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Presentation of an Immunodominant T-Cell Epitope of Hepatitis B Surface Antigen by the HLA-DPw4 Molecule

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Human T cells that recognize a major epitope of the hepatitis B surface antigen were studied for their ability to react with antigen when presented by mouse fibroblasts that express class II products of the human major histocompatibility gene complex after gene transfection. L cells expressing HLA-DPw4, but not those expressing HLA-DR4 or HLA-DR7, induced strong proliferative responses of antigen-specific T cells to either hepatitis B surface antigen or the synthetic peptide S1d, which bears the immunodominant T-cell epitope. These results identified a genetic restriction element of human helper T-lymphocyte responses to a major antigenic determinant of hepatitis B virus and might be important in the design of subunit vaccines to this pathogen. Peptides that induce T-cell responses that are restricted by a frequently encountered major histocompatibility complex molecule in the general population such as DPw4 would be ideal candidates as subunit vaccines.

The activation of T lymphocytes by antigen is an important event in the elimination or prevention of viral infections. T cells participate in immune effector mechanisms such as the production of virus-neutralizing antibodies and cell-mediated cytolysis of virus-infected cells. T lymphocytes have been classified into two main functional groups: (i) T helper lymphocytes (T_h), which produce soluble factors (lymphokines) that promote the synthesis of antibodies by antigen-stimulated B lymphocytes, and (ii) cytotoxic T lymphocytes, which are responsible for the elimination of virus-infected cells and thus prevent the assembly and spread of mature virion particles throughout the organism. Both types of T cells can only be activated by antigen molecules exposed on the surface of another cell, such as an antigen-presenting cell (APC) or a target cell. Products of the major histocompatibility gene complex (MHC) also participate in the recognition of antigen by T cells (4, 27, 36). For the T cells to become activated, the antigenic determinants found within the viral molecules must first interact with the MHC-encoded molecules on the surface of the APC. Thus, T lymphocytes react with a molecular complex on the surface of the APC which is formed by a foreign antigenic determinant (viral) and a self-determinant provided by the MHC molecule. In humans, T_h responses to a particular determinant are restricted by products of the HLA-DR, -DP, or -DQ genes of the MHC.

Protective immunity to hepatitis B virus (HBV) seems to be determined mostly by antigenic epitopes present in the viral envelope protein known as hepatitis B surface antigen (HBsAg). Thus, vaccinated and some previously HBV-infected individuals contain significant amounts of antibodies to HBsAg in their sera (19, 32). The production of antibodies to HBsAg is, as with most antigens, regulated by T_h (12, 26). In our effort to study the recognition of the HBsAg molecule by human T cells, we have established cell lines and have isolated clones of HBsAg-specific T lymphocytes (9, 11). We recently reported that a significant number of HBsAg-reactive T cells from various HBV-immune individuals recognize a determinant localized near the amino terminus of HBsAg (10). This immunodominant region was detected with a series of overlapping synthetic peptides spanning residue positions 4 to 33 of the S gene product of HBV. A single T-cell determinant was identified in a 10-amino-acid peptide corresponding to residue positions 19 to 28 of HBsAg. The synthetic peptide, called S1d, triggered strong proliferative and cytotoxic T cell responses that were restricted by class II MHC molecules of the APC. Experiments with a panel of HLA-typed lymphoblastoid cells as APCs and inhibition studies with anti-class II MHC monoclonal antibodies (MAb) suggested that the T-cell reactivity to peptide S1d is restricted by products of the HLA-DP genes (10).

The present study was undertaken to identify the MHC class II molecule responsible for restricting the recognition of the S1d determinant of HBsAg by T_h derived from three human HBV vaccine recipients. Mouse L cells transfected with individual human MHC class II genes were used as APCs to identify the genetic restricting elements of this T-cell response to HBsAg.

MATERIALS AND METHODS

Antigens. HBsAg was purified from plasma of chronic HBV carriers or from the culture supernatant of the Alexander hepatoma cell line (18) by affinity chromatography as described previously (9). Analysis of the HBsAg preparations by polyacrylamide gel electrophoresis revealed that 95% of HBsAg existed as the gp27 and p24 forms (8). Purified HBsAg was denatured by suspending the protein in 70% formic acid overnight at 4°C (8) followed by extensive dialysis against phosphate-buffered saline (0.15 M NaCl, 0.015 M PO_4; pH 7.4). Peptide S1d (amino acid sequence, Phe-Phe-Leu-Leu-Thr-Arg-Ile-Leu-Thr-Ile) was prepared in a BioSearch automated synthesizer by the solid-phase method.

HBsAg-reactive human T cells. Peripheral blood mononuclear cells (PBMC) from HBV-immune individuals were obtained from heparinized blood by centrifugation on a Ficoll-diazotrizoate density gradient. Long-term T-cell lines

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and clones were isolated and maintained as described previously (9, 11). Long-term T-cell lines (and clones) were shown to be CD4$^+$ by surface immunofluorescence (9). HBsAg-specific T cells from three immune donors were used in the present study. The long-term T-cell line HBL-1.DV and the clone derived from this line, HBC-1.DV, were isolated from a vaccine recipient with HLA-DR2/4, -DPw4, -DQwl/w3. T-cell clone HBC-9.BS was selected from a donor with HLA-DR2/7, -DPw4, -DQwl/w3; and clone HBC-28.VM was from a donor with HLA-DR3/4, -DP?, -DQw2/w3. Medium consisted of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% (vol/vol) human AB serum (Sigma Chemical Co., St. Louis, Mo.), 5×10^{-5} M 2-mercaptoethanol, 50 μg of gentamicin per ml, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 2 mM L-glutamine.

APCs and class II MHC-transfected L cells. The following HLA-D-typed Epstein-Barr virus (EBV)-transformed cell lines were used as APCs: EG (DR4/7, DPw4, DQw2/w3); Priess (12) (DR4/4, DPw2/w3, DQw3/w3); 639 (DR4/4, DPw3/w3, DQw3/w3); and Burkhardt (DR7/7, DPw4/w4, DQw2/w2). Mouse L cells expressing human Ia molecules were produced by cotransfection with cDNAs corresponding to the MHC class II α and β genes (15a). Full-length human MHC class II α- and β-chain cDNAs in the Okayama and Berg (24) expression vector pcD were used for transfection; DRα and DR7β_I (Dw7) from a DR7 cDNA library (15) and DR4β_I (Dw13) from a DR4 library (14). The DPw4α and DPw4β cDNA clones were also isolated from the DR7 library (15b). Cells of the DAP.3 subclone of mouse L-cell fibroblasts were cotransfected with 20 μg each of MHC class II α- and β-chain cDNAs and 1 μg of the pSV2 neomycin resistance plasmid (31) by the calcium phosphate coprecipitation method (13). Transfectants were selected for neomycin resistance in medium containing the neomycin analog G418 (geneticin; GIBCO), and transfectants expressing high levels of human MHC class II molecules were isolated by flow cytometric sorting and cloning. Transfected L cells were maintained in Dulbecco modified eagle medium (GIBCO) supplemented with 10% (vol/vol) fetal calf serum and 250 μg of G418 per ml. Cells were continuously monitored for mycoplasma infection.

T-cell proliferation assays. Proliferative responses to several concentrations of HBsAg and peptide were measured by the incorporation of [^{3}H]thymidine (Dupont, NEN Research Products, Boston, Mass.) into DNA by replicate cultures. Proliferation assays were performed in flat-bottomed 96-well microdilution plates (Costar, Cambridge, Mass.) by incubating 2×10^4 T cells with 5×10^4 autologous irradiated PBMC, 3×10^4 EBV-transformed lymphoblastoid cells, or various numbers of irradiated (3,000 rads) transfected L cells in each well for a total of 3 days. Cultures were pulsed for the last 18 h with 1 μCi of [^{3}H]thymidine per well and harvested onto fiber glass filters for determination of incorporated radioactivity. Results are expressed as mean counts per minute (cpm) of the replicates or as cell proliferation indices, which were calculated by the ratio of cpm obtained in the presence of antigen to cpm obtained in the absence of antigen. Standard deviations of the mean cpm were consistently below 10%. Cell proliferation index values ≥3 were considered statistically significant.

MAb. Mouse MAb to human class I and II MHC antigens were used to inhibit T-cell proliferation assays. Antibody DA6.231 (33) with broad specificity to DR and DP structures (30) was kindly provided as ascites fluid by K. Guy (Medical Research Council, Edinburgh, Scotland). Antibody B7/21,

TABLE 1. Antigen stimulation of HBsAg-specific T cells in the presence of PBMC and HLA-typed lymphoblastoid cells

APC	HLA type			Cell proliferation index[a]	
	DR	DP	DQ	HBsAg[b]	Sld[c]
DV-PBMC[d]	2/4	w4	wl/w3	43.9	80.9
EG[e]	4/7	w4	w2/w3	49.1	41.9
Priess[e]	4/4	w3/w4	w3	10.9	18.6
639[e]	4/4	w3	w3	1.1[f]	2.6[f]
Burkhardt[e]	7/7	w4	w2	16.4	11.9

[a] HBsAg-specific T-cell line HBL-1.DV (DR4/7, DPw4, DQwl/w3) was tested for its proliferative response to antigen in the presence of various APCs. Cell proliferation indices were calculated by the ratio of cpm of [^{3}H]thymidine incorporated in the presence of antigen to the cpm obtained in the absence of antigen. A cell proliferation index ≥3 was considered significant.
[b] Formic acid denatured, tested at 25 μg/ml.
[c] Tested at 1 μg/ml.
[d] Autologous PBMC tested at 5×10^4 cells per well.
[e] EBV-transformed lymphoblastoid cell lines used at 3×10^4 cells per well.
[f] Not significant.

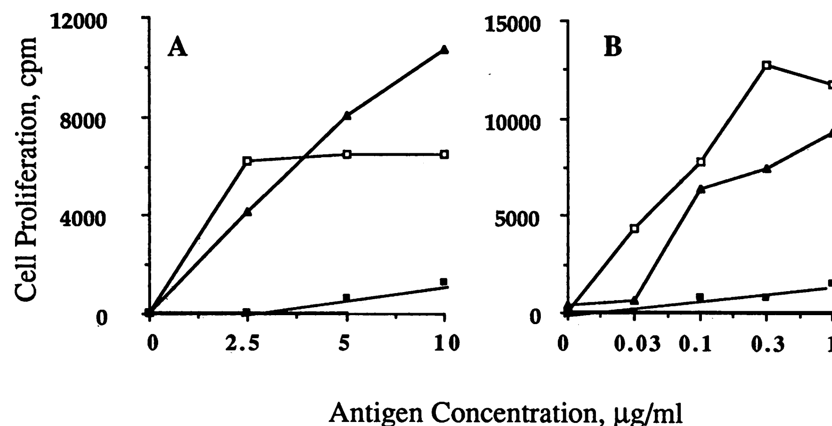

FIG. 1. Ability of transfected cell lines to present antigen to an HBsAg-specific T-cell line. PBMC (▲), DPw4 L-cell transfectants (□), and DR7 L-cell transfectants (■) were compared for their capacity to stimulate the HBL-1.DV T-cell line (HLA-DR2/4, -DPw4, -DQwl/w3) in the presence of various concentrations of formic acid-denatured HBsAg (A) or the synthetic peptide Sld (B). L-cell transfectants and PBMC were used at 6×10^3 and 5×10^4 cells per well, respectively.

TABLE 2. Antigen presentation to HBsAg-specific T cells by class II-transfected APCs

Antigen	Cell proliferation (cpm) by clone[a]:							
	HBC-28.VM				HBC-9.BS			
	PBMC	L-DPw4	L-DR7	None	PBMC	L-DPw4	L-DR7	None
HBsAg[b]	4,627	430	96	90	5,262	544	135	153
Sld[c]	2,710	1,472	102	260	5,162	1,989	250	457
None	158	50	17	60	158	43	30	4

[a] Antigen-induced proliferation of T-cell clones HBC-28. VM (DR3/4, DP?, DQw2/w3) and HBC-9.BS (DR2/7, DPw4, DQwl/w3) was measured in the presence of PBMC (5×10^4 per well), L-DPw4 cells (1×10^4 per well), L-DR7 cells (1×10^4 per well), or no APCs.
[b] Formic acid-denatured tested at 25 μg/ml.
[c] Tested at 1 μg/ml.

specific for HLA-DP molecules (25, 35), was obtained from hybridoma culture supernatants of cells provided by I. Trowbridge (Salk Institute, La Jolla, Calif.). Antibody W6/32 (anti-HLA-A, -B, -C) (6) was obtained from culture supernatants prepared from hybridoma cells purchased from the American Type Culture Collection (Rockville, Md.).

RESULTS

The proliferative responses of an antigen-specific T_h line to HBsAg and to a synthetic peptide representing amino acid positions 19 to 28 of the S molecule of HBsAg were determined with various APCs with known HLA-D-region types. Significant cell proliferation indices to both the antigen and the synthetic peptide (Sld) were observed when autologous PBMC were used as APCs (Table 1). Furthermore, three of the four EBV-transformed lymphoblastoid cell lines tested served as efficient for APCs for these cell responses to both antigens.

To define more precisely the MHC class II molecule responsible for presenting the Sld epitope of HBsAg to human T cells, we performed the following experiments. Mouse L cells transfected with and expressing the α and β genes of HLA-DPw4 were tested for their capacity to trigger an antigen-induced proliferative response of HBsAg-specific human T_h. The DPw4-transfected L cells (6×10^3 cells per well) induced significant proliferative responses similar to those observed with autologous PBMC (5×10^4 cells per well) in the presence of various concentrations of HBsAg (Fig. 1A) or the synthetic peptide Sld (Fig. 1B). L cells transfected with HLA-DR7 genes did not induce proliferative responses of the T_h with either antigen preparation. Similar results were obtained with HBsAg-specific T_h isolated from two additional vaccine recipients (Table 2; Fig. 2). These results indicate that DPw4 can be a restriction element for the T-cell epitope of HBsAg found on peptide Sld.

Comparison of the DPw4-transfected L-cell line with autologous PBMC for efficiency in presenting antigen indicated that approximately 100-fold-fewer DPw4-transfected cells than PBMC were required for efficient stimulation of T_h proliferation in response to HBsAg (Fig. 3A) or peptide Sld (Fig. 3B). The intensity of the proliferative response to HBsAg (measured as maximal cpm) with DPw4 transfectants was approximately 1.5-fold lower than the response observed with PBMC (Fig. 3A). On the other hand, when the DPw4 transfectants were used as APCs, peptide Sld produced slightly higher proliferative responses than those observed with the PBMC (Fig. 3B). L cells transfected with DR4 genes did not present HBsAg or peptide Sld to the human T_h.

MAb specific for human MHC molecules were tested for their capacities to inhibit the antigen-mediated proliferative response of HBsAg-specific T_h. Antibody DA6.231, which reacts with both DR and DP MHC products, was highly inhibitory in the proliferative response of T_h to either HBsAg or peptide Sld when DPw4-transfected L cells were used as APCs (Fig. 4). Furthermore, MAb B7/21, which is highly specific for the products of the HLA-DP genes, was equally effective in inhibiting the reactivity of the T_h to antigen in the presence of the DPw4 transfectants. Similar results were

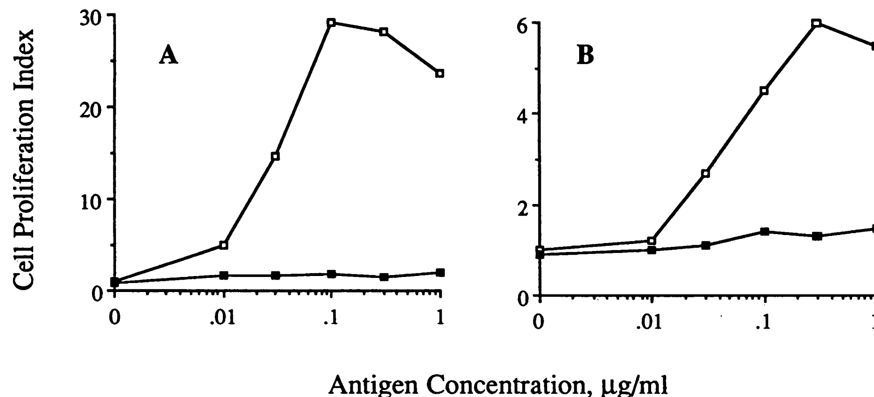

FIG. 2. Recognition of peptide Sld by human T_h clones in the presence of HLA-DPw4-transfected L cells. The proliferative response of clones HBC-28.VM (panel A, DR3/4, -DP?, -DQw2/w3) and HBC-9.BS (panel B, HLA-DR2/7, -DPw4, -DQwl/w3) to various concentrations of peptide Sld was studied with 10^5 DPw4 (□)- and DR4 (■)-transfected L cells per ml as APCs. The cell proliferation index was calculated as the ratio of cpm obtained in the presence of antigen to the cpm observed in the absence of antigen (medium alone).

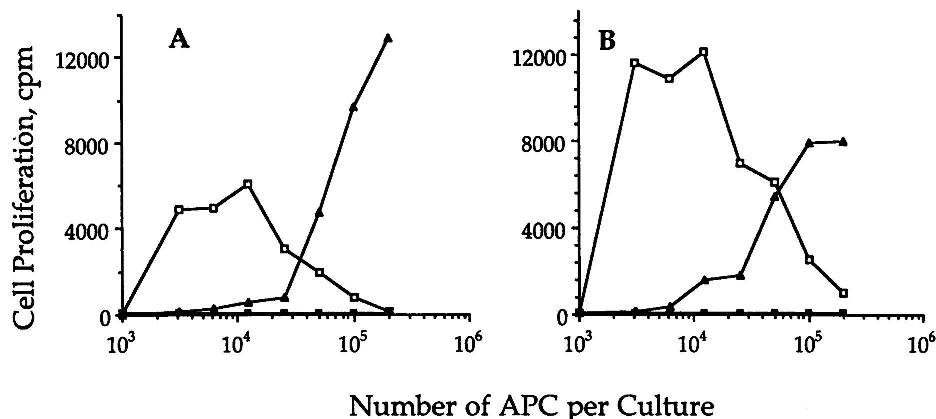

FIG. 3. Efficiency of antigen-presenting function of PBMC and L-cell transfectants on the proliferative response of human T_h to antigen. Various numbers (per culture) of autologous PBMc (▲) or DPw4 (□)- or DR4 (■)-transfected L cells were tested for their ability to stimulate the T-cell line HBL-1.DV (HLA-DR2/4, -DPw4, -DQw1/w3) in the presence of 5 μg of denatured HBsAg per ml (A) or 0.3 μg of peptide S1d per ml (B).

obtained when autologous PBMC were used as a source of APCs (Fig. 5). The anti-class I MHC MAb, W6/32, had no effect on the proliferative response of the T_h to antigen.

DISCUSSION

The recognition of antigen by T lymphocytes requires the active participation of surface molecules encoded by the MHC. Recently, it has been shown that small synthetic peptides containing amino acid sequences of the antigen molecules can effectively stimulate T cells in the presence of APCs (3, 5, 16, 22). In addition, some of the T-cell immunogenic peptides bind specifically to the corresponding purified class II MHC molecules (2, 7). The identification of the class II MHC molecule involved in the presentation of antigen to a T_h cell has been generally achieved by using panels of large numbers of HLA-typed APCs such as EBV-transformed lymphoblastoid cells. In addition, the ability of class II MHC-specific MAbs to block antigen-induced proliferative responses in T_h cells has also been used to determine which MHC molecule is responsible for a particular antigen presentation. Recently, a new approach has been taken to identify the genetic element(s) that restricts T_h responses to antigen by using MHC-transfected cell lines. The genes corresponding to the α and β chains of some of the human MHC class II molecules have been cloned and successfully transfected and expressed in murine cells (1, 17, 23, 28). Fibroblasts that express the human HLA-DPw2 or HLA-DR1 molecule on their surfaces were shown to efficiently present influenza A viral antigens to human T cells (1, 28). We have recently used the same approach to study the T-cell response of humans to rabies virus antigens (7a). The main advantage of this system is that the role of an individual MHC molecule in antigen-mediated activation of human T_h can be clearly analyzed.

An immunodominant T_h epitope situated in the amino-terminal end of the HBsAg molecule was recently described (10). The synthetic peptide S1d, which occupies amino acid

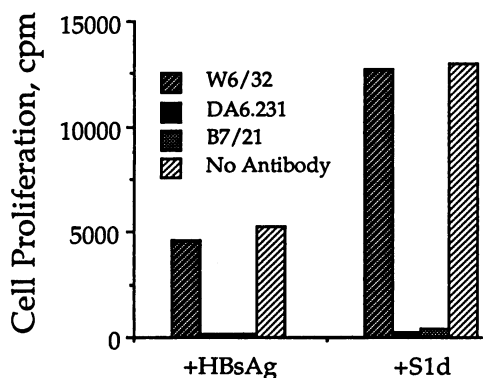

FIG. 4. Effects of MAb specific for human MHC molecules on the induction of the antigen-mediated proliferative responses of T_h by DPw4-transfected APCs. Antibody DA6.231, with broad specificity for DR and DP, and antibody B7/21, specific for DP antigens, were tested for their capacity to inhibit the proliferative response of the HBsAg-specific T-cell clone HBC-1.DV (HLA-DR2/4, -DPw4, -DQw1/w3) to either whole denatured HBsAg or peptide S1d in the presence of 6×10^3 irradiated DPw4 L cells per well. Antibody W6/32, with specificity for class I MHC molecules, was used as a negative control. Antibody concentrations were as follows: DA6.231, 1:100 dilution of ascites fluid; B7/21 and W6/32, 1:20 dilution of hybridoma culture supernatant. Antigen concentrations were as follows: HBsAg, 5 μg/ml; peptide S1d, 0.3 μg/ml.

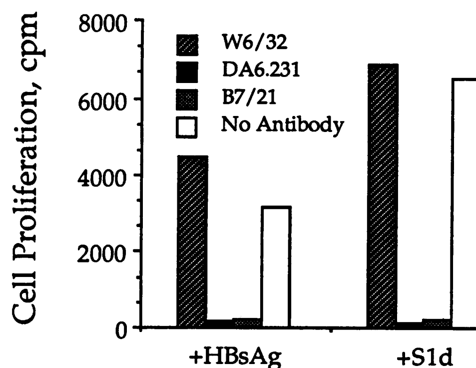

FIG. 5. Inhibitory effects of MAb specific for class II MHC molecules on the antigen-presenting function of autologous PBMC. Conditions for this experiment are as described in the legend to Fig. 4, except that autologous irradiated PBMC (5×10^4 per well) instead of DPw4-transfected L cells were used as APCs.

positions 19 to 28 of the HBsAg molecule (p24-gp27), was recognized by a large number of HBsAg-specific T cells isolated from several vaccine recipients (9). Experiments presented here (Table 1) and previous results (10) suggested that HBsAg-reactive human T cells recognized the S1d epitope in the context of an HLA-DP molecule. To study in more detail the human Ia molecules involved in the presentation of the S1d epitope to HBsAg-reactive T lymphocytes, we used L cells transfected with individual human class II MHC molecules as APCs. L cells expressing the DPw4 molecule, but not those expressing the DR4 or the DR7 molecule, induced a significant T-cell proliferative response in the presence of antigen. It has recently been observed that these DR7 and DPw4 L-cell transfectants can function efficiently as APCs for rabies virus-specific T-cell clones (7a) and that the DR4-transfected L cells can present antigen to HIV-specific T cells (30a). In some of the experiments presented here, low but significant antigen-induced proliferative responses of the HBsAg-specific T-cell clones were observed with the DR7 L-cell-transfected APCs, or even in the absence of APCs (Table 2; Fig. 1); however, in all cases, these responses were considerably lower than those obtained with the DPw4-transfected L cells. A possible explanation for these results is that low numbers of autologous irradiated PBMC (which are used to stimulate and maintain the T-cell clones in bulk culture) could remain in the cultures and thus allow low proliferative responses to antigen. Indeed, in those experiments in which the T-cell lines and clones were maintained in the absence of APCs (but in the presence of interleukin-2) for more than 2 weeks, no proliferative responses to any concentration of antigen were evident (Fig. 2 and 3). Similar results have been observed with rabies virus-specific T cells (7a).

The overall results demonstrated definitely that the HLA-DPw4 molecule can act as a restriction element for the recognition of this immunodominant epitope (S1d) of HBsAg in some T lymphocytes. However, the possibility exists that other MHC class II molecules besides DPw4 may be able to present the S1d epitope to other HBsAg-specific T cells. Compared with PBMC, the DPw4 transfectants appeared to present denatured HBsAg less efficiently to the T_h, since T-cell activation (maximal cpm) obtained with the DPw4-transfected L cells was less than that observed with PBMC. These results can be explained by differences between PBMC and L cells in their capacity to process complex antigens such as HBsAg since the DPw4-transfected L cells were always efficient in stimulating the T_h when peptide S1d was used. Furthermore, native HBsAg stimulated the T cells significantly less (5 to 10 times) than formic acid-denatured HBsAg (which requires less processing [8]) when DPw4-transfected L cells were used as APCs (data not shown). Similar results have been obtained by others (29), indicating that fibroblasts may not be as efficient as monocytes or B cells in processing antigens. A cell-to-cell comparison between PBMC and L-cell transfectants indicated that optimal proliferative responses to both HBsAg and S1d are obtained with approximately 100-fold-fewer DPw4-transfected L cells. Thus, it appears that in the heterogeneous PBMC population not all cells express DPw4 molecules and that only those that do can present HBsAg (or S1d) to the T_h. The inhibitory effect in the antigen-induced proliferative response of T_h observed with high numbers ($>10^4$) of DPw4-transfected cells could reflect the depletion of nutrients in the medium by the high metabolism of these cells (although they are irradiated and do not appear to divide, the L cells remain alive and attached to the bottoms of the microdilution wells for approximately 3 days).

The data presented here could be relevant for the design for subunit vaccines to protect against pathogens such as HBV. It has recently been shown that synthetic peptides containing B- and T-cell epitopes for HBV-derived antigens will induce the production of antibodies in mice (20, 21). Identification of several immunodominant T_h epitopes and their corresponding MHC class II restricting molecules may allow preparation of a synthetic antigen containing several T-cell epitopes that could be stimulatory in the majority of individuals of an outbred population. Such a preparation would constitute an ideal immunogen to boost or enhance the T_h immunological memory to a particular virus. Since it has been reported that the DPw4 specificity is present in approximately 70% of the population (34), the use of peptide S1d as a component of a mixed peptide vaccine would ensure that T-cell responses to HBsAg occur in a significant number of individuals. However, in view of the high degree of polymorphism that exists among all and within each MHC class II molecule, the practicality of this approach compared with conventional vaccines such as inactivated viruses, which contain the total number of T-cell epitopes for that virus, should be considered. Experiments aimed toward studying the immunogenicity of synthetic peptides bearing T_h epitopes in humans must first be performed to evaluate the feasibility of this approach.

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