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Recognition of Major Histocompatibility Complex Antigens on Cultured Human Biliary Epithelial Cells by Alloreactive Lymphocytes

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We have developed an *in vitro* system to study the interactions between biliary epithelium and lymphocytes using cultured human biliary epithelial cells. No class II antigens were detected by immunoperoxidase staining of the normal biliary epithelial cells, but alloactivated lymphocyte culture supernatants were able to induce class II expression. The activity of the supernatants was blocked with an anti-γ-interferon monoclonal antibody. In addition, recombinant human γ-interferon alone induced the expression of class II antigens and increased the intensity of class I staining of cultured biliary epithelial cells. Biliary epithelial cell–induced proliferation of alloreactive T lymphocytes demonstrated that the major histocompatibility complex molecules carry functional lymphocyte-activating determinants. The recognition of major histocompatibility complex determinants was confirmed by monoclonal antibody–blocking studies and by stimulation of an alloreactive T-cell clone. However, the biliary epithelial cells were much less potent stimulators than arterial endothelial cells tested in the same assay system. (HEPATOLOGY 1991;13:239-246.)

The biliary epithelium is a major target of lymphocytic attack in human liver allograft rejection and in many immunologically mediated liver diseases such as PBC (1, 2). The immune damage in these disorders is thought to be mediated primarily by T cells. Major histocompatibility complex (MHC) antigens are likely the target of lymphocytic attack in rejection (3), and, although the target antigens in PBC have not yet been identified, the aberrant expression of class II antigens seen on the bile ducts in PBC livers has been hypothesized to play a role in the disease (4).

The biliary epithelium in normal livers expresses class I but not class II human leukocyte antigens (HLA) (5-7). However, all three class II subregion products, namely HLA-DR, HLA-DP and HLA-DQ, have been detected on bile ducts in transplanted livers during rejection and other complications (5, 8), in hepatic graft vs. host disease after bone marrow transplantation (9) and in PBC (4). It has been hypothesized that this expression is induced by soluble inflammatory mediators released from the invading mononuclear cells. Although the pathogenic role of this aberrant class II expression is unclear, we have previously shown that the increased destruction of bile duct epithelium during rejection is associated with HLA class II–specific lymphocytes invading the allograft (10).

We have established an *in vitro* model system to study the interactions between biliary epithelium and lymphocytes using cultured normal human biliary epithelial cells (BEC). The cultured BEC have been used to investigate the cytokines responsible for the up-regulation of class II MHC antigens and whether these MHC molecules were functionally active (i.e., capable of stimulating T lymphocytes). The results obtained for BEC have been compared with arterial endothelial cells (AEC), which have already been shown to be potent allogeneic stimulators of lymphocyte proliferation.

MATERIALS AND METHODS

Isolation and Culture of BEC. BEC cultures were established using a modification of a technique previously published for the isolation of intrahepatic BEC (11). The use of human tissues in these studies was approved by the internal review board. Because whole donor livers are rarely available for research purposes, an unused section of the common bile duct from donor livers was the source of cells in this study. Any excess connective tissue was removed, and the duct was opened longitudinally and washed free of blood with HBSS obtained from Gibco (Grand Island, NY). The opened duct was placed with the epithelial surface down into a solution of 0.25% collagenase IV obtained from Sigma Chemical Co. (St. Louis, MO) at room temperature for 10 to 15 min to free up the epithelial cells. The duct was then turned over and flushed with Williams E (WE) medium (Gibco) containing 10% heat-inactivated FBS from Gibco to detach loosely adherent BEC. Harvested BEC were pelleted by centrifugation at 1,200 rpm for 5 min, resuspended in culture medium and examined under phase-contrast inverted microscopy. The isolated cells were in organoid clusters as previously described (11) and

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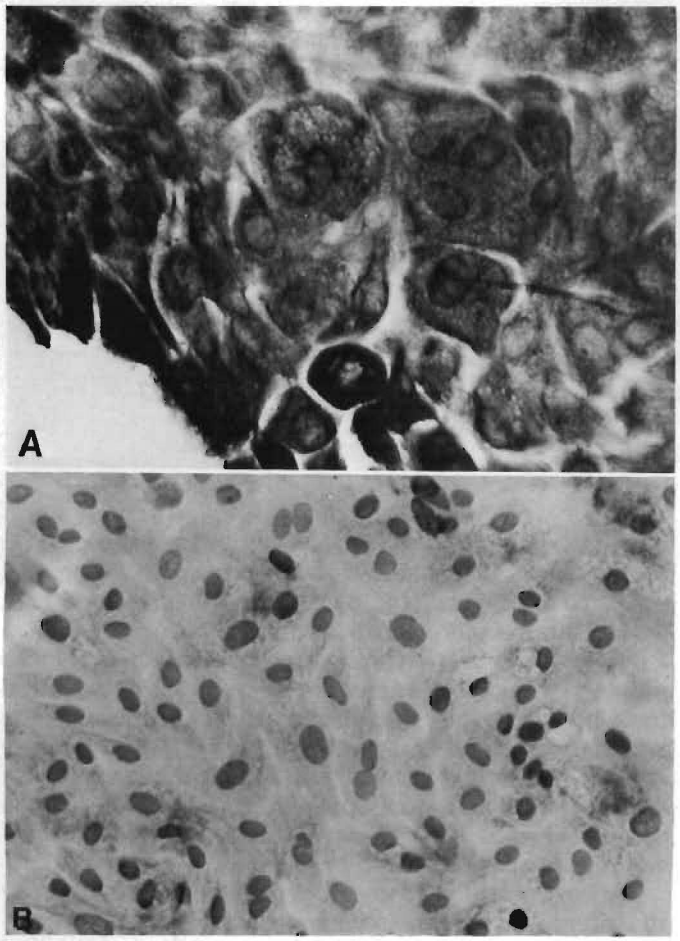

FIG. 1. Immunoperoxidase stain of cultured human BEC showing positive stain for cytokeratin (A, original magnification ×560) and negative stain for factor VIII–related antigen (B, original magnification ×350).

could not be dispersed into a single cell suspension without severely compromising viability and successful expansion in culture. Therefore the BEC clusters were suspended in culture media and plated after estimating the number of cells present. Cell identity was confirmed by their typical structure, with positive staining for cytokeratin (AE1/AE3) (Fig. 1A) and negative staining for factor VIII–related antigen (Fig. 1B) as previously described (11).

BEC were grown in a serum-free medium consisting of WE supplemented with 4 mmol/L L-glutamine obtained from B & B/Scott (Fiskeville, RI), 24 mmol/L HEPES buffer (Gibco), 60 μg/ml gentamycin sulfate (Gibco) and 70 μg/ml bovine pituitary extract (BPE) prepared as described by Hammond, Ham and Stampfer (12). In addition, a commercially supplied mixture of growth supplements (1 ml each of SGF-7 and SGF-9/100 ml of medium obtained from B & B/Scott) was included. Recently, the SGF supplements have become unavailable, but we have found that the BEC grow well in KGM (Clonetics Corp., San Diego, CA), which is a medium developed for the serum-free culture of human keratinocytes (13). It includes many of the same growth factors as the WE media described above, including BPE, and results of functional assays have been similar using both kinds of medium.

Cultures for staining were plated on Lab-Tek (Miles Scientific, Naperville, IL) tissue-culture chamber slides, and 1×10^4 irradiated (22,000 rad) cells of the 3T3 mouse fibroblast line obtained from the American Type Culture Collection (ATCC) (Rockville, MD) were added to each well as feeder cells. Cultures for proliferation assays were plated on 96-well flat-bottom plates (Costar, Cambridge, MA) previously coated with Matrigel (Collaborative Research, Inc., Lexington, MA).

AEC Culture. Human AEC were isolated from intact segments of the distal aorta or common iliac arteries obtained from organ donors. They were harvested and cultured using methods previously described (14). After washing with HBSS, the open ends of the vessel were clamped, infused with a 0.1% collagenase V solution (Sigma Chemical Co.) and incubated for 30 min at 37° C. After flushing with HBSS, the detached AEC were pelleted by centrifugation and cultured in endothelial cell media consisting of M199 (Gibco) supplemented with 10% FBS, 10% pooled human serum, 4 mmol/L L-glutamine, 23 mmol/L HEPES buffer, 56 μg/ml amphotericin B (Fungizone, Gibco), 10 IU/ml heparin and 100 μg/ml endothelial cell growth factor (Collaborative Research, Lexington, MA). Primary cultures were established in 25 cm^2 tissue culture flasks (Costar) incubated at 37° C in a 5% CO_2 humidified atmosphere. AEC were detached for subculture using a 0.125% trypsin/0.05% EDTA solution (Gibco).

Induction of MHC Antigen Expression. Supernatants were harvested from cultures of T lymphocytes expanded from biopsy samples of liver allografts undergoing rejection (15). Approximately 3×10^5 cultured lymphocytes were stimulated with 1×10^6 irradiated (4,000 rad) donor splenocytes in each well of a 24-well tissue-culture plate (Costar) in lymphocyte culture media (LCM). LCM consisted of RPMI-1640 (B & B/Scott) supplemented with 10% normal human serum, 4 mmol/L L-glutamine, 24 mmol/L HEPES buffer and 60 μg/ml gentamycin sulfate. Supernatants were collected after 48 hr. Supernatants from cultures of irradiated splenocytes alone were used as controls.

Supernatants were diluted 1:1 with WE and BPE growth medium and added to BEC grown on tissue-culture chamber slides. B3, a monoclonal antibody specific for γ-interferon (γ-IFN) (16) (a gift from Centocor, Malvern, PA), was added to some wells at the same time as the supernatants. BEC cultures were also treated with human recombinant γ-IFN (a gift from Genentech, San Francisco, CA) at the concentrations indicated.

Immunostaining. Expression of MHC antigens was determined using a standard two-step indirect immunoperoxidase technique. BEC cultures were incubated with the activated T cell supernatants or with recombinant γ-IFN for 3 days and then washed with HBSS. The plastic chambers were removed, and the slides were air dried, fixed in acetone for 10 min and then stained for MHC antigen expression. Slides were frozen if they could not be stained immediately.

Monoclonal antibodies (MoAbs) reactive with monomorphic HLA determinants were used as primary antibodies. MoAb PA2.6 recognizes an epitope present on all HLA class I molecules (17). MoAb PL8 detects a monomorphic epitope found on all HLA-DR molecules. MoAb Tu22 is specific for all DQw molecules. MoAb PL15 detects an epitope found on HLA-DP molecules. These antibodies were purified immunoglobulins available through our laboratory's participation in the Tenth International Histocompatibility Workshop (18). MoAb Leu10, which recognizes a common polymorphic epitope present on HLA-DQ molecules of cells expressing DQwl and DQw3 (19), was purchased from Becton Dickinson & Co.

(Mountain View, CA). Peroxidase-conjugated rabbit anti-mouse antibodies (Dakopatts, Copenhagen, Denmark) were used as the secondary reagent. Color development was achieved with aminoethyl carbazole, and cells were lightly counterstained with Mayer's hematoxylin (Sigma Chemical Co.). For negative controls, the primary antibody was omitted.

Sections of normal livers or livers affected by PBC were frozen in OCT compound (Miles Scientific) and used as biological controls. The biliary epithelium in normal liver tissue did not stain with any of the class II MoAbs tested, whereas it stained strongly in the PBC livers with all four antibodies.

Preparation of Alloreactive Lymphocyte Lines. Human lymphocytes were isolated from peripheral blood or donor spleens by Ficoll-Paque (Pharmacia AB, Uppsula, Sweden) gradient separation. Two different types of alloantigen-primed lymphocyte lines were used as responder cells in the proliferation assays. The first consisted of polyspecific primed lymphocytes (PPLT), which were prepared by stimulating normal lymphocytes with a pool of 10 different irradiated (2,000 rad) panel lymphocytes carrying as many HLA antigens as possible. Mixed lymphocyte cultures (MLC) were set up in upright 25 cm^2 flasks using equal quantities (1×10^7 cells in 7 ml of LCM) of stimulator and responder cells. After 10 days of culture, the lymphocytes were aliquoted and frozen.

The alloreactive lymphocyte clone DB29 was generated from 3-day MLC-activated lymphocytes in the two-layer soft agar culture method as previously described (20). The resultant clone was determined to be specific for HLA-DR2–associated DQwl cells.

Proliferation Assays. The ability of the cultured BEC to stimulate in secondary proliferation assays was determined using the primed lymphocyte test (PLT). BEC were grown to confluence (9 to 14 days) in 96-well flat-bottom tissue culture wells coated with Matrigel. Some wells were treated for 3 days with 100 IU/ml of γ-IFN. The entire plate was irradiated (2,000 rad), and each well was washed with RPMI-1640, 3×10^4 alloantigen-primed lymphocytes in LCM were added to the cultured BEC or to 1×10^5 irradiated (2,000 rad) splenocytes isolated from the same donor as the BEC. Background proliferation was measured in wells containing BEC with LCM alone and in Matrigel-coated wells containing only the primed lymphocytes. Final volume in each well was 200 μl. Plates were cultured for 3 days and, during the final 18 to 20 hr of incubation, each culture was pulsed with 1 μCi of ^{3}H-thymidine (specific activity 20 mCi/mmole, New England Nuclear Products, Boston, MA). The cultures were harvested with a multiple sample harvester (Skatron, Sterling, VA), after which wells were treated with trypsin/EDTA to detach any remaining adherent cells. The plates were harvested a second time, and ^{3}H-thymidine uptake was determined by counting filter papers from both harvestings in the same vial using a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). Results were calculated from the mean cpm in two to four replicate wells ± S.D. Test wells with cpm > 4X background are considered positive.

AEC were tested for their ability to stimulate primed lymphocytes in a similar assay system; 2×10^4 cultured AEC were placed in each well of a 96-well flat-bottom tissue-culture plate coated with Matrigel. Cells were incubated with or without 50 IU/ml γ-IFN, which was the highest concentration able to induce MHC class II expression without cytotoxicity (data not shown). After 3 days, the wells were confluent, and the PLT was done using the method described above for BEC.

MoAb Studies. In blocking studies, anti-HLA MoAbs were tested for their ability to inhibit the PLT responses. Recognition of class I HLA antigens was blocked using MoAb W6/32 (21) (PelFreez, Rogers, AR). Class II recognition was blocked using a pool of three MoAbs specific for monomorphic determinants found on class II molecules (IVA12 [22]; L243 and L227 [23]). Hybridomas producing these MoAbs were purchased from the ATCC. The antibodies were added to the wells at optimal concentrations determined previously. Cell numbers and final volume in each well were as described above for proliferation assays.

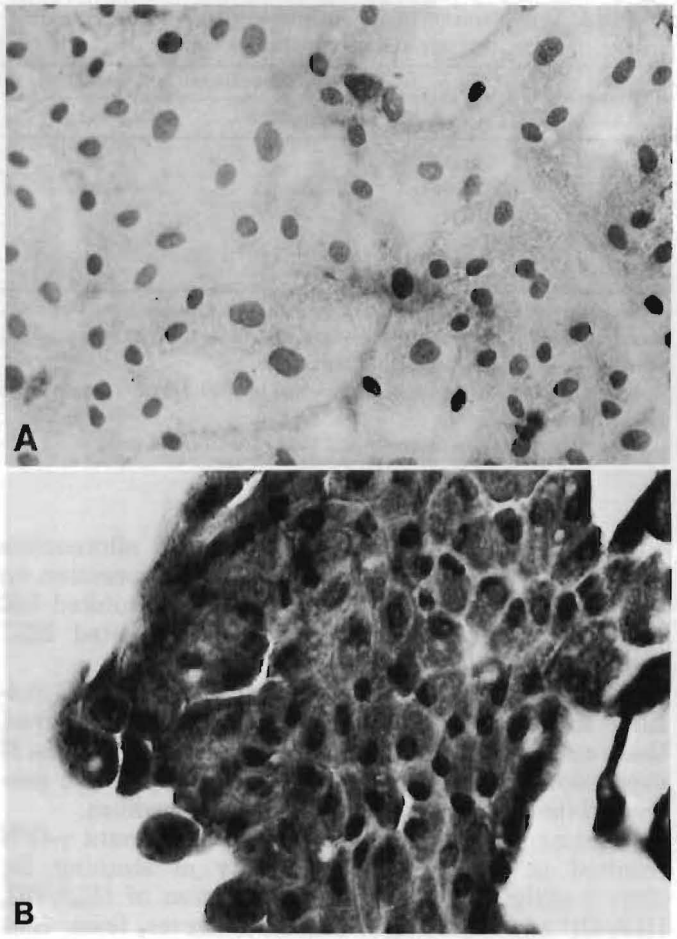

FIG. 2. Immunoperoxidase stain of cultured BEC using a monoclonal antibody to HLA-DR (PL8). Cultures grown in serum-free medium demonstrate only the occasional HLA-DR–positive cell (A, original magnification ×350). Cultures treated for 3 days with supernatants from stimulated alloreactive T-lymphocyte cultures show strong staining for HLA-DR antigens (B, original magnification ×350).

Statistics. Statistical analysis was performed using the two sample *t* test or the Mann-Whitney test on an IBM computer with the "Statistix" software package (NH Analytical Software, Roseville, MN).

RESULTS

Induction of MHC Antigen Expression on Cultured BEC. BEC cultured in serum-free medium showed constitutive expression of HLA class I, whereas only an occasional cell expressed HLA-DR, HLA-DP or HLA-DQ (class II) (Fig. 2A). However, a 3-day treat-

TABLE 1. Induction of MHC antigen expression on cultured human biliary epithelial cells

Monoclonal antibody	HLA specificity	Degree of cultured BEC staining[a]	
		Untreated	γ-IFN treated[b]
PA2.6	Class 1	+ + +	+ + +[c]
PL8	DR	∓	+ + +
Leu10	DQ	−	+
Tu22	DQ	∓	+ +
PL15	DP	∓	+

[a]Staining of cultured BEC was graded as − (negative); ∓ (<5% positive cells); + (20% to 50% positive cells); + + (50% to 80% positive cells); + + + (80% to 100% positive cells).

[b]Cells were cultured in the presence of 100 IU/ml recombinant γ-IFN for 3 days.

[c]Staining was more intense than that of untreated cells.

ment with supernatants from stimulated alloreactive T-lymphocyte cultures induced HLA-DR expression on most cells (Fig. 2B). A MoAb to γ-IFN inhibited DR expression, with staining similar to untreated BEC cultures.

MHC expression was also determined on BEC cultures in WE medium containing 10% FBS. However, these cultures showed a higher baseline level of class II expression; therefore all subsequent assays were performed on BEC cultured in serum-free medium.

Treatment of cultured BEC with recombinant γ-IFN resulted in an increased intensity of staining for class I antigens and induced expression of HLA-DR, HLA-DP and HLA-DQ (Table 1). However, fewer cells stained with HLA-DP– and HLA-DQ–specific antibodies, whereas more than 80% of the cells stained strongly for HLA-DR. A concentration of γ-IFN as low as 10 IU/ml was able to induce some class II antigen expression, although 3 days of treatment with at least 50 IU/ml was needed to induce maximal expression. BEC cultures started to show evidence of toxicity (cell death and detachment) after more than 3 days of treatment with γ-IFN or at levels higher than 2,000 IU/ml.

BEC grown on Matrigel showed the same staining characteristics and induction of MHC as those grown on the 3T3 cells. However, Matrigel is derived from mouse tissue and thus binds the peroxidase-conjugated anti-mouse antibody, resulting in intense nonspecific staining of the matrix behind the adherent BEC.

Stimulation of Alloreactive T Lymphocytes by Cultured BEC. Cultured BEC were able to stimulate lymphocytes primed against multiple HLA antigens (Table 2). However, responses to BEC were much lower than responses to splenocytes from the same donor, and γ-IFN treatment of the BEC made little difference in their ability to stimulate the primed lymphocytes.

To confirm that it was the MHC antigens on the BEC that were inducing the proliferative responses, monoclonal blocking studies were performed (Table 3). The class II MoAbs were able to block the response of the PPLT line to both untreated BEC and splenocytes by more than 50%. A combination of class I and class II MoAbs was required to significantly block the γ-IFN–treated BEC.

A T-cell clone specific for an epitope on the DQwl molecule was able to proliferate in response to cultured BEC from a donor who was HLA typed as DQwl positive (Table 3). These cultured BEC were able to stimulate even before γ-IFN treatment. The response to untreated BEC and splenocytes could be blocked to background levels by the class II–specific MoAbs, whereas the class I–specific MoAb produced no significant inhibition. The anti-class II MoAbs were also able to inhibit the response of the clone to γ-IFN–treated BEC by more than 70%.

Comparison of Stimulation by BEC with Stimulation by Cultured AEC. The proliferative response of the alloreactive T cell lines to cultured BEC was compared with their response to cultured AEC tested in the same assay system. BEC and AEC from the same donor were not available, so the average responses of multiple assays with each cell type were compared. Figure 3 demonstrates that untreated AEC were much stronger stimulators than untreated BEC (24,795 cpm vs. 3,274 cpm; $p < 0.0001$). γ-IFN treatment of the cultured stimulators gave similar results (28,553 cpm vs. 3,083 cpm; $p < 0.0001$). However, splenocytes from AEC donors stimulated at a level similar to splenocytes from the BEC donors (38,238 cpm vs. 43,771 cpm; $p = 0.6$), indicating that the difference in stimulation by the two cell types was not due to a difference in the response to the different HLA types of the donors.

DISCUSSION

Although we and others have published methods for the isolation and culture of human biliary epithelium (11, 24), this study is the first step in a natural line of investigation using BEC in functional assays. We have demonstrated that the MHC molecules on the cultured BEC carry functional lymphocyte-activating determinants (i.e., they can be recognized by alloprimed T lymphocytes in a secondary proliferation assay). The blocking of the proliferation by class II–specific MoAbs and the response of an alloreactive T-cell clone confirm that the stimulation is triggered by recognition of MHC determinants. We have also demonstrated that class I MHC antigen expression on cultured human BEC can be enhanced, and class II antigen expression can be induced in response to alloactivated lymphocyte supernatants. The active constituent of these supernatants is most likely the γ-IFN produced by T cells invading the liver allograft. Levels of recombinant γ-IFN as low as 10 IU/ml were able to induce class II expression, although maximal expression was reached at concentrations of 50 to 100 IU/ml. All three class II subregion products (HLA-DR, HLA-DP and HLA-DQ) were expressed on the γ-IFN–treated BEC.

Although many immunohistochemical studies on MHC expression in the transplanted liver exist, the significance of the detected alterations has not been clear. In normal livers MHC class I antigens are expressed on the sinusoidal cells, large vessel and sinusoidal endothelia, biliary epithelium, dendritic cells

TABLE 2. Response of alloreactive polyspecific primed lymphocytes to cultured biliary epithelial cells as measured in 3-day proliferation assays

	Proliferative response (cpm ± S.D.)			
		Stimulators		
Responders	Matrigel[a] alone	BEC[b] (untreated)	BEC[b] (γ-IFN treated)	Splenocytes[c]
Experiment 1				
Media alone	55 ± 13	451 ± 87	–	65 ± 14
PPLT[d] 1	100 ± 12	2,584 ± 228	–	28,880 ± 642
PPLT 2	79 ± 17	1,821 ± 587	–	42,835 ± 2,081
Experiment 2				
Media alone	49 ± 6	574 ± 111	270 ± 78	49 ± 3
PPLT 1	258 ± 204	4,283 ± 418	3,042 ± 1,152	32,403 ± 3,345
PPLT 3	69 ± 20	5,655 ± 1533	5,707 ± 139	46,413 ± 3,980

[a]Matrigel = matrix coated on wells for BEC culture.
[b]BEC treated with or without 100 IU/ml recombinant γ-IFN for 3 days.
[c]Splenocytes from the same donor as the BEC.
[d]PPLT = polyspecific primed lymphocyte line.

TABLE 3. Monoclonal blocking studies of BEC-induced stimulation of polyspecific primed lymphocytes and a DQw1-specific T-cell clone

		Proliferative response (cpm ± S.D)			
			Stimulators		
Responders	MoAb[a] treatment	Matrigel[b] alone	BEC[c] (untreated)	BEC[c] (γ-IFN treated)	Splenocytes[d]
Experiment 1					
Media alone		52 ± 32	493 ± 129	311 ± 129	86 ± 34
PPLT[e]	None	93 ± 25	3,930 ± 1,305	4,017 ± 1,521	4,366 ± 2,734
PPLT	Class I	93 ± 25	4,326 ± 2,874	3,657 ± 2,755	2,874 ± 228
PPLT	Class II	118 ± 32	1,674 ± 194[f]	2,358 ± 735	857 ± 183[g]
PPLT	Class I + II	109 ± 43	1,325 ± 583[g]	1,358 ± 630[g]	998 ± 479[g]
Experiment 2					
Media alone		–	587 ± 456	180 ± 48	107 ± 71
Anti-DQw1 clone	None	51 ± 18	2,326 ± 795	5,005 ± 2,350	47,124 ± 427
Anti-DQw1 clone	Class I	–	1,799 ± 563	5,565 ± 3,246	31,870 ± 3,380
Anti-DQw1 clone	Class II	–	268 ± 59[g]	1,596 ± 155[g]	128 ± 34[g]

[a]Monoclonal antibody to either class I or class II antigens or a mixture of both antibodies was added to wells at initiation of proliferation assay.
[b]Matrigel = matrix coated on wells for BEC culture.
[c]BEC treated with or without 100 IU/ml recombinant γ-IFN for 3 days.
[d]Splenocytes from the same donor as the BEC.
[e]PPLT = polyspecific primed lymphocyte line.
[f]Indicates >50% inhibition; statistical analysis demonstrates marginal significance ($p = 0.067$) because only duplicate wells were tested.
[g]Indicates wells in which monoclonal antibody treatment blocked the response (>50% inhibition) and that significantly differed from untreated wells with $p \leq 0.05$ by the Mann-Whitney test.

and weakly, if at all, on hepatocytes. Class II antigens are found only on the sinusoidal cells, capillary endothelium and dendritic cells found in the portal connective tissue and in the connective tissue immediately subjacent to the terminal hepatic venules (5-7, 25). After liver transplantation, alterations occur in the distribution of these antigens. Class I expression is increased on hepatocytes and bile ducts, and class II antigens can be detected on the biliary epithelium and large vessel endothelium (5, 8). How these alterations fit into an overall pathogenic scheme in liver allograft rejection has yet to be determined, but a hypothesis is expressed below.

The earliest histological evidence of acute cellular rejection of both the rat and human liver is focal collections of mononuclear cells in the interstitium of the portal tracts (1). Sludging of mitotically active lymphoid and monocytoid cells in the interstitial portal capillaries as early as 2 to 3 days after transplantation suggests that some degree of sensitization occurs within the graft. It is likely that mononuclear cells are activated by antigens on the portal capillaries and dendritic cells. The activated lymphocytes would release cytokines such as γ-IFN that are able to enhance class I and induce class II expression on the vascular endothelium and biliary epithelium. This induced MHC expression would make

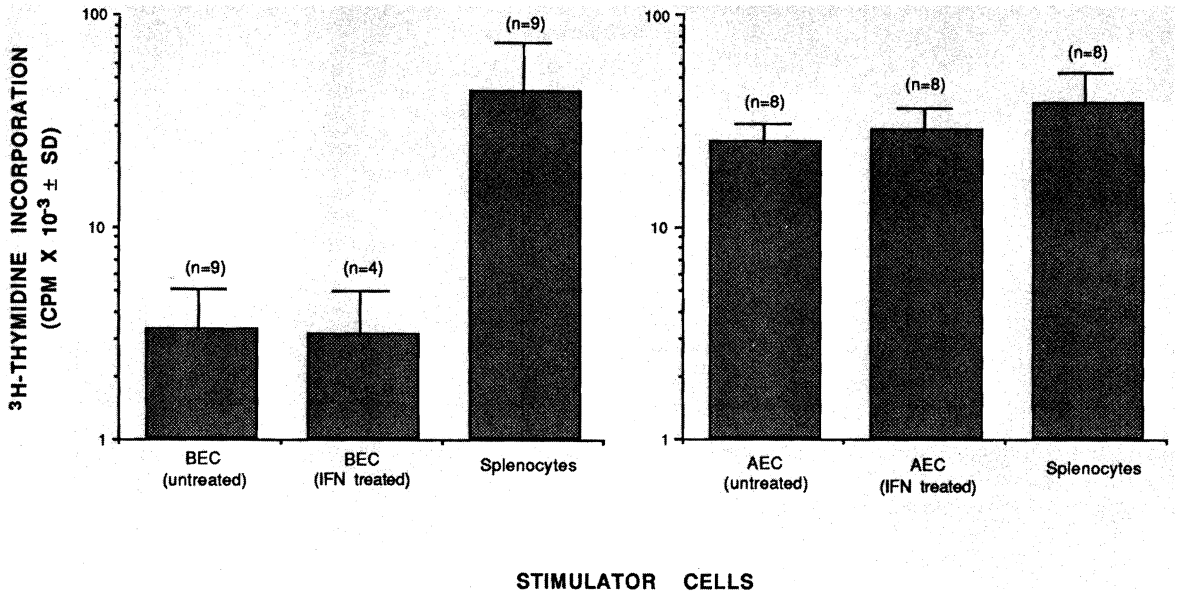

FIG. 3. Comparison of the proliferative response of alloreactive T-cell lines (PPLT) to cultured BEC and AEC. Results are the mean of multiple assays using five BEC lines, three AEC lines and splenocytes from their respective donors as stimulator cells. n = number of stimulator/responder combinations tested. Values shown are net cpm (after subtracting background proliferation) ± S.D. Statistical comparison by t test: BEC vs. AEC (untreated), $p < 0.0001$. BEC vs. AEC (γ-IFN treated), $p < 0.0001$. Splenocytes from BEC vs. AEC donors, $p = 0.6$.

these structures more susceptible to immune recognition and destruction through direct T cell cytotoxicity and indirectly by way of delayed-type hypersensitivity responses (3, 26). It is also possible that γ-IFN or other cytokines released locally may directly injure the biliary epithelium, as shown by our results.

Others in the field of organ transplantation have presented similar hypotheses concerning MHC antigen induction and allograft rejection (27). After studying HLA antigen expression in serial liver graft biopsy specimens, Gouw et al. (28) have also hypothesized that the induction of MHC antigens on bile duct epithelium accelerates the alloresponse leading to rejection.

In vivo, induced expression of MHC antigens has been reported for a number of other inflammatory sites, including transplanted kidneys (27), biliary epithelium in PBC (4), thyroid epithelium in Graves' disease, β cells in the diabetic pancreas and salivary ducts in Sjögren's syndrome (29). The presence of γ-IFN has been demonstrated at the site of inflammation (30). *In vitro*, γ-IFN has been shown to induce *de novo* class II antigen expression on a variety of human cell types, including vascular endothelial cells and fibroblasts (31, 32), AEC (33), glomerular mesangial cells (34) and thyrocytes (35). The class II antigens induced on endothelial cells are functional and can be recognized by cytotoxic T cells (31). They also are able to act as antigen-presenting cells (36).

We were surprised to find that γ-IFN treatment of the cultured BEC or AEC does little to increase lymphocyte stimulation. Similar work with endothelial cells also showed no reproducible difference in the lymphocyte response to those cells that expressed class II antigens or those that did not at the start of coculture (31). However, class II antigens may still have played a role in the T-cell stimulation because many of the endothelial cells were induced to express class II antigens within 24 hr of coculture with lymphocytes, probably because of the γ-IFN released by the T cells. It is likely that a similar situation existed in our assays. This is further supported by our data showing that untreated BEC, which normally express very little or no HLA-DQ by immunohistochemistry, were capable of stimulating the HLA-DQ–specific T-cell clone.

It is also possible that the very low level of class II expression on the untreated BEC was sufficient to stimulate the primed lymphocytes. Whereas many cell types are considered to be "class II negative," in actuality low levels of expression have been demonstrated *in vivo* and *in vitro*. Studies have shown that occasional BEC in normal livers do express HLA-DR (7). Geppert and Lipsky (32) found a small number of DR-positive endothelial cells in their untreated populations that appeared capable of stimulating allogeneic T4 cells.

The proliferative response to cultured BEC, with or without γ-IFN treatment, or to splenocytes was not blocked by the class I MoAb. This is likely due to the nature of the responding cell populations. The PPLT lines respond most strongly to class II MHC antigens, and the T-cell clone tested was specific for an epitope found only on class II molecules. Although the response of the PPLT line to γ-IFN–treated BEC also was not significantly blocked by the class II MoAbs, some blocking was seen. A combination of both class I and class II MoAbs did result in significant blocking. The

broad reactivity of the PPLT line, along with the low levels of proliferation and the high standard deviations inherent with the assay system, likely contributed to these discrepancies.

Studies using endothelial cells have shown that they can stimulate a primary lymphocyte response, whereas fibroblasts can only stimulate primed alloreactive T-cell lines in a secondary proliferation assay (32). Our cultured BEC were also not able to activate fresh T lymphocytes in a primary response (data not shown). However, the responses of the primed T-cell lines were relatively low. Because a primary response is usually much lower than a secondary response, our negative results were not unexpected.

Despite the fact that we have shown that BEC are able to stimulate primed allogeneic lymphocytes and therefore probably contribute to their own destruction during rejection, they were much less potent stimulators than AEC. These results might demonstrate an inherent difference in the immunogenicity of the two cell types, although it is also possible that the BEC are more sensitive to culture conditions that may affect their stimulatory capability.

It is widely recognized that endothelial cells are not only potent stimulators of alloresponses but that they also can function as accessory cells. A similar hypothesis has been forwarded to explain the pathogenesis of the vanishing bile duct syndrome, where DR-compatible BEC could present incompatible class I MHC or viral antigens to the host immune system (37). Although this hypothesis is tenable, the less potent stimulatory capacity of BEC also suggests that other factors may be important in making bile ducts susceptible to destruction. Although speculative at this point, ischemic injury as a result of arterial damage may be of equal or greater importance in the ducts' destruction (38).

Finally, the results of this study demonstrate that cultured human BEC are very useful in the study of immunological diseases of the human biliary tree. Another logical area of investigation is PBC, where it has been hypothesized that the aberrant expression of class II antigens seen on the bile ducts in PBC livers plays an important role in disease pathogenesis (4). Preliminary work with lymphocytes isolated from livers affected by PBC has shown that they are able to recognize autologous BEC *in vitro*. The use of isolated BEC targets in conjunction with MoAb blocking studies may permit a more precise identification of target antigen(s) in this perplexing disease.

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