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Research Article

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Defective Antigen Presentation and Novel Structural Properties of DR1 from an HLA Haplotype Associated with 21-Hydroxylase Deficiency

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Abstract

We have segregated DR1⁺ individuals into two categories according to whether or not their class II⁺ cells stimulated T lymphocyte clones specific for or restricted to DR1. In a majority of cases (87%), failure to stimulate was a property of cells having the B14;DR1 haplotype and/or nonclassical 21-hydroxylase deficiency. Absence of clonal proliferation could not be explained by release of an intercellular suppressor factor or by stimulator cell absorption of interleukin 2. Homozygous cells inheriting both stimulatory ($DR1_n$) and nonstimulatory ($DR1_x$) haplotypes did not successfully mediate clonal expansion, implying that a *trans* acting factor operates intracellularly to modify both DR1 alleles or their products. Other DR alleles did not appear to be affected as evidenced by normal proliferative responses of T lymphocyte clones restricted to DR2 or DR7 and stimulated by $DR1_x$,2 and $DR1_x$,7 cells, respectively. By two-dimensional gel analysis, we have further identified a 50-kD surface glycoprotein contained in anti-DR immunoprecipitates of $DR1_x$, but not $DR1_n$ or non-DR1 cellular lysates. This 50-kD structure had antigenic and peptide identity to DRα and β chains but was resistant to dissociation under conditions that normally separate DRα and β (8 M urea plus 5% 2-mercaptoethanol); boiling in sodium dodecyl sulfate was required to segregate the component polypeptides of the 50-kD heterodimer. We postulate that a product of a novel combinatorial association between constitutive chains of DR may interfere with or compete for normal T cell receptor recognition of DR1 as both an alloantigen and a restricting element. We further propose that gene abnormalities within the class III region of a haplotype associated with nonclassical 21-hydroxylase deficiency may extend into the DR subregion of the major histocompatibility complex with consequent aberrations in DR1 presentation.

Introduction

Class II genes of the human major histocompatibility complex (MHC) encode a set of protein products responsible for mediating activation of T helper/inducer cells of the immune system. The class II heterodimer expressed by cells in which these genes are active is constructed from distinct α (32–34 kD) and β (26–29 kD) chains noncovalently complexed together. T cell receptors of CD4⁺ cells recognize processed antigen coupled with autologous class II complexes displayed at the surface of an antigen-presenting cell (APC)[1] (reviewed in reference 1). The relevant class II complexes involved in this process are derived from genes of the DP, DQ, and DR subregions, as has been shown with appropriate monoclonal antibody (mAb) blocking studies (2) and with the identification of T cell clones specific for epitopes of these products (2–5). However, structural details pertaining to the molecular affiliations and the sequence of interactions between T cell receptor, antigen, and the two constituent chains of class II structures continue to be a provocative issue.

Severe deficiency of 21-hydroxylase (21-OH) causes a recessive inborn error of metabolism known as congenital adrenal hyperplasia. A lack of sufficient 21-OH enzyme activity interrupts normal adrenal steroidogenesis such that the immediate precursor in the pathway, 17-hydroxyprogesterone, is shunted into excessive production of androgens with subsequent symptoms of virilization. Multiple variants of the disease have been described (reviewed in reference 6), which range in severity from the classical "salt wasting" and "simple virilizing" forms present at birth to the nonclassical "cryptic" and "late onset" variants where clinical symptoms fail to become evident or are not apparent until puberty. Different forms of the disease are frequently inherited in genetic linkage disequilibrium with different extended HLA haplotypes (7, 8). In the case of nonclassical 21-OH deficiency, this haplotype includes HLA-DR1 and B14 (8). Two genes for 21-OH, one active and one nonfunctional, have been mapped alternating with the complement C4A and C4B genes within the class III region of the human MHC (9, 10). Although there is extensive evidence documenting a deletion of the active 21-OHB gene in some cases of classical 21-OH deficiency (11), other reports have referred to a duplication of both the C4B gene (12) and one 21-OH gene (11) in some cases of nonclassical 21-OH deficiency. It remains unclear to what extent DNA rearrangements have functionally altered particular genes associated with the disease and whether those modifications are confined to the class III region of the affected haplotypes.

Despite the location of 21-OH genes within the human MHC adjacent and telomeric to DR subregion genes, no consistent associations with immunological abnormalities, in particular antigen presentation by class II structures, have been described in 21-OH–deficient patients or related obligate carriers. In this report, we demonstrate failure of certain DR1⁺

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; ATCC, American Type Culture Collection; 2-D, two-dimensional; EBV, Epstein-Barr virus; IEF, isoelectric focusing; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; SRBC, sheep red blood cell; [³H]TdR, tritiated thymidine; TLC, T lymphocyte clone; TNP, trinitrophenyl.

cells to mediate activation and proliferation of some DR1-restricted or specific T lymphocyte clones. This does not appear to reflect a "split" of the DR1 alloantigen and seems to be inherited in a dominant fashion, usually in linkage disequilibrium with HLA-B14 and nonclassical 21-OH deficiency. Only some epitopes of the DR1 antigenic complex are involved with the defective trait, and successful presentation of other DR allelic products is not obstructed. We further show that deviant antigen presentation is positively correlated with the presence of a novel 50-kD surface structure having DR peptide and antigenic homology.

Methods

Human T lymphocyte clones (TLC). Methods for the derivation, cloning, and screening of T cells used in this study have been detailed elsewhere (13). Clone GM-1C1 was cloned from a bulk population of responder GMc cells (A11,24; Bw58,w60; Cw3,w5; DR1,7; DQwl,w2; DRw53; DPw2,w4) that had been sequentially stimulated with trinitrophenyl (TNP)-modified peripheral blood mononuclear cells (PBMC) of the same donor. Clone RD-2A4 originated from a primary mixed lymphocyte response between PBMC of responder RD (A1,w30; B8,49; DR3,w6; DQw1,w2; DPw2,w4) and irradiated stimulator PBMC from GMc. Clonal expansion was carried out with antigenic stimulation every 7 d in Linbro 24-well plates (Flow Laboratories, Inc., McLean, VA) by using 0.5×10^6 cloned responder cells mixed with 1.0×10^6 irradiated (3,000 rad from a ^{60}Co source) PBMC in a total volume of 1.0 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin sulfate (Gibco, Grand Island, NY), 24 mM sodium bicarbonate (Mallinckrodt, Inc., St. Louis, MO), 25 mM HEPES (Sigma Chemical Co., St. Louis, MO), 10% human serum from defibrinated plasma, and 5–10% interleukin 2 (IL-2)–containing medium. Where the responding clone was specific for TNP, stimulator cells were conjugated with trinitrobenzene sulfonic acid (ICN Nutritional Biochemicals, Cleveland, OH) by methods previously described (14). Cells were passed over Isolymph (Teva, Ltd., Jerusalem, Israel) density gradients to remove cellular debris and residual stimulator cells before cryopreservation in 90% fetal calf serum (FCS; Gibco) with 10% dimethylsulfoxide (DMSO). After 5 mo of in vitro expansion, both clones were > 98% positive by flow cytometric analysis for CD3, CD4, CD2, and DR surface antigens and < 3% positive for the CD8 marker.

IL-2. IL-2–containing medium was prepared from spleens of trauma patients, as previously described (13), or from buffy coats (Gulf Coast Regional Blood Center, Houston, TX) according to existing protocols (15, 16). IL-2–containing supernatants were harvested by centrifugation at 800 *g*, stored at −70°C, and passed through 0.45-μm filters (Becton Dickinson & Co., Oxnard, CA) before use. Quantitation of IL-2 in conditioned medium was assessed by the ability of supernatants to support growth of HT-2 cells (17) relative to a known IL-2 standard (Jurkat human IL-2; Biological Response Modifiers Program, National Cancer Institute, Frederick, MD). Our preparations of IL-2–containing medium generally held 30–60 U IL-2/ml and were used at final concentrations between 5 and 10%.

Leukocyte isolation. Heparinized blood was obtained from consenting donors by venipuncture or leukapheresis. PBMC were fractionated on Isolymph density gradients according to the method of Böyum (18), washed once in Hanks' balanced salt solution (Gibco), resuspended in supplemented RPMI 1640 with 10% FCS, and depleted of platelets by centrifuging at 200 *g* through a solution of RPMI 1640 with 10% FCS and 28.6% Isolymph. Pelleted cells were washed twice and used immediately or cryopreserved.

Epstein–Barr virus (EBV)-transformed lines. Difficulties in procuring blood samples in some cases necessitated the production of EBV-transformed B cell lines. PBMC were resuspended to 1×10^6 cells/ml in supplemented RPMI 1640 with 10% FCS, and a dilution of EBV (B95-8 virus; Showa University Research Institute, St. Petersburg, FL) was added to a final concentration of 6.25×10^6 transforming U/ml. Cells were plated at 200 μl/well of a 96-well flat-bottom plate (Costar Data Packaging Corp., Cambridge, MA) and were cultured for 3 d after which medium was aspirated and replaced. Individual wells were thereafter pooled and expanded as growth permitted. All TLC and EBV-transformed lines were maintained free of mycoplasma and routinely assayed using the Hoechst dye 33258 (Calbiochem-Behring Corp., La Jolla, CA) method of Chen (19).

HLA typing. Donor leukocytes were typed for HLA-A, B, C, DR, DQ histocompatibility antigens using standard serologic techniques (20, 21). Mononuclear cells from venous blood were fractionated on Lymphoprep (Nyegaard Co., Oslo, Norway) density gradients and passed over nylon–wool (Fenwall Laboratories, Berkeley, CA) columns. Nonadherent cells were collected and typed for HLA-A, B, C antigens. Nylon–wool adherent cells were recovered and typed for DR and DQ antigens. DP antigens were typed using standard primed lymphocyte typing procedures (22).

ACTH testing. Individuals consenting to undergo ACTH testing were administered a 0.25-mg bolus of Cortrosyn intravenously between 8:00 and 9:30 a.m. Blood was drawn just before and at 30 and 60 min after Cortrosyn injection. Levels of 17-hydroxyprogesterone were measured using a radioimmunoassay after chromatography of the plasma extract (23). Plasma cortisol concentrations were also measured to ensure that an effective dose of ACTH had been administered.

Stimulation assays. All assays were carried out in 96-well round-bottom plates (Costar Data Packaging Corp.) using clonal expansion medium in the absence of exogenous IL-2. Stimulator cells were irradiated, 3,000 rad in the case of PBMC and 15,000 rad in the case of EBV-transformed lines. For assay, cloned responder T cells (1×10^5 per well) were mixed with an equal number of stimulator cells, 200 μl/well, and cultured 48 h. Proliferative responses were measured by the addition of 1 μCi/well tritiated thymidine ([^{3}H]TdR; 20 Ci/mM sp act; Amersham Corp., Arlington Heights, IL) over the final 20–24 h of incubation. Cultures were harvested onto glass fiber filter mats using a Skatron cell harvesting system (Skatron, Inc., Sterling, VA). Dried filter disks were immersed in liquid scintillation cocktail and counted on a liquid scintillation counter (LS7500; Beckman Instruments, Inc., Irvine, CA). Data are expressed as mean counts per minute of incorporated [^{3}H]TdR from triplicate cultures with the SEM.

Lymphocyte fractionation and activation. Enriched fractions of resting T and B cells were isolated by twice sheep red blood cell (SRBC; Bethyl Laboratories, Montgomery, TX) rosetting as has been described (24). Activated T cell blasts were recovered on day 4 from concanavalin A (Con A; 10 μg/ml; Pharmacia Fine Chemicals AB, Uppsala, Sweden)–treated cultures. Activated B cell blasts were recovered from day 7 lipopolysaccharide (LPS; 50 μg/ml; Difco Laboratories Inc., Detroit, MI)–dextran sulfate (20 μg/ml; Sigma Chemical Co.)-treated cultures. Blasts were passed over an Isolymph density gradient to remove dead cells and debris before subsequent manipulation.

Cellular radiolabeling and immunoprecipitation. Lactoperoxidase-catalyzed cell surface iodination, ^{35}S-methionine metabolic labeling, preparation of detergent lysates, lysate preclearing, and immunoprecipitations were all carried out as has been described (25). $10–20 \times 10^6$ viable cells were washed twice in phosphate-buffered saline (PBS; Gibco) and resuspended in 100 μl of the same, followed by additions over 15 min at room temperature of 1–2 mCi sodium ^{125}I (10–20 μl; Amersham Corp.) in combination with 150 μl lactoperoxidase (1 mg/ml; Sigma Chemical Co.) and 180 μl H_2O_2 (0.03%). Alternatively, $10–20 \times 10^6$ cells were resuspended at 2×10^6/ml in methionine-free RPMI 1640 (Inland Laboratories, Austin, TX) with 15% dialyzed FCS and 0.5–1.0 mCi ^{35}S-methionine (Amersham Corp.) and were incubated 12–18 h at 37°C. Radiolabeled cells were washed 2–3 times in PBS before lysing in 0.5% NP-40 (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY); in PBS supplemented with the protease inhibitors $ZnCl_2$ (50 μM); in aprotinin (33 μg/ml; Boehringer Mannheim GmbH, Mannheim, FRG); and in phenylmethylsulfonylfluoride (1 mM; Boehringer Mannheim GmbH). Preclearing mAbs included

OKT3 or OKT11 (Ortho Pharmaceutical, Raritan, NJ) or P3X63Ag8 myeloma (P3X; American Type Culture Collection (ATCC), Rockville, MD) culture supernatant. Anti–class II immunoprecipitations were conducted with excess L243 (ATCC), L227 (ATCC) (26), Dako-HLA-DR (Dakopatts, Copenhagen, Denmark), 2D6 (27), Genox 3.53 (ATCC) (28), Leu 10 (ATCC) (29), B7/21 (gift from Dr. Nancy Reinsmoen, University of Minnesota, Minneapolis, MN) (30), or L203 (gift from Dr. Ronald Levy, Stanford University Medical Center, Stanford, CA) (26) mAb culture supernatants. Immune complexes were isolated with *Staphylococcus aureus,* Cowan strain I (Bethesda Research Laboratories, Gaithersburg, MD) and were eluted in the presence of 5% 2-mercaptoethanol unless otherwise indicated.

Two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE). Immunoprecipitated molecules were analyzed by 2-D PAGE according to published protocols (25) using a 3.5–9 pH gradient isoelectric-focusing (IEF) gel in the first dimension and a 10% polyacrylamide slab gel in the second dimension. Gels were fixed, unless otherwise stated, dried, and autoradiographed at −70°C using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens.

2-D Western blotting. 2-D slab gels containing unlabeled, immunoprecipitated cellular proteins were electroblotted to nitrocellulose paper (Schleicher & Schuell Inc., Keene, NH) by methods previously described (31). Nonspecific binding sites were blocked by incubation in 0.05 M Tris-buffered saline with 5% nonfat dry milk, followed by an overnight exposure to mAb culture medium, and exposed an additional 6 h to ^{125}I-sheep anti-mouse Ig (Amersham Corp.). Washed and dried blots were autoradiographed as above.

Cleveland peptide mapping. Peptide mapping was performed essentially as has been described (32). 2-D gels were dried and autoradiographed without prior fixation, and the molecules of interest were excised and eluted overnight at 37°C into 0.125 M Tris buffer, pH 6.8, containing 0.1% sodium dodecyl sulfate (SDS) and 20% glycerol. Aliquots of eluted material, boiled or not boiled, were loaded into lanes of a 15% acrylamide gel having a 7-cm 4% acrylamide-stacking gel and were overlayered with 50 μl of diluted enzyme in 0.125 M Tris buffer, pH 6.8, with 10% glycerol, 0.1% SDS, and bromophenol blue. Digestive enzymes included *Staphylococcus aureus* V8 protease (1 or 10 μg/well; Miles Laboratories, Inc., Naperville, IL), chymotrypsin (2 μg/well; Miles Laboratories, Inc.), and papain (0.9 pg/well; Sigma Chemical Co.).

Results

Failure of some DR1⁺ cells to stimulate DR1-restricted and -specific TLC. Previous screening and mAb-blocking studies performed in this laboratory have established that clone GM-1C1 was specific for TNP in the context of DR1 (27), whereas clone RD-2A4 was alloreactive and specific for DR1. However, extensive panel analysis of both clones revealed that stimulator cells from a significant number of DR1$^+$ individuals were not able to mediate clonal expansion as measured in a standard proliferation assay. Representative data illustrating this finding are shown in Table I where PBMC from donors GLG, ReG, and OrB were not able to promote [^{3}H]TdR incorporation by either clone despite the serologically defined presence of DR1 on the surface of these cells. In all instances, equivalent clonal proliferation results were observed whether DR1$^+$ allogeneic stimulators were prepared from PBMC or EBV-transformed lines. Ability or inability to stimulate was always a property equally applicable to both the alloreactive and TNP-specific clones; no antigen-presenting cell ever stimulated one clone but not the other.

We have used the following nomenclature to define this functional dichotomy between some DR1$^+$ stimulators. Cells that were proficient stimulators of either clonal response carried the phenotypic designation DR1$_n$, whereas cells that were ineffective stimulators were designated DR1$_x$.

Table II summarizes the proliferative responses of clones GM-1C1 and RD-2A4 to all stimulator cells of our panel. Non-DR1 stimulator cells were not able to induce clonal activation that was consistent with the restriction specificity of these clones. Dissecting the DR1$^+$ portion of our panel, it became apparent that most cells of the DR1$_x$ phenotype also carried B14 on the same haplotype with DR1 (24 of 33 cases). In contrast, most cells of the DR1$_n$ phenotype failed to type for B14 on either haplotype or carried B14 on the haplotype op-

Table I. Proliferative Responses of DR1-Specific or -Restricted TLC

PBMC stimulator	HLA genotype	[^{3}H]Thymidine incorporation*	
		Clone RD-2A4‡	Clone GM-1C1§
GMc	DR1,7	44,883±941	25,940±5,025
DaL	DR1,7	45,740±699	32,443±1,539
ReG	DR1,4 B14 *cis*	1,733±418	1,790±540
OrB	DR1,2 B14 *cis*	2,089±821	1,542±220
GLG	DR1,3 B14 *trans*	2,037±712	1,423±375
AmCS	DR1,7 B14 *trans*	39,147±687	25,253±1,516
RaD	DR3,w6	2,030±178	2,257±731

* Results are expressed as mean counts per minute [^{3}H]thymidine incorporation for three replicate cultures ±SEM at the peak of the proliferative response at 48 h. Proliferative responses of PBMC stimulators alone or clone alone were consistently < 2,000 cpm.
‡ Clone RD-2A4, an alloreactive clone specific for DR1.
§ Clone GM-1C1, a TNP-specific clone restricted to DR1. Proliferative responses are shown for hapten-modified cells. Responses of unmodified cells with clone averaged < 2,000 cpm.

Table II. Summary of Proliferative Responses by Clones GM-1C1 and RD-2A4

Genotype	No. of individuals that serve as		
	Stimulator (DR1$_n$)	Nonstimulator (DR1$_x$)	Total
Non-DR1	0 (0.0%)	25 (100.0%)	25
DR1			
DR1, not B14	12 (81.5%)	5 (18.5%)	17
DR1, B14 *cis*	4 (14.3%)	24 (85.7%)	28
DR1, B14 *trans*	3 (42.8%)	4 (57.1%)	7
Total	19	33	52
	No. of unrelated haplotypes that serve as		
Genotype	Stimulator (DR1$_n$)	Nonstimulator (DR1$_x$)	Total
Non-DR1	0 (0.0%)	25 (100.0%)	25
DR1			
DR1, not B14	21 (84.0%)	4 (16.0%)	25
DR1, B14 *cis*	2 (11.8%)	15 (88.2%)	17
DR1, B14 *trans*	3 (60.0%)	2 (40.0%)	5
Total	26	21	47

posite that of DR1 (15 of 19 cases). Thus, the $B14^+;DR1^+$ *cis* haplotype had a positive correlation with the $DR1_x$ phenotype ($P < 0.001$), whereas the $B14^-;DR1^+$ haplotype correlated with the $DR1_n$ phenotype ($P < 0.001$). This conclusion was also true when haplotypes from multiple related individuals were excluded in the final statistical analysis (*bottom,* Table II). Exceptions to these generalizations are interesting and may reflect recombination events in the ancestry of these cells such that B14 has segregated away from or recombined with the DR1-bearing haplotype.

Apparent linkage of the $DR1_x$ phenotype with nonclassical 21-OH deficiency. As stated earlier, the B14;DR1 haplotype and its associated complotype (C4A2; C2C; BFS; C4B1,2) are commonly inherited in conjunction with nonclassical 21-OH deficiency (8, 12). Previously reported results indicate that the B14;DR1 supratype can reliably predict the clinical status of individuals not formerly known to be 21-OH deficient carriers (33). Of 21 individuals from our panel whose cells manifested the $DR1_x$ phenotype and who consented to undergo ACTH testing, 19 were carriers or homozygous patients with nonclassical 21-OH deficiency. 12 of these 19 were known to carry B14 on the same haplotype with DR1; of the remainder, genotyping was not possible in three cases and B14 was *trans* to DR1 in three cases (related siblings) or absent from both haplotypes in one case. This high frequency of association (90.5%) between the incompetent DR1 stimulator status and 21-OH deficiency would suggest that both characteristic traits are inherited in strong linkage disequilibrium. However, in one categorical $DR1_x$ individual 21-OH deficiency was apparent without the B14 marker; three other individuals with the $DR1_x$ phenotype did not inherit the B14 allele and were not 21-OH deficient. This would imply that the distance between the 21-OHB gene and the $DR1_x$ gene influencing normal expression of DR1 or between B14 and the $DR1_x$ gene is not sufficiently small to prohibit recombination.

Familial segregation of the $DR1_x$ phenotype. Both the $DR1_x$ and the $DR1_n$ phenotypic traits appeared to follow normal Mendelian rules of inheritance as evaluated in stimulation assays from family studies (Table III). Effective stimulator status always segregated with the $DR1_n$ haplotype (family C), whereas ineffective stimulator status segregated with the B14;$DR1_x$ *cis* haplotype (family W). It should be noted that individual LiW of family W is a homozygous DR1 example inheriting the B14;$DR1_x$ *cis* haplotype of her mother and the $DR1_n$ haplotype of her father. The resulting $DR1_x$ phenotype of this individual's APC suggests that the $DR1_x$ trait is expressed in a dominant fashion. The absence of any stimulatory capacity mediated by these heterozygous $DR1_x$,$DR1_n$ cells further suggests that the phenomenon is not attributable to a serologically undefined split of DR1. Similar results were obtained in another pedigree where cells from the mother expressed the B14;$DR1_x$ *cis* phenotype, cells from the father carried the $DR1_n$ phenotype, yet APC from their $DR1_x$,1_n daughter did not mediate clonal expansion (data not shown).

$DR1_x$ cells do not secrete a suppressor factor. To determine whether failure to promote adequate clonal proliferation was due to secretion of a nonspecific soluble or cellular-bound suppressor factor, irradiated $DR1_x$ stimulator cells from EBV-transformed B cell lines were mixed with a similar irradiated fraction of $DR1_n$ stimulators (Table IV). No obvious suppression was apparent; graded decreases in the overall number of effective $DR1_n$ stimulator cells per well correlated with a concomitant decrease in [^{3}H]TdR incorporation. This suggests that failure to induce clonal expansion resulted from improper or altered presentation of the DR1 antigen and not from a suppressor factor that depressed clonal activation.

Table III. The $DR1_x$ Phenotype Segregates with the B14;DR1 Haplotype

				[^{3}H]Thymidine incorporation*	
Family	PBMC stimulator	Relation	Phenotype	Clone RD-2A4‡	Clone GM-1C1§
G	ReG	Daughter	$DR1_x$,4 B14 *cis*	3,245±711	783±31
	BrH	Son	DR2,4	2,770±653	592±30
	OrB	Mother	$DR1_x$,2 B14 *cis*	3,000±723	582±33
	JoW	Father	DR4,5	2,851±122	559±36
W	RoW	Mother	$DR1_x$,2 B14 *cis*	2,363±436	880±98
	MiW	Father	$DR1_n$,w8	43,367±1,946	21,733±121
	LiW	Daughter	$DR1_x$/1_n B14 *cis*	3,185±548	2,479±145
M	GMc	Brother	$DR1_n$,7	39,891±1,275	26,536±495
	RMc	Brother	DR2,7	1,127±242	770±145
C	AmCS	Father	$DR1_n$,7 B14 *trans*	38,783±1,404	25,583±755
	ToC	Son	DR4,7	1,978±112	6,233±703
P	ViW	Daughter	$DR1_x$,5 B14 *cis*	3,150±429	970±140
	ELP	Daughter	$DR1_x$,2 B14 *cis*	923±54	993±58
	MiG	Mother	DR2,5	2,543±67	917±228

* Results are expressed as mean counts per minute [^{3}H]thymidine incorporation for three replicate cultures ±SEM at the peak of the proliferative response at 48 h. Proliferative responses of PBMC stimulators alone or clone alone were consistently < 2,000 cpm. ‡ Clone RD-2A4, an alloreactive clone specific for DR1. § Clone GM-1C1; a TNP-specific clone restricted to DR1. Proliferative responses are shown for modified stimulator cells. Responses of unmodified stimulator cells with clone averaged < 2,000 cpm.

Table IV. $DR1_x$ Stimulator Cells Do Not Secrete a Suppressor Factor

EBV stimulator (phenotype)	No. cells × 10^5/well	[^{3}H]TdR incorporation* Clone RD-2A4‡
SaR ($DR1_n$)	1.0	35,686±79
	0.75	34,811±2,083
	0.5	35,199±1,834
	0.25	28,908±1,863
NeP ($DR1_x$)	1.0	2,459±197
	0.75	2,088±22
	0.50	1,556±179
	0.25	1,171±275
BLP (non-DR1)	1.0	2,251±208
	0.75	1,767±186
	0.5	1,677±405
	0.25	900±40
SaR + NeP	(0.75 + 0.25)	32,509±2,047
	(0.5 + 0.5)	33,787±4,346
	(0.25 + 0.75)	26,859±865
SaR + BLP	(0.75 + 0.25)	36,857±3,701
	(0.5 + 0.5)	33,962±999
	(0.25 + 0.75)	31,440±1,659

* Results are expressed as mean counts per minute [^{3}H]thymidine incorporation for three replicate cultures ±SEM at the peak of the proliferative response at 48 h. Proliferative responses of EBV stimulators alone or clone alone were consistently < 4,000 cpm.
‡ Clone RD-2A4, an alloreactive clone specific for DR1.

$DR1_x$ stimulators do not absorb IL-2. To ensure that APC of the $DR1_x$ phenotype were not preferentially absorbing IL-2 produced by T cell clones in assay and thus preventing clonal utilization of lymphokines essential for cell cycle progression, EBV-transformed lines of the $DR1_x$ and $DR1_n$ phenotypes were stained with anti-Tac mAb (34) (gift from Dr. Thomas Waldmann, National Cancer Institute, National Institutes of Health, Bethesda, MD) to quantitate expression of IL-2 receptors. No IL-2 receptor was detectable by indirect fluorescence on EBV lines from either the $DR1_n$ or $DR1_x$ categories; fluorescence profiles were consistently equivalent to or below background staining with anti-CD3 (data not shown). Furthermore, crude supernatants of IL-2–containing medium added to assay at final exogenous concentrations of 10 or 50% failed to reverse the inability of $DR1_x$ EBV lines to stimulate clonal proliferation (data not shown). Thus, it is unlikely that the apparent ineffectiveness of $DR1_x$ stimulator cells reflected their competitive absorption of IL-2.

$DR1_x$ cells can present other epitopes of DR1 to other DR1-restricted clones. We questioned whether the inability of some $DR1^+$ cells to serve as efficient stimulators reflected a modification of all potential antigenic determinants of the DR1 complex. To explore this issue, we generated additional clones specific for or restricted to DR1. Clone GM-3G4 was derived from a mixed lymphocyte response using donor GMc as the responding cell type and TNP-modified GMc PBMC as the stimulator cell type. Screening of this clone revealed that all $DR1^+$ cells serve as competent stimulators, including cells with the $DR1_x$ phenotype that are incompetent stimulators of clones GM-1C1 and RD-2A4 (Table V). Consequently, although at least one determinant of the DR1 complex has been deleted or altered, other determinants are unaffected and are capable of being recognized by their corresponding T cell receptors on other clones.

Table V. $DR1_x$ Cells Can Present Other Epitopes of DR1 and Other DR Alleles

		[^{3}H]Thymidine incorporation*			
EBV stimulator	HLA-DR phenotype	Clone 1C7‡	Clone SB-3D5§	Clone GM-3G4‖	Clone RD-2A4¶
SaR	$DR1_n$,2	57,331±4,012	ND	ND	23,488±875
GMc	$DR1_n$,7	2,347±200	14,345±2,055	24,094±458	14,574±411
NaD	$DR1_x$,7	1,679±54	8,787±340	ND	1,586±18
DeM	$DR1_x$,7	1,757±90	18,746±1,486	ND	2,151±94
PaV	$DR1_x$,7	1,422±105	10,276±704	ND	23,299±2,033
OrB	$DR1_x$,2	59,294±3,094	1,548±257	ND	ND
RbR	$DR1_x$,2	57,243±1,836	2,071±130	ND	1,600±624
NeP	$DR1_x$,2	59,082±5,730	ND	ND	4,097±399
JoR	$DR1_x$,7	958±39	14,062±655	47,420±1,372	1,128±44
BLP	DR2,4	63,335±3,339	ND	ND	2,466±202
CeG	$DR1_n$,3	ND	ND	61,507±2,073	ND
AmCS	$DR1_n$,7	ND	24,440±1,717	55,113±1,004	14,373±1,485
ViW	$DR1_x$,5	ND	ND	13,357±327	ND
DiR	DRw6,7	ND	ND	870±67	ND

* Results are expressed as mean counts per minute [^{3}H]thymidine incorporation for three replicate cultures ±SEM at the peak of the proliferative response at 48 h. Proliferative responses of EBV stimulators alone or clone alone were consistently < 4,000 cpm. ‡ Clone 1C7, an alloreactive clone specific for DR2. § Clone SB-3D5, an alloreactive clone specific for DR7. ‖ Clone GM-3G4, a TNP-specific clone restricted to DR1. ¶ Clone RD-2A4, an alloreactive clone specific for DR1.

Heterozygous $DR1_x$ cells effectively present other DR allelic products. $DR1_x$,2 and $DR1_x$,7 cells were tested in proliferative assays with alloreactive DR2- or DR7-specific clones to determine whether heterozygous $DR1_x$ cells could successfully stimulate other class II specific clones or if the $DR1_x$ phenotype interferes in a *trans* fashion with normal presentation of any DR allelic product. Table V demonstrates that DR2 and DR7 complexes were adequately presented on the surface of heterozygous $DR1_x$ APC in a manner that allowed normal stimulation of their respective clones. Therefore, lack of stimulation of the DR1-specific or -restricted clones represents a defect in presentation of some DR1 antigenic determinants, but not other DR allelic products.

Co-precipitation of a 50-kD glycoprotein with DR from some $DR1^+$ cells. We attempted to compare the molecular profile of $DR1_x$ and $DR1_n$ antigens using standard techniques designed to separate these molecules by charge and molecular weight. When radioiodinated cellular lysates were subjected to immunoprecipitation with anti-DR mAbs followed by IEF in the first dimension and SDS-PAGE in the second dimension, we identified a unique species having a molecular weight of 50 kD and an isoelectric point (pI) between 5 and 6 that co-precipitated with α and β chains of DR from $B14^+$;$DR1^+$ *cis* cells (Fig. 1 *B*), but not from $B14^-$;$DR1^+$ cells (Fig. 1 *A*) or $B14^+$;$DR1^-$ cells (data not shown). The presence of this 50-kD moiety correlated well ($P < 0.001$) with the $DR1_x$ phenotype (Table VI). Lysates of cells from 19 of 21 individuals tested who manifested the $DR1_x$ phenotype contained the 50-kD species that co-precipitated with anti-DR mAbs. In contrast, only 1 of 18 cellular lysates from individuals exhibiting the $DR1_n$ phenotype contained the co-precipitating 50-kD species. Because presence or absence of the 50-kD molecule was consistent for any given stimulator cell source, was equally perceptible in PBMC or EBV cellular lysates, and was inherited in a Mendelian fashion much like the $DR1_x$ phenotype, it is unlikely that the 50-kD entity could be accounted for by artifacts of immunoprecipitation or techniques used in the 2-D gel analysis.

Table VI. Correlation Between Clonal Stimulation and 50 kD Glycoprotein

Genotype	No. positive for 50 kD/no. tested	
	Stimulators ($DR1_n$)	Nonstimulators ($DR1_x$)
Non-DR1	0/0	1/10
DR1		
DR1, not B14	0/15	3/3
DR1, B14 *cis*	1/1	15/15
DR1, B14 *trans*	0/2	1/3
Total	1/18	19/21

To incorporate these new findings we have expanded our nomenclature, designating those cells expressing the 50-kD species as originating from individuals of the $DR1^{50}$ phenotype and those lacking the 50-kD species as originating from the $DR1^{null}$ phenotype.

We were able to visualize the 50-kD moiety on 2-D gels using immunoprecipitations from both ^{125}I-lactoperoxidase catalyzed surface-labeled (Fig. 1) and ^{35}S-methionine internally labeled lysates (data not shown). Lysates or solubilized immunoprecipitates processed with or without the reducing agent 2-mercaptoethanol failed to yield any change in the apparent molecular weight or IEF properties of the 50-kD species (data not shown), leading us to conclude initially that the 50-kD molecule represented a single polypeptide chain that was not disulfide bonded to DR α or β chains.

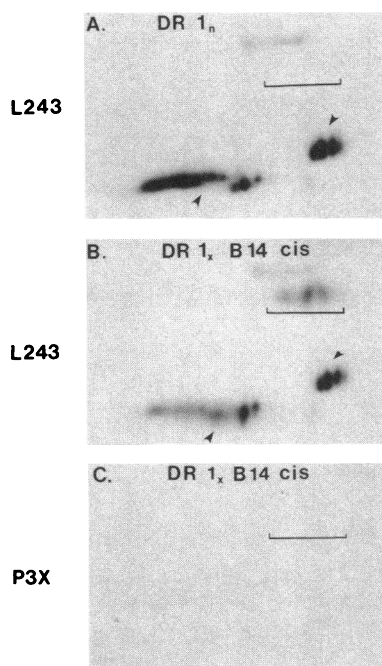

Figure 1. Comparative 2-D PAGE analysis of anti-DR immunoprecipitates from $DR1_x$ and $DR1_n$ PBMC cellular lysates. DR complexes were precipitated with the mAb L243 (*A* and *B*) from a $DR1_n$ PBMC lysate (SaR, *A:* HLA-A3,w23; B7,w35; C–,–; DR1,2) and a $DR1_x$ PBMC lysate (GLG, *B* and *C:* HLA-A28,w34; Bw53,14; Cw4,w5; DR1,w8); culture supernatant from the murine myeloma P3X63Ag8 served as the negative control precipitating reagent (*C*). The portion of each autoradiograph shown represents identical areas of the IEF (pH ~ 3.5–9.5, *right* to *left*) and PAGE (~ 60–20 kD, *top* to *bottom*) dimensions. DR β molecules are designated by an up arrowhead and DR α molecules, by a down arrowhead. Brackets mark areas of the gel in which molecules of 50 kD, pI 5–6 would be expected to appear.

Regulation of expression of the 50-kD protein. Using donor ReG who was known to carry the $DR1_x^{50}$ phenotype, we enriched for resting B and resting T cells using two sequential rounds of SRBC rosetting. Unfractionated PBMC from the same donor were used to generate mitogen-activated B and T cell blasts. Anti-DR immunoprecipitations from ^{125}I-labeled lysates of these populations demonstrated that activated B and T cells and resting B cells all contained the 50-kD protein with DR α and β. Anti-DR mAbs failed to precipitate α and β chains from resting T cells, as expected, and did not contain the 50-kD species (Fig. 2). Also, IL-2–dependent TLC derived from individuals having the $DR1_x$ phenotype consistently expressed the 50-kD protein, whereas lysates of $DR1^-$ or $DR1_n$ clones did not (data not shown). Similarly, gamma interferon–treated fibroblasts (graciously supplied by Dr. David Maurer from our laboratory) from individuals having the $DR1_x$ phenotype expressed the 50-kD complex whereas treated fibroblasts from $DR1_n$ individuals did not (data not shown). Therefore, we believe that the appearance of the 50-kD protein on the surface of these cells is regulated in a fashion similar to other class II proteins, in particular DR.

Exclusive precipitation of the 50-kD protein using anti-DR mAbs. From a panel of anti–class II mAbs, only those with specificity for DR were able to co-precipitate the 50-kD protein from lysates of $DR1_x^{50}$ cells (Fig. 3). Co-precipitation of the 50-kD protein was noticeably absent from immunoprecip-

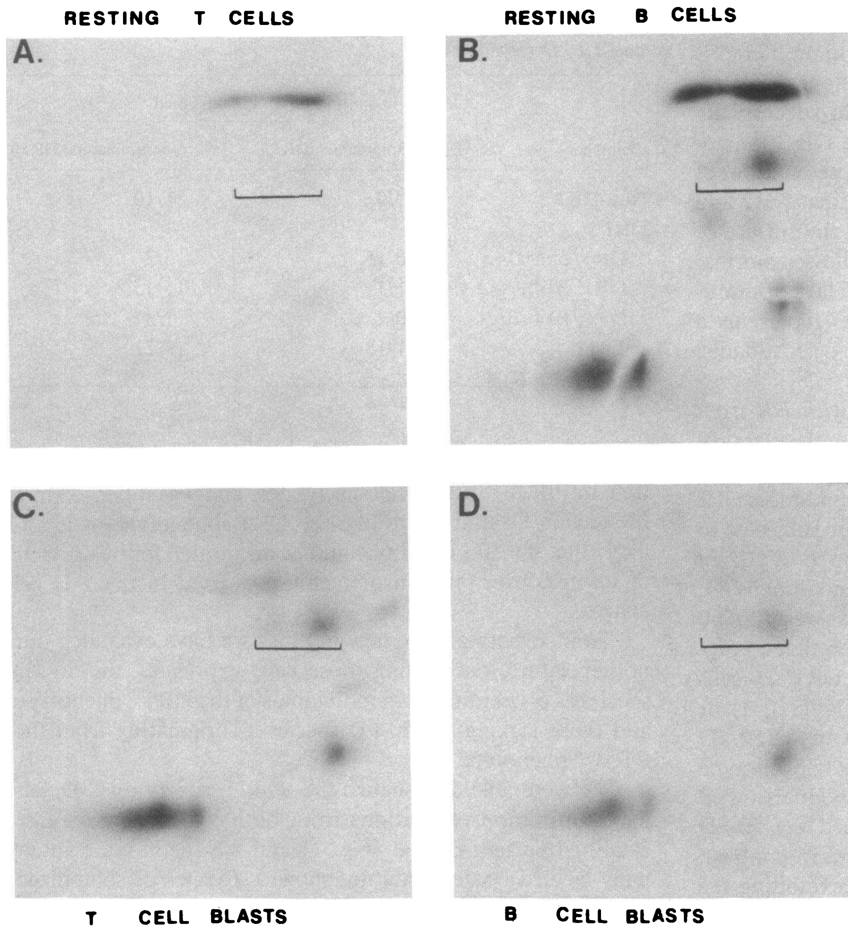

Figure 2. The 50 kD species is regulated like class II molecules. Anti-DR immunoprecipitates (mAb L243) from B14;DR1_x *cis* cellular lysates (ReG) were analyzed by 2-D PAGE. Resting T or B cells from peripheral blood were isolated by SRBC rosetting. T cell blasts were derived from day 4 Con A cultures; B cell blasts were from day 7 LPS–dextran sulfate cultures.

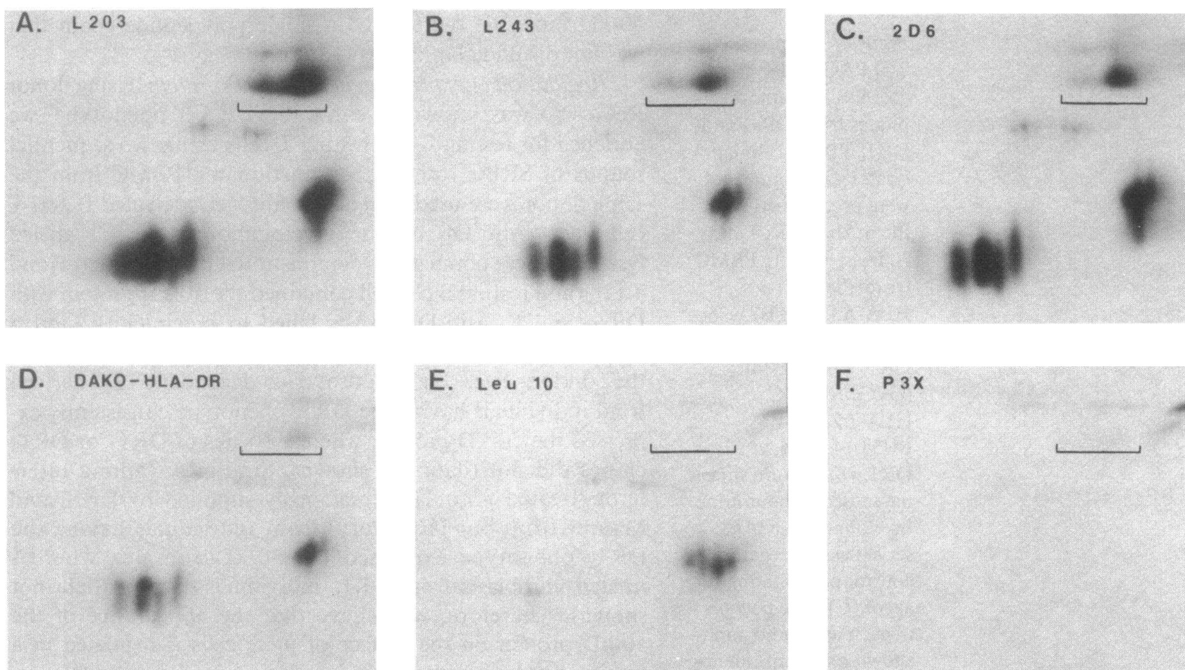

Figure 3. Exclusive precipitation of the 50-kD species with anti-DR mAbs. A B14;DR1_x *cis* EBV cellular lysate (GLG) was immunoprecipitated with mAb against DR (L203, L243, 2D6, and Dako–HLA-DR) or DQ (Leu 10) and the precipitates were analyzed by 2-D PAGE; culture supernatant from the murine myeloma P3X63Ag8 (*F*) was utilized in negative control immunoprecipitations. Orientation of α and β polypeptide chains is as given in Fig. 1. We believe that molecules appearing just below the bracketed 50-kD region represent co-precipitating class I heavy chains.

itations utilizing anti-DQ (Leu 10; Fig. 4 *E*) and anti-DP (B7/21; data not shown) mAbs. We interpret this to mean that the 50-kD molecule shares crossreactive determinants only with DR α and/or β chains or that the 50-kD species noncovalently complexes with DR molecules but not with DQ or DP molecules. In further experiments (not shown), we have found that the 50-kD complex is not a class I antigen because it is not precipitated with mAb against HLA-A, B, C or β-2 microglobulin, nor does it represent a chondroitin sulfate modified invariant chain.

The 50-kD species includes DR antigenic determinants. To pursue this evidence for common antigenic determinants between the 50-kD protein and DR α and/or β chains, we ran unlabeled anti-DR immunoprecipitations on 2-D gels, transferred them to nitrocellulose paper, and immunoblotted with a panel of class II mAbs. All anti-DR mAbs tested had specificity for the 50-kD molecule (L243, Fig. 4 *B*), whereas anti-DQ mAbs did not blot (data not shown).

Dissociation of the 50-kD species by boiling. After excising spots representative of DR α and β and the 50-kD species from 2-D gels followed by elution and rerunning on one-dimensional SDS-PAGE gels under nonreducing conditions, we observed that the 50-kD band dissociated into two constituent chains if the eluted material was boiled before rerunning. If treatment did not include boiling, no separation was apparent (Fig. 5, compare lanes *3* and *6*). The component chains of the 50-kD complex appeared to have molecular weights equivalent to DR α and β chains (Fig. 5, compare lanes *6* and *7*).

Enzymatic susceptibility and peptide mapping. Successful enzymatic digestion of the 50-kD complex could not be achieved unless boiling preceded digestion. When eluted material representative of the 50-kD moiety was subjected to digestion by *Staphylococcus aureus* V8 protease, chymotrypsin, or papain without prior boiling, no peptide fragments were discernible (Fig. 6 *A*, compare lanes *3* and *6*). However, boiling followed by enzymatic digestion produced a peptide map virtually identical to that of purified DR α and β chains mixed together (Fig. 6 *B*, compare lanes *6* and *7*). These data suggest that the 50-kD species is composed of DR α and β chains, but the inherent associative properties or the configurations of these two chains are sufficiently different from conventional DR α and β chains as to preclude separation under conditions that include SDS and 8 M urea or reducing agents such as 5% 2-mercaptoethanol.

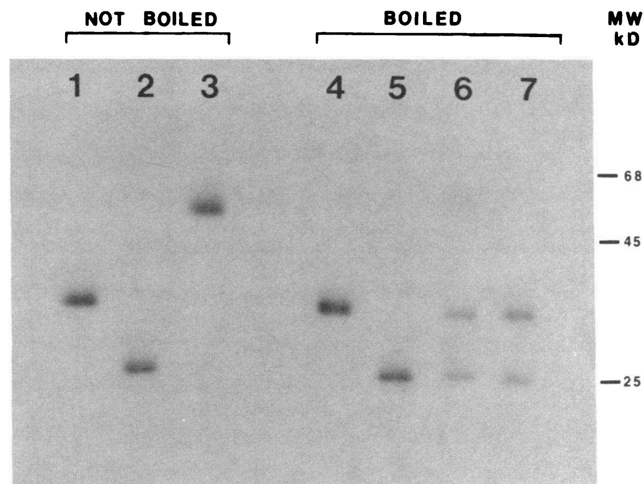

Figure 5. The 50-kD species dissociates after boiling. Material representative of DR α and β or the 50-kD species from a B14;DR1_x *cis* EBV-transformed cell line (TaT) was eluted from 2-D PAGE gels, boiled or not boiled, and analyzed on a 10% acrylamide one-dimensional SDS gel under nonreducing conditions. Lanes *1* and *4*, DR α. Lanes *2* and *5*, DR β. Lanes *3* and *6*, 50-kD species. Lane *7*, DR α and β mixed.

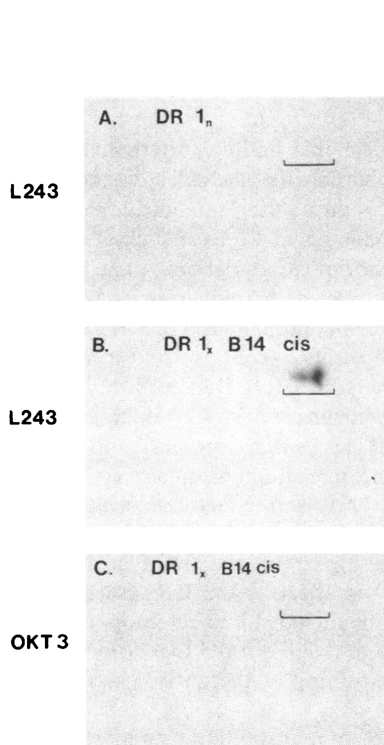

Figure 4. The 50-kD species includes DR antigenic determinants. Anti-DR immunoprecipitates from EBV cellular lysates of DR1_n (JiN, *A:* HLA-A2,11; B44,27; Cw2,5; DR1,4) and B14;DR1_x *cis* (DoH, *B* and *C:* HLA-Aw33/2; B14/w62; C−/w3; DR1/4) individuals were separated by 2-D PAGE and subjected to analysis via Western blotting with L243 or OKT3 mAb followed by ^{125}I-sheep anti–mouse Ig. Failure of individual DR α or β chains to be recognized by these reagents is interpreted to mean that the corresponding determinants were lost after dissociation of the two peptides or after denaturation in SDS.

Discussion

We have observed that DR1$^+$ cells of some individuals failed to mediate activation and proliferation of T lymphocyte clones either restricted to or specific for DR1. An unusually high association (87.0%) of this phenomenon with haplotype inheritance of HLA-B14 and/or nonclassical 21-OH deficiency coupled with previous mapping of both the HLA-B14 and 21-OH genes telomeric to the DR subregion have allowed us to use these secondary features as tentative markers for defective antigen presentation of DR1. However, the identification of DR1_x cells from our panel that were B14$^-$;DR1$^+$ 21-OH normal or of DR1_n cells that were B14$^+$;DR1$^+$ *cis* 21-OH deficient would suggest that the association is not absolute, and that although all three phenotypic traits are tightly linked, they can be separated by recombination. Because defective antigen presentation was always seen in conjunction with DR1_x, we believe that the gene(s) causing abnormal antigen presentation is linked closer to or is identical to some gene(s) of the DR subregion.

Inheritance of the DR1_x phenotype within the families we studied appeared to follow normal Mendelian patterns of segregation. In two instances, we had access to cells from unrelated "natural hybrid" individuals inheriting one parental DR1 haplotype encoding the competent stimulator (DR1_n) phenotype and another, encoding the incompetent stimulator

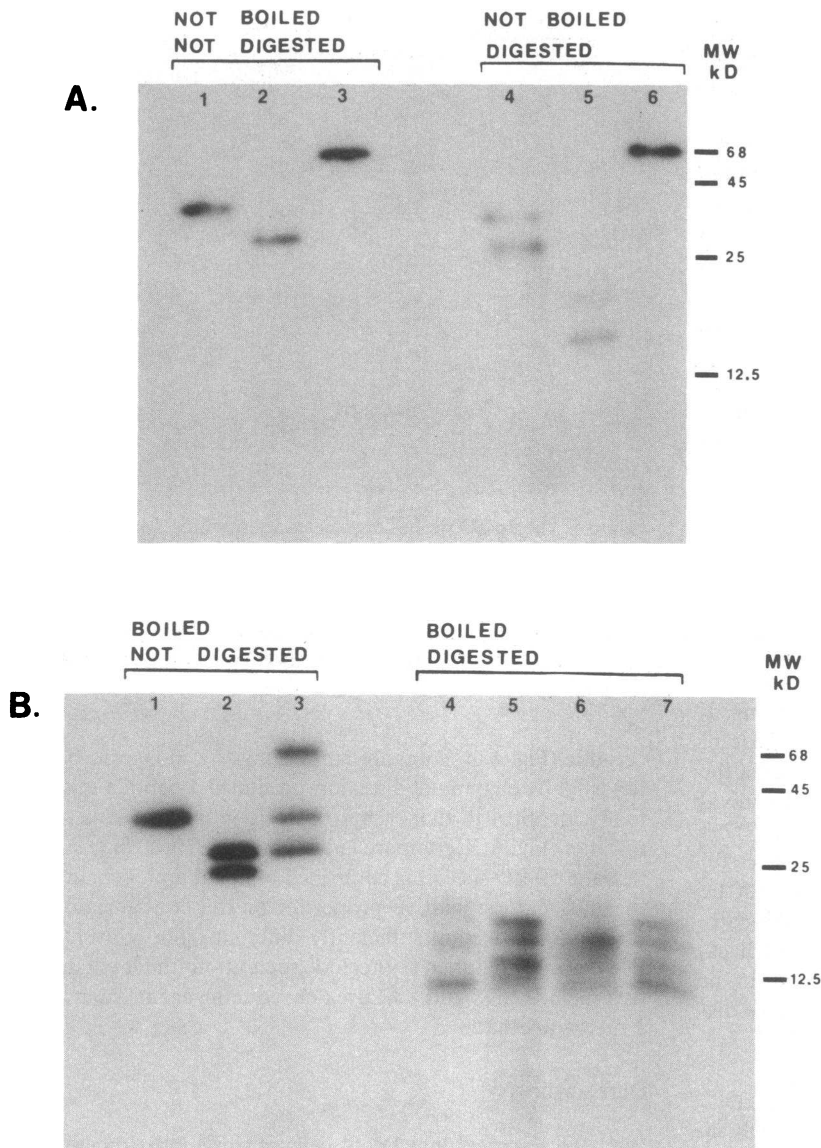

Figure 6. Enzymatic susceptibility and peptide mapping of the 50-kD species. DR α and β chains or the 50-kD species from a B14;DR1_x,1_x *cis* (DiO, *A*) or B14;DR1_x,3 *cis* (ALG, *B*) EBV-transformed cell line were digested with *Staphylococcus aureus* V8 protease with (*B*) or without (*A*) previously boiling the eluted material. Lanes *1* and *4* (both *A* and *B*), DR α. Lanes *2* and *5*, DR β. Lanes *3* and *6*, 50-kD species. Lane *7* (*B* only), DR α and β mixed. We believe that the lower M_r DR β band apparent in immunoprecipitates of some cellular lysates (lane *2*, *B*) corresponds to the DR β_2 protein product.

(DR1_x) phenotype. Overall, cells of these "hybrid" DR1_x,DR1_n individuals expressed the nonstimulatory (DR1_x) phenotype. Repeat testing of the stimulatory capacity of each family member's cells again confirmed these results. Although some caution must be exercised in drawing conclusions from this limited sample, it appears that the DR1_x phenotypic trait is dominant and may contain a *trans*-acting factor affecting expression of both DR1-bearing haplotypes. These particular examples also support our contention that defective antigen presentation is not representative of an as yet unidentified serological "split" of DR1. Testing cells from additional natural hybrid individuals is required to substantiate these conclusions and will be carried out as cells from such rare pedigrees become available. We also intend to construct in vitro somatic cell hybrids from cell lines that combine both phenotypes.

It is especially notable that defective antigen presentation of DR1 is limited to the representative DR1_n-specific and -restricted clones reported here. DR1_x,2 and DR1_x,7 stimulator cells were able to mediate normal proliferative responses from DR2 and DR7 alloreactive clones, respectively. We plan to utilize additional alloreactive clones specific for these and other DR alleles to confirm this finding. Alternatively, the possibility remains that inheritance of the defective trait(s) on the DR1_x haplotype may conduct a *trans* modification of other epitopes or other DR products that we have not yet investigated although the frequency of clones defining these missing or modified determinants may be extremely low. Again, in light of the evidence for normal presentation of DR2 or DR7 from opposing haplotypes of DR1_x cells, we find it puzzling that phenotypically heterozygous DR1_x,DR1_n cells cannot adequately present the DR1_n allelic product. Cell-mixing experiments where DR1_x and DR1_n cells were combined and used as stimulators failed to demonstrate an intercellularly acting suppressive agent. Hence, if a *trans*-acting factor associated with the B14;DR1_x haplotype exists, it must be operative intracellularly. Our collective data suggest that the modifying agent(s) responsible for the defect is affecting the DR1 allele only. Other DR alleles may not be included in a determinant-altering process or may be able to retain normal function by compensating during tertiary folding of the entire class II DR complex.

Not all polymorphic determinants of DR1 itself are modi-

fied or masked in this alteration process since we have generated one T lymphocyte clone capable of responding to all $DR1^+$ stimulator cells regardless of defined $DR1_x$ or $DR1_n$ phenotypes. Serological typings of these cells also tend to support this observation because no serological differences were detected using a large battery of DR1-specific alloantisera that presumably recognize polymorphic epitopes within the $\beta1$ domain of the DR1 antigenic complex.

We have not yet determined a molecular cause for the aberrant structural assembly of the $DR1^{50}$ complex. Since the constitutive chains of the 50-kD complex are very similar if not identical in size to corresponding conventional DR chains, no major deletions or insertions in the protein sequence are predicted. Differential expression of a secondary, linked gene responsible for posttranslational modifications of the $DR1_x$ vs. the $DR1_n$ complex could account for the appearance of the 50-kD heterodimer and consequently the eradication of one or more antigenic determinants. Alternatively, a mutation in one or more DR subregion genes could generate the same result. In either instance, increased avidity of interaction between the two chains could interfere with antigen-presenting properties or with potential binding interactions of the class II complex and a specific T cell receptor complex.

In summary, we have evidence for a novel combinatorial association between constituent chains of the DR1 complex, the product of which may functionally interfere with or compete for normal T cell receptor recognition of DR1 as both an alloantigen and as a restricting element. It is especially noteworthy that a defect of this nature is highly correlated with the B14;DR1–extended haplotype commonly inherited with nonclassical 21-OH deficiency, a haplotype known to contain at least one major rearrangement of DNA downstream of the DR subregion. Others have speculated that an extended haplotype of this nature may be retained if duplications of the C4B and 21-OHB genes carried with B14 and DR1 introduce sufficient misalignments of the chromosomes to suppress recombination in that region and across portions of the adjoining MHC (35). Although deficiencies of immunological function in such patients have not previously been reported, our data suggest that additional HLA gene product structural abnormalities are inherited with this haplotype, that modifications of the DNA extend beyond the class III region, and that at least one consequence may be abnormal surface presentation of the DR1 antigen.

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