Disease-associated Human Histocompatibility Leukocyte Antigen Determinants in Patients with Seropositive Rheumatoid Arthritis

Functional Role in Antigen-specific and Allogeneic T Cell Recognition

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Abstract

The susceptibility to develop seropositive rheumatoid arthritis (RA) has been linked to specific genomic polymorphisms within the HLA complex. Two different haplotypes have been associated with the disease, HLA-DR1 and HLA-DR4. To investigate the link between such phenotypic disease associations and potential immune mechanisms we used alloreactive and antigen-specific human T cell clones. Here we describe a panel of alloreactive T cell clones directed to polymorphic determinants encoded by the third hypervariable region (hvr) of the HLA-DR β_1-chain. T cell determinants defined by these clones are shared among HLA-DR1, HLA-Dw4, HLA-Dw13, HLA-Dw14, and HLA-Dw15, and are frequent in a population of RA patients. To study the role of such disease-associated epitopes in antigen-restricted T cell recognition we generated T cell clones from RA patients specific for mycobacterial antigens, Epstein-Barr virus antigens, and tetanus toxoid. In all three antigenic systems T cell clones were restricted to either HLA-DR1 or HLA-DR4. These data suggest that the polymorphisms within the first and second hvr of the HLA-DR β_1-chain that are distinct in HLA-DR1 and HLA-DR4 and not associated with the disease are crucially involved in the recognition of antigens. Polymorphic determinants encoded by the third hvr are shared among disease-associated haplotypes and may function to mediate the interaction of alloreactive T cell receptor molecules with the HLA complex. (*J. Clin. Invest.* 1990. 85:1051–1057.) T cell clones • Epstein-Barr virus • mycobacterial antigens • HLA-DR β_1-chain • HLA and disease association

Introduction

Although the pathogenesis of seropositive rheumatoid arthritis (RA)[1] is unresolved, multiple factors have been identified that

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1. *Abbreviations used in this paper:* hvr, hypervariable region; LCL, lymphoblastoid cell line; RA, rheumatoid arthritis; TT, tetanus toxoid.

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contribute to the disease. One major element is a genetic predisposition that has been linked to gene products of the human MHC locus, the HLA complex. Initially, cellular and serological typing techniques have documented an association of the disease with HLA-DR4 alleles (1–3). More recently, molecular biology has helped to understand the allelic variation of the serologically defined broad specificity HLA-DR4. Genetic subtypes of HLA-DR4 have been described and have been attributed to the polymorphism of at least five distinct DRβ molecules that combine on the cell surface with a nonpolymorphic DRα-chain (4, 5).

The information on the genetic organization of HLA class II loci might help us to understand the molecular basis of the genetic susceptibility to seropositive RA. Many studies have suggested that the disease is associated with the Dw4, Dw14, and Dw15 subtypes of the HLA-DR4 family, but not with the Dw10 subtype (1, 6–10). We have recently used alloreactive human T cell clones to study HLA-DR molecules expressed on the cells of RA patients and have shown that HLA-Dw14–reactive T cell clones can identify shared T cell recognition sites in patients who do not carry the classical disease-associated haplotype HLA-DR4 (8, 11). We have mapped these T cell epitopes and have correlated the conformational determinants to sequence homologies within the third hypervariable region (hvr) of the DRβ_1-chain (12). These findings opened the possibility that the genetic predisposition was linked to one or more T cell epitopes encoded by HLA-DR genes (13, 14). Such disease-associated T cell epitopes could then be recognized by T lymphocytes in the context of more than one HLA-haplotype explaining the incomplete association of the disease with one specific allele. Such a model would be in support of previous reports describing an association of RA with the HLA-DR1 haplotype that have recently been confirmed by using specific oligonucleotide probes to identify susceptibility alleles (15–18).

The role of disease-associated HLA-molecules in the pathogenesis of seropositive RA is not understood. As one hypothesis we have suggested that the disease-associated molecules might function to select, bind, and present an arthritogenic antigenic fragment to T lymphocytes, thus initiating a chronic destructive inflammatory response in the synovia (8).

The purpose of this study was to identify HLA-encoded T cell recognition sites that are highly frequent in RA patients and investigate the biological function of such determinants in the recognition of antigens. Here we show that alloreactive T cell clones can be used to recognize a cluster of T cell epitopes associated with RA. These T cell epitopes are encoded by the third hvr of the HLA-DRβ_1-chain and are shared among the two disease-associated HLA haplotypes DR4 and DR1. Studies with antigen-specific T cell clones demonstrated that the function of these epitopes was clearly distinct in allorecognition and HLA-restricted recognition of antigens, suggesting

that their biological function in the pathogenesis of RA might be linked to their interaction with T cell receptor molecules and not to their selective binding of an arthritogenic antigen.

Methods

Patients. Patients who fulfilled the American Rheumatism Association 1987 revised criteria for RA were selected for analysis (19). All patients were positive for rheumatoid factor. Patients with longstanding and severe progressive disease were included in the first study group (patients RA1–RA15). MHC class II antigens were analyzed by conventional HLA-DR and HLA-DQ typing. A second group of patients with seropositive RA (RA16–RA39) was randomly selected from patients treated at the outpatient clinic.

Additionally, a distinct subset of 11 patients with seronegative polyarthritis were studied. In these patients the disease started after age 60 and presented with systemic symptoms reminiscent of polymyalgia rheumatica (20). None of these patients developed characteristic bony erosions. The polyarthritis was characterized by a prompt and sustained response to low-dose oral steroid therapy.

Normal controls were chosen from HLA-typed healthy blood donors.

Cell lines. B lymphoblastoid cell lines (LCL) representing the HLA-DR haplotypes HLA-DR1 through HLA-DRw10 were obtained from the core panel of the tenth International Histocompatibility Complex workshop. Additional homozygous typing lines were provided by J. Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA), S. Hsu (Johns Hopkins University Medical School, Baltimore, MD), and N. Reinsmoen (University of Minnesota, Minneapolis, MN). B cells from local patients and controls were immortalized by incubation with EBV-containing supernatant.

Generation of T cell clones. Alloreactive T cell lines from normal donors were generated by stimulation with HLA-DR4$^+$ stimulator cells from patients with seropositive RA. Antigen-specific T cell lines were established from HLA-DR1–positive and HLA-DR4–positive patients by stimulation with tetanus toxoid (TT), *Mycobacterium tuberculosis* soluble extract, and EBV. In different cloning experiments either ultraviolet-irradiated, EBV-containing supernatant of the marmoset cell line B9-558 or EBV-transformed LCL were used as EBV antigens. Established T cell lines were cloned at a cell density of 0.3 cells/well in the presence of 10^5/ml irradiated B lymphoblastoid cells and 20 U/ml recombinant IL-2 (Cetus Corp., Berkeley, CA). Growing clones were maintained in recombinant IL-2–containing medium by restimulation in biweekly intervals (21).

In cross-stimulation experiments PBL from a HLA-DR1/DR4 heterozygous patient were stimulated with antigen. After 10 d recovered T cells were purified by rosetting with 2-aminoethylisothio-uronium bromide–treated sheep erythrocytes and restimulated with antigen in the presence of irradiated macrophages (5,000 rad) from an HLA-DR1/w6$^+$ or an HLA-DR4/7$^+$ patient. Cultured T cells were isolated after 10 d and restimulated with antigen and the opposite stimulator cells.

T cell proliferation assays and MAb blocking studies. The specificities of T cell clones were characterized in target inhibition studies with MAb to monomorphic HLA determinants and in panel studies using defined LCL as allostimulators and as antigen-presenting cells, respectively. 2×10^4 rested T cell clones were stimulated with 5×10^4 irradiated LCL or 1×10^5 PBL (and antigen, if appropriate) and cultured for 60 h. Proliferative responses were determined by [^{3}H]thymidine incorporation. For target inhibition studies, serial dilutions of the following MAb (0.03–5 µg/well) were added to the stimulator cells at the initiation of the culture: L227 (HLA-DR), P4.1 (HLA-DRβ_1), L243 (HLA-DR), L203 (HLA-DR), IVD12 (HLA-DQw3), Genox3-53 (HLA-DQ), and B7/21 (HLA-DP) (22).

Characterized HLA-DR–specific T cell clones were used to define T cell determinants on PBL or LCL from patients with RA in T cell proliferation assays. Results were calculated as the specific thymidine uptake by subtracting the mean thymidine uptake in cultures without

responder cells from the uptake in cultures with responder cells. They were expressed as percent relative response induced by each individual stimulator when compared with the maximal response obtained in the assay. Relative responses of < 20% were considered negative.

Results

T cell recognition sites encoded by the HLA-DR β_1-chain associated with seropositive RA. We have recently described a panel of alloreactive human T cell clones that are specific for a cluster of T cell epitopes expressed by HLA-DR4$^+$ stimulator cells (12). Panel studies on homozygous typing cells representing the different subtypes of HLA-DR4 demonstrated that these T cell clones recognized structures crucially determined by the third hvr of the HLA-DR β_1-chain. A subset of HLA-DR4–specific alloreactive T cell clones crossreacted with HLA-DR encoded molecules on HLA-DR1$^+$ stimulators. Since the HLA-DR β_1-chain of the HLA-DR1 and HLA-DR4 alleles are distinct for the first and second hvr but very similar for the third hvr, these findings suggest that a cluster of T cell recognition sites is shared among HLA-DR1$^+$ and HLA-DR4$^+$ individuals.

We wanted to study whether these T cell epitopes were found on cells of patients with seropositive RA, a disease associated with HLA-DR1 and HLA-DR4. A group of patients with classical seropositive RA and severe disease was selected. Cells of these patients were used to stimulate the proliferation of a panel of clones shown in Fig. 1. All clones were previously characterized and found to be specific for a determinant shared by HLA-DR1 and one or more subtypes of HLA-DR4 (Table I) (12). As shown in Fig. 1, all patients expressed at least one of these crossreactive T cell epitopes defined by the alloreactive T cell clones. Most patients' cells were seen by more than one of the clones, suggesting that the conformational
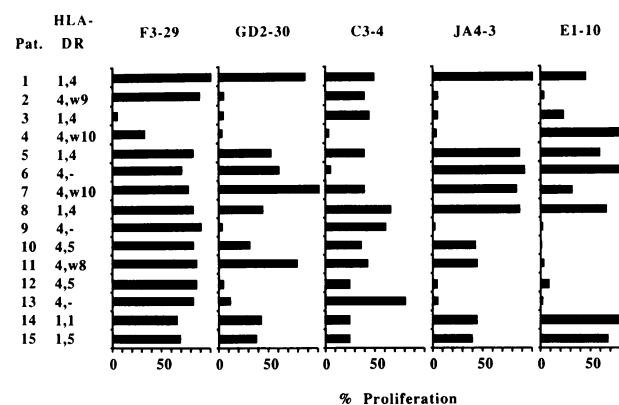

Figure 1. Distribution of allogeneic T cell epitopes in patients with severe seropositive RA. Five T cell clones that recognized shared determinants encoded by the HLA-DR β_1-chains of the HLA-DR1 and HLA-DR4 haplotypes were tested for their proliferative response to cells of patients with severe RA. Specific proliferative responses were calculated by subtracting the mean thymidine uptake from stimulator cells without responder cells from the uptake of cultures with T cell clones and irradiated stimulator cells. In each assay T cell clones were stimulated with HLA-DR1– and HLA-DR4–positive LCL as positive controls and with syngeneic cells as negative controls. Results are expressed as percent proliferative response induced by each individual stimulator, when compared with the maximal response obtained in the assay. Relative responses of < 20% were considered negative.

Table I. T Cell Epitopes Defined by Different Alloreactive T Cell Clones

Clone	HLA-Dw specificity expressing the stimulating determinant
G1-2	Dw14
GD2-30	Dw1-Dw14
C3-4	Dw1-Dw14-Dw15
F3-29	Dw1-Dw13-Dw14
JA4-3	Dw1-Dw13-Dw14
E1-10	Dw1-Dw4
HKB1-11	Dw1-Dw4
HKB1-15	(Dw1)-Dw4

Fine specificity patterns of proliferative alloreactive T cell clones were determined in target inhibition studies with HLA-DR–, HLA-DQ–, and HLA-DP–specific MAb and in panel studies using defined lymphoblastoid cell lines as stimulator cells as previously described.

Table II. Distribution of Allogeneic T Cell Recognition Sites in Patients with Seropositive RA and Seronegative Nonerosive RA of Late Onset

Clone T cell epitope	E1-10 Dw1-Dw4	HKB1-11 Dw1-Dw4	HKB1-15 (Dw1)-Dw4	G1-2 Dw14
Seropositive RA				
RA16	+	−	−	+
RA17	−	+	−	−
RA18	+	+	+	−
RA19	+	−	−	−
RA20	−	−	+	−
RA21	−	−	−	−
RA22	+	+	+	−
RA23	+	+·	−	+
RA24	−	−	−	+
RA25	+	−	−	−
RA26	−	−	−	−
RA27	+	−	−	−
RA28	+	+	−	−
RA29	−	−	−	−
RA30	−	+	+	−
RA31	+	+	−	−
RA32	+	+	+	−
RA33	+	+	+	−
RA34	+	+	+	−
RA35	−	+	+	−
RA36	+	+	+	−
RA37	+	+	+	−
RA38	−	−	−	−
RA39	+	+	n.t.	−
	15/24	14/24	10/24	3/24
Seronegative nonerosive RA of late onset				
SNPA1	+	−	−	+
SNPA2	−	−	n.t.	−
SNPA3	−	−	−	−
SNPA4	−	−	−	−
SNPA5	−	−	−	−
SNPA6	−	−	−	−
SNPA7	−	−	−	−
SNPA8	+	+	+	−
SNPA9	−	−	n.t.	−
SNPA10	−	−	−	−
SNPA11	−	−	−	−
	2/11	1/11	1/11	1/11

Cells of unselected patients with seropositive RA and seronegative polyarthritis of late onset were analyzed for the expression of allogeneic T cell determinants encoded by the third hvr of the β_1-chain of the HLA-DR4 Dw4, HLA-DR4 Dw14, and HLA-DR1 haplotypes. Proliferative responses of T cell clones were scored as positive or negative as defined in Fig. 1.

structure recognized by the T cell clones is highly conserved in patients with severe seropositive RA.

To support the finding that alloreactive T cell recognition sites shared by HLA-DR1 and HLA-DR4 are highly frequent in patients with seropositive RA, we prospectively studied a second group of patients who fulfilled the diagnostic criteria for RA but were not selected for disease severity. Results of RA patients and control patients are shown in Table II. Control patients were seen at our rheumatology clinic and were diagnosed to have seronegative RA of late onset. Both patients' groups were studied for the expression of HLA-DR4– and HLA-DR1–encoded determinants with alloreactive T cell clones. The specificities of the two T cell clones E1-10 and G1-2 have been previously reported and are summarized in Table I (12). T cell clones HKB1-11 and HKB1-15 were generated against an HLA-DR4 Dw4/HLA-DR1 heterozygous patient with RA. Proliferation of both clones could be blocked by HLA-DR–specific MAb, but not by DQ- or DP-specific MAb. In panel studies HKB1-11 recognized a determinant shared by HLA-DR1^{+} and HLA-DR4 Dw4^{+} stimulators, whereas HKB1-15 was predominantly specific for HLA-DR4 Dw4 with only a slight crossreaction on HLA-DR1 stimulators (data not shown, Table I). Again we found that T cell recognition sites defined by these alloreactive T cell clones were highly frequent in patients with seropositive RA (Table II). Only 4 of the 24 patients with seropositive RA were negative for all four clones. Compared with the selected patient group with severe disease (Fig. 1), the epitopes were slightly less frequent in unselected patients. Reactivity pattern of these T cell clones on cells of normal individuals typing HLA-DR1 or HLA-DR4 was similar, suggesting that the expression of such epitopes is not limited to RA patients (data not shown). Interestingly, cells of patients with seronegative RA of late onset were rarely recognized by the T cell clones, suggesting that this subset of patients is immunogenetically distinct from classical seropositive RA.

Antigen-specific recognition in patients with RA: crossreactivity between HLA-DR1 and HLA-DR4 in allorecognition does not predict crossreactivity of antigen-specific T cells. To approach the question of whether the shared T cell recognition sites encoded by the third hvr are used as restriction elements to recognize antigen, we studied antigen-specific proliferation

in patients whose cells were recognized by allogenic HLA-DR1-DR4 crossreactive T cell clones. PBL of an HLA-DR1/4 heterozygous patient were stimulated with either TT or EBV antigen. After 10 d of culture antigen-specific T cells were split into two aliquots. One aliquot was restimulated with antigen-pulsed stimulator cells of an HLA-DR1/w6[+] patient, and the other aliquot was cocultured with cells of an HLA-DR4/7[+]

patient plus antigen. 10 d later the lines were tested for their antigen-specific proliferation and cross-stimulated with the opposite stimulator and the appropriate antigen in parallel. No surviving cells were recovered from any of these cultures, indicating that there were no EBV- or TT-specific T cells that could be stimulated by an HLA-DR1$^+$ as well as an HLA-DR4$^+$ stimulator and could be selected by this experimental approach.

T cell recognition sites used by antigen-specific T cell clones of RA patients. To evaluate whether antigen-specific T cell clones restricted to the T cell recognition sites defined by the crossreactive alloreactive T cell clones are present in the repertoire of patients with seropositive RA, we established antigen-specific T cell clones directed to three different antigens: mycobacterial, EBV, and TT antigens. Antigen-reactive T cell clones were generated from RA patients with the following haplotypes: KE-HLA-DR1/1 Dw1; SA-HLA-DR4/w6 Dw14; DO-HLA-DR1/4 Dw14; KL-HLA-DR1/4 Dw4; and JE-HLA-DR4/5 Dw4. T cell clones whose proliferative responses could be blocked by the addition of HLA-DR–specific MAb were selected. To define the fine specificities DR-restricted T cell clones were stimulated with homozygous typing lines representing the HLA-DR haplotypes HLA-DR1 through HLA-DRw10 preincubated with the appropriate antigen. Reactivity patterns of representative clones specific for the three tested antigens EBV, Myobacterium tuberculosis soluble extract, and TT are shown in Fig. 2, a–c. T cell clones restricted to HLA-DR1 were strictly specific for the antigen when presented by HLA-DR1$^+$ stimulator cells. None of these clones could recognize the antigen when it was presented by HLA-DR4$^+$ cells. Vice versa, antigen-reactive T cell clones restricted to HLA-DR4 only proliferated when stimulated with antigen-pulsed stimulator cells of the HLA-DR4 haplotype. They did not recognize the antigen in conjunction with HLA-DR1 molecules. Some of the clones were specific for only one subtype within the HLA-DR4 family (JEE1-1, SAP2-1, DOT2-1). The majority of antigen-specific T cell clones used restriction elements shared by different subtypes of the HLA-DR4 family.

Frequency of allogenic and antigen-specific T cell clones reactive to shared and allele-specific recognition sites expressed by HLA-DR1 and HLA-DR4 molecules. In multiple cloning experiments we found a high frequency of alloreactive T cell clones that were specific for determinants shared among HLA-DR1 and HLA-DR4 molecules, while we were unable to establish antigen-restricted T cell clones reactive to HLA-DR1$^+$ and HLA-DR4$^+$ antigen-pulsed stimulators. To support this observation we studied alloreactive and antigen-reactive T cell clones in parallel cloning experiments using cells of an RA patient to stimulate PBL from a normal donor and as responder cells to generate antigen-specific T cell clones.

The results of a representative experiment are shown in Table III. PBL of a patient with seropositive RA (HLA-DR1/4 Dw4) were separated and used as stimulator cells for T lymphocytes of a non–HLA-DR4 donor. Alloproliferative T cells were cloned at a density of 0.3 cells/well. Growing T cell clones were expanded by repetitive stimulations with EBV blasts of the RA patient in the presence of IL-2. Established T cell clones were tested for their fine specificity on a panel of homozygous typing lines representing the different HLA-Dw specificities. In parallel cultures, the patient's PBL were incubated with UV-inactivated EBV antigen to initiate an antigen-specific line. After 10 d recovered T cells were restimulated
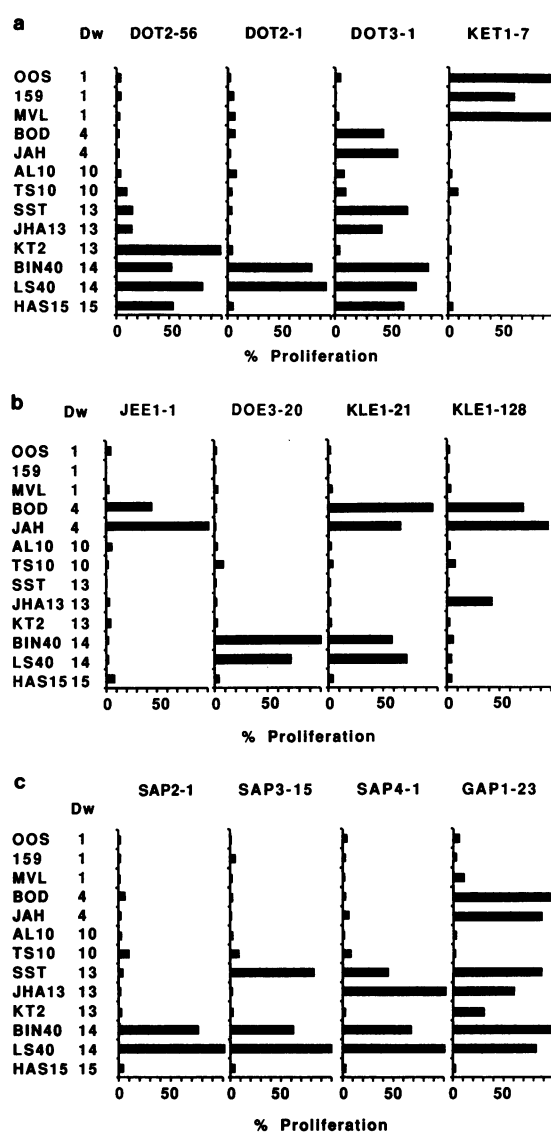
Figure 2. Restriction patterns of antigen-specific T cell clones. T cell clones specific for TT (a), EBV (b), and mycobacterial antigens (c) were established from RA patients with the following haplotypes: KE-HLA-DR1/1 Dw1; SA-HLA-DR4/w6 Dw14; DO-HLA-DR1/4 Dw14; KL-HLA-DR1/4 Dw4; and JE-HLA-DR4/5 Dw4. Clones that proliferated to antigen-pulsed HLA-DR1$^+$ or HLA-DR4$^+$ cells and that could be inhibited by MAb to monomorphic HLA-DR determinants (MAbs P4.1, L243, and L203) were tested on LCL representing the haplotypes HLA-DR1 through HLA-DRw10, as described in Fig. 1. Antigen-specific T cell clones were either restricted to HLA-DR1 or to HLA-DR4 subtypes. None of the clones crossreacted to HLA class II antigens distinct from HLA-DR1 and HLA-DR4 (data not shown).

with antigen-presenting cells of an HLA-DR4 Dw4$^+$ RA patient in the presence of EBV antigen. T cell blasts from the antigen-specific and alloreactive cultures were cloned in parallel. As shown in Table III, 46 alloreactive T cell clones were generated. 21 of them were specific for stimulator cells expressing HLA-DR4/Dw4. Two of the clones recognized a determinant shared by Dw4, Dw10, Dw13, and Dw14. 15 of the clones crossreacted on HLA-DR4 Dw4$^+$ and HLA-DR1$^+$ stimulator cells. Eight of the clones could not be blocked by

Table III. Crossreactivity Pattern of Dw4-specific Alloreactive and Dw4-restricted EBV-specific T Cell Clones

Specificity	No. of alloreactive clones	No. of EBV-specific clones
DR4 Dw4	21	28
DR4 Dw4-Dw14	0	2
DR4 Dw4-Dw13	0	2
DR4 Dw4-Dw10-Dw13-Dw14	2	0
DR4 Dw4-DR1 Dw1	15	0
Non–HLA-DR	8	9
	46	41

T cell clones were raised in parallel from an HLA-Dw4–specific and an HLA-Dw4–restricted EBV-specific T cell line, respectively. PBL of the same patient with RA were used as responder cells to initiate the antigen-specific line and as stimulator cells for the alloreactive line. Growing T cell clones were characterized in target inhibition studies and in panel studies as described in Table I. Crossreactive patterns were confirmed after subcloning.

MAb to monomorphic determinants on HLA-DR molecules, indicating that they were directed against non–HLA-DR molecules. In the parallel cloning experiment 41 clones reactive to EBV antigens were established. The vast majority of them, 28 clones, recognized the antigen when presented by HLA-DR4 Dw4$^+$ antigen-presenting cells. Four clones were restricted to determinants shared amongst Dw4 and Dw14 and Dw4 and Dw13, respectively. None of the 41 EBV reactive clones proliferated when the antigen was presented by an HLA-DR1$^+$ stimulator cell. Again, a subset of clones was not restricted to cell surface molecules encoded by the HLA-DR subregion. Thus, the analysis of Dw4-specific alloreactive and Dw4-restricted EBV-specific T cell clones demonstrated that T cell determinants shared among HLA-DR1 and HLA-DR4 were sufficient to stimulate alloreactive T lymphocytes, but not to restrict antigen-specific T cell clones. Similar results were obtained in an HLA-DR4 Dw14$^+$ patient with RA in whom TT-specific responses were studied (data not shown).

Discussion

We have used alloreactive and antigen-specific human T cell clones to analyze the biological functions of HLA class II determinants associated with seropositive RA. Although it has long been known that the genetic predisposition to develop seropositive RA is carried by HLA molecules or genes closely linked to the HLA complex the precise role of HLA molecules in the pathogenesis of the disease is not understood. Recent efforts have concentrated on pinpointing the genetic region which causes the susceptibility to the disease. Further characterization of the encoded protein structure transferring disease susceptibility may help to understand the pathological events leading to RA.

Currently HLA molecules are considered to be membrane-bound receptor structures that function in the selective binding and presentation of antigenic fragments to mediate the activation of antigen-specific T lymphocytes (23–25). Recent reports on the three-dimensional structure of an HLA class I molecule have provided a model suggesting an antigenic binding groove on the top of the molecule. The polymorphic regions of the HLA molecule form the areas of direct contact to the antigenic fragment and the interacting T cell receptor molecule (26, 27). To understand the function of HLA class II molecules in RA patients we initially generated alloreactive T cell clones specific for HLA-DR4–encoded molecules (Fig. 1, Table II) (11). We have identified multiple T cell recognition sites that are frequently expressed on cells of RA patients. The expression of these epitopes is not limited to patients who type HLA-DR4 by classical serology. HLA-DR4$^-$ patients, especially patients who carry the HLA-DR1 haplotype, share these T cell recognition sites (Fig. 1) (8, 11). Panel studies on stimulator cells exhibiting a limited sequence polymorphism have allowed us to map these T cell epitopes more precisely (12). These studies have shown that the alloreactive T cell clones are reactive to structures that are determined by the third hvr of the HLA-DR β_1-chain. Provided that the three-dimensional structure of HLA class I molecules can be applied to HLA class II molecules, we predict that these T cell epitopes are located on an alpha-helical structure that forms the wall of the antigen binding groove and may have direct contact to the T cell receptor and/or the bound antigenic fragment (12).

The T cell recognition sites that are seen by alloreactive T cell clones represent the molecular basis of the gene sequences associated with the disease. The strength of the association seems to be correlated to the severity of the disease. These findings are in accordance with earlier reports that severe seropositive RA has a strong association to HLA-DR4 and that patients with a severe variant of the disease, Felty's syndrome, are almost all HLA-DR4$^+$ (28).

Having defined and characterized a series of multiple T cell recognition sites that are seen by alloreactive T lymphocytes and linked to disease susceptibility, we were interested in understanding the function of these epitopes for the binding of antigenic fragments to the HLA molecule. Recent reports have provided evidence that allorecognition is similar to antigen-restricted recognition in the sense that alloreactive T cells do interact with a bimolecular complex formed by the foreign HLA molecule and a self-antigen (29). Provided that alloreactive T cell clones recognizing HLA-DR1$^+$ and HLA-DR4$^+$ cells were reactive to an antigen-HLA complex, then multiple antigenic fragments should be found that are able to bind to HLA-DR1 as well as HLA-DR4 molecules. To test this hypothesis we have generated antigen-specific clones from RA patients in three different antigen-driven systems. We have chosen T cell reactivity to TT as an antigenic system that allows the generation of antigen-specific T cells in the majority of individuals. A potential causative role in the pathogenesis of RA has been suggested for mycobacterial and EBV infections (30–33). In none of these systems were we able to establish a T cell clone that recognized antigen in restriction to both HLA-DR1$^+$ and HLA-DR4$^+$ antigen-presenting cells (Fig. 2). These findings suggest that the high frequency of crossreactive alloreactive T cell clones is unlikely to be explained by widely spread antigen fragments bound in the groove of the HLA-DR1 and HLA-DR4 molecules (Table III).

Although we did not find antigen-reactive T cell clones from RA patients crossreactive for both disease-associated haplotypes HLA-DR4 and HLA-DR1, it can not be excluded that there exist antigenic fragments that can be presented in the context of HLA-DR1 as well as HLA-DR4 molecules. An

example of such a peptide has recently been described by Lamb et al. (34). These authors have identified a peptide of the 19-kD protein of *Mycobacterium tuberculosis* that was recognized by individuals expressing the HLA-DR1, DR4, DRw6, or DR3 haplotype. They have also described a T cell clone from an HLA-DR1/3 heterozygous donor specific for the 19-kD mycobacterial peptide that proliferated in response to HLA-DR1$^+$ and HLA-Dw15$^+$ stimulator cells. The role of such a peptide in the pathogenesis of RA remains to be established. Studying HLA-Dw4$^+$ and HLA-Dw14$^+$ RA patients we did not find antigen-specific T cell clones that were restricted to HLA-DR1 as well as DR4, indicating that immunogenic fragments that are presented by both disease-associated haplotypes are not a frequent event in the three antigenic systems tested.

Data presented in this paper suggest that alloreactive human T cell clones are useful tools for the study of the biological function of disease-associated HLA determinants in RA, whereas the crossreactivity patterns of antigen-specific T cell clones have been limited. Mapping of the T cell epitopes recognized by the alloreactive T cell clones demonstrated that they are directed to nonlinear conformations encoded by the third hvr. In a model of HLA class II molecules based on sequence and structure homologies to class I molecules, the third hvr would adopt a helical structure exposed on the surface. Additional studies with transfected HLA class II molecules or site-directed mutagenesis may provide information about which role self peptides play in allorecognition and whether antigenic fragments are corecognized by alloreactive T cell clones that crossreact on HLA-DR1$^+$ and HLA-DR4$^+$ stimulators. Our interpretation that alloreactive human T cell clones have a direct contact to sites determined by polymorphic residues of the third hvr is supported by findings reported by groups using MAb. Several MAb have been characterized that bind to crossreactive epitopes expressed by HLA-DR1 and HLA-DR4 molecules. Studies of RA patients with these antibodies have demonstrated a high association of the disease with these shared HLA determinants. Thus, serological data and data presented in this paper suggest that the genetic susceptibility to develop seropositive RA is associated with polymorphic binding sites exposed on the top of the alpha-helical part of HLA molecules encoded by the β_1-chain. Biological functions of HLA molecules distinct from their role in the selective binding of immunogenic peptides might prove to be important in the etiopathogenesis of RA. Molecular mimicry between host proteins and microbacterial antigens has been presented as a hypothesis to explain the breakdown of self-tolerance (35). Support for this mechanism comes from a recent report describing a sequence homology between a hexapeptide of the EBV-encoded gp110 and the third hvr of disease-associated haplotypes (36). Alternatively, the pathogenic role of disease-associated T cell epitopes might be to directly interact with T cell receptor molecules and thus shape the T cell repertoire (37).

Acknowledgments

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