BIOCHEMICAL CHARACTERIZATION OF A SECOND FAMILY OF HUMAN Ia MOLECULES, HLA-DS, EQUIVALENT TO MURINE I-A SUBREGION MOLECULES*

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The generation of immune responses is dependent upon interactions among three types of cells: T, B, and macrophages. These interactions are regulated by genes located within the immune response or I region and HLA-D region of the murine and human major histocompatibility complexes (MHC),[1] respectively, (1–8). The mechanisms by which these I or HLA-D region genes regulate cellular interactions is not well understood. However, they are believed to be mediated by I or HLA-D region glycoprotein products expressed on the cell surface of B cells and macrophages and known as Ia (I region-associated) molecules (9–14). T cells possess allele-specific receptors that recognize Ia molecules on B cells and macrophages (15, 16). Recognition of these molecules by T cells, either in combination with antigen or alone, is a requisite for development of T cell function, i.e., helper function for antibody production by B cells and cell-mediated immunity. Because Ia molecules are highly polymorphic, with more than 20 and 12 alleles having been described in mouse and man, respectively, isolated T cells from one individual possessing receptors for its own Ia molecules will not interact with B cells and/or macrophages from an individual with a different set of Ia molecules. However, differentiation of bone marrow or spleen stem cells in an environment containing "foreign" Ia molecules will result in the development of T cells that can now specifically recognize the "foreign" Ia molecules (17–20). This differentiation, with concomitant development of receptors specific for Ia, apparently takes place in the thymus (21–23).

In mice, two families of Ia molecules encoded by the I-A and I-E subregions have been identified and characterized (24). Both I-A and I-E molecules consist of two noncovalently associated polypeptide subunits. The large (Aα) and small (Aβ) subunits of I-A have molecular weights of 35,000 and 26,000, respectively, while the large (Eα) and small (Eβ) subunits of I-E have molecular weights of 32,000 and 29,000, respectively (25). Despite connotations of similarity denoted by both being generally referred to as Ia molecules, the I-A and I-E molecules are structurally

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[1] *Abbreviations used in this paper:* IEF, isoelectric focusing; MAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; 2-D, two dimensional.

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distinct based on partial N-terminal sequence and peptide map analysis (26–29). Furthermore, I-A and I-E molecules display different patterns of structural variability. When allotypic I-E molecules are structurally compared, their small (β) subunits are found to be very different, whereas their large (α) subunits are generally invariant (25, 30). I-A molecules, in contrast, display structural variability in both subunits when allotypes are compared (25, 31). In this respect, it is intriguing to note that the genes coding for the three variable subunits, Aα, Aβ, and Eβ, are tightly linked, whereas the gene encoding the invariant Eα subunit is apparently separate and distinct (30–33).

Until recently, only one group of Ia molecules had been identified in man. This family of Ia molecules, known as HLA-DR (for D region-related), is structurally homologous to the murine I-E subregion molecules on the basis of amino acid sequence analysis (27). We have recently been able to identify the human equivalents to the murine I-A subregion molecule using a monoclonal antibody prepared in our laboratory (34). We describe here the isolation and further biochemical properties of these molecules, which we have designated HLA-DS for "second D-region locus."

Materials and Methods

Cell Lines. All human cell lines used in this study are homozygous by consanguinity at the HLA locus. Their HLA phenotypes are described in Table I. The human lymphoblastoid cell lines with a GM designation were obtained from the Human Mutant Cell Repository in Camden, NJ, as was the marmoset cell line GM3158(M). The human lymphoblastoid cell lines with an LG designation were obtained from the Leibold-Gatti collection (Department of Pediatrics, UCLA Medical Center, Los Angeles, CA).

Isolation of Ia Molecules. Cells (2×10^8) were radiolabeled in overnight culture in 5 ml of RPMI 1640 (lacking the amino acid that was added in radioactive form) containing 10% dialyzed fetal bovine serum and 1–5 mCi of radioactive amino acid. After washing the cells in phosphate-buffered saline (PBS), membrane proteins were solubilized with 5 ml of 0.5% Triton X-100 in 0.01 M Tris, pH 7.4 (buffer A). The supernatant was incubated overnight at 4°C with lentil lectin coupled to Sepharose 4B. After extensive washing of the lentil lectin column, glycoproteins were eluted with 2% α-methyl mannoside in buffer A. The glycoprotein fraction was incubated overnight at 4°C with 0.2 ml of immunoadsorbent prepared by covalently coupling monoclonal antibody to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) at a ratio of 1 ml ascites fluid/g Sepharose followed by suspension in 0.01 M Tris, pH 8.2, containing 0.15 M NaCl and 0.5% deoxycholate (buffer B). After extensive washing of the immunoadsorbent with buffer B, followed by two washes with buffer B lacking salt, the purified Ia molecules were eluted with 0.05 M diethylamine containing 0.5% deoxycholate. The samples were lyophilized, redissolved in 1.0 ml of H_2O, and precipitated with 3 ml acetone in the presence of 100 μg of human gamma globulin (HGG). The precipitate was washed once with acetone, air-dried, and redissolved in 100 μl

TABLE I

HLA Phenotypes of Lymphoblastoid Cell Lines

Cell Line	Name designation	HLA-A	HLA-B	HLA-C	D	DR
GM3107	PGF	3	7		2	2
GM3163	LBF	30	13	2		7
LG2	JB	2	27		1	1
LG10	MAu	29	12		7	7
LG14	ALT	1	8	6	3	3
LG36	HMs	23	12	4	7	7

Laemmli nonreducing sample buffer (35). After electrophoresis on 12.5% polyacrylamide gels, proteins were eluted from 1-mm gel slices by overnight incubation with 0.5 ml of a 0.01% sodium dodecyl sulfate (SDS) solution. The Ia α and β subunits were localized by counting aliquots of the eluted material.

Peptide Map Analysis. Ia polypeptide subunits purified by SDS-PAGE and labeled with 50,000 cpm of [^{3}H]phenylalanine or 40,000 cpm of [^{14}C]phenylalanine were combined and lyophilized.

REDUCTION/ALKYLATION. The lyophilized samples were dissolved in 800 μl H_2O and combined with 100 μl of a dithiothreitol (DTT) solution (15.4 mg/100 μl) before incubation at 37°C for 3 h. Recrystallized iodoacetamide (200 μl of a 46 mg/200 μl solution) was added, and the samples were incubated at 37°C. After 10 min, 50 μl of 2 N NaOH was added, followed 10 min later by a further addition of 25 μl of 2 N NaOH. Finally, the samples were incubated for 10 min followed by the addition of 7.8 mg of DTT.

TRICHLOROACETIC ACID (TCA) PRECIPITATION. After the addition of 1 mg of HGG, the samples were vortexed and mixed with 0.15 ml of 100% TCA. After incubation at 4°C for 1 h, the samples were centrifuged at 3,000 rpm for 5 min. The resultant precipitates were washed once with 2 ml of 5% TCA, washed twice with acetone, and air dried overnight.

TRYPSIN DIGESTION. After reduction/alkylation and TCA precipitation, the dried precipitates were dissolved in 0.5 ml of 0.1 N NH_4HCO_3, pH 8.0. 10 μl of a solution of 10 mg TPCK trypsin/ml H_2O were added to each sample, followed by incubation at 37°C for 1 h. Afterward, an additional 100 μl of TPCK trypsin was added, and incubation continued another 2 h, followed by the addition of 100 μl of 10 M isoelectric focusing (IEF) sample buffer (a 10 ml solution contains 0.4% Triton X-100, 0.5 ml ampholine [LKB] mixture (37.5% pH 3.5–5.0, 25% pH 4.0–6.0, 37.5% pH 5.0–8.0), 1.05 ml H_2O, 5.82 g urea). The samples were then lyophilized.

IEF

(*a*) *Preparation of gels.* Formula for seven gels: 16.94 g urea, 1.16 ml Triton X-100, 1.46 ml ampholine mixture, 5.0 ml of a solution containing 20% acrylamide 1% Bis, and 9.88 ml H_2O. Heat and stir to dissolve, bring to room temperature, add 0.15 ml 10% ammonium persulfate, and fill 7-mm × 13-cm gel tubes to the 10-cm level. Overlay with IEF sample buffer diluted fourfold.

(*b*) *Running buffers.* These buffers are made fresh. The upper buffer is 0.1 N NaOH. The lower buffer is 0.01 M H_3PO_4.

(*c*) *Preparation of samples.* Dissolve lyophilized sample in 60 μl H_2O and 5 μl 2-mercaptoethanol.

(*d*) *To run gels.* Cover bottom of each gel tube with dialysis tubing held in place with a rubber band. Place tubes in electrophoresis chamber, add 100 μl 6.7 M IEF sample buffer to each, and prerun for 1 h at constant power, 0.1 W/gel. Apply the sample under the 6.7 M IEF sample buffer and run as above for 6½ h.

(*e*) Freeze gels, slice in 1-mm sections, and elute in 0.5 ml H_2O overnight. Determine pH gradient, add 4 ml scintillation fluid, and count in scintillation counter. Adjust for channel spillover, and plot cpm against slice number.

Amino Acid Sequence Analysis. Partial N-terminal sequencing using microamounts of radiolabeled protein was performed as described (36). Ia subunits radiolabeled with single tritiated amino acids were purified by SDS polyacrylamide gel electrophoresis (PAGE) as described above. After elution from the polyacrylamide gel, samples were dialyzed extensively against 0.01% SDS. 3 mg of sperm whale myoglobin was added to each sample, which was then sequenced on an 890C Beckman sequenator (Beckman Instruments, Inc., Fullerton, CA). The sequentially released thiazolanones were converted to thiohydantoins with 0.1 N HCl and extracted with ethyl acetate. Small aliquots of the extract (5%) were analyzed by high-pressure liquid chromatography to ascertain repetitive yield. The remainder of the sample was counted in a liquid scintillation counter.

Complete N-terminal sequence analysis was performed as described by Shively et al. (37). 1–2 nmol of Ia molecules was purified from 10^{10} cells by immunoadsorbtion using a small amount of radiolabeled protein as tracer. After lyophilization and dissolution in water as described above, Ia molecules were precipitated with acetone without the addition of carrier HGG. Isolated α and β subunits were obtained after further purification by SDS-PAGE.

Two-dimensional (2-D) Gel Electrophoresis. Samples radiolabeled with [^{35}S]methionine were

purified by immunoadsorbtion as described above, except that the final washes and elution from the immunosorbent were done in the presence of Triton X-100 instead of deoxycholate. The samples were lyophilized, resuspended in 25 μl sample buffer, and electrophoresed as described by O'Farrell (38) with the following modifications, some of which were adapted from McMillan et al. (39): (*a*) 2-mercaptoethanol was eliminated from all reagents; (*b*) the sample buffer and IEF gel were prepared according to McMillan et al.; (*c*) the gel overlay solution is composed of sample buffer diluted to 6 M urea; (*d*) the sample overlay buffer is composed of 6 M sample buffer containing 0.05% aspartic and glutamic acid; (*e*) samples were electrophoresed according to McMillan et al. (39); (*f*) SDS-PAGE was performed according to O'Farrell (38) on 12.5% polyacrylamide gels.

Results

Identification of Marmoset and Human I-A Subregion-like Molecules. The studies described here were initiated with the intention of isolating and characterizing the human homologs of murine Ia antigens. To achieve this goal, we immunized BALB/c mice with a mixture of lymphoblastoid cell lines GM3158(M), GM3107(DR2), and GM3163(DR7), obtained from the Human Mutant Cell Repository in Camden, NJ, to produce monoclonal antibodies (MAb) directed against human Ia molecules. Of four monoclonal antibodies produced by this immunization, which were reactive with these cell lines, studies with just two monoclonal antibodies, designated SG157 and SG171, will be described here.

When used in an indirect immunoprecipitation assay, these two antibodies bound molecules having SDS-PAGE profiles depicted in Fig. 1. Although the profiles obtained using SG157 or SG171 with either GM3107(DR2) or GM3163(DR7) cells were basically identical, the differences in profiles observed when these two antibodies were tested with GM3158(M) cells suggested that SG157 and SG171 were reacting with different molecules. This was confirmed by peptide map analysis and 2-D gel electrophoresis (34). To determine whether SG157 and SG171 were reacting with molecules related to HLA-DR, both MAb were used in conjunction with a radiolabeled glycoprotein fraction from GM3158(M) cells to isolate molecules that were then subjected to partial N-terminal sequence analysis. The results of this analysis (34) indicated that SG157 reacts with an HLA-DR (I-E-like) molecule, whereas SG171 reacts with a molecule structurally homologous to the murine I-A subregion molecule.

During the course of these initial studies, it became apparent that GM3158 (M) had properties quite distinct from GM3107 and GM3163 and from other human lymphoblastoid cell lines. Although the other cell lines were pleomorphic and grew only in suspension, GM3158(M) grew both in suspension as round cells and on the surface of the tissue culture flasks, where they took on a fibroblast-like morphology. Furthermore, GM3158(M) was the only cell line that secreted large quantities of Epstein-Barr virus. This latter property is especially unusual for human cell lines and made us suspicious of the origins of GM3158(M). When GM3158(M) was subsequently karyotyped, it was found to be of marmoset and not human origin. In contrast, both GM3107(DR2) and GM3163(DR7) were found to be of human origin. Because we had not previously worked with marmoset cells, it was unlikely that our laboratory was the source of contamination, and, indeed, when GM3158(M) was karyotyped immediately after receipt from the Human Mutant Cell Repository, it was found to be of marmoset origin. Thus, these cells were most likely already of marmoset origin when originally donated to the repository. Marmoset cell contamination is apparently due to the fact that Epstein-Barr virus used to transform human

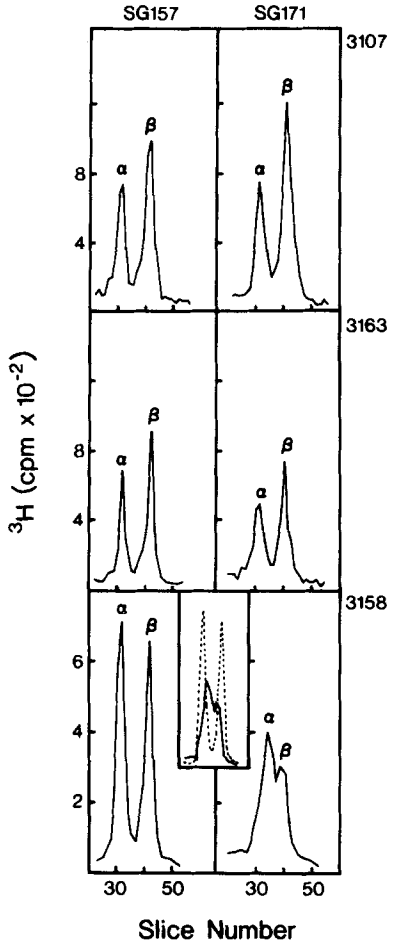

FIG. 1. SDS-PAGE profiles of molecules isolated from cell lines GM3107(DR2), GM3163(DR7), and GM3158(M) by immunoprecipitation (40) with monoclonal antibodies SG157 (left column) and SG171 (right column). The inset shows the SDS-PAGE profile obtained from a mixture of SG157-precipitated material ([^{3}H]phenylalanine labeled) and SG171-precipitated material ([^{35}S]methionine labeled) isolated from GM3158(M) and established the differences in molecular weights between these two complexes. All samples were electrophoresed under nonreducing conditions. The α and β subunits have molecular weights of ~37,000 and 29,000, respectively.

lymphocytes is routinely obtained from transformed marmoset cells from which it is secreted in large quantities. Inadequate removal of marmoset cells from the virus extract before transformation may result in contamination with marmoset cells that rapidly outgrow the human lymphocytes. We have since found that several other cell lines being used by other investigators and believed to be of human origin are actually marmoset. All displayed the unusual growth properties of GM3158(M) and possessed the I-A subregion-like molecule.

Because our intention now was to isolate the human equivalent to the murine I-A subregion-like molecule, we turned our attention to the two human cell lines, GM3107(DR2) and GM3163(DR7), that were under study in our laboratory. 2-D gel analysis of GM3107(DR2) molecules binding to SG157 and SG171 revealed virtually

identical profiles (Fig. 2). This was quite surprising in view of the fact that the DR-like and I-A subregion-like molecules from the marmoset cell line, GM3158(M), could easily be distinguished by 2-D gel analysis. The 2-D gel results suggested, therefore, that in contrast to GM3158(M), where SG157 and SG171 reacted with different molecules, these MAb reacted with identical molecules when used with GM3107(DR2) cells. Furthermore, because we knew from our analysis of the marmoset cell line GM3158(M) and from other previous studies using human cell lines (40) that SG157 reacts with HLA-DR molecules, the identity in 2-D gel profiles suggested that SG171 was also reacting with HLA-DR molecules. Indeed, when the

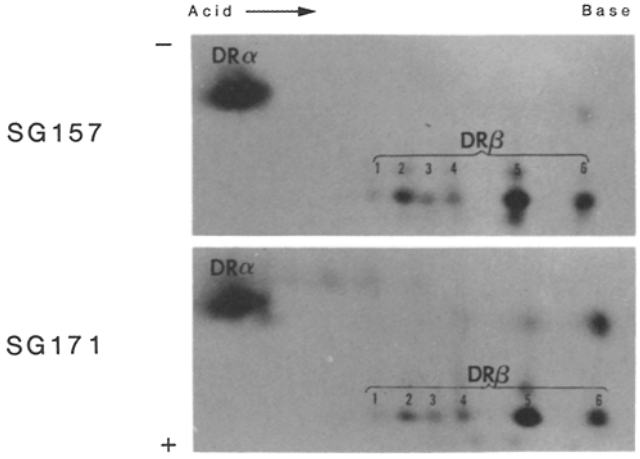

FIG. 2. 2-D gel analysis of proteins isolated from GM3107(DR2) with SG157 (top) and SG171 (bottom). It is important to point out that the α subunit, although appearing as a single spot, is actually represented by four closely migrating spots when the x-ray film is exposed for a shorter period.

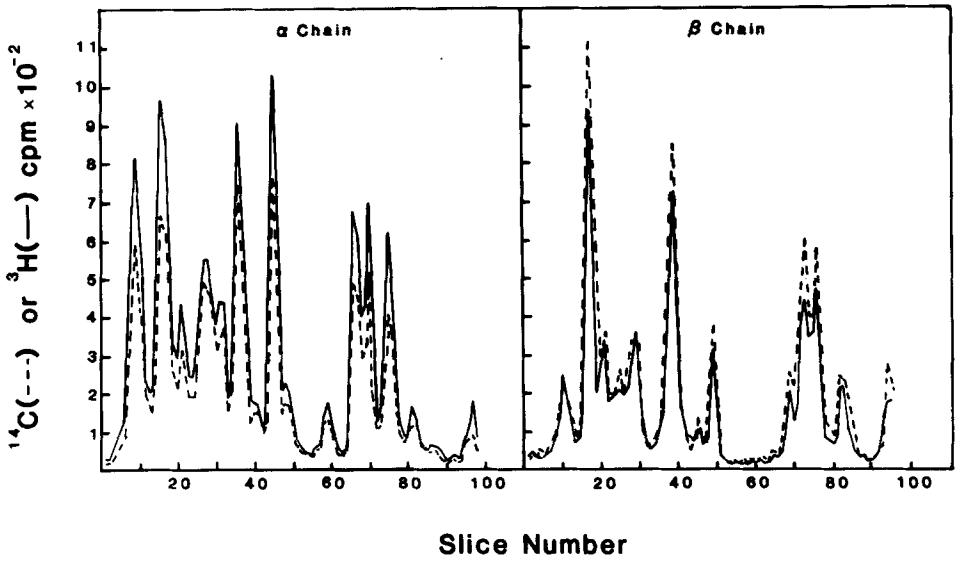

FIG. 3. Tryptic peptide comparison of α (left) and β (right) polypeptides isolated from GM3107(DR2) with SG157 (——) and SG171 (- - -).

peptide maps of molecules from GM3107(DR2) binding to SG157 and SG171 were compared, they were found to be identical (Fig. 3). Thus, although SG171 bound I-A subregion-like molecules from the marmoset, it not only failed to bind any easily detectable I-A subregion-like molecules from the human cell line GM3107(DR2), but also cross-reacted with HLA-DR. To rule out the possibility that SG171 was composed of two different monoclonal antibodies, one reacting with marmoset I-A and the other with human DR molecules, we performed a competitive inhibition test. The human cell line GM3107(DR2) completely absorbed the reactivity of SG171 against the marmoset cell line GM3158(M).

The results of studies with the human cell line GM3163(DR7) were, however, quite different from those with GM3107(DR2). Peptide map profiles of molecules binding to SG157 and SG171 were very different from each other (Fig. 4). There were two obvious ways to account for these differences: (*a*) SG157 and SG171 were binding to totally unrelated molecules, or (*b*) SG157 was binding HLA-DR molecules while SG171, which cross-reacted with human HLA-DR and marmoset I-A, was binding both HLA-DR and the human equivalent to the I-A subregion-like molecules. The results of 2-D gel analysis (Fig. 5) confirm the latter interpretation. The 2-D gel profiles of molecules from GM3163(DR7) cells binding to SG157 and SG171 were identical except for three additional basic spots corresponding to a molecule of 29,000 mol wt (i.e., the β subunit) that was present in the SG171 profile. These three more basic spots presumably represent the small subunit of I-A subregion-like molecules (DSβ), while the adjoining series of acidic spots presumably represents the DRβ subunit. This hypothesis was confirmed in a depletion experiment in which the glycoprotein extract from GM3163(DR7) was first depleted as much as possible of SG157 (DR-binding) reactivity and then reacted with SG171. As can be seen, SG157 nearly completely removes the more acidic (DRβ) spots while leaving behind the more basis (DSβ) spots. In contrast to the two series of low molecular weight spots corresponding to the DRβ and DSβ subunits, only a single spot was observed in the 37,000 mol wt range, suggesting that the DRα and DSα subunits had identical molecular weights and charge. Confirmation of this proposal as well as the dual specificity of SG171 (i.e., DR and DS) was obtained by N-terminal sequence analysis

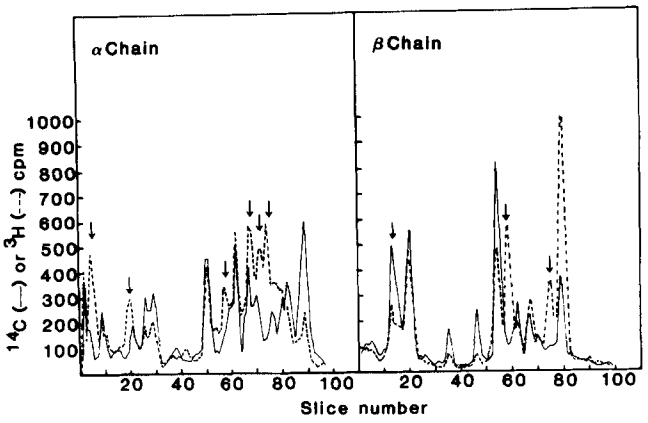

FIG. 4. Peptide map comparisons of α (left) and β (right) polypeptides isolated from GM3163(DR7) with SG157 (——) and SG171 (- - -). Arrows denote peptide differences.

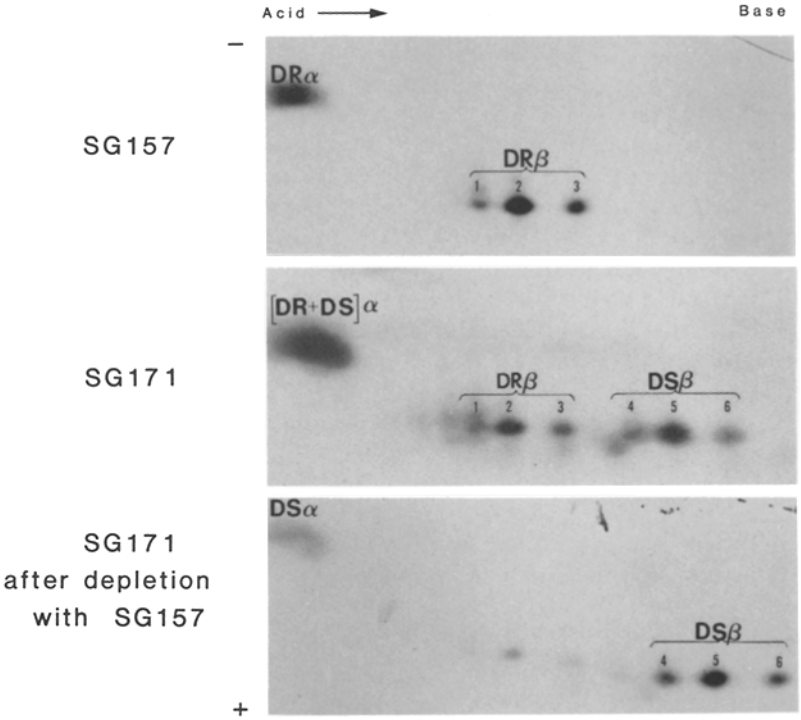

FIG. 5. 2-D gel analysis of proteins isolated from GM3163(DR7) with SG157 (top), SG171 (middle), and SG171, after depletion of the glycoprotein extract of SG157-binding protein (bottom). Depletion was accomplished by passing a GM3163(DR7) glycoprotein extract over an SG157-immunoadsorbent twice before using an SG171-immunoadsorbent.

TABLE II

*Amino Acid Sequences of Large (α) Subunits**

		Step number																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
GM3158(M)	DSα	E	D	I	V	A	D	H	V	A	A	Y	G	V	N	L	Y	[E]	S	Y	G	P	M	G	Q
GM3163	DSα	E	D	I	V	A	D	H	V	A	A	Y	G	V	N	L	Y	[Q]	S	Y	G	P	M	G	Q
	DRα	I	K	E	E	H	V	I	I	Q	A	E	F	Y	L	N	P	D	Q	S	G	E	F	M	F
LG2	DRα	I	K	E	E	H	V	I	I	Q	A	E	F	Y	L	N	P	D	Q	S	G	E	F	M	F

* Glycoprotein extracts from 10^{10} cells of the human cell lines LG2(DR1) and GM3163(DR7) and the marmoset cell line GM3158(M) were adsorbed onto SG171-immunoadsorbent columns, and HLA-DR and DS molecules were isolated as described in Materials and Methods. The component α and β subunits were purified by SDS-PAGE, eluted from the gels, dialyzed, and sequenced in the presence of polybrene as described (37). The marmoset (GM3158) and human (LG2) α chains gave single, although very different, N-terminal sequences. The α subunit isolated from GM3163(DR7) gave a mixed sequence, one corresponding to the α subunit of LG2 (DRα) and the other corresponding almost perfectly to the marmoset α sequence (DSα). Sequences are given using the single-letter amino acid code. Boxed residues indicate differences between human and marmoset DSα.

of the large molecular weight component (37,000 mol wt) isolated from GM3163(DR7) cells with SG171. Results of this analysis and a comparison with the amino acid sequences of the DRα and the marmoset Aα subunits reveal that this large molecular weight component (37,000 mol wt) from GM3163(DR7) consists of two molecules, one that has a sequence identical to that of the DRα subunit isolated from human cell lines and a second molecule whose sequence is identical to the marmoset Aα subunit in 23 of 24 residues (Table II).

Specificity of the MAb SG171. The ability of SG171 to isolate I-A subregion-like molecules from GM3163(DR7) but not from GM3107(DR2) could be accounted for in two possible ways: (*a*) only some human cell lines express I-A subregion-like molecules, possibly because of their being at different stages of differentiation, or (*b*) SG171 is a polymorphic antibody, i.e., it recognizes only certain allotypes of I-A subregion-like molecules. To distinguish between these two possibilities, we examined, by peptide mapping and/or 2-D gel analysis, several other human lymphoblastoid cell lines, LG2(DR1), LG14(DR3), LG10(DR7), and LG36(DR7), for their ability to express I-A subregion-like molecules reactive with SG171. When the molecules from LG2(DR1), reactive with SG157 and SG171, were compared by peptide mapping and 2-D gel electrophoresis, they were found to be identical. Similarly, the molecules from LG14(DR3), reactive with SG157 and SG171, displayed identical 2-D gel patterns. Thus, just as was found for cell line GM3107(DR2), SG171 bound only HLA-DR molecules from cell lines LG2(DR1) and LG14(DR3). In contrast to these observations, when SG171 was tested on LG10(DR7) and LG36(DR7), the 2-D gel profiles obtained were virtually identical to those observed with the cell line GM3163(DR7), i.e., three extra basic spots characteristic of the small subunit of I-A subregion-like molecules that were absent when these cells were tested with SG157 (Fig. 6). It is crucial to the interpretation of this data that we point out that all three cell lines that express I-A subregion-like molecules reactive with SG171 have been typed as DR7, and, indeed, we confirmed by peptide mapping (40) that they express identical DR molecules. Thus, SG171 reacts only with I-A subregion-like molecules found on DR7 cell lines. This observation has several important implications that will be discussed later.

Isolation of I-A Subregion-like Molecules from Other Human Cell Lines. The specificity of SG171 for I-A subregion-like molecules present only on DR7 cell lines made SG171 of limited use for isolating I-A subregion-like molecules from cell lines of other DR types. To circumvent this obstacle, we decided to prepare a more broadly reactive antiserum by immunizing rabbits and mice with I-A subregion-like proteins purified from the marmoset cell line GM3158(M). This antiserum, Rb03, reacts solely with the I-A subregion-like molecules [i.e., no evidence of cross-reaction with DR from the DR7 cell line GM3163(DR7)] (Fig. 7). Furthermore, when used in conjunction with GM3107(DR2) cells, the rabbit antiserum binds a molecule represented by a series of spots of ~29,000 mol wt that are distinguishable from the DRβ subunit isolated with SG157 (Fig. 8). However, the DSα and DRα subunits are still indistinguishable, as was observed for the GM3163(DR7) cells. Partial N-terminal sequence analyses of the large (α) and small (β) subunits from GM3107(DR2) and LG10(DR7) binding to the rabbit antiserum confirm their assignment as HLA-DS (Table III). Except for the absence of phenylalanine residues at position 14, which is present in the marmoset molecule, the sequences obtained are consistent with their assignment as DS molecules. Furthermore, no evidence of DR-like sequences is observed. Also, note that the DSα subunits isolated from GM3107(DR2) and LG10(DR7) differ at position 11, indicating that DSα subunits are polymorphic. These results clearly demonstrate that GM3107(DR2) cells express an I-A subregion-like (DS) molecule distinct from HLA-DR and detectable with the rabbit antiserum.

Discussion

An understanding of the basic mechanisms that underlie immune regulation requires a detailed knowledge of the structures of molecules that mediate cellular

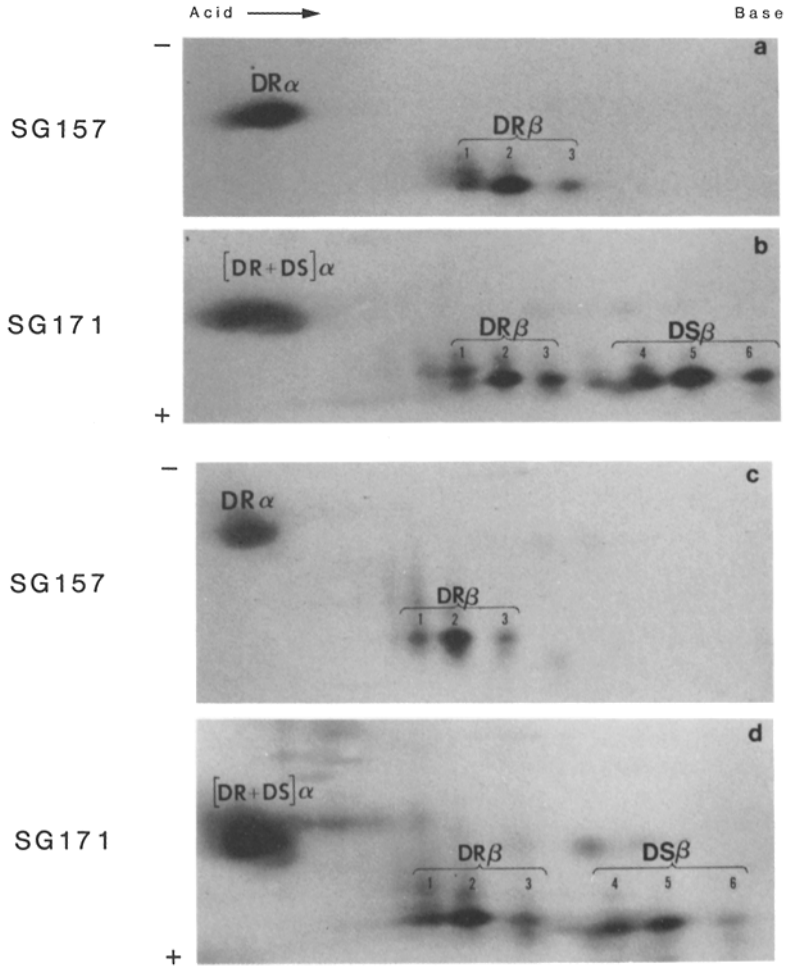

FIG. 6. 2-D gel analysis of proteins isolated from LG10(DR7) and LG36(DR7) with SG157 (a and c, respectively) and SG171 (b and d, respectively).

interactions among immunocompetent cells. Having biochemically defined two such families of molecules in mice, I-A and I-E, known collectively as Ia for immune-response associated, we extended our studies to man. We have previously shown that human HLA-DR molecules initially described by Springer et al. (41) are structurally equivalent to the murine I-E molecules. This paper describes the isolation and biochemical properties of the human equivalent to the murine I-A molecule, henceforth known as HLA-DS (for second D-region locus).

The two monoclonal antibodies used in this study, SG157 and SG171, display very different specificities for human Ia molecules. SG157 reacts with HLA-DR from all human cell lines tested (40) and with its structural equivalent in marmoset. In contrast, SG171 reacts with DS molecules from marmoset and from human cell lines that are DR7. Thus, SG171 is a polymorphic antibody in that it reacts with only one allotype of DS molecule (henceforth known as DS7); i.e., those associated with HLA-DR7 cell lines. However, the situation is complicated by the fact that SG171 also

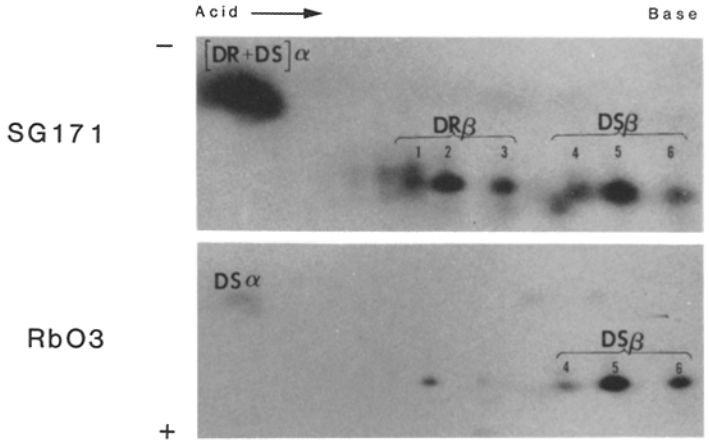

FIG. 7. 2-D gel analysis of proteins isolated from GM3163(DR7) with SG171 (top) and rabbit anti-marmoset I-A serum (bottom).

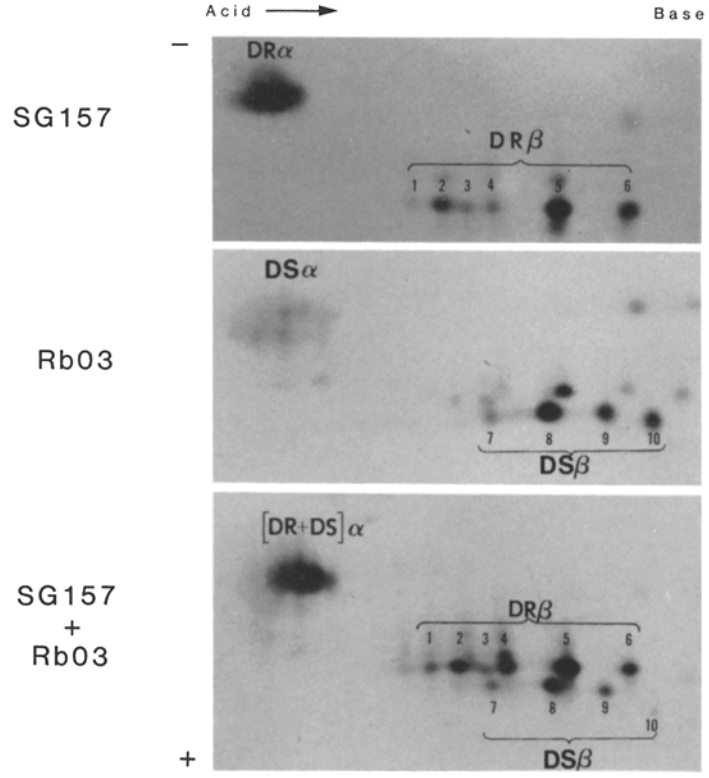

FIG. 8. 2-D gel analysis of proteins isolated from GM3107(DR2) with SG157 (a) or rabbit anti-marmoset I-A serum (b). Panel c represents a mixture of samples a and b and shows that, although DR2 and DS2 are very similar by this method of analysis, they are nevertheless distinguishable.

cross-reacts with HLA-DR molecules from all human cell lines, although this cross-reaction is not observed in the marmoset. Thus, some antigenic determinant or epitope is common to DS7 and all HLA-DR allotypes.

TABLE III

*Partial N-Terminal Sequences of HLA-DS Molecules Isolated with Rb03**

	Step number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
GM3163(DR7)α‡	E	D	I	V	A	D	H	V	A	A	Y	G	V	N	L	Y	Q	S	Y	G
LG10(DR7)α											Y					Y			Y	
GM3107(DR2)α			I	V				V			—					Y			Y	
GM3158(M)α§							F	V			F			F		Y	F			
LG10(DR7)β							F				F			—			F			
GM3107(DR2)β							F	V			F			—		Y	F			

* HLA-DS molecules were isolated from LG10(DR7) cells radiolabeled with [^{3}H]tyrosine and [^{3}H]phenylalanine and from GM3107(DR2) cells radiolabeled with [^{3}H]isoleucine, [^{3}H]valine, [^{3}H]tyrosine, and [^{3}H]phenylalanine, using Rb03 antiserum and protein-A agarose. DSα and β subunits were purified by SDS-PAGE and sequenced as described in Materials and Methods. Sequence identities are enclosed, and sequence differences are denoted by dashes.

‡ This sequence was obtained from protein purified with the monoclonal antibody SG171, as described in the text.

§ This was taken from ref. 34 and represents the marmoset I-A subregion-like molecule.

The allotype specificity of SG171 and its cross-reactivity with HLA-DR limited the usefulness of SG171 for identifying I-A-like molecules from other cell lines. Consequently, we prepared a monomorphic (recognizing all allotypes) antibody for DS by immunizing rabbits with purified I-A molecules from marmoset. Although the studies described here only demonstrate the reactivity of Rb03 with DS molecules from GM3107(DR2) and GM3163(DR7) cells, additional studies (unpublished) demonstrate that Rb03 also reacts with DS molecules from DR1, DR3, and DR4 cell lines.

Biochemical analysis of human DS molecules using both SG171 and the rabbit anti-I-A antiserum provides an explanation for the failure of others to detect it. Although the murine I-A and I-E molecules are easily discernible by SDS-PAGE when analyzed under appropriate conditions, the same is not true for their human equivalents. Based on 2-D gel analysis, the human DSβ subunits have virtually the same size as the DRβ subunits and are also closely related in charge. We were fortunate that the DS7 β subunit is quite a bit more basic than the DR7 β subunit, allowing us to easily distinguish the two. In contrast, the DS2 β subunit (from GM3107, a DR2 cell line) has virtually the same charge as the DR2 β subunit. As for the α subunits, the DS and DR α subunits are indistinguishable under the conditions used in this study. In view of the similarity in size and charge of human DR and DS molecules, it is quite likely that other investigators have produced monoclonal antibodies to DS and incorrectly deduced that they were directed against DR. Indeed, it should be pointed out that others have described the existence of multiple human Ia-like molecules (42–47); however, it is not clear whether these represent additional families of DR-like molecules or whether some of them are equivalent to the DS molecules described here.

The observation that SG171 reacts with DS molecules found only in conjunction with DR7 and that these DS7 molecules isolated from all three DR7 cell lines are identical strongly suggests that DS is closely linked to DR. This is entirely consistent with the genetic organization of murine Ia molecules, where the polymorphic (β) subunit of I-E molecules is tightly linked to I-A molecules (31, 32). Furthermore, the

observed polymorphism of DSα subunits is consistent with the observed polymorphism of murine I-A subregion α subunits (25). The existence of a second group of Ia molecules (DS) closely linked to DR but nevertheless structurally distinct may provide an explanation for observations suggesting nonidentity of the HLA-D locus (defined by MLR reactions) and the HLA-DR locus (defined serologically) (48–50), i.e., some HLA-D determinants may represent antigenic specificities expressed by the closely linked DS molecules. Biochemical analysis of Ia molecules isolated from such individuals should provide an answer to this enigma. Of equal importance is the question of whether the DS molecules defined here are equivalent to the DCl, MB, and MT molecules defined by others as Ia-like molecules closely linked to HLA-DR (51–53). Experiments directed at resolving this question are presently under way.

The cross-reaction of SG171 with DS7 and DR molecules is surprising in view of their structural dissimilarity. However, there are several other examples in the literature of cross-reactions between murine I-A and I-E molecules (54–56). These cross-reactions suggest that, despite apparent structural dissimilarity, the DS and DR molecules might have evolved from a common ancestral gene.

The cross-reactivity of DS7 with HLA-DR points out a dilemma confronted by the immune system of individuals possessing the DS7 allotype. If, as data in mice suggest, specific recognition of self I-A is a requisite for cell cooperation, then, clearly, the epitope seen by SG171 on DS7 cannot be used for specific recognition of the DS7 molecules by T cell receptors specific for self I-A. Otherwise, I-A restriction, i.e., recognition of self I-A molecules in an allotype-specific fashion, would not be observed among DS7 individuals. This reasoning, extended to its logical conclusion, argues that although a particular DS allotype may consist of several epitopes that distinguish it from other allotypes, only some of these may be used for recognition by T cell receptors. This proposal is supported by the observations of others (57, 58) demonstrating the nonequivalency of different epitopes on H-2K or Ia molecules recognized by H-2K or I region-restricted T cells. The functional significance and consequences of this sort of constraint placed upon the immune system is as yet unclear.

Summary

In mice, two families of structurally distinct Ia molecules, one designated I-A and the other I-E, have been identified and characterized. The HLA-DR molecules represent one family of human Ia molecules equivalent to the murine I-E molecules on the basis of amino acid sequence homology. We describe the isolation and biochemical characterization of a second family of human Ia molecules, designated HLA-DS for second D-region locus, equivalent to the murine I-A molecules. The human HLA-DS molecules consist of two polypeptide chains, DSα (37,000 mol wt) and DSβ (29,000 mol wt), with 73% amino acid sequence identity to the murine I-A molecules. Furthermore, the HLA-DS molecules are closely linked genetically to HLA-DR molecules, a situation analogous to that observed in mice. The similarity in molecular weights of the DR and DS molecules might explain why others have failed to identify the latter in man.

Note added in proof: Results of recent 2-D gel experiments (S. M. Goyert, M. Krumpton, and J. Silver, manuscript submitted for publication) indicate that the "supertypic" specificities, DCl and MT3, detected with the monoclonal antibody

SDR1 and the alloantiserum Hon 7, respectively, are present on the HLA-DS family of molecules. Futhermore, Bona and Strominger [*Nature (Lond.,)* in press] have found that the heavy chain of the DC1 molecule isolated with the monoclonal antibody Genox 3.53 is homologous to the heavy chain of HLA-DS molecules described above.

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