SEROLOGICAL AND IMMUNOCHEMICAL ANALYSIS OF THE PRODUCTS OF A SINGLE HLA DR-α AND DR-β CHAIN GENE EXPRESSED IN A MOUSE CELL LINE AFTER DNA-MEDIATED COTRANSFORMATION REVEALS THAT THE β CHAIN CARRIES A KNOWN SUPERTYPIC SPECIFICITY

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The products of the major histocompatibility complex (MHC)[1] class II genes are surface glycoproteins, consisting of noncovalently associated α and β chains, which are involved in the presentation of antigen to T cells during the immune response. The class II products are highly polymorphic and this polymorphism is the basis for the restriction of antigen presentation to T cells of the same haplotype, as well as for haplotype-specific variation of the immune response to different antigens (1). Thus, the polymorphic regions of class II products are implicated in interactions with T cell receptor and antigen. They also define the epitopes responsible for serologic and immunochemical specificities.

The region encoding the human class II or Ia antigens represents a complex genetic system of at least three subregions, HLA-DP, -DQ, and -DR, which have recently been further divided into multiple individual loci. There are currently estimated to be seven β chain genes (2) and five or six α chain genes (3, 4). This multiplicity at the genetic level correlates with the existence of multiple α and β chains at the cell surface (5, 6).

Because of this complexity, an understanding of the nature and functional role of each of these molecules and their correlation with established serological and immunochemical specificities has become an important challenge. One approach is based on the possibility of expressing individual class II genes independently by DNA-mediated transformation. We have reported (7) the construction of a mouse fibroblast cell line transformed with one of the DR-β chain genes, the DR-α chain gene, and the gene for the DR-associated invariant chain. This cell line synthesizes HLA-DR antigen and expresses it on the surface where it can be

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; cDNA, complementary DNA; DRpub3, DR public 3; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; SSC, standard sodium citrate.

identified with anti-DR antibodies. In this paper we analyze the DR antigen product of a single DR-β chain locus as expressed in these transformed mouse cells.

The polymorphism associated with the DR antigens is predominantly carried by the β chain. Two kinds of polymorphic determinants have been attributed to HLA-DR products: (a) the DR antigen proper, controlled by a highly polymorphic DR-β chain gene, of which at least 14 distinct allelic specificities have been described so far, and (b) supertypic specificities that are shared by groups of different haplotypes. Two of these supertypic specificities, BR3 and BR4, have been shown to be carried by β chains distinct from those carrying the DR specificity (8, 9). We show here that the product of the cloned DR-β gene used in the transformation of mouse cells (7) carries one of the supertypic specificities that has been attributed to a specific locus (8). This allows us to establish the correlation between a given serological specificity and the DR-β chain gene encoding that specificity. The evolutionary implications of these results are also discussed.

Materials and Methods

Sequence of the DR-β Chain Gene. The first, second, and transmembrane domains of the DR-β chain gene used for the transformation were subcloned in M13 phage vectors (10). Sequences were performed by the chain termination method (11). Only the first domain and transmembrane region were entirely sequenced.

Southern Blotting for Typing the DR-β Chain Gene Used for the Transformation. Southern blot hybridization was performed basically as described by Jeffries and Flavell (12). DNA from the cell line from which the DR-β chain gene had been cloned (typed as DR4,w6)[2] as well as DNA from (a) a DR4 homozygous typing cell, (b) a DRw6 homozygous typing cell, and (c) the genomic DR-β chain clone, were digested with Hind III, electrophoresed, and transferred to nitrocellulose. The blot was hybridized using a genomic fragment corresponding to the second domain and flanking intron sequences. Hybridization was in 4× standard sodium citrate (SSC) (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 5× Denhard's solution (1× Denhard's: 0.1% Ficoll, 0.1% polyvinyl-pyrilidone, 0.1% bovine serum albumin [BSA]) at 68°C. The final wash was in 0.25× SSC at 68°C.

Antibody-binding Assays. The transformed mouse fibroblasts and the parental DR4,w6 cell line were grown, harvested, and washed in phosphate-buffered saline (PBS)/BSA (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 0.2% BSA). They were then aliquoted into microtiter wells, pelleted, and reacted with the appropriate monoclonal antibody (mAb) for 2 h at 4°C with agitation. The cells were then washed and reacted with ^{125}I-labeled sheep anti–mouse Ig for 2 h at 4°C with agitation. After further washing they were resuspended and counted. All assays were performed in triplicate.

In Vivo Labeling and Analysis. Cells were in vivo labeled with [35S]methionine and lysed, and the lysates were reacted with the appropriate mAb. After precipitation using protein A–Sepharose, the precipitates were analyzed by two-dimensional electrophoresis as described (13). For the B cell line, the supernatants were then reprecipitated with the same mAb to assure that the first precipitation was quantitative, followed by a precipitation with the second mAb of the pair.

Analysis of Antigenic Specificities. An inhibition assay was used based on the reaction of antisera or antibodies with ^{125}I-labeled class II molecules. These were purified from the membranes of lymphoblastoid cell lines according to a previously described method (14).

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[2] The old DRw6 nomenclature is retained because the cells used have not been analyzed to determine their new typing. We use the local names BR3, BR4, and DRpub3 to describe certain supertypic specificities.

The following cell lines were used as starting material for class II molecule purification: U698M (DR2,4; DR3 = BR4; DQw1,3) and Daudi (DRw6; DR52 (DRpub3 + BR3); DQw1). Class II molecules from U698M were used either as such or after digestion with papain (EPD preparation). Such treatment has been shown to abolish the DR4 specificity (15). The untreated U698M preparation was used for testing DR4, the U698M-EPD preparation for testing BR4 (DR53).

Alloantisera were kindly provided by Dr. G. B. Ferrara, Genoa, Italy. Their specificity has been previously defined as follows: Fe59/15, anti-DR4 (16); Fe27/26, anti–DR7 + BR4 (17); Fe88/37, anti-MT2 (18). mAb MCS7 was also used, which recognizes an MT2-like specificity that is present exclusively on DR, and not BR, molecules (9). Rabbit antiserum anti–human Ia 7147 was raised against a preparation of Ia molecules purified from Daudi cells and has been shown to contain antibodies reactive with both isolated α and β chains (19).

The binding inhibition assay was performed as follows: cells were lysed with 2% Renex 30 at a cell concentration of 20 × 10^6/ml. Varying dilutions of each cell lysate were incubated with a fixed amount of antibody for 24 h at 20°C and again, for 24 h at 20°C, with an aliquot of ^{125}I-labeled class II preparation. The reaction mixture (100 μl) contained 0.075 M Tris-HCl, pH 7.8, 0.2% Renex 30, and 0.02% BSA. Then, sheep anti–human (or rabbit or mouse) Ig serum was added at equivalence and incubated 1 h at 37°C. The resulting immune precipitate was washed and the radioactivity determined. A control with normal serum was included in each assay. Results are expressed as the percentage decrease (percent inhibition) of maximum binding, i.e., the radioactivity precipitated in the absence of inhibitor.

Results

Amino Acid Sequence and Haplotype of the DR-β Chain Expressed in Transfected Mouse Fibroblasts. The use of cells transformed with a single cloned DR-β chain gene in biochemical, serological, and functional studies has the advantage that all the results can be referred directly to the protein sequence derived from the DR-β chain gene used for the transformation. We have determined that the nucleotide sequence of the exon encoding the polymorphic first domain of this DR-β chain gene is identical to that of a complementary DNA (cDNA) clone isolated from the same cell line which we had sequenced previously (20). The amino acid sequence of the first domain (Fig. 1) is extended to include the entire β chain by using the cDNA data.
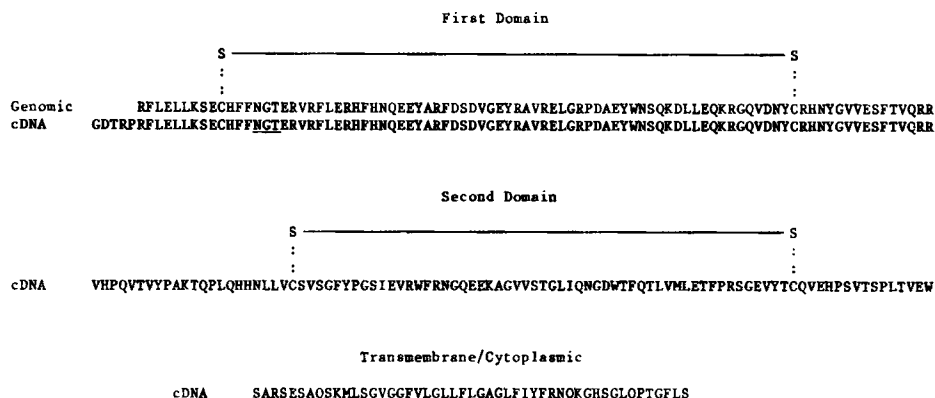

Figure 1. Amino acid sequence of the first domain of the DR-β chain gene used for transfecting mouse L cells. The amino acid acid sequence is extended using the published sequence of the cDNA clone (18) that corresponds to this gene.

Since the gene was cloned from a DR4,w6 heterozygous cell line, we used Southern blot hybridization to determine whether it belonged to the DR4 or DRw6 haplotype. DNA from a DR4,4 homozygote, a DRw6,w6 homozygote, or from the DR4,w6 cell line from which the gene was isolated, together with DNA from the cloned gene itself, was digested with Hind III, electrophoresed, transferred onto nitrocellulose, and hybridized with a probe specific for the second domain of the DR-β gene. The DR4 and DRw6 patterns can be easily distinguished (Fig. 2). The DR4,w6 pattern is the sum of the bands present in DR4 and DRw6 DNA. The gene used for the transformation experiment (Fig. 2A) gave a Hind III fragment characteristic of DRw6 and is therefore of the DRw6 haplotype. Neither DP-β nor DQ-β hybridized with the probe.

Analysis of the DR Gene Products Synthesized in Transfected Fibroblasts by mAb.
The immunochemical analysis of the products of a single DR gene expressed in mouse fibroblasts had two objectives, to investigate the specificity of anti-DR mAb and to establish the fidelity of the gene expression in transformed mouse fibroblasts.

There have been several reports (21–24) that certain mAb recognize different subsets of DR molecules. Since the members of these subsets could represent the products of different DR loci it was important to compare the reactivities of
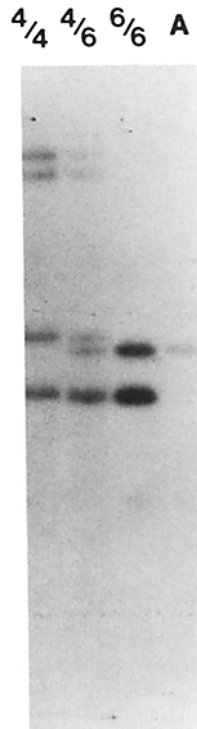

FIGURE 2.   Identification of the haplotype of the DR-β chain gene used in DNA-mediated L cell transformation by Southern blot analysis. DNA from reference cells, the DR4,w6 line, and the DR-β genomic clone were digested with HindIII, and analyzed by Southern blot hybridization. The typing of cells whose DNA was analyzed is given above each lane. (A) The DR-β genomic clone.

these mAb. We investigated two pairs of antibodies, L243 and L227 (21) and D1 and D4 (22), by cell-binding studies using iodinated protein A. D1 and L243 showed (Fig. 3) a concentration-dependent increase in binding to the transformed fibroblasts, homologous to the binding of the parental cell line from which the DR-β gene was derived. L243 is reported (21, 23) to be a DR-specific mAb whereas L227 only precipitates a subset of DR molecules. D1 and D4 are both reported to immunoprecipitate nonoverlapping subsets of DR molecules (22). Consequently, the product of this DR-β gene corresponds to the D1-precipitable subset but not to the L227 or to the D4 subsets. These results also confirm that the gene products expressed in mouse fibroblasts are recognized as true DR molecules.

To extend the correlation between the unique DR-β gene and the subsets defined immunochemically, the in vivo labeled products from the fibroblasts and the parental B cell line were immunoprecipitated and analyzed by two-dimensional electrophoresis. The choice of mAb, D1 and D4, was based on the results of the previous binding experiments. The results are shown in Fig. 4. The D1 mAb precipitated three intense β chain spots from the DR4,w6 line (Fig. 4, broad arrows), which were also precipitated from the transfected fibroblasts (T22$_9$). D4 precipitated five major β chain spots from the parental line, of which three migrated in the same position as those precipitated by D1. None of the D4-specific parental spots were seen in the transformed fibroblasts. Both the D1 and D4 mAb precipitated low amounts of protein from the transfected mouse fibroblasts that did not correspond to products made by the parental B cell line
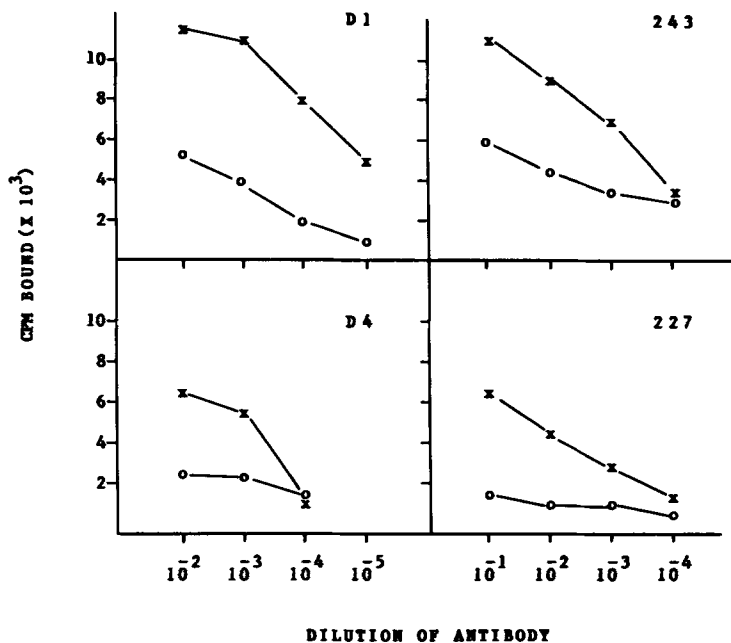

FIGURE 3.   Binding assays using the DR-transfected L cells and the parental DR4,w6 cell line. The mAb used are shown in the corner of each graph. The binding to nontransformed mouse fibroblasts (average, 500 cpm) has been subtracted from each point.
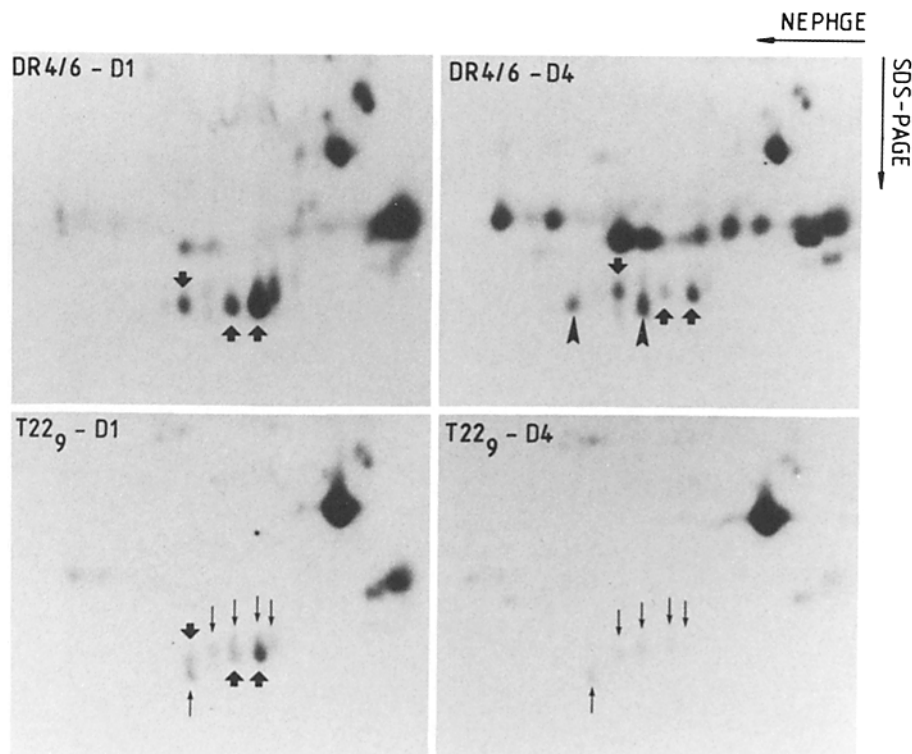
FIGURE 4.   Two-dimensional gel analysis of the immunoprecipitated products of in vivo labeled cells. The cell and mAb used are indicated. T22$_9$, DR-transfected mouse L cell line; DR4/6, B cell line from which the DR-β gene used for transfection was derived. The thick arrows point to spots present only in D4 precipitations, the medium arrows to spots present in both D1 and D4 precipitations, and the thin arrows to spots only precipitated from the transformed fibroblast, irrespective of antibody used.

(Fig. 4, thin arrows). These faint spots are probably posttranslationally modified DR-β chains not representative of DR-β chains found in B cells. The absence of dose-dependent binding of D4 to the transfected fibroblasts indicates that either these molecules are not present at the cell surface or they are responsible for the low-level nonspecific binding.

To ensure that the two mAb really distinguished nonoverlapping subsets of DR, the supernatants from the B cell immunoprecipitations were reacted with the second of the paired mAb (see Materials and Methods). When the D1 supernatant was precipitated with D4, the resulting 2D gel was identical to the direct D4 precipitation. Identical results were obtained with D1 (results not shown). This indicates that the three spots (Fig. 4, broad arrows) common to D1 and D4 represent nonoverlapping sets of DR molecules. The results with the transfected fibroblasts confirm this. If the spots precipitated from the B cell and the fibroblast (Fig. 4, broad arrows) are a subset of D4, then D4 should precipitate these same spots from the transfected fibroblasts. It does not. Therefore, the DR polypeptide encoded by the gene used here is precipitated by D1 and runs in the

same position on a 2D gel as another DR product found in the B cell line. This other polypeptide is precipitated by D4.

As in the case of the parental B cell line, we observed two α spots in the transfected fibroblasts although only a single α DR gene had been introduced into the cell. The lack of association of the invariant chain with D1-precipitable DR chains (Fig. 4) will be discussed in detail elsewhere (M. Strubin, J. Gorski, and B. Mach, manuscript in preparation).

Inhibition Studies for Typing the Transfected Cells and Correlation With Serological Supertypic Specificities. The transfected fibroblasts were used in an inhibition assay (Fig. 5) to determine their serological typing and to see if the DR-β gene present was responsible for any of the reported DR-associated serological super-specificities. The assay consisted of labeling surface Ia molecules from a target cell and determining whether the transfected fibroblasts inhibited the reaction of the antigen with a number of antisera or antibodies. For each series of experiments, the labeled antigen, and the sera or antibody with its specificity are given (Fig. 5). For each pair of antigen and serum, the inhibitors were the HHK cell line (DRw6,w6), the DR4,w6 parental B cell line, and the transfected fibroblasts. In Fig. 5*A*, the antigen was the Ia surface product of cell line U698M (DR2,4) and the antibody was a rabbit anti–human Ia serum with broad specificity for class II antigens. Both HHK and the parental line inhibited at equivalent concentrations (Fig. 5*A*). The transformed fibroblasts inhibited at a 10-fold greater concentration and a nontransformed mouse cell line showed negligible inhibition. The difference in concentration needed for inhibition reflects not only the different concentration of DR molecules on the cell membrane and the nonclonal nature of the transformed cells, but also the overall complexity of the Ia molecules on the B cells compared with the transfected fibroblasts.
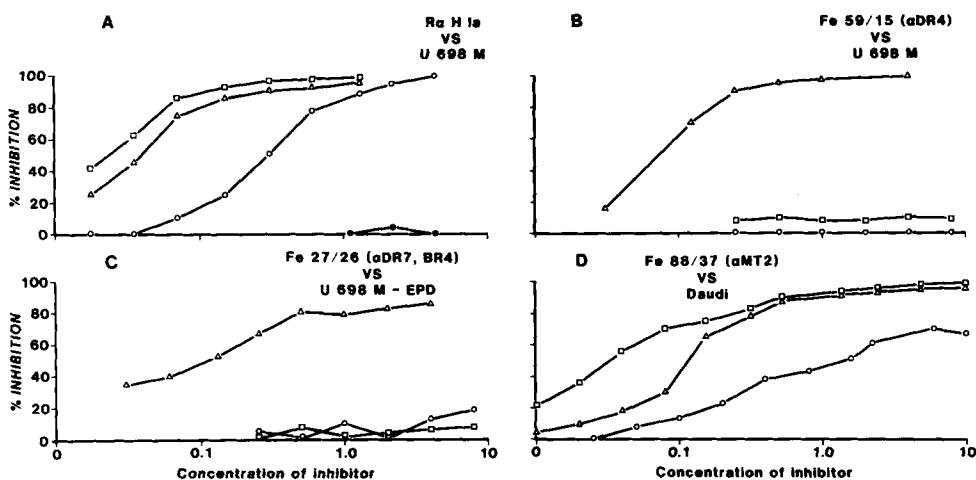

FIGURE 5. Test of antigenic specificity. Inhibition reactions performed as in Materials and Methods except that incubation times of the rabbit anti–human Ia serum (*R*α *H Ia*) (*A*) were shortened to 1 h at 37°C. The following volumes of antisera were used: rabbit anti-Ia (serum 7147), 0.016 μl; Fe59/15, 7 μl; Fe 27/26, 1 μl; Fe88/37, 3 μl. ~15,000 cpm aliquots of Daudi and U698M and 7,000 cpm of U698M-EPD were used. Inhibitors: HHK (DRw6,6) (□); DR4,w6 (△); 22/9 (transfected fibroblast) (○); untransfected fibroblasts (●).

Fig. 5B used the same DR4,2 antigen but an anti-DR4 serum. The inhibition was evident, as expected, only with the DR4,w6 parental cells.

Fig. 5C used papain-treated U698M cells as targets. The papain treatment destroys the DR4 determinant but leaves intact a supertypic specificity associated with DR4 and DR7, called BR4 (DRw53). The sera were anti-DR7 and -BR4. Again, only the DR4,w6 cell line acted as inhibitor to this test system, ruling against either a DR4 specificity or a BR4 superspecificity on the transformed mouse line.

In Fig. 5D, the target antigen was Daudi (DRw6) and the serum was anti-MT2 (DRw52). An anti-MT2 response indicated the presence of supertypic determinants DR public3 (DRpub3) and BR3. In fact, MT2 is composed of two specificities that have similar association patterns (DR3,5,w6,w8,w13) but are localized on different DR subsets (8). There was inhibition by the transformed fibroblasts (Fig. 5D), strongest with HHK, half as strong with the DR4,6. A 10-fold-increased concentration of the transfected fibroblasts was necessary to reach a plateau of inhibition. Interestingly, this plateau was not at 100%, implying that there are some determinants on Daudi that were not being blocked by the DR-expressing fibroblasts.

Since two superspecific determinants can be defined by an anti-MT2 response, it was of interest to see if the DR-β chain expressed on the fibroblast corresponded to the DRpub3 or BR3 superspecificity. mAb MCS7, directed against the DRpub3 determinant, was used in the inhibition assay shown in Fig. 6. The HHK and the parental lines were potent inhibitors, but the transfected mouse fibroblasts showed no inhibition. The product of the DR-β chain gene expressed in the mouse fibroblast thus corresponds to the BR3 superspecificity.

Discussion

Cells that, as the result of DNA-mediated transformation with cloned class II genes, express only one of the multiple human Ia products, are useful experimental models. They can be used to correlate antibody specificities to the products of individual loci and to study the functional aspect of individual class II genes. They can also provide a means of raising specific mAb for any single class II molecule.

The present work deals with the correlation of a single DR-β chain gene to
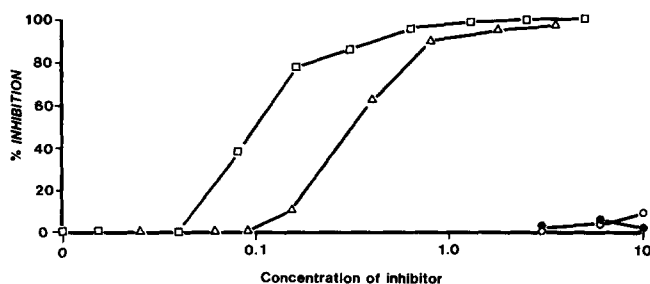

FIGURE 6.   Inhibition assay with MCS7 mAb. A 5% dilution of MCS7 culture fluid (concentrated fivefold) was used. 10 μl of normal mouse serum was added as carrier. The labeled antigen preparation (15,000 cpm) was from Daudi cells (DRw6). Inhibitors are as in Fig. 5.

different serological and immunochemical properties of DR-β chains. The DR-β chain gene used here is one of three DR-β chain genes present in the DRw6 haplotype and only one of at least seven class II β chain genes in the human MHC. We have determined (Fig. 1) the primary structure of the product of this DR-β chain gene and identified the gene as belonging to the DRw6 haplotype by both molecular and serological approaches.

The differential precipitation of the DR-β chain indicates that, at least for the DRw6 haplotype, some locus-specific products can be differentiated by mAb. This kind of analysis has been extended recently (25) to mAb of the panel from the First International Workshop on Monoclonal Antibodies to Human MHC Class II Antigens.

Our two-dimensional gel analysis of the products of the transformed fibroblasts showed that the three predominant forms of β chains expressed in the transformed fibroblast correspond to molecules present in the parental B cell. The faint spots, precipitated indiscriminately by either D1 or D4 mAb from fibroblasts but not from parental B cells, could reflect DR-β chains that result from a processing specific to mouse fibroblasts. From the low intensity of these spots it is clear that they are only a small portion of the products. One feature of this system is the fidelity with which most chains are processed in mouse fibroblasts. The presence of two α chain spots in both B cells and in fibroblasts transformed with a unique α chain gene indicates that these are alternate forms derived from one gene.

From the two-dimensional gel analysis, it is evident that the products of a single locus can give rise to multiple spots representing several forms of β chains. The gel pattern of the products of one DR locus was complex, and the complexity was much greater in experiments with normal cells where the products of all the DR loci were analyzed together.

The identity of the epitopes responsible for serological specificities and the distribution of these epitopes across the products of different DR-β chain loci are questions amenable to study using transformed cells. We have investigated the ability of DR-positive mouse fibroblasts to inhibit interactions between cells of known DR specificity and various sera or antibodies. DR4- or BR4-specific sera were not blocked by the fibroblast membrane preparations. This indicates that the gene used for transformation does not belong to the DR4 haplotype.

The specificities defined by the DRw6 haplotype were then analyzed. DRw6 cannot be considered as a defined antigen since antisera reacting specifically with DRw6 have not yet been found. Thus the only testable specificity was MT2, which is a supertypic specificity, that is, associated with several haplotypes, DR3, 5, w6, w8, and w12. Most supertypic specificities have been shown to be correlated with distinct class II subregions, either DR or DQ. MT2 has been more difficult to define. Markert and Cresswell (26) first recognized the existence of "MT2 + DR" and "MT2 only" molecules, which was subsequently confirmed (9, 27). MT2 thus can be considered as a bi-locus specificity with both loci probably belonging to the DR subregion (9). The two MT2 supertypic determinants have been called DRpub3 and BR3 (8). Our experiments have shown that the DR-β gene studied here encodes the MT2 supertypic specificity. The MT2 nature of this gene has been corroborated by binding studies (28) with 7.3.19.1,

a mAb directed against MT2. It remained to be seen whether this gene corresponds to the locus carrying the DRpub3 determinant or the BR3 determinant (8). Presently, the only way to differentiate between these two possibilities is based on reactivity with a polymorphic mAb, MCS7, that recognizes the DRpub3 determinant (9). The failure of transfected mouse cells to inhibit an MCS7 reaction indicates that the MT2-positive DR-β chain produced by these cells carries only the BR3 determinant. This is unambiguous evidence that the locus postulated to encode the BR3 determinant is one of the DR loci.

Our results, along with Southern blotting data of the various DR haplotypes, suggest a simple model for the evolution of supertypic families based on the existence of several DR-β chain loci that show different degrees of allelic polymorphism. Southern blot data (C. DePreval, G. Angellini, B. Boogh, G. B. Ferrara, and B. Mach, manuscript in preparation) has shown that the DNA from haplotypes within a supertypic group often share most and sometimes all the same restriction fragments. Such conservation of restriction sites argues heavily in favor of evolutionary relatedness. The present data assign a given supertypic specificity, BR, to a distinct DR-β chain locus. DNA restriction fragments associated with this gene were also seen in the DR3 and DR5 haplotypes. We postulate that all the DR haplotypes that presently define a given supertypic group arose from a common progenitor and that the different DR loci within these haplotypes subsequently diverged at various rates. This evolution may proceed by the addition of new determinants to those already present or by a concomitant destruction of old determinants during the mutation events that give rise to the newer determinants. The most polymorphic locus today defines individual DR specificities (e.g., DR3, DR5, and DRw6). It remains linked to other less polymorphic loci, which constitute the supertypic loci.

There are precedents for the existence of more or less polymorphic regions within the class II subregions of both mouse and man. In the mouse, a comparison of restriction maps of clones from different haplotypes shows regions of little or no polymorphism and other regions of high polymorphism (29). In man, genomic blotting analysis (30) indicates that the DR subregion shows a much higher level of restriction site polymorphism than the DQ region, whereas the DP region is relatively nonpolymorphic by this criteria (31). Therefore, it is likely that, even within the DR subregion, the different DR loci could show different degrees of polymorphism.

If the locus carrying the supertypic specificity is less polymorphic than other DR loci, one would predict that molecular analysis of other haplotypes within the MT2 supertypic group will show the existence of a DR-β chain gene that is very similar and perhaps identical to the one analyzed here. This gene should encode the protein carrying the BR3 supertypic specificity of these other haplotypes.

Summary

Using a mouse cell line transformed with and expressing a single HLA DR-α and DR-β chain gene, we present evidence that the product of the DR-β chain gene carries a supertypic determinant, BR3, previously defined by serology. The amino acid sequence of this β chain gene is determined from the DNA sequence.

Another DR-associated supertypic specificity defined by monoclonal antibody MCS7 was not encoded by this DR-β chain gene. This provides formal proof that a supertypic specificity can be associated with a product of a distinct DR-β locus. We propose that haplotypes sharing such specificities are evolutionarily related.

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