$^+$ T clones can efficiently present HBenvAg only after processing in endosomal compartments, because presentation is inhibited by chloroquine. The finding that HBenvAg presentation was drastically inhibited when presenting T cells were cold-pulsed argues against the possibility that denatured HBenvAg fragments directly bind MHC molecules without processing and indicates that both internalization and processing are required. These results suggest that HBenvAg uses a surface receptor to enter T cells. Our investigations support the hypothesis that TfR represents one of the membrane molecules, which play a fundamental role in HBenvAg internalization and presentation. Indeed, only the anti-TfR/CD71 (OKT9), from a large series of randomly selected mAbs, was able to inhibit killing of both CD4$^+$ and CD8$^+$ T cells by specific class

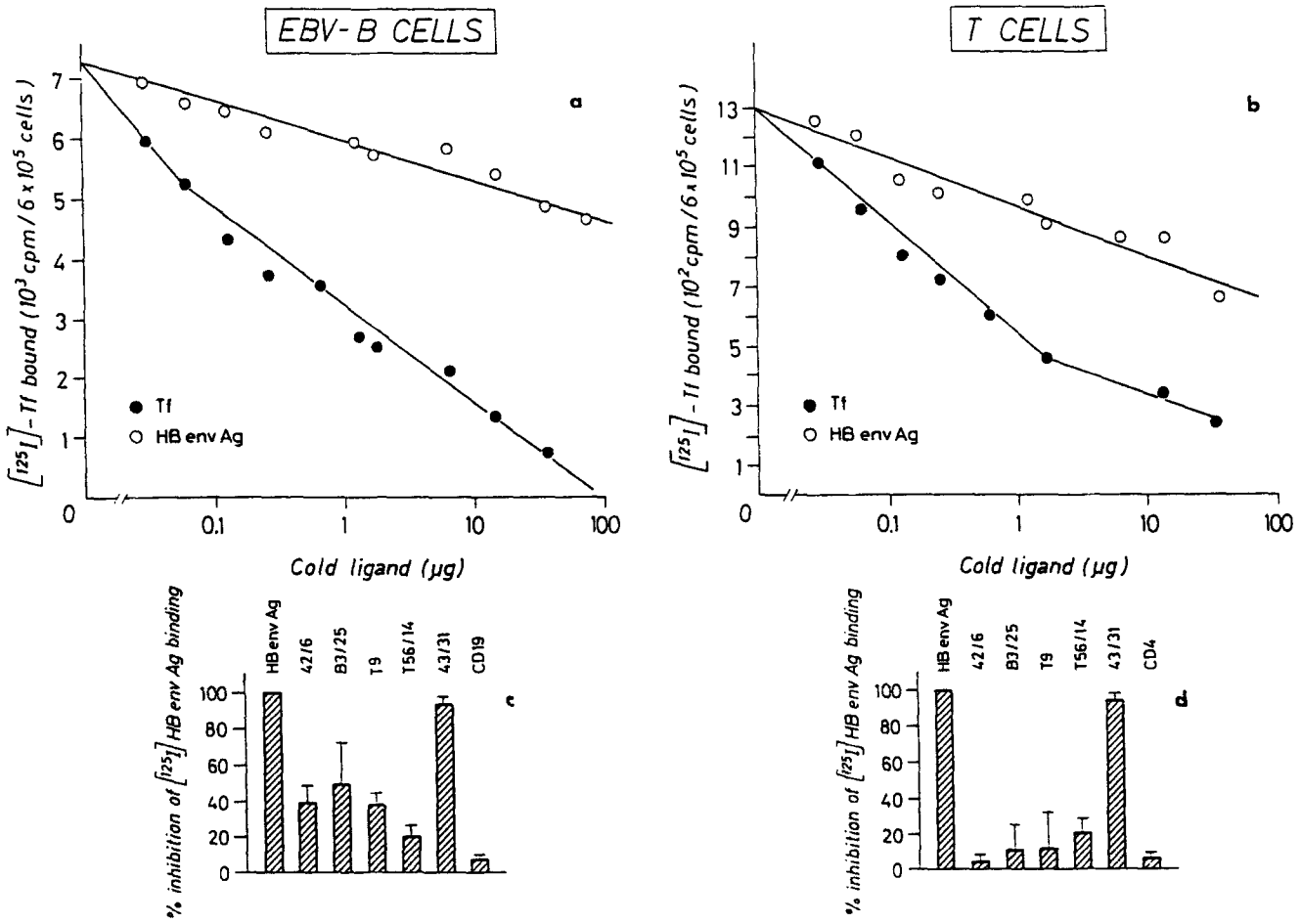


Figure 5. (*a* and *b*) Competition binding assay between ^{125}I-Tf and either unlabeled Tf or HBenvAg (S,L construct). EBV-B cells or T cells (nonspecific CD4$^+$ T clones) were incubated for 120 min at 4°C with a fixed concentration of ^{125}I-Tf (500 ng/ml) alone or in the presence of either unlabeled Tf (0.1–200 μg/ml) or unlabeled HBenvAg (0.1–400 g/ml ^{125}I). Results are the mean values observed in three separate experiments. (*c* and *d*) Effect of different anti-CD71 mAbs on the binding of ^{125}I-HBenvAg to EBV-B cells and T cells (nonspecific CD4$^+$ T clones). EBV-B or T cells were preincubated for 1 h at 4°C in the presence of 10 μg/ml of different purified anti-CD71 mAbs (42/6, B3/25, T9, T56/14, and 43/31) in the presence of anti-CD4 (T cells) or anti-CD19 (EBV-B cells), or in the presence of unlabeled HBenvAg (S,L construct, 200 μg/ml). ^{125}I-HBenvAg (S,L construct, 1.25 μg/ml) was then added, and the cells were incubated for 120 min at 4°C. Cell-bound radioactivity was evaluated after centrifugation and a cushion of oil phtalate. Results represent mean values ± SEM observed in three separate experiments.

II–restricted CTL when added before and not after antigen pulsing. This suggests that OKT9 was the only mAb blocking the HBenvAg capture by T cells. OKT9 also inhibited antigen uptake by EBV-B cells, but to a lesser degree than that observed with T cells, thereby suggesting that HBenvAg may in part use the CD71 receptor to enter B lymphocytes. However, B lymphocytes may also capture the HBenvAg either aspecifically (27) or through interaction with other membrane structures.

The anti-TfR mAb selectively interfered with HBenvAg uptake by APC, since it did not inhibit the presentation of either the 1–21 peptide (which does not need processing) or the unrelated TT antigen. Conversely, the aspecific interactions between APC and T cells, rather than the initial HBenvAg uptake, were inhibited by adhesion molecule mAbs (24). They indiscriminately inhibited specific lysis when added before or after antigen pulsing of APC. These mAbs also blocked presentation of both the 1–21 peptide and unrelated TT antigen.

The selective blocking of specific lysis, observed by pulsing the presenting T or EBV-B cells with HBenvAg after treatment with soluble Tf, further suggests that TfR/HBenvAg interaction is a crucial, initial step required for antigen processing and presentation. Endocytosis and recycling of the TfR with either its ligand or anti-TfR mAbs have been extensively analyzed in both lymphoid and nonlymphoid cells (30–33). In agreement with these findings, we found that OKT9 does not inhibit Tf binding to its receptors, but downregulates surface TfR on T cells when incubated at 37°C. This is followed by a decrease of iodinated Tf and HBenvAg bindings, confirming that this mAb blocked HBenvAg presentation by interfering with its internalization (data not shown).

The involvement of TfR in HBenvAg interaction with lym-

phocytes is also supported by binding experiments. In this regard, HBenvAg only partially inhibits Tf binding. Furthermore, among the anti-CD71 mAbs inhibiting the binding of Tf to its receptor, only one (43/31 mAb) markedly decreases iodinated HBenvAg binding to the cells. This clearly suggests that the epitope involved in the interaction between HBenvAg and Tf receptor is similar but not identical to that involved in the binding of Tf. To the best of our knowledge, this is the first study indicating that TfR may be involved in the binding of a ligand different from Tf.

We evaluated whether the system of antigen presentation by T cells could also be used for identification of the sequence relevant for HBenvAg interaction with T cells. Our results clearly indicate that this sequence mapped to the pre-S2 region, based on the ability of T cells to present both the entire (pre-S1, pre-S2, S) protein and the protein containing pre-S2/S regions without pre-S1 to a S-specific T clone (VB27), and on their failure to present the S protein alone to the same clone. Moreover, the finding that T cells presented the r(S,L) protein, containing the only 133–145 sequence of pre-S2 region, to lesser extent than r(pre-S2, S) containing the entire pre-S2, suggests that HBenvAg epitope binding to T cells overlaps the 133–145 pre-S2 peptide. Comparative analysis of the capacity of different HBenvAg molecular constructs to inhibit Tf binding provided evidence strictly in line with results derived from antigen-presentation studies, i.e., the HBenvAg sequence interacting with TfR on T cells is contained in the pre-S2 region. More precise mapping of this epitope may be useful for the design of innovative synthetic vaccines. Studies on T and B cell recognition of pre-S2 epitopes suggest that the 120–132 and 132–149 peptides are particularly crucial for virus neutralization (26, 34, 35). Moreover, the pre-S2 domain, as well as the pre-S1 region, have been proposed to be involved in attachment of HBV to hepatocytes and to other cells that are susceptible to infection (26).

It has been hypothesized that antigen presentation by T cells plays a role in immunoregulation (36, 37). In our system, antigen-specific $CD4^+$ T clones killing presenting T cells may participate in generalized immunosuppression by elimination of T lymphocytes. In particular, $CD71^+$ cells expressing class II molecules, as macrophages or activated T cells, could be susceptible to lysis by HBenvAg presentation to class II–restricted CTL.

The in vivo relevance of cytotoxic activity mediated by $CD4^+$ T cells is controversial, but the finding that these cells act as CTL has been reported for a number of viral infection models, including HBV, measles, herpes, and coronavirus (14, 15, 38–42). In our hands, $CD4^+$ CTL did not elicit bystander lysis, indicating that they killed by a direct target/CTL contact mechanism, and not aspecifically by secreted soluble cytotoxic mediators (25, 43). The finding that $CD4^+$ CTL are able to lyse both $CD4^+$ and $CD8^+$ CTL clones in a HLA-restricted manner are in contrast with reports suggesting that CTL are resistant to self-mediated lysis (44). These studies used murine CTL instead of human $CD4^+$ CTL, utilized in the present study, thus, possibly explaining this discrepancy (6). Experiments with human class II–restricted CTL, specific for the HIV gp120, indicated that lysis of gp120-presenting $CD4^+$ T cells represent an immunosuppression mechanism (10, 37). Analogously, class II–restricted CTL specific for HBenvAg can lyse themselves or other HBenvAg-presenting $CD4^+$ or $CD8^+$ T cells, thereby downregulating the T cell response and posing a selective advantage for HBV persistence (45).

In conclusion, our data indicate that both $CD4^+$ and $CD8^+$ activated T cells can process and present HBenvAg with high efficiency. More importantly, HBenvAg uptake via TfR seems to be an essential initial step for the presentation of this antigen. Human TfR (20) is expressed on a variety of cell types, including hepatocytes (21, 46–53), which represent the privileged target cells of HBV infection. The wide TfR distribution may thus explain the broad tissue tropism of HBV (54). Other pathogens enter cells using the binding site for a known physiological ligand (55–58); e.g., HIV uses the CD4 receptor, rhinoviruses enter through ICAM-1 molecules, herpes simplex virus 1 through the fibroblast growth factor receptor, and *Pneumocystis carinii* through the mannose receptor (59–62).

The identification of both the receptor used by HBV to enter cells and the HBenvAg epitope relevant for this interaction may have fundamental implications, not only for the design of alternative vaccines, but also on therapeutic approaches to chronic HBV infection.

We thank L. Adorini, A. Alberti, M. Ferrarini, and A. Lanzavecchia for criticism and suggestions.

This work was supported in part by grants from Ministero dell'Università della Ricerca Scientifica and by "Programma Terapia dei Tumori," Istituto Superiore di Sanità, Rome, Italy.

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Received for publication 30 December 1991.

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