MIXED ISOTYPE CLASS II ANTIGEN EXPRESSION
A Novel Class II Molecule is Expressed on a Murine B Cell Lymphoma

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Class II molecules encoded by the MHC regulate the recognition of foreign antigens on the surfaces of APC by T cells through a phenomenon known as MHC restriction (1, 2). These molecules, also known as Ia antigens, play a major role in the mixed lymphocyte response (3). In the mouse there are two class II isotypes, I-A and I-E, whereas in man three major class II isotypes are expressed, DR, DQ, and DP (reviewed in reference 4). Each of the class II isotypes is a glycoprotein composed of an α and a β subunit. The high level of allelic polymorphism in the molecules is believed to reflect the variation required for the generation of specific immunity to infectious agents.

Sequences in the NH_2 terminus, primarily in the β chain (5, 6), appear to regulate the intracellular assembly of the heterodimers, and generally, interisotypic pairing is prevented in cells with a full complement of α and β chains. It has been shown that α and β chains of a given haplotype can transassociate in certain heterozygotes (7, 8). Recently, through the use of DNA-mediated gene transfer, the expression of mixed isotype dimers has been demonstrated in L cells transfected with certain class II gene pairs (9, 10). In these studies, L cells transfected with A_β^d and $E_\alpha^{a/k}$ (9) or E_α^d (10) genes produced β and α chains that paired successfully and were expressed at the cell surface. Furthermore, in this latter study, it was shown that although several GAT-specific T cell hybridomas restricted to I-A^d failed to respond to antigen presented by this transfectant, another T cell hybridoma, AODH-7.1, specific for human gamma globulin (HGG)[1] in the context of I-E^d (11), was stimulated by the transfected L cell in the presence of antigen. Norcross et al. (12) reported that the introduction of a mouse A_β^d gene into a human lymphoblastoid cell line resulted in the expression of the mouse $A\beta$ chain at the cell surface as part of a molecule containing the endogenous DR_α chain. Since the DR is homologous to I-E, the pairing of the mouse $A\beta$ chain to the DR_α chain was not anticipated. The most direct evidence to date for the assembly and surface expression of mixed isotype class II molecules in a nontransfected cell was presented by Lotteau et al. (13). A mixed isotype molecule, $DR_\alpha DQ_\beta$, was shown to be expressed by a human EBV-

[1] *Abbreviations used in this paper:* HGG, human gamma globulin; NC, nitrocellulose.

transformed cell line, PGF. This cell line also expressed the appropriate class II isotypic pairs, however, no $DQ_\alpha DR_\beta$ molecule was detected.

In this study, we present evidence that the mouse B cell lymphoma, 2PK3, expresses a mixed isotype molecule, $E_\alpha^d A_\beta^d$, as a major class II molecule at the cell surface. A normal I-E heterodimer is also expressed, but no I-A isotype is detected. The mixed isotype molecule is capable of presenting antigens restricted by I-A^d, although the responses induced are consistently weaker than responses generated by the normal I-A^d isotype. Furthermore, antigen presentation by the mixed isotype molecule is inhibited by mAb reagents specific for I-A and I-E, respectively. The 2PK3 lymphoma cells induce a strong proliferative response by syngeneic T cells, which is inhibitable by an anti-I-A^d mAb.

Materials and Methods

Animals. BALB/c and CBA/J mice were bred at the National Jewish Center for Immunology and Respiratory Medicine (Denver, CO) and were 8-10 wk of age when used. Armenian hamsters were obtained from Cambridge Diagnostic Laboratory (Cambridge, MA).

Cell Lines. The B cell lymphoma line A20-1.11 (A20) (14) was originally obtained from Drs. K. J. Kim and R. Asofsky (National Institutes of Health, Bethesda, MD), and 2PK3 was obtained from American Type Culture Collection (Rockville, MD). Both lymphomas are of BALB/c origin, express Ia antigens, and have been shown to function as APC (15). B cell lymphoma variants expressing only I-A^d (M12.B5) or I-E^d (M12.A2) were originally obtained from Dr. L. Glimcher (Harvard School of Public Health, Boston, MA) (16). Alloreactive T cell hybridomas AC5, CG4, DG11, EE10.D8, and JB9 recognizing either I-A^d or I-E^d have been characterized previously (17). T cell hybridomas recognizing chicken OVA in context of I-A^d (DO-11.10, 3DO-18.3, 3DO-20.10, 3DO-54.8, and 4DO-44.1) have been described (18) and were kindly provided by Drs. J. Kappler and P. Marrack (National Jewish Center for Immunology and Respiratory Medicine). The T cell hybridomas ME1, recognizing λ repressor peptide 12-26 (λ-rep [12-26]) in context of I-E^d, and LFE-1, recognizing sperm whale myoglobin peptide 132-153 (Myo[132-153]) in context of I-E^d, were provided by Dr. M. Gefter (Massachusetts Institute of Technology, Cambridge, MA). The T cell line AODH-7.1 recognizes HGG in context of I-E^d (11). A20 and 2PK3 were grown in RPMI 1640 supplemented with 10% FCS (HyClone Laboratories, Logan, UT) and 5×10^{-5} M 2-ME (Eastman Kodak, Rochester, NY). All other cell lines were maintained in MEM (formula 78-5067; Gibco Laboratories, Grand Island, NY) plus a nutrient cocktail as described (19).

Antibodies. mAbs MK-D6 (reactive to A_β^d [19]) and 14-4-4S (reactive to E_α^d[20]) were produced as ascites in pristane-primed, sublethally irradiated (600 rad) BALB/c mice. Antibodies were purified via passage over protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) columns. K24.199, specific for A_α^d (21, 22), was used as culture supernatant. Polyclonal antibodies to the individual subunits of the Ia antigens were produced in hamsters by injecting electroeluted α or β chains from specifically purified I-A or I-E isolated from A20 or 2PK3. Ia antigens were subjected to electrophoresis on SDS-PAGE and gel slices containing α or β chains were electroeluted using an electroelution apparatus (C. B. S. Scientific Co., San Diego, CA) as described previously (23). Hamsters received 5-10 μg antigen per injection subcutaneously, the first injection in CFA and subsequent injections in IFA.

Flow Cytometric Analysis. The mAbs were protein A purified and used at a concentration of 10 μg/ml. Approximately 10^6 B cells were incubated with ~0.5 μg primary mAb. After washing, FITC protein A (Sigma Chemical Co., St. Louis, MO) was used as the developing reagent. Cells were routinely fixed in PBS/1% formaldehyde before cytofluorometic analysis on an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Affinity Purification of Ia Antigens. This procedure was carried out as described (17). Several passages and elutions were performed to remove all of the I-A, followed by specific purification of I-E in the same manner.

Chemical Deglycosylation of Ia Antigens. The complete removal of carbohydrate was accomplished using acid hydrolysis of glycosidic linkages following the procedure of Edge et al.

(24). Briefly, ~20 μg protein was precipitated with acetonitrile, dried under a stream of N_2, suspended in 45 μl of anhydrous trifluoromethanesulfonic acid and 5 μl anisole, and incubated on ice for 2 h. The mixture was then added to 1 ml of pyridine/ethyl ether (1:9) in a dry ice/acetone bath. After centrifugation at 10,000 g for 10 min, the pellet was dissolved in 0.1 M NH_4HCO_3 and dialyzed against PBS.

Cell Surface Labeling with ^{125}I for Immunoprecipitation. Cells were surface labeled with $Na^{125}I$ (ICN Biomedicals, Costa Mesa, CA) using Iodogen (Pierce Chemical Co., Rockford, IL), followed by solubilization of membrane proteins with 1% NP-40 lysing buffer (19). For immunoprecipitation, 10 μl of whole antiserum or protein A-purified mAb (1 mg/ml) were added to 2-4 $\times$ 10^6 cpm of detergent lysate and the mixture was incubated overnight at 4°C. Protein A-Sepharose (Sigma Chemical Co.) (200 μl of a 50% suspension in PBS) was added and mixed at room temperature for 2 h, followed by washing five times with 0.5% NP-40 in PBS. Complexes were eluted by boiling in Laemmli sample buffer containing either iodoacetamide or 2-ME at concentrations of 0.5 M and 5% for unreduced and reduced conditions, respectively. Immunoprecipitates were analyzed by SDS-PAGE using 10% polyacrylamide minigels. Dried gels were autoradiographed using Kodak XAR-5 film and intensifying screens at -70°C.

Solid Phase RIA. Approximately 4 μg of specifically purified Ia antigen in 50 μl was allowed to bind to wells of flexible polyvinyl chloride round-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) for 1 h at room temperature followed by a wash with PBS and blocking of unbound sites with 5% nonfat dry milk in PBS (M/PBS) with 0.02% sodium azide. Antibody reagents (appropriate dilutions of culture supernatant or protein A-purified antibody from ascites) were added in 50-μl amounts and incubated 1 h followed by washing with PBS. The binding of mAbs was detected by the addition of ^{125}I-labeled protein A (25,000 cpm), followed by cutting of the wells from the plates, and counting in a gamma counter.

Immunoblotting. The reactivity of hamster polyclonal sera to I-A and I-E antigens was analyzed by immunoblotting. Approximately 1 μg of specifically purified I-A or I-E was subjected to electrophoresis under nonreducing conditions on a 10% SDS-PAGE gel. After this, the proteins were electrophoretically transferred from the gel to nitrocellulose (NC) at 150 mA for 1 h in a Polyblot apparatus (American Bionetics, Hayward, CA). After the transfer was completed, nonspecific sites on the NC were blocked with M/PBS for 15 min, followed by incubation with the appropriate dilution of antibody (usually 10 μl serum to 10 ml of M/PBS), and incubated overnight on a mechanical shaker at 4°C. The NC was washed extensively with PBS and then incubated with 10^6 cpm ^{125}I-labeled protein A for 2 h at 25°C, washed, dried, and placed on film for autoradiography.

Tryptic Peptide Mapping of A20 and 2PK3 Ia α and β Chains. Specifically purified A20 and 2PK3 I-A and I-E molecules were subjected to electrophoresis on 10% polyacrylamide gels under nonreduced conditions to separate the α and β chains. The gels were fixed and stained with Coomassie blue. Gel segments corresponding to the α and β subunits were excised and the proteins were recovered and processed for trypsin digestion as previously described (23). The digested proteins were injected onto a 4.6-mm $\times$ 30-cm C_{18} 300 Å reversed phase column installed on an HPLC system (model 721; Waters Associates, Milford, MA). The peptides were eluted with a flow rate of 1 ml/min with a linear gradient of 0.1% TFA in acetonitrile to 50% over 90 min. The elution was monitored at 214 nm and fractions were collected at 30-s intervals. The sequencing of selected peptides was performed on a protein sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) using a modified Edman chemistry.

Stimulation of IL-2 Production by T cell Hybridomas. For alloreactive T cell hybridoma responses, microculture wells were prepared containing 200 μl of culture medium, 2 $\times$ 10^5 hybridoma cells, and 10^5 B lymphoma cells. For antigen presentation, microculture wells were prepared containing 300 μl of culture medium, 10^5 hybridoma cells, 10^5 B lymphoma cells as APC, and 100 μg of appropriate antigen. A tryptic digest of chicken OVA (Sigma Chemical Co.) was used in most antigen stimulation assays requiring OVA. HGG was purified from Cohn fraction II by DEAE cellulose chromatography. Peptides OVA(323-339), λ-rep(12-26), and Myo(132-153) were synthesized as described (25) and were added at 10 μg per culture. The cultures were incubated at 37°C in flat-bottomed, 96-well microtiter plates. Supernatants from these cultures were harvested 24 h later and then were assayed for the presence of IL-2 (19).

Stimulation of Autologous T Cells by A20 and 2PK3 in a Primary MLR.   T cells were obtained from the spleens of nonimmune BALB/c or CBA/J mice. Accessory cells were depleted by passing the spleen cells over nylon wool and G-10 columns as described (26). The T cells (4 × 10^5 cells per well) were cultured with various numbers of mitomycin C–treated B cells in a final volume of 0.3 ml of complete medium in flat-bottomed, Linbro 96-well microtiter plates (Flow Laboratories, Inc., McLean, VA). The cultures were incubated at 37°C for a total of 5 d. Approximately 8 h before harvesting the cultures, 1 μCi of [^{3}H]thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) was added to each well. The wells were harvested by using a Titertek cell harvester (Flow Laboratories, Inc.), and [^{3}H]thymidine incorporation was determined by using a liquid scintillation spectrometer.

Results

Comparison of the Antigen-presenting Capabilities of 2PK3 and A20.   Previous studies have shown that the A20 cell line functions well as an APC to antigen-specific MHC-restricted T cells (15) and as a stimulator cell for alloreactive T cells (17). When the functional capacity of 2PK3 cells to serve as APC was analyzed in comparison with that of A20, differences were noted (Tables I and II). Most notably, I-A^d-specific alloreactive T cell hybridomas reacted poorly to 2PK3 but responded well to A20. However, 2PK3 stimulated I-E^d-specific T cell hybridomas well, and in some cases, more effectively than did A20 (e.g., EE10.D8). Differences in the ability to present antigen to antigen-specific, MHC-restricted T cell hybridomas were also observed particularly in the case of I-A^d-restricted responses. As shown in Table I, OVA-specific responses restricted by I-A^d were stimulated when A20 was used as the APC, whereas responses to antigen presented by 2PK3 to the same T cell hybridomas were much lower or undetectable. Three I-E^d-restricted, antigen-specific T cell hybridomas examined, specific for HGG, λ-rep(12–26) peptide, and Myo(132–153) peptide, did respond well to antigen presented by either 2PK3 or A20. These results

TABLE I

Stimulation of Alloreactive and Antigen-specific T Cell Hybridomas by A20 and 2PK3

Hybridoma*	Specificity	IL-2 titer	
		Stimulator cell	
		A20	2PK3
		U/ml	
AC5	I-A^d	>1,280	<20[‡]
DG11	I-A^d	640	80
JB9	I-A^d	320	<20
EE10.D8	I-E^d	40	1,280
CG4	I-E^d	640	1,280
3DO-18.3	OVA[§]/I-A^d	160	<20
3DO-20.10	OVA/I-A^d	320	<20
4DO-44.1	OVA/I-A^d	320	20
AODH-7.1	HGG[‖]/I-E^d	320	640

* 2 × 10^5 alloreactive or 10^5 antigen-specific T hybridoma cells were incubated for 24 h with 10^5 A20 or 2PK3 B lymphoma cells. For antigen presentation, antigen was added to the cultures as described below. Supernatants were then harvested and assayed for IL-2 content.

[‡] A titer of <20 indicates undetectable quantities of IL-2.

[§] Tryptic digest of OVA, 1 mg/ml; 100 μl added.

[‖] Whole HGG, Cohn fraction II, 1 mg/ml; 100 μl added.

TABLE II

Inhibition of Antigen Presentation with mAb Specific for I-A or I-E

Hybridoma	Antigen	mAb*	IL-2 titer when stimulated by:			
			A20		2PK3	
			Exp. 1	Exp. 2	Exp. 1	Exp. 2
DO-11.10	OVA[t]/I-A^d	–[s]	640	640	80	40
	OVA/I-A^d	MK-D6	<20	20	<20	<20
	OVA/I-A^d	14-4-4S	640	640	<20	<20
3DO-54.8	OVA/I-A^d	– ˙	80	160	80	80
	OVA/I-A^d	MK-D6	<20	20	<20	<20
	OVA/I-A^d	14-4-4S	80	160	<20	20
ME1	SWM[ll]/I-E^d	–	640		320	
	SWM/I-E^d	MK-D6	640		320	
	SWM/I-E^d	14-4-4S	40		40	
LFE-1	λ-rep[q]/I-E^d	–	320		320	
	λ-rep/I-E^d	MK-D6	160		160	
	λ-rep/I-E^d	14-4-4S	20		20	

* Protein A–purified MK-D6 (A$^d_\beta$ specific) or 14-4-4S (E$_\alpha$ specific); 50 μl at 1 mg/ml added to cultures.

[t] Tryptic digest of OVA; 50 μl, 2 mg/ml added.

[s] Irrelevant mAb added.

[ll] Sperm whale myoglobin (132-153) peptide; 50 μl, 0.2 mg/ml added.

[q] λ repressor (12-26) peptide; 50 μl, 0.2 mg/ml added.

indicate that A20 and 2PK3 differed in their ability to perform MHC-restricted functions.

The differences in Ia-stimulatory capacity between A20 and 2PK3 could be due to variations in Ia antigen density or to structural differences in the Ia antigens themselves. We had previousy quantified the I-A^d and I-E^d expression by an RIA using radiolabeled MK-D6 and 14-4-4S mAbs, respectively, and found that A20 cells on an average expressed ~10^6 I-A molecules per cell and ~4–5 × 10^5 molecules of I-E per cell, whereas 2PK3 cells expressed only 50% of the amount of "I-A" but twice the number of I-E molecules per cell as did the A20 cells (S. Fobare and R. Kubo, unpublished results). These differences in Ia antigen densities alone were probably not sufficient to account for the poor I-A-presenting capacity of 2PK3.

The effect of the addition of MK-D6 or 14-4-4S on the presentation of OVA by A20 and 2PK3 to the T cell hybridomas DO-11.10 and 3DO-54.8 was analyzed (Table II). As expected, the addition of MK-D6 inhibited the presentation of OVA by both A20 and 2PK3, and the addition of 14-4-4S had no effect on the ability of A20 to present OVA to the two T cell hybridomas. In addition, presentation of λ-rep(12–26) and Myo(132–153) to I-E^d-restricted T cell hybridomas LFE-1 and ME1, respectively, was markedly inhibited by 14-4-4S and not MK-D6. Taken together, the results indicate that MK-D6 and 14-4-4S were specific in their ability to inhibit antigen presentation by A20. Unexpected results were observed with 2PK3; the stimulation of both DO-11.10 and 3DO-54.8 was completely inhibited or markedly reduced by the addition of either MK-D6 or 14-4-4S. This inhibition by MK-D6 and 14-4-4S provides strong evidence that the class II restriction element on 2PK3 involved with presenting OVA to the T cell hybridomas was not a conventional I-A^d molecule.

It has been previously reported that DO-11.10 and 3DO-54.8 can recognize OVA(323–339) in the context of I-A^d (18) and it has also been shown that this peptide can bind to I-A^d but not to I-E^d (27). Furthermore, we have shown that a variant B cell lymphoma M12.A2, which expresses I-E^d but not I-A^d (16), was not able to present OVA(323–339) to either DO-11.10 or 3DO-54.8, whereas the variant M12.B5, which expresses I-A^d but not I-E^d (L. Glimcher, personal communication), presented the OVA peptide effectively (data not shown). It would appear that 2PK3 uses the unusual class II molecule to present the OVA(323–339) peptide and that this molecule bears epitopes recognized by both MK-D6 and 14-4-4S. A mixed isotype structure with the composition of E$^d_\alpha$A$^d_\beta$ would have this feature. The structural analysis of this molecule presented below supports this notion.

Both A20 and 2PK3 induced strong allogeneic responses using CBA/J splenic T cells as responders (Table III). Quite interestingly, 2PK3 induced a strong syngeneic response with BALB/c splenic T cells, whereas the A20 cells appear only marginally effective as syngeneic stimulators. Both allogeneic and syngeneic stimulatory responses mediated by 2PK3 were inhibited by the addition of MK-D6 to the cultures, suggesting that class II molecules were primarily responsible for the stimulations observed.

Structural Analysis of Ia Antigens from A20 and 2PK3.   The structure of the I-A and I-E molecules of A20 and 2PK3 was initially characterized using the cell surface labeling technique. Each of the cell lines was surface labeled with Na^{125}I, lysed with detergent, and the clarified lysate subjected to immunoprecipitation with either MK-D6 or 14-4-4S mAbs. SDS-PAGE analysis of the immumoprecipitates in an unreduced state (which allows for better resolution of the subunits) revealed that both the α and β chains of A20 I-A were radiolabeled, while preferential labeling of the β chain of the 2PK3 I-A was observed (Fig. 1). In the case of the I-E molecules, the A20 and 2PK3 patterns were essentially identical to the 2PK3 I-A pattern in that only the β chains were labeled (Fig. 1). These results again suggested that the I-A molecules of A20 and 2PK3 were different. The lack of incorporation of radiolabel into

TABLE III

Comparison of A20 and 2PK3 to Act as Stimulator Cells in a One-way MLR

Responder T cells[*]	Stimulator B cells[*]	mAb[‡]	Incorporation of [^{3}H]thymidine[§]		
			Stimulator cells per culture		
			2 × 10^5	2 × 10^4	2 × 10^3
			cpm		
BALB/c	A20	–	2,014 ± 214	1,546 ± 632	1,132 ± 397
BALB/c	2PK3	–	37,249 ± 6,400	10,074 ± 2,497	2,274 ± 475
CBA/J	A20	–	154,067 ± 26,495	16,287 ± 3,730	420 ± 58
CBA/J	2PK3	–	122,880 ± 15,383	124,769 ± 26,732	58,761 ± 12,302
BALB/c	2PK3	MK-D6	5,201 ± 1,551	5,649 ± 568	3,179 ± 463
CBA/J	2PK3	MK-D6	8,256 ± 1,026	18,662 ± 2,957	3,732 ± 689

[*] Splenic T cells and stimulator B cells were prepared as described in Materials and Methods.

[‡] mAb was added in the form of 100 µl culture supernatant.

[§] 4 × 10^5 T cells were added to the indicated number of mitomycin C-treated B cells in 0.3 ml and were incubated for 5 d. They were pulsed with 1 µCi [^{3}H]thymidine for the last 8 h of culture. Values represent the mean of triplicate cultures ± SD. Thymidine incorporation in the absence of stimulator cells was <600 cpm.
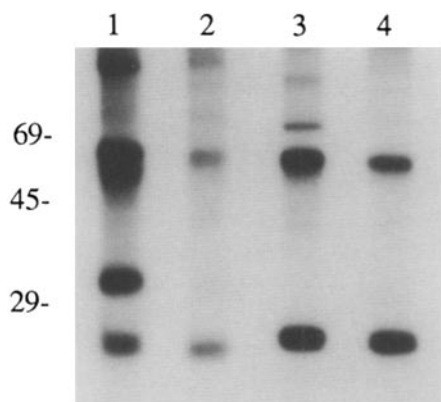
FIGURE 1. SDS-PAGE analysis of radiolabeled I-A and I-E antigens from A20 and 2PK3 cells. Cells were subjected to surface radioiodination, detergent lysis, and subsequent immunoprecipitation with mAbs specific for I-A (MK-D6) (lanes *1* and *2*) and I-E (14-4-4S) (lanes *3* and *4*). Immunoprecipitates from A20 are in lanes *1* and *3*, and those from 2PK3 are in lanes *2* and *4*. Samples were analyzed under non-reducing conditions. Numbers at the left margin indicate the positions of protein molecular weight standards: 69, BSA; 45, OVA; 29, carbonic anhydrase, with apparent molecular weights of 69,000, 45,000, and 29,000, respectively.

the Eα chain and the α chain of the 2PK3 I-A molecule limited the use of this technique for further structural studies.

The Ia antigens were preparatively purified by affinity chromatography using MK-D6- and 14-4-4S-conjugated Sepharose beads as described in Materials and Methods. Approximately 5 μg of each Ia antigen preparation was analyzed by SDS-PAGE under nonreducing conditions and the protein bands were revealed by staining with Coomassie Brilliant Blue (Fig. 2). Resolution of the α and β subunits is readily obtained for both I-A and I-E. After reduction, the I-E α and β subunits have similar mobilities and are poorly resolved (data not shown), whereas the α and β subunits of the I-A antigen are still well separated under these conditions. The I-E molecules from the two B cell lymphomas have similar patterns. Attempts at enzymatic deglycosylation (N-glycanase or endoglycosidase F) resulted in only partial removal of carbohydrate on some chains. For this reason, we chose to use chemical deglycosylation, which completely removed carbohydrate from all subunits. The slight differences seen in the I-E patterns could be eliminated by chemical deglycosylation of these proteins, suggesting that variations in carbohydrate moieties accounted for the differences in subunit mobilities. The patterns of the I-A molecules also show differences in the subunits. After chemical deglycosylation, the 2PK3 "I-A" α chain comigrated with the I-E α chain and not with the A20 Aα chain. The Aβ chains of A20 I-A and 2PK3 "I-A" comigrated after deglycosylation (data not shown).

To determine whether the α chain associated with the molecule that we had presumed to be 2PK3 "I-A" by virtue of the reactivity with MK-D6 was identical to or related to Eα chain of A20 I-E, the α and β chains of the I-A and I-E molecules from A20 and the "I-A" from 2PK3 were isolated by preparative SDS-PAGE and
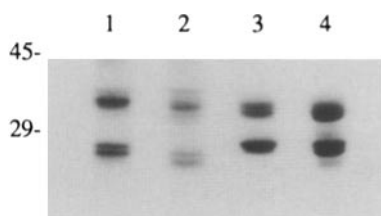

FIGURE 2. SDS-PAGE analysis of unreduced I-A and I-E specifically purified from A20 and 2PK3 on MK-D6 and 14-4-4S Sepharose affinity columns. Approximately 5 μg of affinity-purified I-A from A20 (lane *1*) and 2PK3 (lane *2*), and affinity-purified I-E from A20 (lane *3*) and 2PK3 (lane *4*) were subjected to electrophoresis and subsequently visualized by Coomassie Brilliant Blue staining. Positions of molecular weight standards are indicated on the left margin as in Fig. 1.

subsequent electroelution of the protein from excised gel segments as described in Materials and Methods. Each of the subunits was digested with trypsin and the digests were subjected to peptide mapping by HPLC chromatography. The elution profiles of the digests of the 2PK3 "I-A" α chain and A20 Eα chains were almost identical with the exception of minor variations in the regions of several peaks, which may be due to differences in glycosylation (Fig. 3 A). Each of these patterns, however, is distinct from that of the A20 Aα chain. The patterns of the Aβ chains were essentially identical.

The peptides in the peaks labeled a, b, and c in Fig. 3 A were each subjected to amino acid sequence analysis to help confirm the identification of the 2PK3 α subunit. Fig. 3 B shows the four peptide sequences obtained. Peaks b and c each yielded single unambiguous sequence that could be aligned with the known sequence reported for E$_\alpha^d$ (28). The sequence of peptide 3 terminated with the identification of the serine residue. In the predicted sequence this is followed by an arginine residue, however, this residue could not be reliably identified in our analysis of peptide 3. Sequence analysis of the material in peak a indicated the presence of two peptides in significant molar ratios. A comparison of the amino acid residues obtained at each cycle with the predicted amino acid sequence of the E$_\alpha^d$ chain was used to determine these two sequences. Neither of these sequences occur in the A$_\alpha^d$ chain. The results of these structural data lead us to conclude that the α chain associated with the Aβ chain in 2PK3 is Eα, indicating that a mixed isotype class II molecule, E$_\alpha^d$A$_\beta^d$, is the major (if not exclusive) MK-D6-reactive molecule expressed by 2PK3.

Antigenic Analysis of the Ia Antigens. Studies on the antigenic analysis of the Ia antigens of A20 and 2PK3 further supported the notion that the 2PK3 cell line expressed a mixed isotype class II molecule. The affinity-purified I-A and I-E molecules were analyzed using a solid phase RIA that used MK-D6 and 14-4-4S mAbs (Table IV). The A20 I-A reacted only with MK-D6 and the I-E molecules of A20 and 2PK3 (both of which had been purified using 14-4-4S after the cell lysates were depleted of I-A on MK-D6-Sepharose) reacted only with 14-4-4S. The 2PK3 "I-A" molecule reacted with both MK-D6 and 14-4-4S.

The I-A and I-E molecules from A20 and 2PK3 were further analyzed by immunoblot using chain-specific hamster antisera prepared against the individual subunits. Hamsters were individually immunized with electroeluted Aα chains from A20 I-A, Aβ chains from A20 I-A, Eα chains from 2PK3 I-E, or Eβ chains from A20 and 2PK3 I-E. The specificity of the antisera probes was determined by immunoblot against I-A and I-E antigens from A20 and 2PK3 (Fig. 4 A). The anti-Aα antiserum detected the α chain of A20 I-A but did not react with the α chain of I-E or 2PK3 "I-A". Anti-Eα detected the α chain of A20 and 2PK3 I-E but not the α chain of A20 I-A. Anti-Eβ detected the β chain of A20 and 2PK3 I-E. Antiserum against Aβ reacted strongly with Aβ (Fig. 4 A, lanes 7 and 8) but also showed a weak cross-reactivity to Eβ (data not shown). Using these antisera, analysis of 2PK3 "I-A" yielded the anticipitated result: the α chain was recognized by Eα-specific (Fig. 4 A, lane 4), but not Aα-specific antiserum (Fig. 4 A, lane 1), while the β chain was recognized by only the Aβ- (Fig. 4 A, lane 8), and not Eβ- (Fig. 4 A, lane 10), specific antiserum. This analysis confirmed that the 2PK3 "I-A" is composed of an Eα and an Aβ. In addition, the apparent differences in mobilities seen between the Eα chains
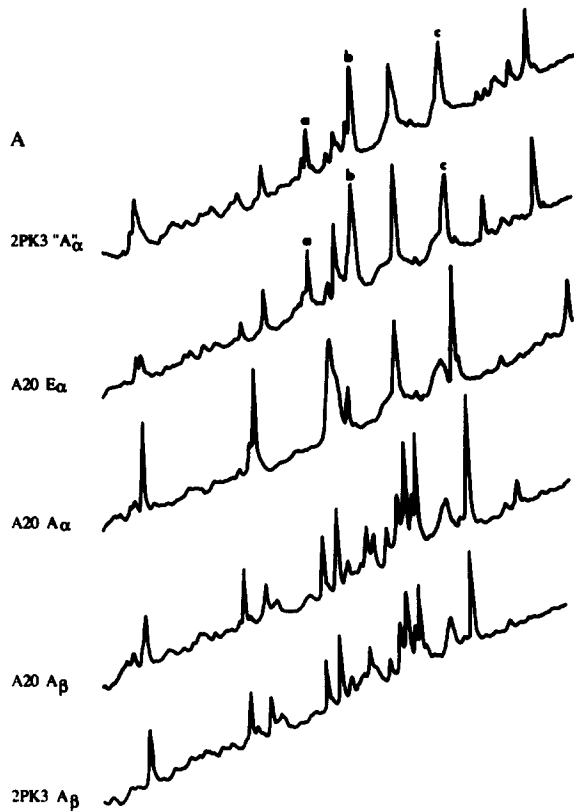
A

2PK3 "A$_\alpha$"

A20 E$_\alpha$

A20 A$_\alpha$

A20 A$_\beta$

2PK3 A$_\beta$

FIGURE 3. Evidence for the 2PK3 I-A α chain being an E$_\alpha$ chain. (A) Tryptic digests of 2PK3 "A$_\alpha$," A20 E$_\alpha$, A20 A$_\alpha$, A20 A$_\beta$, and 2PK3 A$_\beta$ chains individually isolated from preparative SDS-PAGE were fractionated on a 4.6-mm × 30-cm C$_{18}$ 300 Å pore reversed phase column by HPLC, with the resulting elution profiles shown for each subunit as indicated. The x-axis represents time while the y-axis represents the OD measured at 214 nm. (B) Peaks a, b, and c from 2PK3 "A$_\alpha$" and A20 E$_\alpha$ were subjected to amino acid sequence analysis. Peaks b and c each yielded single unambiguous sequences that could be aligned with the known sequence reported for E$_\alpha^d$. The sequence of peptide 3 terminated with the identification of the serine residue. Although this should be followed by an arginine residue, we could not reliably identify this in our analysis. Peak a contained two peptides in significant molar ratios and, therefore, the two sequences had to be resolved by comparing the amino acid residues obtained at each cycle with the predicted amino acid sequence of the E$_\alpha^d$ chain.

B

Peak	Peptide	Sequence	Position in E$_\alpha^d$	Domain
a	1	DDHLFRK	145–151	α_2
a	2	TLLPETK	188–194	TM
b	3	SNNTPDANVAPEVTVLS	81–97	α_1-α_2
c	4	NGRPVTTGVSETVFLPR	128–144	α_2

of the mixed isotype and I-E molecules as well as mobility differences between the Aβ chains of the mixed isotype and A20 I-A molecules were eliminated after deglyco-sylation of the chains (Fig. 4 B).

The mAb K24.199 (21) has been recently reported to be specific for A$_\alpha^d$ (22). Thus, the reactivity of this mAb to either A20 or 2PK3 was compared with that of MK-D6 and 14-4-4S by immunofluorescent staining analysis. Both A20 and 2PK3 stained brightly with both MK-D6 (A$_\beta^d$ specific) and 14-4-4S (E$_\alpha$ specific) (Fig. 5). A20 reacted well with K24.199, however, no staining of 2PK3 with this mAb was detected. Thus, by this analysis as well, no expression of A$_\alpha^d$ on the surface of 2PK3 cells could be demonstrated.

TABLE IV

Solid Phase RIA Identifying Determinants Found on Affinity-purified A20 and 2PK3 I-A and I-E

Coat antigen*	mAb used	
	MK-D6	14-4-4S
	cpm	
A20 I-A	13,500‡	420
A20 I-E	450	13,370
2PK3 I-A	13,050	11,960
2PK3 I-E	750	11,090

* Approximately 4 μg of each antigen was coated onto wells of microtiter plates.
‡ Binding of mAb detected with ^{125}I-labeled protein A. Values (cpm) represent the mean of duplicate wells. Binding of an irrelevant mAb gave a background of <500 cpm.
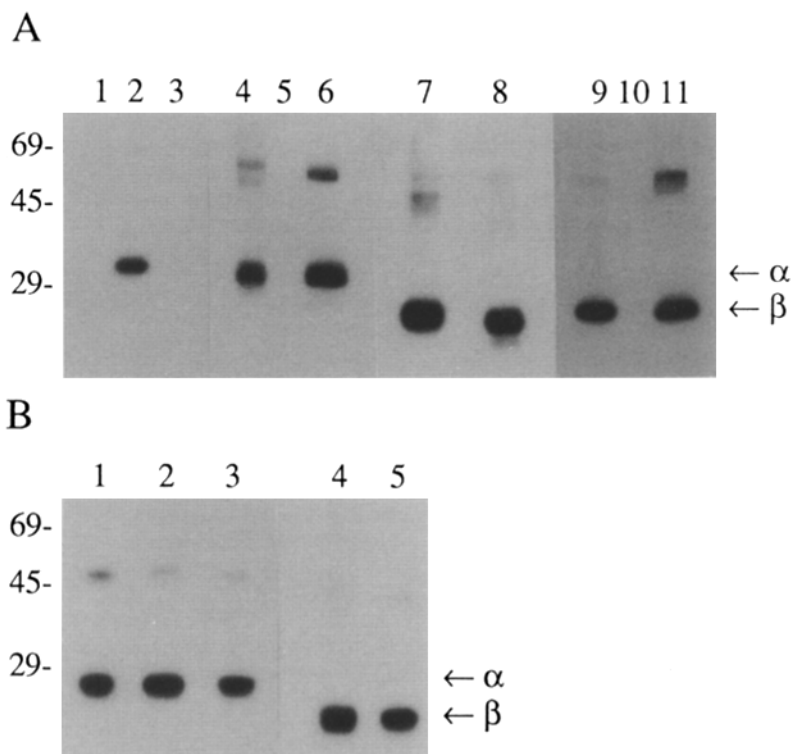
FIGURE 4.   Immunoblot analysis of I-A and I-E specifically purified by MK-D6 and 14-4-4S from A20 and 2PK3 (*A*). 2PK3 "I-A" (lane *1*), A20 I-A (lane *2*), and 2PK3 I-E (lane *3*) were probed with an Aα-specific hamster antiserum. 2PK3 "I-A" (lane *4*), A20 I-A (lane *5*), and A20 I-E (lane *6*) were probed with an Eα-specific antiserum. In lanes *7* and *8*, A20 I-A and 2PK3 "I-A", respectively, were probed with an Aβ-specific antiserum. A20 I-E (lane *9*), 2PK3 "I-A" (lane *10*), and 2PK3 I-E (lane *11*) were probed with an Eβ-specific antiserum. (*B*) The differences observed in the mobilities of the A20 I-E (lane *1*), 2PK3 "I-A" (lane *2*), and 2PK3 I-E (lane *3*) Eα chains were eliminated after chemical deglycosylation. Similarly, mobility differences in the Aβ chains of A20 I-A (lane *4*) and 2PK3 "I-A" (lane *5*) were also eliminated.

A20   2PK3

Control

K24.199

MK-D6

14-4-4S

Cell number

100 200   100 200
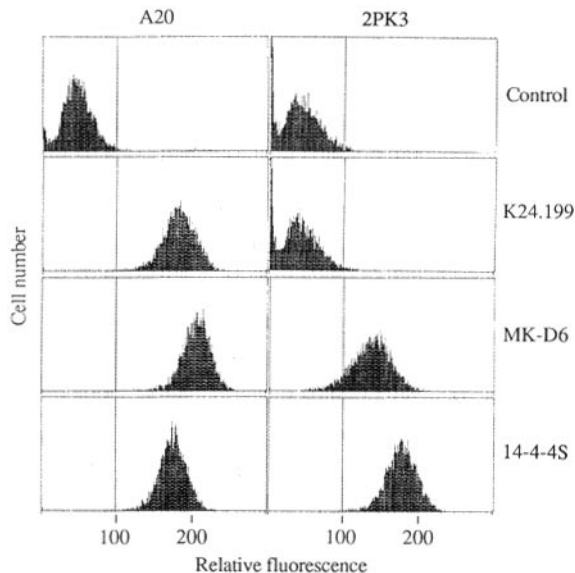
Relative fluorescence

FIGURE 5. 2PK3 does not express $A\alpha$ on its surface. Flow cytometric histograms are shown for A20 and 2PK3 stained with K24.199 (A_α^d specific), MK-D6 (A_β^d specific), and 14-4-4S ($E\alpha$ specific). The secondary reagent was FITC protein A. 2PK3 did not show any staining over background with the $A\alpha$-specific reagent.

Discussion

For the expression of functional Ia molecules on the cell surface, proper pairing of α and β class II gene products is required (29). The bulk of data on the protein chemistry of class II molecules has indicated that these molecules exist as intraisotypic pairs. Thus, in the mouse, $A\alpha$ and $A\beta$ chains associate to form the isotypic heterodimer, I-A, and $E\alpha$ combines with $E\beta$ to form the second isotypic pair, I-E. While it is generally held that only intraisotypic pairing is possible, there have been a few reports that have suggested that mixed isotype class II molecules exist. In the analysis of immunoprecipitates formed with the anti-I-E mAb, Y-17, which recognizes a conformational epitope expressed by certain $E\alpha E\beta$ complexes, Lerner et al. (30) observed radiolabeled products that had the characteristics of $A\alpha$ chains. This observation could be explained by the immunoprecipitation of an $A\alpha E\beta$ molecule with Y-17, however, no formal proof of such a molecule was offered. In another situation, the cells of the A.TFR5 mouse show the surface expression of $E\alpha$ determinants, although there is no synthesis of $E\beta$ chains (31). The possibility exists that the low amounts of $E\alpha$ detected were due to the expression of a molecule composed of $E\alpha$ and $A\beta$ chains. Finally, Maloy et al. (32) reported the possible existence of mixed isotype class II molecules in spleen cells of $H-2^d$ haplotype mice through the use of sequential immunoprecipitation in combination with radiochemical amino acid sequencing.

The results of this study provide what we believe is the first report of the expression of a mixed isotype class II molecule at high density on a murine B cell lymphoma. Malissen et al. (10) presented data that indicated that a mixed isotype molecule was apparently expressed at a low but significant level on the B cell lymphoma, A20.2JAG^R, although no biochemical characterization of the molecule was presented. Studies have shown that polymorphic residues in the NH_2 terminus of the β chain regulate the efficiency of expression of Ia antigens (5, 6). Under normal cir-

cumstances in B cells with a full complement of α and β subunits, assembly of intra-isotypic pairs would be favored. Certain conditions may predispose a cell to the expression of isotypically mismatched α and β pairs. Imbalanced class II antigen expression has been observed in murine B cell lymphomas (33). It may also be possible that in the up-regulation of class II expression resulting from lymphokine stimulation, nonstoichiometric production of the different class II subunits may allow for significant levels of mixed isotype pairing.

In addition to the mixed isotype molecule, the 2PK3 cells express normal isotypic I-E molecules. It is unlikely that 2PK3 expresses any normal I-A since we were not able to detect surface expression of $A\alpha$ by immunofluorescence using the mAb K24.199, and an immunoblot analysis of the MK-D6-purified molecule from 2PK3 using polyclonal hamster antisera specific for the $A\alpha$ chain was also negative. Preliminary experiments have indicated that the level of A_α mRNA in 2PK3 is comparable with the level expressed in A20 (J. Spencer, unpublished observations). Whether or not the A_α mRNA is functional in 2PK3 is currently being investigated. The lack of expression could be accounted for by a mutation(s) in either the A_α or $A\beta$ gene that interferes with normal isotypic chain pairing or perhaps to some other as yet to be elucidated factors. We have noted some differences in the migration pattern of the 2PK3 $A\beta$ chain in comparison with that of A20 $A\beta$ by one- and two-dimensional gel analysis. These differences appear to be primarily due to differences in glycosylation. It has been previously described that the assembly and expression of class II molecules are not dependent on glycosylation, nevertheless, it could be possible that unusual changes in carbohydrate processing in a tumor cell line could influence assembly and/or transport of some multimeric proteins. The 2PK3 $E\alpha$ chains associated with the $A\beta$ and $E\beta$ also show differences in migration that are eliminated on deglycosylation. This finding is interesting because it suggests that $E\alpha$ is glycosylated differently depending on which β chain it is associated with.

Since 2PK3 expresses no normal I-A, then the presentation of OVA(323-339) to the OVA-specific, I-A^d-restricted T cell hybridomas, DO-11.10 and 3DO-54.8, must be mediated by the mixed isotype class II molecule. The ability of both MK-D6 and 14-4-4S to completely inhibit the responses of the two T cell hybridomas to OVA(323-339) presented by 2PK3 supports this notion. Furthermore, a B cell lymphoma variant that expressed only I-E^d, M12.A2 (16), does not present the OVA peptide to the T cell hybridomas. The responses of the T cell hybridomas to different doses of antigen presented by 2PK3 are generally lower than responses mediated by A20 cells, however, the difference in the density of MK-D6 reactive epitopes on 2PK3 as compared with A20 (50%) does not appear to adequately account for the lowered responses to 2PK3. It would be expected, however, that the OVA peptide may display a lower binding affinity to the mixed isotype molecule as compared with the normal I-A^d molecule. This would account for the lower efficiency in the presentation of the OVA peptide by 2PK3. In a preliminary study the binding of OVA(323-339) to an affinity-purified mixed isotype molecule was measured and the results indicated undetectable levels of binding (S. Buus and J. Spencer, preliminary observations). We assume that the level of sensitivity of the peptide binding assay is considerably less than that seen with the stimulation assay. Thus, the peptide binding assay might yield negative results, although low levels of IL-2 are detected in a stimulation assay.

The ability of the $E_\alpha^d A_\beta^d$ molecule to present the OVA peptide is intriguing. The recent description of the three-dimensional structure of a human class I molecule indicates that the $\alpha 1$ and $\alpha 2$ polymorphic domains interact tightly to form a unique quarternary structure within which antigenic peptides could bind and be recognized by appropriate T cells (34). It has been suggested that a similar binding site could be formed by the $\alpha 1$ and $\beta 1$ domains of Ia antigens (34). The same T cell hybridomas recognize OVA(323–339) in the context of normal I-A or $E_\alpha^d A_\beta^d$ mixed isotype molecule. Thus, the A_β^d chain may play a dominant role for both peptide interaction and MHC restriction in this particular situation, while the A_α^d chain may only play a role in stabilizing the binding site, a role that could be subserved by the E_α^d chain in the case of the mixed isotype molecule. In the latter situation, however, the peptide binding site may be slightly altered such that a lowering in the affinity of peptide for the binding site is seen. Nevertheless, significant binding and restricted antigen presentation results. Further investigations with known peptides restricted by either I-A^d or I-E^d are needed to extend the observations.

Combinatorial associations would appear to benefit the individual by expanding the I region immune response capabilities, although in certain instances, unusual pair formations or the expression of modified products could perhaps result in an increased incidence in disease. The reticulum cell sarcoma, a pre-B cell lymphoma, arises spontaneously in SJL/J mice (35). This lymphoma, unlike other lymphomas, induces strong proliferative responses by Ly-1$^+$2,3$^-$ T cells (36). The I-A molecule of the reticulum cell sarcoma has been reported to differ from the I-A expressed by normal SJL spleen cells. The major differences appear to be due to more acidic A$_\alpha$ chains (37), and it has been suggested that the RCS I-A molecule is responsible for the proliferation of the syngeneic T cells and the accompanying tumor growth. Interestingly, 2PK3 elicits a strong in vitro syngeneic MLR, which, in light of the novel nature of the determinants formed by the association of E$_\alpha$ and A$_\beta$, may not be totally unexpected. In man, there have been reports of lymphocyte stimulation among HLA-identical siblings induced by neoplastic lymphoid cells in an MLR (38, 39). It has been assumed that tumor-specific antigens are responsible for these responses, however, it is possible that a mixed isotype class II molecule or some other novel form of Ia may be the stimulating molecule. The observation that certain disease associations correlate with haplotype rather than with a single locus opens the possibility that mixed isotype molecules may play a role in such instances. In addition, infectious agents may alter class II expression on APC, possibly generating increased expression of mismatched pairs that could then be recognized by autoreactive T cell clones. Further examination of the extent of mixed isotype class II molecule expression, particularly in the case of normal Ia$^+$ cell populations, will provide insight into the physiological significance of these novel class II structures in immune responses and disease.

Summary

The structures of Ia molecules expressed by two BALB/c B cell lymphoma lines, A20-1.11 (A20) and 2PK3, were analyzed in an effort to explain the differences in antigen-presenting capacity displayed by these cells. Alloreactive T cell hybridomas specific for I-A^d and antigen-specific, I-A^d-restricted T cells responded well to A20 as the APC. The same alloreactive T cell hybridomas responded weakly or not at

all to 2PK3 and the responses of the antigen-specific, I-A^d-restricted T cells were consistently lower to antigen presented by 2PK3 as compared with A20. T cells restricted to I-E^d responded equally well to either A20 or 2PK3 as APC. Additionally 2PK3, but not A20, stimulated a strong syngeneic mixed lymphocyte response. Structural analyses of the Ia antigens revealed that I-A and I-E molecules were expressed by A20, whereas an I-E and a novel I-A-like molecule were expressed by 2PK3. The novel class II molecule was affinity purified from 2PK3 cells using an mAb specific for A$_\beta^d$ (MK-D6), and this molecule was subsequently shown by an RIA to react with an E$_\alpha$-specific mAb (14-4-4S) as well. Chain-specific polyclonal antisera raised against I-A and I-E α and β chains indicated that the 2PK3 "I-A" α chain reacted in immunoblot with E$_\alpha$-specific and not A$_\alpha$-specific antisera, whereas the β chain reacted with A$_\beta$- and not E$_\beta$-specific antisera. Peptide map and partial amino acid sequence analyses indicated that the "I-A" molecule expressed by 2PK3 represented a mixed isotype structure resulting from the pairing of E$_\alpha^d$ with A$_\beta^d$. By immunofluorescence staining analysis, 2PK3 did not react with an mAb specific for A$_\alpha^d$. 2PK3 was capable of limited antigen presentation through the mixed isotype molecule to I-A^d-restricted OVA-specific T cell hybridomas, although the responses induced were low compared with presentation through I-A on A20. Previous descriptions of the expression of mixed isotype class II molecules in the mouse have resulted primarily from DNA-mediated gene transfer experiments. The results presented indicate that a mixed isotype class II molecule can be expressed naturally.

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