ANALYSIS OF HLA-DR GLYCOPROTEINS BY DNA-MEDIATED GENE TRANSFER

Definition of DR2β Gene Products and Antigen Presentation to T Cell Clones from Leprosy Patients

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The observation that T helper cells recognize antigen in the context of an MHC class II product has generated enormous interest in the structure and function of these cell surface glycoproteins. Studies demonstrating that, in many cases at least, the antigen recognized is in the form of a short peptidic fragment (1) and that such peptides bind directly to a single site on solubilized class II molecules (2), have clarified our understanding of the interactions between the T cell receptor (TCR), antigen, and class II product. These binding studies have suggested that some of the immune response (Ir) gene effects observed in mice of different H-2 type might reflect differences in the ability of their class II molecules to bind the relevant antigenic peptide (3). A more precise analysis of the molecular interactions between the class II product, antigen and TCR would be of obvious value in understanding the basis for MHC disease associations and in vaccine design.

Much work in murine experimental models has been devoted to the introduction by DNA-mediated gene transfer of individual MHC class II products into nonexpressing cells, so that the requirements for presentation of antigen by these molecules can be studied in a defined, manipulable system (4). Such studies have shown that transfected H-2 gene products can be expressed on the surface of mouse L cells and that this expression is sufficient to confer the property of antigen presentation upon the L cell, with respect to synthetic peptides, and in some cases, whole protein antigens (5, 6). Furthermore, analysis of the molecular basis of the interactions involved in presentation using exon-shuffled, transfected Ia molecules has led to the suggestion that separate sites may be responsible for peptide binding and for interaction with the TCR (7).

The HLA class II region encodes at least three expressed products; DP, DQ,

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and DR (8), each of these being highly polymorphic and capable of acting as restriction elements for T cell recognition (9). Given the association of many diseases with particular HLA types, and the view that products of the different subregions may perform differential roles in the immune response (10), the development of an in vitro system where the function of defined gene products can be examined in isolation is a desirable objective. While the cloning and sequencing of human, HLA class II genes has generated a large amount of information about the organization and polymorphism of the HLA class II genetic region, experiments reporting the use of DNA-mediated gene transfer to investigate the function of the encoded products have been limited (11, 12).

We have used transfection to analyze biochemical and functional aspects of the DRβ genes of the HLA-DR2Dw2 haplotype, an HLA type of considerable interest owing to its association with a number of diseases such as narcolepsy (13), Goodpasture's syndrome (14), tuberculoid leprosy (15), and multiple sclerosis (16). There are two expressed DRβ genes in DR2 haplotypes and on the basis of cDNA sequencing and biochemical studies, one appears to vary markedly between the cellularly defined subtypes of DR2 (Dw2 and Dw12) while the other is more conserved (17–19). These genes have been termed DR2β a (or β_1) and DR2β b (or β_2), respectively. The concomitant expression of these two genes, together with the lack of a clear serological distinction between their encoded products, has prohibited a clear understanding of their relative contribution to HLA serology, biochemistry, and function. Using cDNA clones, we have produced L cell transfectants separately expressing high levels of these two HLA-DR products in order to address these questions.

We report here the separate expression of cDNA clones representing the two DR2β genes in mouse L cells, allowing unequivocal assignment of the biochemically defined protein products to the individual cDNA sequences. Furthermore, such L cell transfectants expressing high levels of HLA class II products can efficiently present either peptidic or whole antigens to human T cells and have enabled us to define the restriction element used by T cell clones from DR2 patients with leprosy. This is a disease where, due to the association of different clinical symptoms with HLA-type (20), there has been considerable interest in defining the mycobacterial and HLA epitopes involved (21).

Materials and Methods

Isolation of cDNA Clones and Expression Vector Constructions. Double-stranded cDNA was prepared from 5 μg of poly(A)$^+$ mRNA (22) extracted from the B-lymphoblastoid cell line (B-LCL)[1] ROF (DR2Dw2, DR4Dw4). The cDNA was ligated using Eco RI linkers to Eco RI–digested bacteriophage λgt10 arms (Vector Cloning Systems, San Diego CA) and a library of 400,000 recombinants propagated on *Escherichia coli* NM514 cells. The cDNA library was screened by filter hybridization (23) using a radioactively labeled oligonucleotide probe specific for the 5′ end of DRβ genes. DRβ cDNA clones were sequenced in both directions by the chain termination method (24) and two full-length clones corresponding to the two expressed DRβ genes from the DR2 haplotype were identified.

[1] *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; HMT, hypoxanthine, methotrexate, and thymidine.

DRβ clones corresponding to the two expressed DR2 β chains (designated DR2β a and DR2β b) and a full-length DRα cDNA clone (donated by H. Ikeda, Imperial Cancer Research Fund, London) were ligated into the Eco RI site of the Moloney Leukemia virus–based cDNA expression vector pJ4 (Fig. 1) and subclones were restriction mapped for the orientation of the cDNA clones in the expression vector.

Transfection of DR2β a and DR2β b cDNA Clones into Mouse L Cells. The DR2β a/pJ4 and DR2β b/pJ4 constructs were separately cotransfected together with the DRα pJ4 construct and the plasmid pOPF (25) containing the HSV thymidine kinase gene, into mouse Ltk$^-$ cells using the calcium phosphate technique (26). Transfectants were grown in DME (Flow Laboratories, Rickmansworth, United Kingdom) supplemented with 10% FCS (Gibco Laboratories, Paisley, Scotland) and selected using HMT (10^{-4} M hypoxanthine, 10^{-5} M methotrexate, and 1.6×10^{-5} M thymidine). HMT-resistant colonies resulting from each transfection were pooled, stained with the pan-class II mAb SG465 (27), analyzed by flow microfluorimetry, and repeatedly sorted to obtain transfectants expressing high levels of cell surface expression of DRα/DR2β a and DRα/DR2β b heterodimers.

Flow Microfluorimetric Analysis. 10^6 cells were washed with cold PBS containing 5% FCS and incubated with 100 μl of antibody (diluted in PBS + 5% FCS) for 30 min on ice. mAbs were used either as neat supernatant, ascites diluted 1:100 or purified Ig at a concentration of 50 μg/ml. After two more washes in PBS + 5% FCS, the cells were resuspended in 50 μl of a 1:10 dilution of FITC-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Malvern, PA) and incubated for a further 30 min on ice. After two final washes, the cells were analyzed on the FACS (constructed at The Imperial Cancer Research Fund) with log amplifier.

Northern Blot Analysis. 20-μg samples of total RNA were loaded onto a 1% agarose gel containing 2.2 M formaldehyde (28) and electrophoresed for 20 h at 20 mA. Gels were blotted onto Hybond N (Amersham, Slough, United Kindgom) and probed with full-length DRα or DRβ cDNA probes. The labeled blots were then exposed overnight at −70° to Kodak XAR5 film.

Monoclonal Antibodies. The specificities of the mAbs used in the FACS analyses, T cell inhibition assays, and immunoprecipitations are as follows: SG465 (27), TDR31.1 (29), and CA2 (30): broadly reactive anti-HLA class II–specific mAbs. DA6.164 (31): anti-HLA-DR (except DR7). HU-30 (32): anti-HLA-DR2 and DR1. TAL1B5 (33): anti-DRα. B7.21 (34): anti-HLA-DP. Leu-10 (Becton Dickinson & Co., Mountain View, CA): anti-HLA-DQ. Genox 3/53 (35): anti-HLA-DQw1. W6/32 (36): anti-HLA class I. Leu-3a (Becton Dickinson & Co.): anti-CD4. MHM.23 (37): anti-LFA-1 β chain (CD18). MHM.24 (37): anti-LFA-1 α chain (CD11a)

Two-Dimensional Gel Electrophoresis. Briefly, 5×10^6/ml cells were incubated for 4 h in methionine-free medium supplemented with 10% FCS and 1 mCi/ml [^{35}S]methionine (Amersham) and then lysed in 5 mM Tris, pH 7.4/0.1 M NaCl/4 mM EDTA/0.5% NP-40. After 30 min on ice, the lysates were cleared of solid material by centrifugation, precleared twice with fixed *Staphylococcus aureus* (The Enzyme Center, Boston, MA), and incubated at 4°C with mAb TAL1B5 (33) before precipitation with rabbit anti-mouse Ig/*S. aureus*. Immunoprecipitates were dissolved in IEF sample buffer (9 M urea, 4% NP40, 2% 2-ME, 2% ampholine (pH 6–8, 80%; pH 3.5–10, 20%) at 50°C, centrifuged, and the supernatants were loaded onto the gel (38). The first dimension IEF was performed in tube gels in 4% polyacrylamide, 9.2 M urea, 2% wt/vol NP-40 and 2% ampholines. After focusing for 17 h at 800 V, tube gels were extruded and loaded for second dimension SDS-PAGE on 11% gels. Finally, gels were fixed, immersed in Amplify (Amersham), vacuum dried, and exposed to Kodak XAR-5 film.

Cells. The B-lymphoblastoid cell lines ROF-LCL (cDNA clone donor), REIZ-LCL (T lymphocyte clone donor [DR2Dw2, DR3]), and TOKUNAGA (TOK.LCL; homozygous DR2Dw12) were propagated in RPMI 1640 (Gibco Laboratories) + 10% FCS. The T lymphocyte clones 2F9 and 2F10 specific for *Mycobacterium leprae* antigen and restricted by DR2 have been extensively described elsewhere (39). Clone 2D6, also specific for *M. leprae* and restricted by DR2, was derived from a DR2Dw2,DR4Dw13 individual. The T

cells were propagated in Iscove's Modified Dulbecco's Medium (Gibco Laboratories), supplemented with 10% human serum and 10% Lymfocult T as a source of IL-2 (Biotest, Frankfurt, Federal Republic of Germany).

Proliferation Assays. For use in proliferation assays, transfectants grown to confluence were washed in RPMI 1640, resuspended at 5–10 × 10^6 in cells/ml in mitomycin C (Sigma Chemicals, Poole, United Kingdom) at a final concentration of 100 μg/ml, and incubated at 37°C for 1 h before washing several times. B-lymphoblastoid cell lines were irradiated with 80 Gy. 10^4 cloned T lymphocytes and 5 × 10^4 transfectants or B-lymphoblastoid cell lines were added to 96-well, flat-bottomed microtiter plates (Greiner, Nurtingen, FRG). Peptide (p17, a 24-residue *M.leprae* 65-kD antigen peptide added at 0.01 μg/ml unless otherwise indicated), recombinant 65-kD *M. leprae* antigen (Y4178 added at 10μg/ml [40]), or *M. leprae* antigen (soluble *M. leprae* antigen purified from armadillo according to Immunology of Leprosy (IMLEP) protocol 1-79 and obtained from IMLEP *M. leprae* Bank by courtesy of Dr. R. Rees; added at 1–20 μg/ml as indicated), were added as a source of antigen. PHA was used at a concentration of 4 μg/ml (Wellcome Diagnostics, Beckenham, United Kingdom). Some assays contained mAbs added as dialyzed and filtered ascites. Assays were set up in triplicate, cultured for 72 h with the addition of 1 μCi/well [^{3}H]thymidine (Amersham) for the final 6 h, and harvested on filter discs using an automated cell harvester, before adding scintillant and counting in a scintillation counter (G. D. Searle and Co., Chicago, IL).

Results

Isolation of HLA-DR2β a and DR2β b cDNA Clones and Transfection into Mouse L Cells. Two full-length cDNA clones were isolated that correspond exactly in sequence to the published DR2β a (or β_1) and DR2β b (or β_2) sequences for the DR2Dw2 homozygous cell line PGF (18, 19). These cDNA sequences will be referred to as DR2β a and DR2β throughout (Fig. 1 a). The DR2β a and DR2β

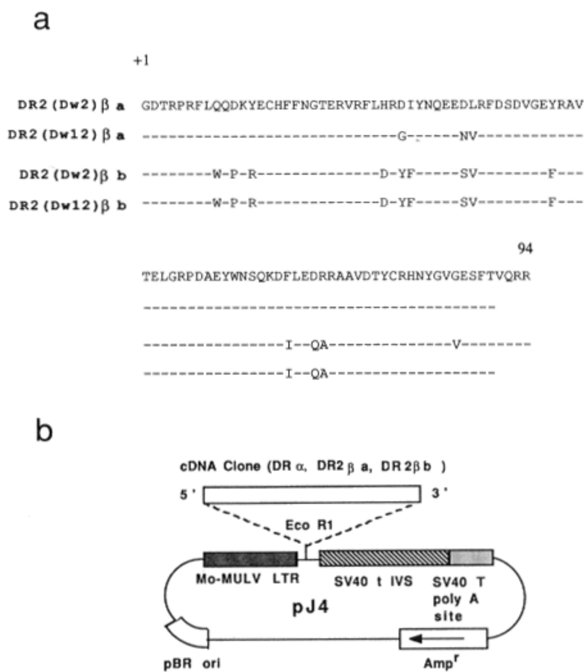

FIGURE 1. β1 domain amino acid sequences of the cDNA clones DR2β a and DR2β b from the DR2 haplotype and subcloning of these sequences into the expression vector pJ4. (*a*) The sequences of the cDNA clones isolated are identical to the published sequences for the DR2β a and DR2β b cDNA clones from the DR2Dw2 haplotype (18, 19). The equivalent sequences of the DR2Dw12 haplotype are shown for comparison. Sequences are shown using the one-letter amino acid code. (*b*) Both cDNA clones and a cDNA clone for DRα (sequence not shown) were subcloned into the cDNA expression vector pJ4, which contains the Moloney murine leukemia virus long terminal repeat (MoMULV LTR) as promoter, the SV40 small t intervening sequence (SV40 t IVS), and SV40 large T polyadenylation site (SV40 T poly A site).

b cDNA clones were subcloned into the Moloney leukemia virus-based expression vector pJ4 (a vector shown to give high levels of transcription in L cells, our unpublished observations) and then separately transfected into mouse L cells together with a DRα/pJ4 construct (Fig. 1 *b*). The resultant transfectant cell lines, LDR2a and LDR2b, were subjected to four rounds of flow fluorimetric sorting to obtain cells expressing high levels of surface expression (>50% of an EBV-transformed B-LCL, as measured by flow microfluorimetry) of the transfected gene products. Southern analysis confirmed the presence of both DRα and β gene sequences in genomic DNA from the transfectants and Northern analysis demonstrated the presence of high levels of RNA transcripts from both genes (data not shown).

Flow Microfluorimetric Analysis of L Cell Transfectants Expressing Products of the HLA-DR2β a and HLA-DR2β b Genes. Analysis of the LDR2a and LDR2b transfectants using flow microfluorimetry and a panel of anti-HLA class II-specific mAbs clearly shows them to be expressing HLA class II products (Fig.2), as evidenced by their binding of the pan-HLA class II mAbs SG465 and CA2 and more specifically, HLA-DR products, as indicated by their binding of the HLA-DR-specific mAb DA6.164 and lack of reactivity with B7/21 (anti-HLA-DP) and Leu-10 (anti-HLA-DQ). Of all the mAbs tested, TDR31.1 was the only mAb to discriminate between the transfectants, having a higher affinity for the DRα/DR2β a heterodimer. Interestingly, this mAb has been shown to recognize deter-

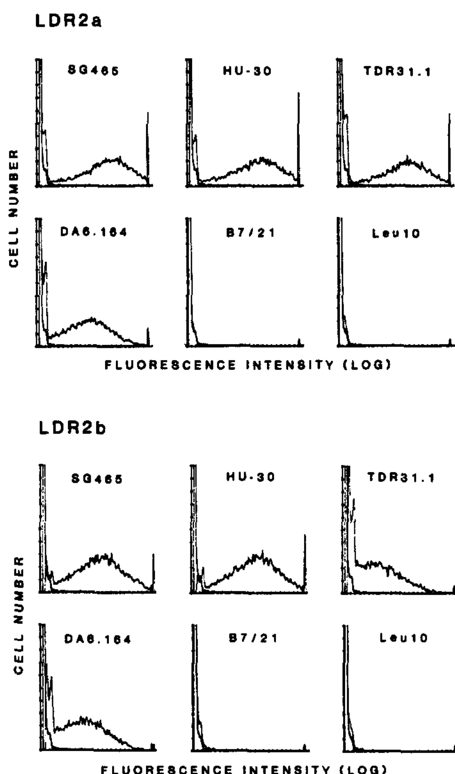

FIGURE 2. Flow microfluorimetric analysis of the transfectant cell lines LDR2a and LDR2b (which express cell surface DRα/DR2β a and DRα/DR2β b heterodimers, respectively) using a panel of anti-HLA class II mAbs. The reported specificities of the mAbs shown are: SG465 (anti-HLA-DP/DQ/DR), TDR31.1 (anti-HLA-DP/DR), DA6.164 (anti-HLA-DR, except DR7), HU-30 (anti-HLA-DR2 and DR1), B7/21 (anti-HLA-DP), and Leu-10 (anti-HLA-DQ). 10^4 cells were analyzed in each profile and results are plotted as log fluorescence intensity (abscissa) against cell number (ordinate). In each profile, the negative control is the transfectant cell line stained with FITC-rabbit anti-mouse Ig alone.

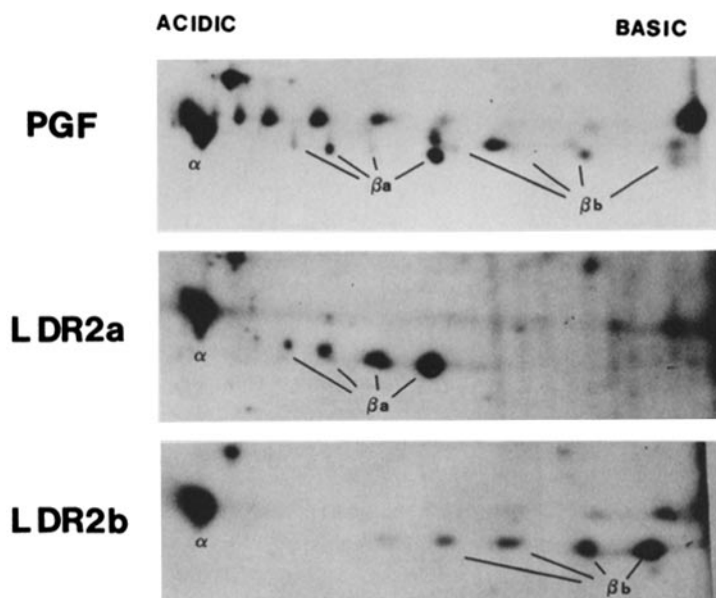

FIGURE 3. Two-dimensional gel analysis of the immunoprecipitated products from the biosynthetically labeled cell lines PGF (homozygous DR2Dw2 B-cell line), LDR2a, and LDR2b, using the anti-HLA-DRα mAb TAL1B5. Isoelectric focusing was performed as the first dimension and SDS-PAGE as the second. The coprecipitated α and β spots are labeled. The set of spots corresponding to the β chain that is electrophoretically variable among DR2 subtypes is designated β a, and those corresponding to the electrophoretically constant β chain are designated β b.

minants on the β1 domain (Jill Maddox, personal communication). The fact that HU-30 bound to both transfectants is surprising in the light of previous immunoprecipitation and two-dimensional (2D)-PAGE studies which suggested that this mAb is specific for the DRα/DR2β b heterodimer (41, 42).

2D-PAGE Analysis of the DR2β a and DR2β b Chains. 2D-PAGE analysis of biosynthetically labeled extracts from the DR2Dw2 homozygous B-LCL PGF after immunoprecitation with the DRα specific mAb TAL 1B5, reveals the presence of two coprecipitated β chains (Fig. 3). The electrophoretic mobilities of these two expressed DR2 β chains are related to the DR2 subtype: the more acidic a (or β_1) chain has a variable position of migration dependent upon the Dw type of the cell line, whereas the b (or β_2) chain has an invariant position of migration irrespective of the Dw type (18, 19, 42). When TAL1B5 immunoprecipitates from the LDR2a and LDR2b transfectants were examined, the DRα chain and a set of spots corresponding to the different glycosylated forms of the β chain were clearly visible. Comparison of the localization of the DRβ chains in PGF and in the transfectants unequivocally shows that of the two β chains present in PGF, the more acidic, variable β chain is the only β chain present in the LDR2a transfectant, the more basic, constant β chain the only one present in the LDR2b transfectant. The variable β chain can thus formally be defined as the product of the DR2β a cDNA sequence and the more constant β chain as that of the DR2β b sequence, as has been proposed on the basis of biochemical and sequencing studies (18, 19).

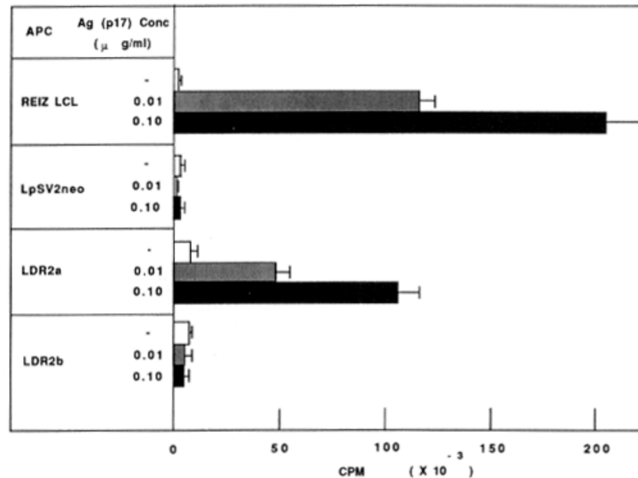

FIGURE 4. DR2 transfected L cells present *M.leprae* peptide to T cells. L cell transfectants and the autologous B-LCL (REIZ) were tested as APC for the T cell clone 2F10. Bars represent the standard errors from the mean of triplicate cultures.

DR2-Transfected L Cells Present Peptide to T Lymphocyte Clone 2F10. T lymphocyte clone 2F10, derived from a tuberculoid leprosy patient is specific for the *M.leprae* 65-kD antigen and has been shown from mapping with B-LCL and antibody blocking to be DR2 restricted (39). The clone recognizes a peptide, p17, synthesized from the 65-kD antigen sequence and initially localized by screening 2F10 on deletion mutants of the 65-kD antigen expressed in *E.coli* (Thole, G., et al., submitted for publication). Five other peptides from the 65-kD sequence shown to be stimulatory for T lymphocyte clones (TLCs) restricted by other DR alleles were not recognized by 2F10. TLC 2F10 responds equally well to antigen whether presented by the autologous B-LCL, REIZ(DR2Dw2, DR3), by the cDNA donor B-LCL ROF(DR2Dw2, DR4) (data not shown) or by the DR2Dw12 homozygous B-LCL TOK (39). Since the Dw2 and Dw12 subtypes of DR2 are largely similar with respect to the DR2β b sequence but also show important regions of sequence homology in the DR2 β a gene (18, 19), these B-LCL presentation studies could not define the DR2β chain used by 2F10. We were therefore interested to see whether either of the L cell transfectants could present antigen to this clone.

When the two DR2 transfectants were tested for the ability to present p17 to clone 2F10, LDR2a was shown to present efficiently (Fig. 4). The LDR2b transfectant, in spite of expressing a similar level of surface DR, had no stimulatory effect on the T cells, generating a response as low as the mock transfectant. This inability to present was not overcome by increasing the peptide concentration 10-fold to 0.1 μg/ml. Thus, DR2-transfected mouse L cells can present peptide to human T cells and clone 2F10 is restricted by the DRα/DRβ a heterodimer. Antigen presentation by the LDR2a transfectant and the autologous B-LCL were then compared with respect to susceptibility to mAb blocking (Fig. 5). Presentation to 2F10 by the B-LCL was largely abrogated by the class II framework mAb, CA2, with the anti-CD4 mAb Leu-3a able to suppress the response by ~60%. Antibodies against the LFA-1α (CD11a) and LFA-1β (CD18) chains blocked the response almost as well, the anti-HLA-DQ antibody Genox 3/53

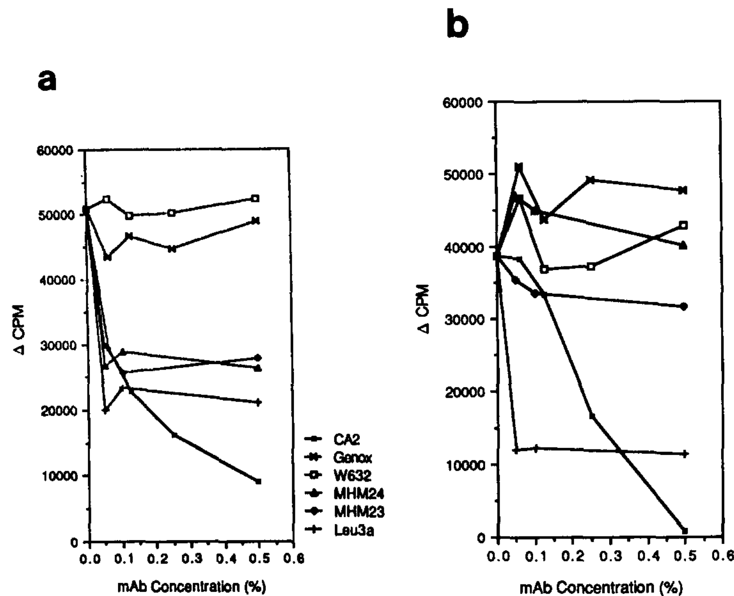

FIGURE 5. Antibody blocking of presentation to clone 2F10. The autologous B-LCL (REIZ) (*a*) or the LDR2a transfectant (*b*), were tested for presentation of p17 (added to cultures at 0.01 μg/ml) in the presence of mAbs at the indicated concentrations. Results are given as Δ cpm, that is [^{3}H]thymidine incorporation in the presence of peptide minus incorporation by T cells plus APC in the absence of peptide. (*a*) Background incorporation in the absence of peptide was 803 ± 232 cpm and the specific response was 51,802 ± 3,724 cpm. (*b*) background incorporation was 1,998 ± 259 cpm and the specific response was 40,744 ± 1,823 cpm.

and the anti-class I antibody W6/32 having no effect. Presentation by the transfectant was almost totally blocked by CA2, and Leu-3a inhibited the response by ~70%. MHM 24 (anti-CD11a) had no effect, while MHM23 (anti-CD18) caused a marginal reduction in the response. Thus, in the case of clone 2F10, presentation by the transfectant is inhibitable by blocking of the DR molecule or of CD4 but is apparently not dependent on any interactions between LFA-1 on the T cell and molecules expressed on the L cell.

Presentation of M. leprae and Recombinant 65-kD Antigen by the LDR2a Transfectant. Since studies with L cells transfected with mouse class II genes have differed in their conclusions regarding the ability of the L cells to process native antigens (5–7), we then tested the ability of the LDR2 a transfectant to present nonpeptide antigens. As can be seen in Table I, the L cells were able to present both the 65-kD recombinant *M.leprae* antigen and the mycobacterium to clone 2F10. Since previous experiments with *M.leprae* in this system showed that it could be presented by paraformaldehyde-fixed B-LCL only if the cells were pulsed with antigen before fixation, it can be concluded that the antigen is one that requires processing and that these preparations are not contaminated by immunogenic quantities of breakdown peptide (43). Interestingly, the L cell transfectants were unable to act as accessory cells in the response of clone 2F10 to PHA. However, we observed heterogeneity with respect to mitogenic stimu-

TABLE I
Presentation of Native M.leprae Antigen, Recombinant 65-kD M.leprae Antigen, and Peptide to T Cell Clone 2F10 by the LDR2a Transfectant

APC	Medium	*M.leprae**	65-kD antigen‡	Peptide§	PHA‖
REIZ.LCL	1,431 + 261	15,082 ± 1,803	26,001 ± 818	88,664 ± 2,678	28,358 ± 1,460
LDR2a	4,597 ± 431	26,001 ± 818	49,809 ± 1,789	185,173 ± 12,540	2,831 ± 334
LDR2b	3,117 ± 1,563	1,891 ± 452	4,090 ± 296	2,493 ± 386	1,795 ± 218
LpSV2neo	213 ± 212	47 ± 16	99 ± 16	53 ± 4	813 ± 125

Results represent the means of triplicate cultures ±SE.
* *M.leprae* antigen was added to cultures at 20 μg/ml.
‡ 65-kD recombinant antigen was added to cultures at a final concentration of 10 μg/ml.
§ Peptide p17 was added to cultures at a final concentration of 0.1 μg/ml.
‖ PHA was added to cultures at a final concentration of 4 μg/ml.

lation, some T cell clones responding to PHA in the presence of either transfected or untransfected L cells (data not shown).

The DRα/DR2β a Heterodimer Appears to be the Dominant Restriction Element Used by DR2-restricted T Cells. The transfectant cell lines LDR2a and LDR2b were then tested for their ability to present antigen to other DR2-restricted T cells. T cell clone 2F9, which is derived from the same individual as 2F10, is also DR2 restricted, and recognizes the 36-kD antigen of *M.leprae* (Elrerink, D., and R. de Vries, unpublished observations). Clone 2D6 derived from a DR2Dw2,DR2Dw13 individual is also DR2 restricted, and is specific for *M.leprae.* Both of the anti-*M. leprae* T cell clones respond to *M.leprae* antigen when presented by the LDR2a transfectant but not the LDR2b transfectant (Table II). Thus, of the three T cell clones we tested from two different individuals and specific for two different *M.leprae* antigens, all used the DR2β a chain as their restriction element. Experiments in another system, the response to *Plasmodium falciparum* circumsporozoite peptides by DR2-restricted T cell clones, similarly showed that 4 of 4 clones used the DR2β a chain for presentation and none the DR2β b chain (Altmann, D., D. Wilkinson, and F. Sinigaglia, unpublished observations).

TABLE II
Use of DR2β a Restriction Element by DR2-restricted T Cell Clones

Clone	Donor HLA DR	APC	Medium	Antigen*
2F9	DR2Dw2,DR3	REIZ.LCL	1,290 ± 48	9,499 ± 698
		LDR2a	4,951 ± 3,036	41,506 ± 1,954
		LDR2b	1,937 ± 402	3,488 ± 257
SC2D6	DR2Dw2,DR4,Dw13	SC MNL‡	123 ± 38	45,977 ± 9,655
		LDR2a	4,695 ± 469	14,523 ± 3,051
		LDR2b	7,395 ± 296	6,928 ± 277

Results represent the means of triplicate cultures ±SE.
* *M.leprae* antigen was added to cultures at a final concentration of 1 μg/ml
‡ Autologous mononuclear leucocytes were used as APC.

Discussion

Numerous studies on the transfection of mouse MHC class II genes into H2 class II–negative cells have generated a wealth of information on the structure and function of these cell surface glycoproteins (reviewed in reference 4). In contrast, relatively little work has been reported on the use of DNA-mediated gene transfer to investigate the function of human, HLA class II genes (8, 9). Although much of the information gained from the murine system is relevant to the study of human MHC class II structure and function, certain aspects of the biology of HLA make an independent study of the function, serology, and biochemistry of HLA class II products an important endeavor. Specifically, the immunological role of these products in resistance to human diseases and the basis of HLA/disease associations are questions of both fundamental and clinical relevance. The very complexity of HLA makes the study of the encoded products a challenging task. At least three products are expressed from the class II region, HLA-DP, DQ, and DR, with two different DR products being expressed in most haplotypes (10). The linkage disequilibrium of some of these genes, together with the scarcity of allele-specific mAbs, makes the contribution of the individual gene products to HLA function less than unequivocal. The DR2 haplotype exemplifies these problems: from biochemical and sequencing studies, there are clearly two DRβ genes expressed, but the relative importance of these two products as restricting elements for class II–restricted, antigen-specific T cells is obscure. The transfection of defined HLA class II genes into cells not normally expressing these products, is a means of dissecting this complexity and offers the opportunity of studying unambiguously the functional, biochemical, and serological characteristics of their encoded products.

An essential requirement for the work presented here was to produce transfectants functionally capable of presenting antigen to human T cells. Studies by Austin et al. (12) demonstrated that mouse L cells transfected with HLA-DP genes could present antigen to an HLA-DP-restricted T cell clone, while another report observed that L cells transfected with HLA-DRw52 were not capable of presenting antigen to human T cell lines (11). Such seemingly contradictory data may hinge on the observation that there is a quantitative dependence of T cell proliferative responses on the density of class II products on the APC surface (5, 44), and that individual T cell clones differ in their requirement for accessory molecule interactions (45). We have used L cells as the recipient cells for transfection of HLA class II genes, due to their class II–negative phenotype, and also because the use of mouse cells offers the possiblity of using stepwise gene transfection to explore the roles of other human gene products (e.g., LFA-1) in antigen presentation. Given the importance of the level of class II expression on the APC, we experimented with different cDNA expression vectors in order to generate transfectants stably expressing high levels of the transfected gene products. The mouse Moloney leukemia virus long terminal repeat (LTR)–based vector pJ4 is very efficient in L cells, giving significantly higher levels of cell surface expression of class II genes than SV40-based vectors, in both transient and long-term transfections (our unpublished observations). Transfection of L cells with HLA class II α and β genes cloned into pJ4 followed by microfluorimetric sorting and single cell cloning has enabled us to produce transfectants expressing

HLA class II products at a level approaching that seen on human B-LCL.

Transfectants expressing a single defined HLA class II product represent an ideal means of defining unequivocally the specificities of anti-HLA class II mAbs, an exercise normally made difficult by the expression of multiple products from the class II region. When the two transfectants LDR2a and LDR2b were screened with a panel of anti-HLA class II mAbs, the only mAb to distinguish between the transfectants was the mAb TDR31.1, and even in this case the mAb bound to both, but with a higher affinity for the LDR2a transfectant. This observation serves to illustrate the present limitations in the human system of using mAbs as reagents for functional analysis of class II products. Although many mAbs have been generated that are broadly reactive with HLA class II products, comparatively few allele-specific mAbs are available, presumably because the polymorphic regions of human class II molecules are not the most immunogenenic in the immunized mouse.

Although serologically similar, the migratory properties of the DR2β a and DR2β b products when analyzed by 2D-PAGE are very different, the DR2β a protein having a more acidic isoelectric point than the DR2β b protein. Comparison of the 2D gel patterns obtained for the DR2Dw2 B-LCL, PGF, and the LDR2a and LDR2b transfectants clearly demonstrates that: (*a*) The individual β chains in the transfectants have electrophoretic mobilities indistinguishable from the β chains in the human B cell, suggesting that there are no gross differences in posttranslational modification of the human proteins when expressed in human and mouse cells, in agreement with previous observations (46); and (*b*) the more acidic set of β spots in the DR2Dw2 B-LCL immunoprecipitate are encoded by the DR2β a sequence, and the more basic set of spots by the DR2β b sequence. This result is a formal proof of the predictions made from studies correlating cDNA sequences with electrophoretic mobilities for the DRβ genes of the DR2 haplotype (18, 19).

Leprosy is a disease where infection of different individuals with the same bacillus leads to a wide range of clinical phenotypes, from lepromatous leprosy, where there is a state of immunological unresponsiveness, to tuberculoid leprosy, where there is a state of immunological hyperresponsiveness (47). The observation that the type of pathology developed is at least partly dependent upon the HLA type of the individual (20) makes leprosy an exciting model for looking at human Ir gene effects. The DR2 allele is overrepresented in both tuberculoid (15) and lepromatous patients, the association in the latter group possibly reflecting linkage disequilibrium with DQw1(48). Given these associations and the clear evidence of pathology caused by a problem of immune regulation, there has been enormous interest in mapping the epitopes on *M. leprae* seen by T cells from leprosy patients and the association of *M. leprae* antigens with HLA class II molecules (39, 40) with a view to advanced vaccine design (21). Substantial progress has been made in the analysis of epitopes on mycobacterium recognized by DR2-restricted T cell clones (49), but precise mapping of the interaction between peptide, HLA class II molecule, and T cell receptor has been hampered by the inability to identify unambiguously the restriction element used by the T cell.

Studies using homozygous B-LCL as APC for clone 2F10, a T cell specific for

a peptide (Anderson, D., et al. manuscript in preparation) from the 65-kD antigen of *M.leprae* in association with DR2Dw2 showed that Dw2 or Dw12 B cells served equally well for presentation. Thus, it was unclear whether the restriction element for presentation of the peptide lay on the DR2β a or DR2β b chain. Our attempt to answer this question using transfectants independently expressing each of the expressed DR2 products definitively showed that the LDR2a transfectant presented peptide efficiently to clone 2F10, while the LDR2b transfectant was unable to do so. Thus, in spite of the ability of Dw2 and Dw12 B cells to present antigen to clone 2F10, it is the more variable DR2β a chain that acts as the restriction element. Comparison of β1 domain sequences of DRβ alleles shows that there are essentially three areas of hypervariability, these being in the regions of amino acid residues 4–14, 25–33, and 67–74. The Dw2β a and Dw12β a chains have similar sequences in the first and third hypervariable regions, but differ markedly in the second. Thus, it is likely that the ability to bind and present peptide to clone 2F10 by both Dw2 and Dw12 B-LCL is dependant upon residues in the first and/or third hypervariable regions. In this connection it is noteworthy that the DR2 B-LCL AZH, which has undergone a recombination event such that its β a chain is identical to the Dw2/Dw12 β b chain in the third hypervariable region, is unable to present peptide to this clone (de Vries, R., D. Elferink & N. Reinsmoen, unpublished observation).

Of two other anti–*M. leprae* clones known to proliferate in response to antigen presented by DR2 cell lines, both use the DR2β a chain as restriction element. Similarly, our more recent observations on DR2-restricted T cell clones against a *P. falciparum* peptide, together with the studies of Jacobson et al. (50) who correlated the more electrophoretically variable DRβ chain in DR2 haplotypes with the restriction element used by DR2-restricted, measles-specific CTL, implicate the DR2β a chain as the dominant DR2β restriction element. The role of the DR2β b chain in DR2 restriction may thus be analogous to the DRw52 and DRw53 supertypic specificities, which probably as a consequence of their low cell surface expression compared with the more variable DRβ chain products (51), rarely function as T cell–restricting elements. In a similar vein, our preliminary studies on alloreactivity using the LDR2a and LDR2b transfectants suggest that the DRα/DR2β a heterodimer is the immunodominant DR product in the mixed lymphocyte response (data not shown).

The ability of the LDR2a transfectant to present peptide to clone 2F10 unequivocally demonstrates that expression of an HLA-DR α/β heterodimer on a mouse L cell is necessary and sufficient for recognition by the T cell. Furthermore, the response elicited was of a similar magnitude whether using the B-LCL or transfectant. As previously reported for presentation of antigen by I-A-transfected L cells to mouse T cell clones, presentation by LDR2a to clone 2F10 led to specific cytolysis of the L cell (52), although we have been unable to detect killing in short-term ^{51}Cr-release assays (data not shown).

Our antibody blocking studies showed that presentation by the transfectant could be blocked by the anti-class II framework mAb CA2 or by an anti-CD4 mAb but not irrelevant mAbs (anti-DQ, anti-HLA class I). The similar pattern of CD4 blocking seen on transfectants and B cells corroborates the findings of others using HLA-DR transfectants that the HLA class II molecule is the ligand

for CD4 and that this interaction plays an important part in the interaction between $CD4^+$ lymphocytes and Ia^+APCs (53). While presentation by the B-LCL was significantly inhibited by mAbs against CD11a (LFA-1α) or CD18 (LFA-1β), these mAbs had no effect on presentation by the transfectants. This is in line with the notion that the function of LFA-1 in T cell/APC interactions represents a nonobligatory means of enhancing intercellular avidity (54), and that the ligand for LFA-1, probably I-CAM-1, is not expressed on the surface of the L cell. These observations confirm those made in studies of presentation by mouse transfectants to mouse T cell clones (5, 54). While it is hard to reconcile this view with the findings of Cowan et al. (55) who reported inhibition of human CTL lysis of mouse HLA-A3 transfectants with antibodies to LFA-1, it could be that even weak interactions resulting from binding of human LFA-1 to xenogeneic ligands on the L cell are important in the adhesion steps leading to cytolysis.

Experiments using mouse T lymphocytes and mouse Ia transfectants have not produced a clear consensus as to whether L cells can serve all the normal antigen processing requirements of a conventional APC. While studies using some whole protein antigens, for example keyhole limpet hemocyanin, have suggested that L cells can indeed process antigen normally (5), other antigens, for example hen eggwhite lysozyme, were only recognized when presented as synthetic peptides (6). We found the LDR2a transfectants were efficient at presenting synthetic peptide, recombinant 65-kD *M. leprae* antigen or whole *M. leprae.* Antigens that can be presented in this way by cells that are not of any specialized antigen presenting lineage might be expected to be in the majority, since the cathepsins which have been implicated in lysosomal degradation of protein antigens by conventional APCs (56) are also found in many other cell types, including L cells (57).

Despite the efficient presentation of antigen to established T cell clones, we have so far found it difficult to demonstrate antigen presentation to freshly isolated T cells or stimulation of a primary MLR by the transfectants. This is somewhat reminiscent of earlier models looking at L cell presentation to mouse T cells where primary MLRs, when detectable, have been low, and most work has been done with T cell hybridomas (5). Furthermore we found that some *M.leprae* T cell clones which from panel studies on HLA-typed APC were expected to be restricted by one of the two DR products from the DR2Dw2 haplotype, failed to proliferate to antigen when presented by either LDR2a or LDR2b. Given that these transfectants express high levels of HLA class II products and are capable of presenting native antigens to human T cells, this observation may be a reflection of a heterogeneity amongst human T cells in terms of TCR affinity and dependence upon accessory molecule function. We are currently investigating this notion by introducing ICAM-1 (58), a known ligand of LFA-1, into the transfected cell lines.

The establishment of a system whereby transfected human DR molecules can be studied in a clinically relevant, functional model represents an exciting development in analysis of the HLA complex. Having developed a model whereby the role of HLA class II molecules presenting antigen to human T cell clones can be studied in a defined way, we are now investigating the nature of the

interaction between Ia, peptide and the TCR using site-directed mutagenesis of the tranfected HLA class II genes and substituted antigenic peptides.

Summary

We have used DNA-mediated gene transfer to express HLA class II molecules in mouse L cells for serological, biochemical, and functional analysis. cDNA clones encoding the DR2β a and DR2β b products of the DR2Dw2 haplotype were subcloned into a mouse Moloney leukemia virus–based expression vector (pJ4) and transfected separately into mouse L cells together with a HLA-DRα/pJ4 construct. These transfectants have allowed differential analysis of the two DR2β products in a manner normally prohibited by the concomitant expression seen in B cells. Two-dimensional SDS-PAGE analysis of the transfectants defines the more acidic β chain as the product of the DR2β a sequence, and the more basic chain as the product of the DR2β b sequence. The LDR2a transfectants present antigen efficiently to *M.leprae*–specific T cell clones and are capable of presenting synthetic peptide, 65-kD recombinant mycobacterial antigen and *M.leprae*. Of the DR2Dw2-restricted T cell clones we have tested, all use the DR2β a chain as their restriction element. Inhibition studies with mAbs demonstrate the dependence of presentation by the transfectant on class II and CD4, while mAbs against LFA-1, which substantially inhibit presentation by B-lymphoblastoid cell lines, do not inhibit transfectant presentation.

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