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# Susceptibility to rheumatoid arthritis maps to a T-cell epitope shared by the HLA-Dw4 DR β-1 chain and the Epstein–Barr virus glycoprotein gp110

**(histocompatibility antigen)**

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ABSTRACT **Rheumatoid arthritis is associated with the HLA antigen HLA-DR4. Disease susceptibility maps to the amino acid sequence QKRAA located in the third hypervariable region of the DR β-1 chain. This region is thought to be a site of recognition for the T-cell antigen receptor. We searched for an antigen in the human environment that could induce T-cell recognition of this sequence. An analysis of protein and DNA databases revealed that the Epstein–Barr virus glycoprotein gp110, which is encoded by the BALF4 open reading frame, contains the sequence QKRAAQRAA, which is highly homologous to the rheumatoid arthritis susceptibility determinant. Experiments using antibodies to synthetic peptides showed that the QKRAA determinant is expressed on the gp110 protein. Humans with serologic evidence of Epstein–Barr virus infection had serum antibodies to gp110 and peripheral blood T cells that recognized peptides from gp110 and HLA-Dw4 encompassing the QKRAA determinant.**

The B lymphocytes and macrophages from 70% of patients with seropositive rheumatoid arthritis express HLA-DR4 as defined serologically (1). The HLA-DR4 specificity is present on different antigens, which can be divided by cellular typing into HLA-Dw4, HLA-Dw14, HLA-Dw13, and HLA-Dw10. Among those HLA-DR4 subtypes, HLA-Dw4 is strongly associated with rheumatoid arthritis, whereas HLA-Dw10 and HLA-Dw13 are not (2–4). Whether or not HLA-Dw14 is associated with rheumatoid arthritis is still not clear (3, 4). The amino acid residues of the DR β-1 chain that distinguish HLA-Dw4 from HLA-Dw10 and HLA-Dw13 have been mapped to amino acids 70–74 in the third hypervariable region (5). The sequence of this region is glutamine-lysine-arginine-alanine-alanine (QKRAA, using the one-letter amino acid code) in HLA-Dw4. Recent experiments have suggested that the class II molecules of the major histocompatibility complex have two functional sites, an antigen binding site and a T-cell recognition site (6). The third hypervariable region of the β chain, which contains the QKRAA sequence, represents a potential recognition site for T cells. In the present experiments, we have searched for an antigen in the human environment that could induce T-cell recognition of the QKRAA sequence of the DR β-1 molecule. The results show that the Epstein–Barr virus (EBV) glycoprotein gp110 contains the QKRAA sequence and that EBV infection in normal people induces T cells that react with peptides from gp110 or HLA-Dw4 encompassing the QKRAA determinant.

## MATERIALS AND METHODS

**Peptides and Antibodies.** The peptides used in this study were synthesized by the solid-phase method of Merrifield, modified slightly as described (7). The sequences of the peptides (p) are as follows: Dw4p, KDLLEQKRAAVDTYC; Dw14p, KDLLEQRRAAVDTYC; Dw10p, KDILEDERAAVDTYC; gp110p, EQNQEQKRAAQRAAGC; gp110p short, EQKRAAQRRA. When used to raise antisera, the peptides were conjugated to keyhole limpet hemocyanin by using *m*-maleimidobenzoyl-*N*-hydroxysuccinamide ester, as described (8, 9). New Zealand White rabbits were injected subcutaneously with 1 mg of conjugate emulsified in complete Freund's adjuvant and then were given booster injections three times at 3-week intervals with 1 mg of conjugate in incomplete Freund's adjuvant. The rabbits were bled 4 days after the last injection and sera were stored at −20°C. The mouse monoclonal antibody to HLA DR β chain was SG171 (10).

**Enzyme-Linked Immunosorbent Assay (ELISA).** Antibody responses to the EBV glycoprotein gp110 were measured by ELISA, using the procedure developed by Luka *et al.* (11). Briefly, gp110, purified by affinity chromatography from P3HR1 infected lymphoblasts (12), was used to coat ELISA plates. Various dilutions of preimmune or immune rabbit sera and human sera diluted in isotonic borate-buffered saline (BBS) were added in 100-μl samples to the wells, which were incubated overnight at 4°C. After the washing with BBS/0.2% Tween-20, bound antibody was detected by using alkaline phosphatase-conjugated goat anti-rabbit IgG (Tago) or alkaline phosphatase-conjugated goat anti-human IgG (Boehringer Mannheim). The results are expressed as absorbance ratios, corresponding to optical densities at 405 nm for the immune serum, divided by that of the preimmune rabbit serum or by that of a serum from an EBV-negative control (11). The antibody titer for each serum was defined as the highest dilution yielding an absorbance ratio of at least 2. For inhibition experiments, peptides were added to the antisera and were allowed to react overnight at 4°C, prior to performance of the ELISA.

**Immunoblots.** Membrane protein extracts were prepared from Molt 4 T-cell lines and from lymphoblastoid cells established from HLA-Dw4 or HLA-Dw2 homozygous donors (GM03161 and GM06821A, respectively, obtained from the Human Genetic Mutant Cell Repository, Camden, NJ), using Triton X-114 extraction according to the method developed by Bordier (13). A membrane extract from $2 \times 10^6$ cells was loaded into each lane of a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) and 1 mM 2-mercaptoethanol. After electrophoresis, the reduced and denatured membrane polypeptides were electrophoretically transferred to nitrocellulose sheets (14, 15). The filters were preincubated for 1 hr in a solution containing 0.05 M borate, 0.15 M NaCl (pH 8.3), and 3% powdered milk; this was followed by overnight incubation at 4°C with the antisera

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Abbreviations: EBV, Epstein–Barr virus; VCA, viral capsid antigen.

diluted in the same solution. For inhibition experiments, peptides were added to the antibody solutions prior to their incubation with the nitrocellulose sheets. After extensive washing of the sheets with borate buffer, bound antibody was detected by using $^{125}$I-labeled protein A (1 mCi/ml; 1 Ci = 37 GBq; ICN). The sheets were incubated for 1 hr with the detecting reagent and then were washed with BBS, dried, and exposed to Kodak XAR film overnight at 70°C using an intensifier screen.

**Enumeration of Peptide-Reactive T Lymphocytes.** The relative precursor frequencies of T cells reactive with gp110p and Dw4p were determined as described (16, 17). Briefly, peripheral blood mononuclear cells were obtained from two normal subjects with no history of EBV infection (JP and BF), as indicated by the absence of antibody to the viral capsid antigen (VCA), as well as from five normal subjects with high-titer anti-VCA antibodies (ED, DS, RS, JF, MD). Mononuclear cells were isolated by isopycnic centrifugation through Ficoll-Hypaque and were washed three times in RPMI 1640 medium. Then the cells were suspended at a density of $10^6$ cells per ml in the same medium supplemented with 10% pooled human AB serum, 1% L-glutamine, 100 units of penicillin per ml, and 10 $\mu$g of streptomycin per ml in the presence of 1 $\mu$g of stimulatory peptide per ml. After 1 week of culture at 37°C, $10^5$ stimulated cells were taken from the bulk culture to evaluate the primary proliferative response to the peptide. The remaining cells were counted and diluted in fresh medium supplemented with the same peptides. The cells were distributed in 100-$\mu$l samples in 96-well round-bottom culture plates. The number of cells in each well was adjusted to $10^5$ by the addition of irradiated (3000 rad; 1 rad = 0.01 gy) peripheral blood mononuclear cells from the same donor. For each cell density, 18 wells with peptide and 6 control wells without peptide were assayed. After 4 days of secondary culture, 1 $\mu$Ci of [5-$^3$H]thymidine was added to each well 18 hr before cell harvesting on glass fiber filters. For each cell dilution, positive wells were defined as having cpm higher than the mean value + 2 SD of the cpm obtained for the 6 control wells. Precursor frequency was evaluated by plotting the percentage of negative wells for each dilution against the number of cells per well, as described (16).

In VCA-positive donors DS, RS, JF, precursor frequency to gp110p short was evaluated as described above. To test for possible cross-reactivity between gp110p short and Dw4p, the primary culture was performed with gp110p short, and then the limiting dilution was performed with Dw4p.

**HLA DR and HLA Dw Typing.** HLA DR typing of the lymphocyte donors was performed by the complement-mediated microlymphocytotoxicity method. Dw4 subtyping was kindly performed by Barbara Nepom, using an HLA-Dw4-specific oligonucleotide probe, as described (4).

## RESULTS

We searched the National Biologic Research Foundation protein sequence database and the GenBank database for a perfect match with the rheumatoid arthritis susceptibility sequence QKRAA (18). As a control, the sequences from the same region in the two HLA-DR4 antigens that are not associated with rheumatoid arthritis, DERAA (Dw10) and QKRAE (Dw13), were also sought. We found a perfect match between HLA-Dw4 (QKRAA) and the predicted sequence for an EBV glycoprotein, gp110, that is encoded by the BALF4 open reading frame of the EBV genome (19–21). The segment 807–816 of the EBV glycoprotein gp110 contains a 6-amino acid stretch, EQKRAA, that perfectly matches HLA-Dw4 and that is followed by a nearly identical second copy, QRAA, of the rheumatoid arthritis susceptibility determinant. The relevant segments are listed below with the amino acid positions shown above and below the two respective proteins.

```
                      807        816
EBV gp110     EQNQ EQKRAA QRAA
HLA-Dw4       KDLL EQKRAA VDTY
                   69    74
```

The sequences QRRAA (HLA-Dw14), DERAA (HLA-Dw10), and QKRAE (HLA-Dw13) were not found in any known human pathogen.

The EBV glycoprotein gp110 has an estimated molecular mass of 110 kDa and contains 854 amino acid residues. It is a late product of the viral infectious cycle (19, 20). The protein is thought to have a transmembrane region (19, 20). The 807–816 segment, which contains the sequence homologous to HLA-Dw4, is at the carboxyl-terminal end of the molecule, on a predicted $\alpha$-helix (19, 21).

To confirm that gp110 contains the QKRAA sequence, we synthesized a 15-amino acid peptide, designated gp110p, that encompasses the predicted sequence of gp110 from amino acid residues 803 to 817. Rabbit antibody against this peptide bound to the purified gp110, as measured by ELISA (Fig. 1). This binding was specific and was blocked by preincubation of the serum (diluted 20-fold) with gp110p (100 $\mu$g/ml).

Similarly, a 15-amino acid peptide, Dw4p, containing the sequence of the DR $\beta$-1 chain of HLA-Dw4 from residues 65 to 79, was used to raise a rabbit antiserum. This antiserum (no. 84) was shown to bind to a membrane protein present on HLA-Dw4 homozygous cells but absent on HLA-Dw2 homozygous cells (Fig. 2). The two DR $\beta$ chains from the Dw2 haplotype, DR $\beta$ a and b, differ from the HLA-Dw4 DR $\beta$-1 chain by 3 and 2 amino acids, respectively, in their third hypervariable region.

```
HLA-Dw4 DR β 1     KDLLE QKRAA VDTYC
HLA-Dw2 DR β a     KDFLE DRRAA VDTYC
HLA-Dw2 DR β b     KDILE QARAA VDTYC
```

Binding of antiserum 84 to the HLA-Dw4 DR $\beta$-1 chain could be inhibited by preincubation with the Dw4p but not with Dw10p, and only partially with Dw14p.

```
HLA-Dw4p      KDLLE QKRAA VDTYC
HLA-Dw10p     KDILE DERAA VDTYC
HLA-Dw14p     KDLLE QRRAA VDTYC
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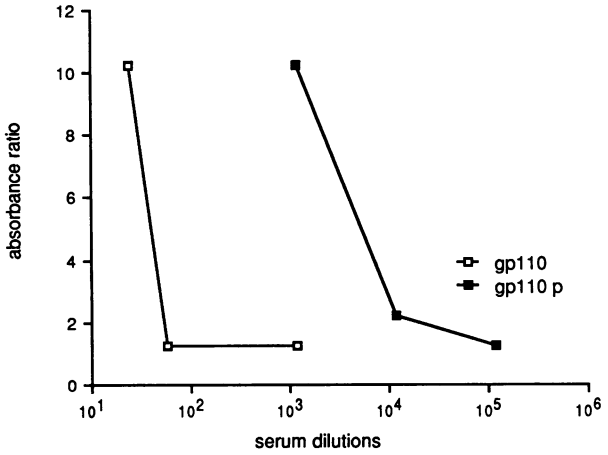
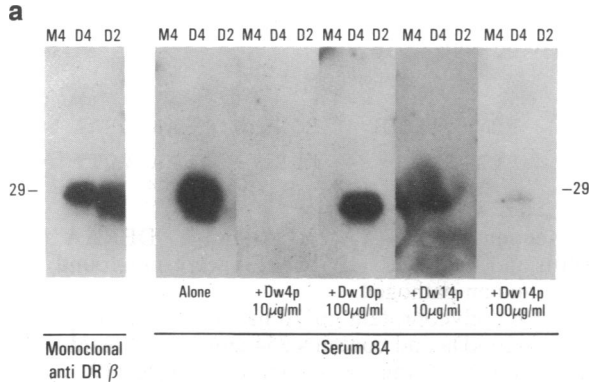



FIG. 1. A rabbit antiserum against gp110p recognizes both gp110p and gp110 in ELISA. The absorbance ratio is the ratio of the optical density at 405 nm for the sample divided by the optical density at 405 nm of the preimmune serum.




**b**

```
                    70 71
HLA-Dw4       KDLLE QKRAA VDTYC
HLA-Dw2 a     KDFLE DRRAA VDTYC
HLA-Dw2 b     KDILE QARAA VDTYC
HLA-Dw4p      KDLLE QKRAA VDTYC
HLA-Dw14p     KDLLE QRRAA VDTYC
HLA-Dw10p     KDILE DERAA VDTYC
                    70 71
```

FIG. 2. (*a*) Immunoblot of membrane extracts from Molt 4 T cells (M4), HLA-Dw4 homozygous lymphoblastoid cells (D4), and HLA-Dw2 homozygous lymphoblastoid cells (D2) electrophoresed on a 10% polyacrylamide gel under reducing conditions and transferred to nitrocellulose. The filter was then incubated with the anti-HLA DR monoclonal antibody SG171 diluted to 1:1000 (3 lanes on the left) or with serum 84 (rabbit anti-Dw4p) diluted to 1:20 (15 lanes on the right) preincubated with different peptides. Molecular masses are indicated in kDa. (*b*) The sequences of the third hypervariable region of the DR β-1 chain of HLA-Dw4, of the two DR β chains of HLA-Dw2, and of the peptides.

These results show that serum 84 recognizes the central part of Dw4p, with particular importance of the residues in positions 70 and 71. As expected, serum 84 showed binding to the purified gp110 protein (Fig. 3). Thus, the QKRAA determinant that carries susceptibility to rheumatoid arthritis is expressed both on the HLA-Dw4 DR β-1 chain and on the EBV glycoprotein gp110.

To determine whether exposure to EBV induces immune recognition of gp110, we studied the antibody response to

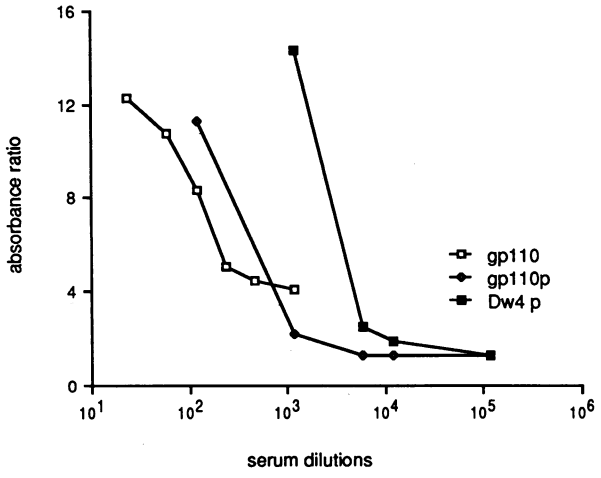



FIG. 3. Rabbit antiserum against Dw4p recognizes Dw4p, gp110p, and gp110 in ELISA. The absorbance ratio is defined as in the legend to Fig. 1.

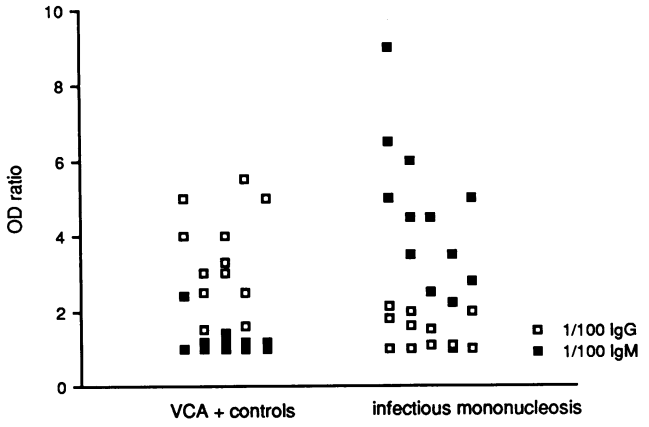



FIG. 4. ELISA for IgM and IgG anti-gp110 antibodies in the serum (diluted 1:100) of VCA-positive controls and patients with infectious mononucleosis. The absorbance ratio is defined as in the legend to Fig. 1.

affinity-purified gp110 in 6 patients with acute mononucleosis (IM) (12 sera) and in 13 VCA-positive controls (13 sera) by ELISA. All IM patients had IgM anti-gp110 antibodies. Conversely, 11 of 13 VCA-positive controls had IgG anti-gp110 antibodies (Fig. 4). These results demonstrate that EBV infection triggers antibody formation against gp110 in most people (J.L., unpublished data).

Having shown that gp110 is recognized by the immune system during and after EBV infection, we determined whether VCA-positive subjects had T lymphocytes recognizing the QKRAA determinant on gp110. Limiting dilution precursor studies were performed in five VCA-positive (ED, DS, RS, JF, MD) and two VCA-negative (BF, JP) normal adults, none of whom was HLA-Dw4 positive. As shown in Table 1, four of the five VCA-positive individuals had T-cell precursors reactive with peptides from gp110 encompassing the QKRAA determinant. Such T cells were not detected in the two VCA-negative controls.

We compared T-cell responses to gp110p and Dw4p in three subjects, ED, MD, and JP, and found they were the same. ED had the same precursor frequency to both Dw4p and gp110p; MD and JP did not have any T-cell precursor to gp110p or Dw4p (Table 1). Thus, we wondered if the same T-cell population could recognize both peptides. To test this possibility in the absence of T-cell cloning, the peripheral blood mononuclear cells from four individuals (DS, RS, JF, BF) were first stimulated with gp110p short and then were cultured under limiting dilution conditions with Dw4p. The results were indistinguishable from those obtained after stimulation with gp110p short in primary and secondary culture (Table 1), suggesting that the populations of T cells reacting with Dw4p and gp110p short overlap.

## DISCUSSION

HLA-DR4 has been subdivided into several different subtypes. The HLA-Dw4 subtype, but not the HLA-Dw10 and Dw13 subtypes, has been associated with rheumatoid arthritis (2, 3). Sequence analysis of HLA DR cDNA clones derived from cells expressing the different HLA-DR4 subtypes has shown that they differ mainly in residues 70–74 of the third hypervariable region of the DR β-1 chain (5). This region, which has the predicted amino acid sequence QKRAA in the HLA-Dw4 antigen, is a recognition site for alloreactive T cells (6, 22). How it influences susceptibility to rheumatoid arthritis is still unknown.

Our survey of sequence databases revealed that the QKRAA sequence is part of a predicted α-helical region in the gp110 protein of EBV (21). To prove that the QKRAA

Table 1. Reactivity of T cells with peptides from gp110 and HLA-Dw4

| | Reactivity | | | | | | |
|---|---|---|---|---|---|---|---|
| | VCA$^+$ | | | | | VCA$^-$ | |
| Peptide | ED (HLA DR2/4 non-Dw4) | DS (HLA DR1) | RS (HLA DR3/7-9) | JF (HLA DR2/7) | MD (HLA DR6/4 non-Dw4) | BF | JP (HLA DR5/4 non-Dw4) |
| gp110p | 1:70,000 | | | | None | | None |
| gp110p short | | 1:60,000 | 1:20,000 | 1:50,000 | | None | |
| Dw4p | 1:70,000 | * | * | * | None | None | None |
| Dw10p | None | | | | None | | None |

Peripheral blood mononuclear cells were stimulated in primary culture with gp110p, gp110p short, Dw10p, or Dw4p. In the VCA-positive subjects ED and MD and the VCA-negative subject JP, precursor frequency was assayed by using gp110 (EQNQEQKRAAQRAAGC), Dw4p (KDLLEQKRAAVDTYC), and Dw10p (KDILEDERAAVDTYC). In all experiments, the peripheral blood mononuclear cells that were recovered from the primary cultures were distributed in microtiter plates at densities of 50,000, 25,000, 10,000, and 5000 viable cells per well. In addition, each well received irradiated autologous mononuclear cells to achieve a final density of $10^5$ cells per well. For each dilution, 18 wells were cultured with peptide and 6 control wells were cultured without peptide. After 4 days, 1 $\mu$Ci of [$^3$H]thymidine was added to each well and incorporation was assessed 18 hr later. For each dilution, wells were considered positive if they had a cpm higher than the mean value + 2 SD of the cpm obtained for the 6 control wells.

*In the VCA-positive controls DS, RS, and JF, cross-reactivity between gp110p and Dw4p was tested by first culturing with gp110p short and then by incubating the cells under limiting dilution conditions with Dw4p (EQKRAAQRAA). The percent nonresponding wells did not differ from cultures in which gp110p was used throughout (results not shown).

sequence is actually expressed on both gp110 and the DR $\beta$-1 chain of HLA-Dw4, we generated antibodies to synthetic peptides encompassing these regions on gp110 and HLA-Dw4. They bound to gp110 and HLA-Dw4, respectively. In addition, the anti-Dw4p antibody bound to gp110. In conjunction with rapid DNA sequencing (3) and oligonucleotide hybridization methods (4), such a sequence-specific antibody may prove useful in identifying individuals whose cells express the QKRAA rheumatoid arthritis susceptibility determinant.

The 175-kilobase EBV genome contains ≈100 potential open reading frames (23). However, antigens that induce protective immunity following EBV infection have not been characterized completely. The gB protein of human cytomegalovirus, which is structurally analogous to the gp110 of EBV, induces neutralizing antibodies (24).

Our experiments showed that EBV infection triggered the production of IgM and IgG anti-gp110 antibodies. Most important, four of five individuals with serologic evidence of EBV exposure had peripheral T cells reactive with short synthetic peptides corresponding to the region of gp110 encompassing the QKRAA sequence. Cross-stimulation studies under limiting dilution conditions suggested that some of these T cells also recognized a peptide corresponding to the third hypervariable region of the HLA-Dw4 DR $\beta$-1 chain. Hence, it seems probable that EBV infection can induce T-cell reactivity to both gp110 and HLA-Dw4. Substantiation of this conclusion will require the characterization of multiple T-cell clones against gp110, derived from donors with and without the HLA-Dw4 antigen. The mechanisms by which particular HLA haplotypes may induce susceptibility to autoimmunity include effects on antigen presentation, on T-cell repertoire selection, and on the immune response to viral and bacterial antigens. Most likely, rheumatoid arthritis represents the final outcome of several different phenomena that are altered in the HLA-Dw4 haplotype. Future experiments will be needed to ascertain how the sharing of the QKRAA sequence by gp110 and HLA-Dw4 influences the immune response to EBV. Conceivably, self tolerance in HLA-Dw4, VCA-positive subjects may prevent the emergence of high-affinity T cells against gp110, thereby altering the course of EBV infection. Alternatively, some HLA-Dw4, VCA-positive people may develop HLA-Dw4 autoreactive T cells through exposure to gp110. The ability to stimulate human T cells with synthetic peptides from gp110 and HLA-Dw4, as demonstrated here, should permit evaluation of these opposite hypotheses.

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