MOLECULAR LOCALIZATION AND POLYMORPHISM OF HLA CLASS II RESTRICTION DETERMINANTS DEFINED BY *MYCOBACTERIUM LEPRAE*-REACTIVE HELPER T CELL CLONES FROM LEPROSY PATIENTS

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The activation of helper T lymphocytes requires the recognition of foreign antigen in association with a self HLA class II molecule. This phenomenon is known as HLA class II restriction (1). The polymorphic class II epitopes that are corecognized by Th cells are functionally defined as restriction determinants (RDs)[1] (2). There are three groups of HLA class II molecules: DP, DQ, and DR (3). These molecules are expressed as heterodimeric glycoproteins on the cell surface of immunocompetent cells and are composed of a heavy (α) and a light (β) chain (3). The DP and DQ regions each contain 2α and 2β genes, whereas the DR region is known to encode 1α and 3β (β_1, β_2, β_3) genes (3). It has been reported (see references 1, 2, 4) that RDs for Th cells are carried by each group of class II products, namely D/DR, DP, and DQ. However, the exact molecules carrying these RDs as well as the epitopes involved have remained poorly characterized.

The major histocompatibility class II molecules have been defined as the products of class II immune response (Ir) genes in experimental animals (5). Such Ir genes determine the ability of an individual to generate T cell–dependent immune responses against specific antigens (5). The polymorphism of these class II Ir genes results in genetically controlled differences in such T cell–dependent immune responses. An important human example of class II Ir genes may be provided by leprosy, a chronic infectious disease that is caused by *Mycobacterium leprae* (6). HLA class II–linked genes are known to control the type of leprosy that develops upon infection (reviewed in 7, 8), as well as the cell-mediated immunereactivity against *M. leprae* and related mycobacteria as measured by skin testing (7, 8). Since both leprosy type and skin test responsiveness strongly correlate with *M. leprae*-specific Th cell reactivity, HLA class II Ir genes may

[1] *Abbreviations used in this paper:* EBV-BC, EBV-transformed B cells; HS, human AB serum, IMDM, Iscove's modified Dulbecco's medium; RD, restriction determinant; TLC, T lymphocyte clone.

regulate these Th cell responses against *M. leprae* antigens. If so, the mechanism of such HLA class II Ir genes might be the differential presentation of *M. leprae* antigens to Th cells by HLA class II RDs.

In this study we have systematically explored the nature of the RDs for *M. leprae* by presenting *M. leprae* antigens to T cell clones (TLC) from leprosy patients. The antigen specificity of several of these TLC has been reported recently (9–11). The molecular localization of the RDs was determined by inhibition studies with HLA class II–specific mAbs, whereas the polymorphism of the RDs was analyzed by the presentation capacity of large panels of fully class II–typed allogeneic APC. The results show that the majority of the RDs for *M. leprae* reside on DR molecules and not on DP or DQ molecules. Since DR molecules have a much higher expression than DP and DQ molecules, this result suggests that quantitative differences in the expression of class II molecules correlate with their function in the immune response. The same explanation holds true for the observation that RDs on DR molecules coded by a DR4Dw13 haplotype were located only on a subgroup of DR molecules with the highest expression. The data also indicate that multiple distinct RDs are present on one DR molecule.

Materials and Methods

Cells.   PBMC were isolated from heparinized venous blood from three leprosy patients (BC, R, and SC) by Ficoll-Isopaque density centrifugation (specific gravity, 1.077 g/ml), washed three times in HBSS (Gibco Laboratories, L. Paisley Scotland), and resuspended in Iscove's Modified Dulbecco's Medium (IMDM; Gibco Laboratories) supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml), both from Flow Laboratories, Inc., Paisley Scotland, and 10% pooled human AB serum (HS). EBV-transformed B cells (EBV-BC) were generated from 5×10^6 autologous PBMC. Cells were frozen in 1-ml ampules (Nunc, Roskilde, Denmark) containing $1–5 \times 10^6$ cells, 70% RPMI 1640 (Gibco Laboratories), 20% screened pooled human AB plasma, and 10% DMSO and were stored at $-196°$C.

Antigen.   *M. leprae* antigen (Dharmendra) was kindly provided by Dr. R. C. Good (Centre for Infectious Diseases, Centers for Disease Control, Atlanta, GA). The preparation consisted of bacilli that had been isolated from human lepromas.

Antigen Reactivation and Cloning of M. leprae Reactive T Lymphoblasts.   This was performed as described recently (10). In brief, 5×10^6 PBMC of two tuberculoid patients (BC and R) and one borderline lepromatous leprosy patient (SC) were restimulated in vitro with *M. leprae* in IMDM supplemented with 10% HS. The cultures were incubated for 5 d in 24-well tissue culture trays (Falcon 3047; Becton Dickinson & Co., Mountain View, CA) at 37°C in a fully humidified 5% CO_2-air mixture. Enrichment for T cell blasts was obtained either by Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density centrifugation or by extending the cultures for another 3–10 d in the presence of 10% IL-2-containing medium (Lymfocult-T; Biotest, Serum Institute, Frankfurt, Federal Republic of Germany). After the isolation of the blasts, a cell suspension was made containing five blasts/ml in a mixture consisting of (*a*) PBMC from three to four random donors (10^6 cells/ml, 30-Gy irradiated), (*b*) autologous EBV-BC (10^5 cells/ml, 50-Gy irradiated), and (*c*) an optimal concentration of *M. leprae* antigen, all in IMDM supplemented with 10% HS. This suspension was plated in 96-well, flat-bottomed microtiter plates (Falcon 3072; Becton Dickinson & Co.) (0.1 ml/well; i. e., 0.5 T lymphoblast/well) and was incubated as described above. Growing cultures were transferred to 24-well tissue culture trays (Falcon 3047; Becton Dickinson & Co.) and restimulated with 1 ml/well of the cell/antigen mixture described above. 3–4 d later IL-2 (10%) was added. After an additional 4–7 d, the cultures were restimulated again until a minimum of 2×10^6 cells per culture was

obtained. The cells were then frozen or expanded further by restimulation as described above for 4 d, except that Leuko Agglutinine (Pharmacia Fine Chemicals) was added to the cell/antigen mixture (final concentration, 1 μg/ml) to increase the yield of cells. This was followed by culturing for 3–5 d in the presence of IL-2.

Proliferative Assays. 10^4 TLC (0.05 ml) and 5 × 10^4 irradiated (40-Gy) autologous or allogeneic PBMC as APC (0.05 ml) were cultured in IMDM with 10% HS with 0.1 ml of *M. leprae* antigen (1/120 dilution) in 96-well, flat-bottomed microtiter plates (Greiner, Nürtingen Federal Republic of Germany). PHA (4 μg/ml; Wellcome Diagnostics, Beckenham, United Kingdom) and plain IMDM were used as controls. The cultures were set up in duplicate or triplicate and incubated as described above for 72 h. 18 h before termination, 1.0 μCi of [^{3}H]-thymidine (sp act, 5.0 Ci/mmol; Radiochemical Centre, United Kingdom) in 0.05 ml RPMI 1640 was added. The samples were harvested on glass-fiber filters using a semiautomatic sample harvester. [^{3}H]Thymidine incorporation was assessed by counting in a liquid scintillation counter (G. D. Searle & Co., Chicago, IL). All cells had been typed for HLA-A, B, C, DR, (including DRw52 and DRw53), DQ, and DP, as mentioned in reference 2. In addition, a number of cells were also typed for the cellularly defined HLA-D determinants, using homozygous typing cells and primed lymphocyte typing reagents as described in references 12 and 13.

Monoclonal Antibodies. The mAbs used in this study were generously provided by F. Koning (from our department), unless mentioned otherwise, and were PdV5.2 (anti–class II monomorphic; recognizing DR, DP, and approximately half of the DQ molecules; IgG1), B9.12.1 (anti–class I monomorphic; IgG2a; a gift of B. Malissen, Marseille, France), B8.11.2 (anti-DR monomorphic; IgG2b; also a gift of B. Malissen), 7.3.19.1 (anti-DRw52-like; IgG2b), SPV-L3 (anti-DQ monomorphic; IgG2a; a gift of H. Spits, Lyon, France), B7/21 (anti-Fa or DP monomorphic; IgG; gift of F. Bach, Minneapolis, MN), IIB3 (anti-DQw1-like; IgG2b), TA10 (anti-DQw3; like IgM; gift of H. Maeda, Tokyo, Japan), 109d6 (anti-DRw53-like; IgG2a; gift of R. Winchester New York, NY), IC2 (anti–class II monomorphic; IgG2a), LD1.1 (anti-DR monomorphic; IgM), Tü22 (anti-DQ monomorphic; IgG2a; gift of A. Ziegler, Tübingen, Federal Republic of Germany), Genox 3.53 (anti-DQw1-like; IgG1; gift of J. Bodmer, London, United Kingdom), OKT3 (anti-CD3; purchased from Ortho Diagnostic Systems Inc., Westwood, MA), RIV-6 (anti-CD4; IgG2a; National Institute of Public Health, Bilthoven, The Netherlands), FK18 (anti-CD8; IgG3), FK24 (anti-CD11; IgG1), anti-Leu-7 (anti–human NK cell–like; IgM; purchased from Becten Dickinson & Co.), and PL15 (anti-DP monomorphic; gift of R. Knowles, Sloan Kettering, New York). All mAbs consisted of mouse-derived ascites except for Tü22, which was the supernatant of a hybridoma culture. All mAbs are described in reference 14.

Inhibition of Antigen-specific TLC Proliferation by mAbs. Cultures were set up as described above, except that the same amount of antigen was added in 0.05 instead of 0.1 ml. At the start of the culture, 0.05 ml IMDM with mAb was added. All mAbs were filter-sterilized through 0.22-μm filters (Gelman Sciences, Inc., Ann Arbor, MI) and tested in a final completely saturating concentration of 1:50–1:200 from the original ascites.

IFN-γ Assay. Culture supernatants of antigen-activated TLC as described above were measured in duplicate for levels of IFN-γ after 90 h by a solid-phase RIA (Centocor, Malvern, PA), by using two distinct anti-IFN-γ mAbs. The first mAb had been coupled to polystyrene beads, the second had been labeled with ^{125}I and was added to the first mAb after the addition of culture supernatant and washing the beads. Unbound, labeled mAb was then removed by washing. Bound radioactivity was determined by gamma scintillation counting. IFN-γ concentrations of the measured samples were derived from a standard curve and expressed as units/milliliter.

Results

Cell Surface Marker Phenotype of, and IFN-γ Production by Activated TLC. To characterize the nature of the TLC obtained, we first studied the cell surface antigens expressed by these TLC. All TLC had the CD3$^+$CD4$^+$CD8$^-$ phenotype

TABLE I

Activated M. Leprae–reactive T Cell Clones Produce IFN-γ

TLC	IFN-γ production		Proliferative response (cpm × 10⁻³)	
	+	−	+	−
	U/ml			
RI 1G5	1.9	0.1	14.2	0.0
RI 1 E4	40.3	0.1	57.9	0.1
RI 2 F9	27.2	0.1	88.4	0.1
RI 3 B4	6.0	0.1	42.2	0.2
RI 1 F9	14.5	0.2	10.0	0.1
RI 3 E8	24.8	0.1	70.5	2.3
Polyclonal T-LB	22.4	0.1	53.7	0.1

M. leprae antigen was presented to six TLC and one polyclonal T lymphoblast culture of the same patient by class II–compatible APC in quadruplicate. Two cultures were assayed for [³H]TdR incorporation by the T cells whereas the supernatant of the two remaining cultures was tested for IFN-γ production (see Materials and Methods).

and were strongly positive for HLA-DR. In contrast, the expression of DQ varied from negative to strongly positive (data not shown).

Helper TLC are known to produce IFN-γ upon antigen activation. IFN-γ is a major macrophage-activating factor and as such an important mediator for the induction of killing of intracellular parasites such as *M. leprae* (15). The correlation between *M. leprae*–induced IFN-γ production in vitro and T cell–mediated immunoresponsiveness in vivo and in vitro has been clearly established (16). The data presented in Table I show that the TLC tested (*n*, 6) and the parental polyclonal T lymphoblast culture produce IFN-γ upon activation with *M. leprae*. We saw a poor correlation between proliferation and IFN-γ production (*p*, 0.45).

Based upon the membrane-phenotypes, IFN-γ production, class II–restricted proliferative responses to *M. leprae* antigens (*vide infra*), and the inability to suppress other Th cell responses against *M. leprae* (11), the TLC described in this paper were defined as helper TLC.

Definition of Distinct Restriction Determinants on DR Molecules Coded by a HLA-DR4/Dw13 Haplotype.   Nine *M. leprae*–reactive proliferative TLC of patient BC were selected at random for further studies addressing their RD repertoire. All these TLC were specific for distinct antigenic determinants expressed by *M. leprae*, as had been determined previously (10; our unpublished observations). The HLA class II phenotype of BC is DR3,4; Dw13; DRw52,53; DQw3; DPw1,5.

The data presented in Table II show that six of the nine TLC tested are activated by antigen in association with DR4 or Dw13 but not DR3 related RDs. DR4 behaves like a supertypic specificity for the cellularly defined Dw4,10,13,14, and 15 determinants (12). These six TLC could all be inhibited completely by the same set of HLA-DR framework (DR⁺) reactive mAbs, but not at all by DQ-, DP-, or class I–specific mAbs. Interestingly, a DRw53-specific mAb (109d6) that has been reported (17, 18) to block completely the responses of other TLC in these highly saturating concentrations (17, 18), did not block those *M. leprae*–reactive helper TLC that clearly have to recognize a determinant on a DR

TABLE II

M. leprae-reactive T Cell Clones Are Restricted by Several Restriction Determinants Associated with DR and/or Dw Specificities Coded by a DR3 and a DR4Dw13 Haplotype

APC					TLC derived from patient BC								
APC	DR(w)	Dw*	DQw	DPw	VI 1E8	II 1E3	VI 5B11	VI 5E7	VI 4F3	II 1D4	II 1E10	II 2F10	II 4A4
Aut‡	3,4	13	3	1,5	34.1 ± 0.0	11.2 ± 2.8	30.6 ± 0.3	29.6 ± 1.5	19.4 ± 5.2	11.7 ± 0.9	5.5 ± 0.3	5.9 ± 1.0	26.6 ± 5.1
2	3,4	4	2,3	4	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	44.6 ± 0.0	0.1 ± 0.3	1.2 ± 0.5	0.1 ± 0.0
3	3,4	4	2,3	ND	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	13.2 ± 1.3	3.8 ± 0.3	13.4 ± 5.9	25.6 ± 1.8
4	3,4	4	2,3	2,4	1.1 ± 0.2	0.3 ± 0.0	0.1 ± 0.1	0.3 ± 0.0	0.8 ± 0.0	45.9 ± 0.9	0.1 ± 0.0	2.7 ± 0.3	0.1 ± 0.0
5	3,11	ND	2,3	1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	2.7 ± 1.3	29.2 ± 0.6
6	3	ND	2	ND	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	6.8 ± 0.3	23.8 ± 1.2	8.6 ± 4.1
7	3,3	ND	2	4	0.3 ± 0.0	ND	ND	0.1 ± 0.0	0.1 ± 0.0	ND	ND	ND	ND
8	2,3	ND	1,2	5	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.1
9	2,3	ND	2	ND	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	ND	ND	ND	ND
10	2,4	13	2,3	ND	17.1 ± 0.9	16.5 ± 0.7	21.1 ± 0.4	43.0 ± 0.4	35.4 ± 2.1	19.7 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	24.6 ± 0.2
11	4,7	ND	2,3	ND	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	32.1 ± 1.9	46.0 ± 3.2	63.7 ± 1.9	0.1 ± 0.0	0.7 ± 0.2	0.3 ± 0.1
12	4,13	13	1,3	ND	0.7 ± 0.1	0.3 ± 0.0	7.3 ± 1.3	32.3 ± 3.2	33.8 ± 0.3	6.5 ± 1.6	0.1 ± 0.0	0.7 ± 0.3	0.2 ± 0.0
13	4,12	13	3	4,5	51.8 ± 0.0	30.8 ± 0.6	27.1 ± 2.7	39.0 ± 0.0	54.5 ± 1.6	49.6 ± 2.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
14	4	13	3	ND	26.3 ± 0.5	9.5 ± 2.5	43.8 ± 0.9	29.5 ± 1.5	17.2 ± 0.2	ND	ND	ND	ND
15	4,8	13	3	ND	0.1 ± 0.1	3.4 ± 0.6	14.3 ± 1.7	79.9 ± 0.0	31.6 ± 0.9	ND	ND	ND	ND
16	4	4,13	3	4	0.9 ± 0.3	3.7 ± 2.6	1.0 ± 0.6	73.3 ± 0.7	26.5 ± 5.6	ND	ND	ND	ND
17	4,13	13	1,3	ND	0.7 ± 0.0	0.5 ± 0.3	8.6 ± 2.1	61.1 ± 3.1	21.0 ± 3.1	ND	ND	ND	ND
18	4,13	4	1,3	ND	0.2 ± 0.0	0.4 ± 0.3	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	ND	ND	ND	ND
19	4,4	4	3	2	0.3 ± 0.1	ND	ND	0.3 ± 0.0	0.3 ± 0.1	ND	ND	ND	ND
20	4,13	4	1,3	2,5	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	1.3 ± 0.0	0.1 ± 0.0	ND	0.1 ± 0.0	0.9 ± 0.1	0.1 ± 0.0
21	2,4	4	1,3	ND	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.7 ± 0.1	ND	ND	ND	ND
22	4,8	non-13 non-4	3	4	0.3 ± 0.1	0.1 ± 0.0	ND	0.9 ± 0.4	0.2 ± 0.1	ND	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
23	4,11	14	3	4	ND	0.1 ± 0.0	ND	2.3 ± 0.3	0.1 ± 0.0	42.6 ± 1.7	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
24	2,4	14	1,3	ND	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.4 ± 1.0	ND	ND	ND	ND
25	4,12	10	3	4	0.7 ± 0.3	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	11.0 ± 0.6	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.5
26	2,9	ND	1,3	3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
27	1,7	ND	1,2	4	0.3 ± 0.0	0.8 ± 0.7	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	ND	0.1 ± 0.0	8.5 ± 0.3§	ND
28	11,11	ND	3	2	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	ND	ND	ND	ND
29	—	—	—	—	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

M. leprae antigen was presented to several TLC derived from patient BC by a panel of allogeneic APC. The HLA class II phenotypes of the APC donors are shown. Results are expressed as cpm $\times 10^{-3}$ ± SD of antigen-stimulated cultures. Background proliferation was 0.5 ± 0.3 $\times 10^3$ cpm. Cultures were regarded positive when the observed cpm was >2,000 cpm and >15% of the cpm seen in case of autologous APC. Boxes designate positive responses.
* Only HLA-DR4-associated Dw specificities are indicated, i. e., HLA-Dw 4, 10, 13, and 14.
‡ Autologous APC and class II phenotype.
§ Background proliferation in the absence of antigen, 3.9 ± 1.9.
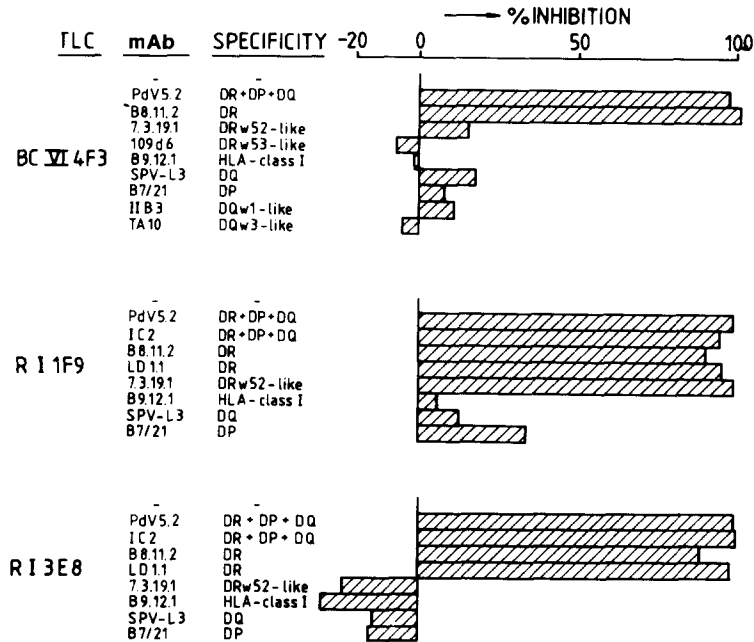
FIGURE 1. Localization of restriction determinants for *M. leprae*–reactive TLC on HLA-DR molecules. Inhibition of *M. leprae*–induced T cell responses in the presence of autologous or class II–compatible APC. The results shown for TLC BCVI4F3 (see Table II), RI1F9, and RI3E8 (see Table III) are expressed as the percentage inhibition of the TLC responses against *M. leprae* as calculated with the formula: (1-cpm of TLC cultured in the presence of mAb/cpm of TLC cultured in the absence of mAb) × 100%. SD, ≤10%. cpm in the absence of mAb for VI4F3 was 31,565; for I1F9 15,075; and for I3E8 11,885.

molecule from the DR4Dw13 haplotype. Since all these inhibition patterns were identical, only one representative example (TLC VI4F3) is shown in Fig. 1. These blocking studies indicate that the RDs for these TLC have to be located on the DR⁺DRw53⁻ molecules and not on the DR⁻DRw53⁺ ones, which are low in expression compared with the DR⁺DRw53⁻ molecules (19, 20). The DR⁺DRw53⁻ molecules carry the DR4 and the DR4-related Dw allospecificities (19–24). Biochemical studies (19–24) have shown the presence of only one such a DR⁺DRw53⁻ ($\alpha\beta_1$) complex in DR4Dw13 homozygous individuals, which implies that the RDs also have to reside on that same molecule.

We saw five different clusters of TLC responses that were reproducible. In the case of TLC II1D4, antigen-induced responses closely followed the presence of the DR4 epitope on the APC ($p = 0.002$) irrespective of the corresponding Dw phenotype. The responses of the other five TLC (Table II, left five) were closely associated with the Dw13 determinant. These latter TLC could not be activated by APC expressing the other DR4-associated Dw specificities, namely Dw4, Dw10, and Dw14. No Dw15⁺ APC were tested since this specificity is only seen in Oriental populations (12). In this group of five TLC, we saw four related but distinct Dw13-associated clusters; in one cluster, (VI5E7; VI4F3) TLC responsiveness followed exactly the presence of the Dw13 specificity on the APC, whereas in the other three cases, respectively, six (VI5B11), five (II1E3), and

three (VI1E8) of the seven Dw13$^+$ APC could activate these TLC in the presence of optimal concentrations of *M. leprae* antigens. These latter three different clusters thus are associated with, but clearly not identical to, the Dw specificity.

It should be mentioned that TLC restricted either by the serologically defined DR specificities or by Dw specificities subdividing the associated DR antigen have been described also for other haplotypes, including DR4Dw14 (25) and DR2Dw12 (26). However, the RDs described in these and other studies correlated with the known Dw types and did not detect an additional Dw-related RD heterogeneity, as shown in the present study.

In conclusion, our results indicate that in the DR4Dw13 haplotype both the serologically defined DR4 and the cellularly defined Dw13 allospecificity may be closely related to if not identical with RDs for *M. leprae*. In addition, five TLC define four Dw13-related clusters, most probably representing four distinct Dw13-associated RDs. All RDs in this haplotype were situated on the DR$^+$-DRw53$^-$ ($\alpha\beta_1$) molecule and not on the DR$^-$DRw53$^+$ ($\alpha\beta_3$) one, which is low in expression compared with the $\alpha\beta_1$ molecule.

Definition of Different Restriction Determinants on DR Molecules Coded by HLA-DR3 and -DR2 Haplotypes. To determine the repertoire of RDs for *M. leprae* on molecules expressed by DR3, DRw52 (DQw2) haplotypes, we studied a number of TLC derived from patient R (class II phenotype: DR2,3; DRw52; DQw1,2; DPw5), as well as the three TLC of patient BC not restricted by DR4Dw13-associated determinants. So far, all TLC from patient R have been found to recognize distinct *M. leprae* protein antigens (9).

As shown in Table II, the three TLC of BC (II1E10, II2F10, and II4A4) recognized different determinants on several but not all DR3$^+$ APC. In addition, TLC II4A4 was activated by a DR3$^-$ APC, which was derived from a DR4Dw13 haplotype-identical sibling. In Table III are shown four TLC from patient R that responded to *M. leprae* plus DR3$^+$ APC. Four other TLC showed an identical pattern and therefore are not shown. Inhibition studies revealed that all TLC of BC and R restricted by DR3-associated determinants could be inhibited by mAbs reactive with DR framework (DR$^+$) structures, as well as by mAb 7.3.19.1, which recognizes a DRw52-like specificity. In Fig. 1 one representative example for these TLC is shown (R11F9). No, or only weak inhibition was seen in case of DP, DQ, or class I–specific mAbs. Using another DR-specific mAb, the RDs of the TLC from patient R, which in panel as well as in the mentioned inhibition studies reveal identical patterns, could be subdivided on the base of differences in inhibition (data not shown here). Taken together, these results indicate that all DR3-related RDs reside on molecules that carry both DR- and DRw52-like determinants.

To determine the RDs for *M. leprae* expressed by class II molecules on DR2, DQw1$^+$ APC, TLC of patient R and of patient SC (class II phenotype: DR2,4; Dw13; DRw53; DQw2,3) were studied in more detail. The results are summarized in Tables III and IV. It is evident from Table III that several TLC are restricted by DR2-associated determinants. Only 4 of 14 TLC, all displaying a similar DR2-associated pattern, are shown in the table. Although only one Dw12$^+$ APC could be tested, the TLC responses seem to be associated with the DR2 specificity rather than with the Dw2 or 12 specificities on the APC.

TABLE III

HLA-DR Is Associated with the Main Restriction Determinants for M. leprae–reactive TLC in a DR2 and a DR3 Haplotype

APC					TLC derived from patient R								
APC	DR(w)	Dw*	DQw	DPw	12E4	11E4	11E3	11F9	12F10	12F9	13E8	12G4	13F10
Aut‡	2,3	52	ND	1,2	5	44.7 ± 2.7	45.9 ± 4.5	22.3 ± 2.0	21.2 ± 2.3	27.2 ± 0.3	54.6 ± 2.8	38.2 ± 1.5	15.2 ± 3.6	37.4 ± 0.4
2	2,2		2	1	4	0.4 ± 0.0	0.9 ± 1.0	0.3 ± 0.1	0.1 ± 0.0	41.7 ± 1.6	74.7 ± 11.2	39.7 ± 4.4	33.3 ± 0.3	0.4 ± 0.1
3	2,2		2	1	4	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	31.7 ± 1.5	57.8 ± 1.2	70.3 ± 2.8	25.1 ± 4.2	0.3 ± 0.0
4	2,2		2	1	2	0.4 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	ND	31.0 ± 2.2	52.7 ± 0.6	ND	18.3 ± 2.0	0.3 ± 0.2
5	2,2		2	1	2	0.5 ± 0.3	0.1 ± 0.0	0.3 ± 0.2	ND	36.1 ± 1.9	60.1 ± 1.3	ND	21.7 ± 2.8	ND
6	2,2		12	1	ND	0.8 ± 0.4	0.3 ± 0.0	0.3 ± 0.2	ND	49.7 ± 0.0	118.0 ± 1.2	ND	4.1 ± 0.0	0.2 ± 0.1
7	3,3	52	3	2	2	35.3 ± 2.1	41.9 ± 2.9	24.2 ± 1.7	41.4 ± 2.5	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.7 ± 0.2
8	3,3	52	3	2	1,4	77.3 ± 0.7	67.2 ± 0.7	44.1 ± 0.4	29.5 ± 1.4	0.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.8 ± 0.6	0.2 ± 0.1
9	3,3	52	3	2	4	45.4 ± 3.6	48.3 ± 1.9	28.7 ± 2.0	ND	0.1 ± 0.0	0.6 ± 0.7	ND	0.7 ± 0.6	0.2 ± 0.0
10	3,3	52	3	2	4	7.7 ± 1.1	8.5 ± 3.0	2.8 ± 1.4	ND	0.1 ± 0.0	0.2 ± 0.1	ND	0.2 ± 0.0	ND
11	1,1			1	2	0.4 ± 0.2	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.2	0.0 ± 0.0
12	13,13	52		1	2,4	0.4 ± 0.3	0.3 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.0 ± 0.0
13	4,7	53		2,3	2,4	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	8.0 ± 3.9	ND
14	4,7	53		2,3	4	2.1 ± 1.1	1.1 ± 1.1	ND	ND	0.1 ± 0.1	ND	ND	22.7 ± 5.4	ND
15	5,5	52		3	2	0.3 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	ND	0.1 ± 0.0	0.2 ± 0.0	ND	0.2 ± 0.1	0.9 ± 0.4
16	10,13	52		1	5	0.3 ± 0.1	0.3 ± 0.1	ND	ND	0.1 ± 0.0	ND	ND	0.1 ± 0.0	0.9 ± 0.9
17	4,13	52,53		1,3	5	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	9.0 ± 0.6
18**	2,3	52	ND	1,2	ND									0.1 ± 0.0
19	2,3	52	ND	1,2	1,4									0.2 ± 0.1
20	2,3	52	ND	1,2	3,4									0.2 ± 0.1
21	2,3	52	ND	1,2	1,4									0.8 ± 0.9
22	2,3	52	ND	1,2	ND									0.4 ± 0.2

HLA class II restriction of *M. leprae*–activated TLC of patient R. See legend to Table II.

* Only DR2 or DR3 related HLA-Dw specificities are shown
‡ Autologous APC and class II phenotype.
§ Without *M. leprae*: 8.6 ± 1.7.
‖ Without *M. leprae*: 21.9 ± 7.3.
¶ Without *M. leprae*: 6.7 ± 1.5.
** For APC 18–22, only TLC 13F10 was tested.

TABLE IV

HLA-DR-associated Restriction of M. leprae–reactive TLC

TLC*	HLA class II antigen analyzed	Effect of sharing or mismatching of class II antigen between T cell and APC on T cell responsiveness[‡]				
		Class II antigen shared: yes / TLC response observed: yes	yes / no	no / yes	no / no	p value
SCII2B2	DR2[§]	5	0	0	18	0.00006
SCII2F5	DR2[§]	3	0	0	12	0.004
SCII2F9	DR2[§]	3	0	0	12	0.004
SCII2F6	DR2	1	4	12	6	0.176
	DR4	5	0	8	10	0.076
	DQw2	6	4	7	6	1.000
	DQw3	10	2	3	8	0.020
	DRw53	13	2	0	8	0.0002
	DPw1-7[‖]					>0.30

* M. leprae antigen was presented by allogeneic APC to TLC derived from leprosy patient SC. The HLA class II phenotype of this patient was DR2,4; Dw13; DRw53; DQw2,3.

‡ The TLC-APC combinations in which the analyzed class II antigen was shared between TLC and APC were compared with regard to the observed TLC proliferative responses to the responses observed in the TLC-APC combinations mismatched for that class II antigen. The significance of the results are given as Fisher's exact p values. Positive responses ranged from 1.2 ± 0.2–40.2 ± 0.4 cpm; negative responses ranged from 0.0 ± 0.0–0.2 ± 0.0 cpm.

§ These TLC were also tested for sharing of DR4, DQw2, DQw3, DRw53, and DPw4 antigens; all p values were >0.25 (data not shown).

‖ Since the T cell donor was not typed for DP, all known DP (w1-7) allospecificities were analyzed in the same way. The p values obtained varied between 0.30 and 0.72, as summarized in the table.

Also, the polyclonal T cell line of this patient responded equally well to Dw2+Dw12− as to Dw2−Dw12+ APC (data not shown here). The results for three TLC of patient SC that were (also) restricted by DR2-associated determinants in panel studies are summarized in Table IV (II2F5, II2F9, II2B2). The antigen specificity of these TLC was described recently (11). In this case, no DR2+Dw2−Dw12+ APC were tested. Inhibition studies performed with these TLC from patient R and SC pointed out that all DR framework–reactive mAb (n, 4) completely inhibited the proliferation of these TLC, whereas we saw no inhibition in the case of mAbs specific for DP, DQ, class I, or DRw52. Therefore, only one representative example is shown in Fig. 1 (RI3E8).

Recently, evidence has been presented (26) suggesting that the DR2 determinant is situated on DR$\alpha\beta_1$ complexes that are distinct from those carrying the Dw2/12 determinants, namely the $\alpha\beta_2$ complexes. Moreover, RDs for streptococcal as well as measles antigens displayed a preference for the $\alpha\beta_2$ and not the $\alpha\beta_1$ complex (26, 27). Our data suggest that in contrast, the M. leprae RDs show a preference for the $\alpha\beta_1$ complex.

In conclusion, our results suggest that also in the case of the class II molecules expressed by DR2, (DQw1) haplotypes, RDs for M. leprae reside almost exclusively on DR molecules. It is likely that most RDs for M. leprae are located on the DR$\alpha\beta_1$ molecules that express the DR2 but not the Dw2 or Dw12 determinants.

New Restriction Determinants Defined on DQ and DP Molecules. TLC 13F10, shown in the last column of Table III, was activated only by M. leprae plus
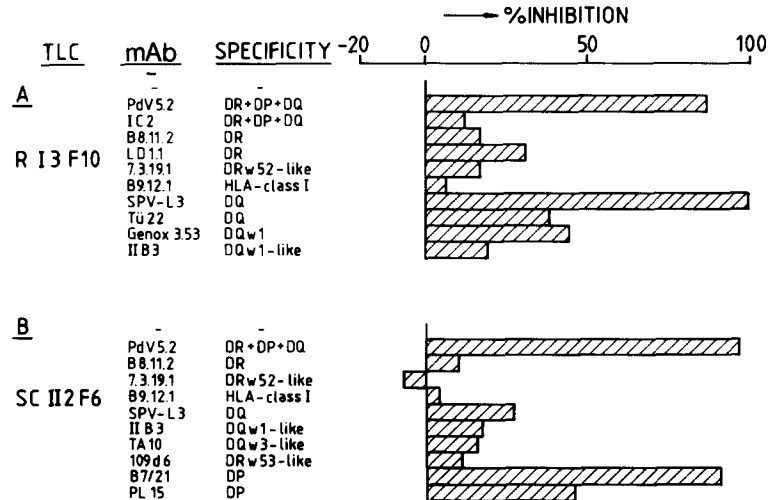
FIGURE 2.   Inhibition patterns of an HLA-DQ and an HLA-DP-restricted *M. leprae*-reactive TLC. Inhibition of *M. leprae*-induced T cell responses in the presence of autologous or class II–compatible APC. The results are expressed as the percentage inhibition (see legend to Fig. 1). SD, ≤10%. cpm in the absence of mAb for I3F10 were 40,545 and for II2F6 5,400.

autologous but not allogeneic APC (n, 32; only 17 are shown), including cells derived from individuals from the same ethnic group. Inhibition studies showed that antigen-induced responses were inhibited completely by an mAb reactive exclusively with DQ molecules (SPV-L3; Fig. 2A), and by only one of the two mAbs reactive with monomorphic class II determinants. We saw partial inhibition in the case of mAb Tü 22 and genox 3.53, reactive respectively with a mono-morphic DQ and a DQw1 determinant. The weak inhibition seen in the case of mAb LD1.1 may be due to crossreactivity with DQ determinants. In conclusion, this TLC recognizes a RD on DQ molecules that is only expressed by autologous APC, and not by the 32 other allogeneic APC.

TLC II2F6, shown in Table IV, recognizes *M. leprae* in association with a variety of allogeneic APC. Positive responses correlated best with the sharing of the DRw53 specificity between APC and TLC (p = 0.0002), less significantly so with DQw3 (p = 0.02), and not at all with DR2 or DR4. Since this T cell donor had not been typed for DP, we analyzed the T cell responses against all known DP allospecificities on the APC tested, namely DPw1-7. None of the DP deter-minants were associated with T cell responsiveness (p = 0.30), excluding an association between the RD and one of the known DP specificities. Unexpectedly, however, blocking studies revealed that the RD is situated on a DP molecule, since mAb B7/21 could inhibit proliferation completely (Fig. 2B). mAb PL15, which is also directed against DP determinants, showed only marginal inhibition. In conclusion, the RD recognized by this TLC is situated on a DP molecule, does not correlate with currently known DP allospecificities, and is frequently expressed among the population of APC tested (13 of 23).

Alloreactivity of Class II–restricted M. leprae–reacted TLC.   Three regular class II–restricted *M. leprae*-reactive TLC were found to crossreact with some but not all allogeneic APC in the absence of antigen. These TLC were BCII2F10,

shown in Table II (APC 27); RI2G4 (Table III, APC 13 and 14); and RI3F10 (Table III, APC 17). One of these three TLC, namely RI2G4, was analyzed in more detail by means of panel and inhibition studies, and was found to recognize a DPw4-related allodeterminant (our unpublished observations).

Discussion

In this report, we have defined the HLA class II molecules and characterized the polymorphic epitopes or RDs on these molecules, which in association with *M. leprae* antigens, are recognized by cloned *M. leprae*-reactive Th lymphocytes from leprosy patients. The molecular localization of the RDs was defined by inhibition studies with HLA class II-specific mAbs, whereas the polymorphism of these RDs was determined in panel studies with fully class II-typed allogeneic APC. The reason why we were interested in defining these RDs is based on two facts. First, polymorphic HLA class II-linked Ir genes have been shown to determine T cell-mediated immune responsiveness against *M. leprae* and related mycobacteria in vivo (reviewed in 7, 8), as well as the type of leprosy that develops upon infection in susceptible individuals (7, 8). Second, MHC class II molecules are involved in the restriction and regulation of antigen presentation to Th cells and as such have been defined as the products of class II Ir gene products (references 5, 8). We reasoned therefore that the Ir genes that regulate the in vivo immune response against *M. leprae* may actually code for RDs that restrict and regulate the (in vitro) presentation of *M. leprae* antigens to Th cells. The definition of such RDs is crucial for the unraveling of the mechanism of this HLA disease association.

Our first important observation was that the majority of the RDs for *M. leprae* are located in the polymorphic domains of HLA-DR molecules, and not on DP or DQ molecules. Of course, one has to be cautious in drawing general conclusions from data obtained from three patients. However, we have also studied the restriction of polyclonal Th cell lines from 22 leprosy patients that show a similar preferential DR restriction (Ottenhoff, T. H. M., D. G. Elferink, J. Kobesson, D. L. Leiker, R. F. M. Lai, A. Fat, and R. R. P. de Vries, manuscript submitted for publication). Thus DR molecules play a major role in the presentation of *M. leprae* antigens to T cells from leprosy patients. Since these same molecules also express the DR allospecificities that are associated with the regulation of immune responses against *M. leprae* in vivo (*vide supra*), it is very likely that those HLA-DR-coded RDs are closely associated with, if not identical to, the *M. leprae*-specific HLA class II Ir gene products. It has been established that the expression of DR molecules is much stronger than that of DP (14, 28, 29) and DQ (14, 29) molecules. Quantitative differences in the expression of the different class II molecules can result in profound differences in their function in immune responses, as has been shown by studies of mice (30). Recently, we have presented evidence that human polyclonal T cell responses to PPD of *Mycobacterium tuberculosis* are restricted preferentially by those DR molecules that show the highest expression on APC (31). However, the precise molecular localization of RDs is only possible with the use of cloned T cells, as was done in the present study. In the case of DR molecules in DR4+ cells, two types of DR molecules are expressed, namely DR+DRw53− ($\alpha\beta_1$) and DR−DRw53+ ($\alpha\beta_3$) molecules (19–24),

the DR4 β_2 gene being a pseudogene (32). Our inhibition studies localized the *M. leprae* RDs only on the $\alpha\beta_1$ and not on the $\alpha\beta_3$ molecules. These DR4 $\alpha\beta_1$ molecules carrying the *M. leprae* RDs have a significantly higher expression than the DR4 $\alpha\beta_3$ molecules (19, 20), which would fit with the preferential localization of *M. leprae* RDs on DR and not on DP or DQ molecules as a consequence of quantitative differences in the expression of class II molecules.

In the case of DR molecules expressed by DR2$^+$ cells, qualitative rather than quantitative differences between different DR molecules may also be of importance for the localization of RDs for *M. leprae*. Recently, two distinct DR2-related DR molecules were described, one probably carrying the DR2 determinant ($\alpha\beta_1$), the other one expressing the Dw2 or Dw12 specificities ($\alpha\beta_2$; 26). It was shown that both molecules could stimulate allogeneic mixed lymphocyte cultures as well as presenting antigen to Th cells. Of interest here is that in this as well as in a second study (27) the RDs for respectively, streptococcal and measles virus antigens were mapped mainly to the $\alpha\beta_2$ and not to the $\alpha\beta_1$ molecules. Our results suggest that RDs for *M. leprae* also may show a preference for the $\alpha\beta_1$ complex. In the case of DR molecules expressed by DR3$^+$ cells, inhibition studies (31) showed that the RDs for *M. leprae* were situated on DR molecules expressing both DR- and DRw52-like determinants. These DR$^+$-DRw52$^+$ molecules have been described previously and have been shown to carry RDs for *M. tuberculosis* as well (31).

Because information concerning the sequences of several DRβ_1 alleles studied by us has recently become available (19–23, 34, 35), we were in a unique position to analyze the relation between structure and function of HLA class II molecules, notably the relationship between allodeterminants and determinants restricting antigen presentation. Comparison of the sequences of DRβ_1 cDNA clones from Dw4$^+$, Dw13$^+$, and Dw14$^+$ individuals has revealed only one to three different nucleotides that result in amino acid substitutions between residues 71–86 of the first domain, which are likely to be situated on the outer face of the DRβ_1 molecule (33, 34). These DRβ_1 differences may explain completely the Dw allelic differences (19–24). Our results show (see Table II) that one of six *M. leprae*–reactive TLC restricted by the DR4$^+$ haplotype recognized a *M. leprae* antigen in association with an RD that was closely associated with DR4, and not related to the Dw specificity of the APC, whereas the other five were restricted by determinants associated with the Dw specificity of the TLC, namely Dw13. Therefore, these five TLCs can distinguish one (Dw13 vs. Dw14, see reference 33) to three (Dw13 vs. Dw4, see reference 33) amino acid differences between DR$\alpha\beta_1$ molecules like alloreactive donors (33, 34). However, four of the five TLC restricted by a Dw13-associated determinant clearly showed a distinct restriction specificity in the panel studies. Although we cannot exclude the possibility that differences in antigen processing might result in the four different Dw13-associated clusters, we consider such a highly antigen-specific processing defect an unlikely explanation. Therefore, we think that the four distinct Dw13-associated restriction specificities defined by these five *M. leprae*–reactive TLCs represent in all probability four different RDs on one DR $\alpha\beta_1$ molecule. The fact that only two of the five TLCs show an identical restriction specificity suggests that the potential of RDs on this DR $\alpha\beta_1$ molecule is much larger.

Because all the *M. leprae*-reactive TLC tested thus far recognize different *M. leprae* antigenic determinants (epitopes), the different processed peptides carrying these epitopes might bind to distinct class II determinants ("desetopes") with their "agretopes" (35). This differential binding might then give rise to the expression of different "histotopes" recognized by the T cell receptor.

Whatever the mechanism generating it, it is clear from our data that a minimal difference between class II molecules (only one AA residue between the DR$\alpha\beta_1$ molecules of Dw13 vs. Dw14) combined with different antigens may result in an almost infinite number of RDs. This confirms data from experimental animals and implies that conformational changes of class II molecules are important in the generation of RDs (36, 37).

A third conclusion is that these *M. leprae*-specific TLC define novel epitopes on DP and DQ molecules, which may be more relevant than those detected with alloantibodies, allospecific T cells, or biochemical techniques. Although *M. leprae*-reactive Th cells are apparently mainly restricted by determinants on DR molecules, some clones do use RDs on DP and DQ molecules. Thus one TLC recognized a RD on a DP molecule not associated with a known DP allospecificity. Even more interesting was that another TLC (RI3F10) defined a RD on a DQ molecule which was only expressed by autologous APC but not by 32 allogeneic APC. The DQ region may therefore be much more polymorphic than assumed thus far.

This may well be biologically quite important. In fact, the low frequency of DP- and DQ-restricted, *M. leprae*-reactive helper TLC seen by us may well be a considerable underestimate of in vivo situations, because of an in vitro selection for clones restricted by class II molecules with the highest expression on the APCs used for restimulation. Moreover, not all T cells are Th cells and it may be that for suppressor T cells, qualitative differences in expression of HLA class II molecules are more important.

Finally, we saw that several *M. leprae*-reactive, class II–restricted helper TLC showed crossreactivity with a minority of allogeneic APC in the absence of antigen. Such a dual specificity has been described extensively for murine TLC, and recently (38) also for a human TLC. One of the three TLC displaying this dual specificity (namely RI2G4) was studied in more detail, and was found to crossreact against a DPw4-like class II determinant. However, we could not extend our studies of this interesting phenomenon because both the original TLC as well as subclones derived from that TLC lost their antigen specificity upon further expansion of the cultures. In our hands, this has been an exception for *M. leprae*-reactive TLC. Whether this loss of antigen specificity preceded by the appearance of alloreactivity has any biological significance, and if so what, remains to be seen.

Summary

MHC class II molecules carry the restriction determinants (RDs) for antigen presentation to antigen-specific Th lymphocytes. This restriction of T cell activation endows those molecules with a key role in the induction and regulation of antigen-specific immune responses. Moreover, class II molecules are the products of class II immune response (Ir) genes. The polymorphism of these Ir

genes leads to genetically controlled differences in immuneresponsiveness between different individuals. An important human example is leprosy, in which HLA class II–linked Ir genes determine the immune response against *Mycobacterium leprae*, the causative organism of the disease. Since the immune response against *M. leprae* is entirely dependent on Th cells, the HLA class II–linked Ir gene products may well regulate the immune response by controlling the presentation of *M. leprae* antigens to Th cells. We therefore have investigated the HLA class II RD repertoire of *M. leprae*–reactive Th cell clones (TLC) by means of extensive panel and inhibition studies with fully class II–typed allogeneic APCs and well-defined HLA class II–specific mAbs. The TLC studied (n, 36) proliferated specifically towards *M. leprae*, produced IFN-γ upon activation, and had the $CD3^+CD4^+CD8^-$ phenotype.

The results show in the first place that the majority of the RDs for *M. leprae* reside on DR and not on DP or DQ molecules. This indicates a major role for DR molecules in the immune response to *M. leprae* and suggests that these molecules are the main products of *M. leprae*–specific Ir genes. Furthermore, since the expression of DR molecules is much stronger than that of DP and DQ molecules, these findings suggest that the localization of RDs for *M. leprae* on class II molecules correlates with the quantitative expression of these molecules. The observation that the RDs on DR molecules coded by a DR4 haplotype were situated only on those DR molecules that are known to be highest in expression can be explained in the same way.

Second, four distinct RDs related with but not identical to the Dw13 allodeterminant were carried by the DR^+DRw53^- $(\alpha\beta_1)$ molecules of a DR4Dw13 haplotype. Since the known amino acid residue differences between the allelic DR4 related $Dw\beta_1$ chains cannot explain the observed RD-polymorphism, this observation suggests that multiple distinct RDs unique for the DR4Dw13 haplotype are expressed by these molecules.

Only 2 of 36 TLC were not restricted by DR. One of these TLC recognized a new DP determinant, whereas the other TLC defined a remarkably polymorphic RD on a DQ molecule, which was distinct from the known DQ-related allospecificities. These TLC therefore define novel and functionally relevant polymorphisms on class II molecules. Finally, 3 of the 36 TLC reacted also with a restricted number of allogeneic APC in the absence of *M. leprae* antigen, indicating crossreactivity between self class II RD in combination with *M. leprae* antigens and allodeterminants.

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