Preparation and Isolation of Antibodies to Human MHC Class II alpha Chains by Aid of Synthetic Peptides

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Synthetic Peptides, Histocompatibility Antigens

Antibodies against HLA Class II alpha chains were prepared by using as immunogens synthetic peptides selected from the HLA-DQ1 alpha chains sequence.

Antibodies raised against peptide E2, a 15-residue fragment of the polymorphic first domain, reacted preferentially with cells with the DQ1 phenotype; however, despite the low sequence homology of this fragment with corresponding segments in DQw2 and DQw3 alpha chains, a partial crossreactivity with cells not expressing the DQw1 specificity was detected. Antibodies to peptide H, selected from the monomorphic frame, might be specific for DQ alloantigens, and presumably do not react with DR antigens.

The two peptides, in addition, bind anti-Class II antibodies from the serum of a rabbit immunized with human cells, and appear to represent immunogenic linear determinants in the native glycoprotein molecule.

Introduction

Over the last four years, research laboratories and private institutions have produced a relevant number of monoclonal antibodies against Class II histocompatibility antigens, which are membrane protein dimers made of two non-covalently associated chains, alpha and beta.

The large majority of these antibodies are reactive only with the beta chain, or with the $\alpha\beta$ dimer [1]; this has led to the conclusion that the complex, in its native state, is folded with the beta chain on the outside, while the immunogenic epitopes of the alpha chain are normally concealed by the three dimensional folding of the protein molecule, and therefore less accessible [2]. The relative paucity of anti-α MoAbs can be also explained by the fact that, in most protocols, hybridoma supernatants are screened by indirect binding assays with intact cells as targets. There are however several indications that the majority of anti-α MoAbs does not bind to living cells [3, 4]. Anti-α MoAbs might therefore be missed in conventional screening assays. Only by using sophisticated miniaturized Western Blot screening protocols, a panel of anti-α antibodies has been finally prepared and characterized [2].

As an alternative approach for preparing specific reagents for the alpha chain, one might attempt to raise an immune response against synthetic fragments selected from the glycoprotein primary structure: over the last five years it has been widely demonstrated that a peptide from almost any region of a protein may elicit antibodies capable of recognizing the entire protein, if originally located on the surface of that protein, and that this property is not dependent on the immunogenicity of the fragment in the complex antigen [5]. This approach might therefore provide an additional set of anti-alpha antibodies to employ for the study of relationships between structure and function of the alpha chain.

Following the same approach used for HLA-class II beta chains [6, 7], we synthesized five peptides corresponding to known amino acid sequences of DQ1 alpha chain glycoproteins, and tested which of the resulting antipeptide antibody would be reactive with intact cells expressing Class II histocompatibility antigens. In addition, we immunized a rabbit with intact cells, and attempted the isolation of anti-Class II antibodies by the use of immunoadsorbents prepared from Sepharose and three of the five synthetic fragments.

This investigation moreover might provide the location of buried and exposed regions of the native protein, and define which of the surface stretches contains an immunogenic determinant.

Abbreviations: MoAb, monoclonal antibody; KLH, Keyhole Limpet hemocyanin; PBS, phosphate-buffered saline; ELISA, enzyme linked immuno sorbant assay.

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Methods

Peptides

The five peptides were selected from the primary sequence of DQ1 alpha chain (Table I), as reported from the literature [8]. Fragments E1 (54–61), E2 (47–61), M (85–93) and N (77–84) were synthesized manually, peptide H (132–144) by aid of a Dupont-Vega Coupler, Model 1000. Peptides E1 and E2 represent polymorphic regions, and are overlapping for the C-terminal eight residues. Peptides M, N and H were selected from monomorphic amino acid stretches. An additional peptide (Q: DQ1 beta chain, 82–93), known to correspond to an exposed immunogenic region of DQ glycoproteins (Chersi *et al.* in preparation), was also used as a control in binding assays.

Antibodies

5 Rabbits were immunized with KLH-peptide complexes using the same procedures reported in previous investigations [7]. A sixth rabbit (566) was injected with whole M14 (HLA-DR4, w6; DQw1, w3) cells with the same schedule previously described for Namalwa cells [6].

Specific antipeptide antibodies were obtained from the sera of all rabbits by chromatography of the IgGs, precipitated by ammonium sulfate at 33% saturation, on 0.8×4 cm columns packed with affinity adsorbents prepared by linking Sepharose-AH to the synthetic fragments by aid of glutaraldehyde [6]. Antibodies were eluted by 0.2 M glycine-HCl buffer, pH 2.6, dialyzed against PBS, and stored at −20 °C until used.

Binding test

The binding of the antibodies to the antigens was routinely assayed with an ELISA. Wells of microtiter plates were coated with 1 μg of synthetic peptide, or 2×10^4 cells, and dried carefully at 30 °C. Immune sera were diluted 1:100 or 1:500, antibodies to 10 μg/ml, and used in the amount of 50 μl. The antigen-antibody complex was then reacted with goat antirabbit-horseradish peroxidase Ig (GAR-HRP).

o-Phenylendiamine was used as the substrate. The reaction was stopped after 10 min, by addition of 25 μl of 4 N sulforic acid, then the plates were read at 492 nm in a Titertek Multiskan.

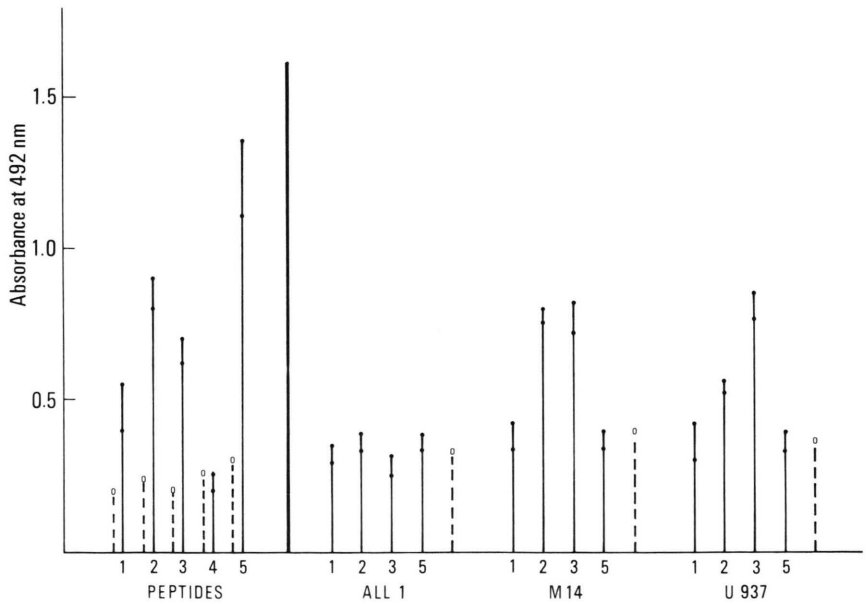

Fig. 1. Binding of antipeptide sera to the inciting fragments (left), and of affinity-purified antipeptide antibodies to ALL1, M14, and U937 cells (right), as evaluated by ELISA. Immune sera were diluted 1:500, antibodies to 10 μg/ml, and used in the amount of 50 μl. Rabbit preimmune serum, or preimmune IgG, were used as negative controls (dotted lines). The assay was performed in duplicates. 1: anti-E1; 2: anti-E2; 3: anti-H; 4: anti-M; 5: anti-N. Controls with preimmune serum or IgG are indicated with dotted lines.

The phenotypes of the cells routinely used as antigens, as defined by RIA assay [9], were the following:

M14: HLA-DR4, w6; DQw1, w3;
U937: HLA-DR3,3; DQw2, w2;
Namalwa: HLA-DR2,4; DQw1, w3.

ALL 1 cells, that do not express Class II antigens, were occasionally tested as negative controls.

An additional set of cells expressing different Class II specificites was employed in a single experiment. The phenotype of these lines is reported under Fig. 3.

Immunoprecipitation

Immune sera and affinity-purified antibodies were also tested in immunoprecipitation of highly purified, iodine-labeled Class II glycoproteins, using rabbit preimmune serum or IgG as negative controls. The sera were used in the amount of 1.25 µl, with five serial dilutions, antibodies were adjusted to 100 µg/ml and used in the amount of 5 µl, with three serial dilutions.

Results

After four immunizations with KLH-peptide complexes, the five rabbit antisera were tested with an ELISA against the five inciting fragments. All sera but anti-M were able to react with the immunogen (Fig. 1): in addition, anti-E1 and anti-E2 partly crossreacted, as predictable from the fact that fragments E1 and E2 share a complete sequence homology between positions 54 and 61 (data not shown).

Serum anti-M was not processed further. The IgGs of the other four immune sera were then loaded onto columns packed with Sepharose-peptide affinity adsorbents. The recovery of antibodies eluted with 0.2 M Gly-HCl buffer, pH 2.6 was in the range 0.25–1.7 mg/5 ml serum.

The four affinity-purified antibodies, adjusted to the same protein concentration, were then tested with an ELISA on ALL1, U937 and M14 cells; rabbit preimmune IgG, at the same dilution, was used as negative control. Results are reported in Fig. 1. None of the antibodies bound ALL1 cells, that do not express Class II antigens; anti-E2 and anti-H reacted however with U937 and/or M14 cells, suggesting therefore that peptides E2 and H might have corresponded to surface segments in the intact alpha-beta dimer. However, no conclusions could be drawn about the real immunogenicity of the two fragments in the native glycoproteins.

In order to answer the point, a rabbit (566) was immunized with intact M14 cells (HLA-DR4, w6; DQw1, w3). After six injections, the anti-class II activity of the immune serum was first assessed by testing its binding to highly purified, iodine-labeled class II molecules (data not shown), then with an ELISA, at 1:100 dilution, on fragments E2, H, and N, as well as on peptide Q, which corresponds to an exposed immunogenic epitope in HLA-DQ (Chersi *et al.*, in preparation), as well as in DR glycoproteins [10]. The antiserum apparently did not react with any peptide: this result was interpreted as due to the extremely low concentration of antibodies directed to sequential determinants, that might have prevented their direct quantitation by the immunoenzymatic method.

IgG aliquots of serum 566 were therefore chromatographed onto Seph-E2, Seph-H, Seph-M and Seph-Q in PBS, and, as a negative control, also onto Seph-N. Gly-HCl buffer, pH 2.6, eluted from all columns adsorbed proteins (range: 0.01 mg, from Seph-N; 0.12 mg from Seph-Q). The eluted samples, adjusted to the same protein concentration (0.010 mg/ml) were then tested in ELISA on the panel of cells, using preimmune serum IgG as negative control.

The binding data, summarized in Fig. 2, indicate that samples 566-E2, 566-H and 566-Q exhibited remarkable affinity for cells. Sample 566-N and 566-M, on the contrary, were devoid of antibody activity, since their binding to cells was low and comparable to that of preimmune rabbit IgG. 566-H bound also U937 cells that do not express the DQ1 phenotype.

The specificities of antibodies 566-E2 and 566-H were further defined by testing in ELISA their binding to a panel of lymphoblastoid cells with known phenotype. The results, as shown in Fig. 3, suggest that antibody 566-E2 reacts preferentially with cell lines expressing to DQ1 phenotype, and is apparently more specific, although less reactive, than antibody 566-Q used as positive control, which appears to crossreact extensively also with cells with DQ2 and DQ3 specificity. 566-H reacted with all cell lines.

Finally, in the immunoprecipitation of highly purified, iodine-labeled class II proteins extracted

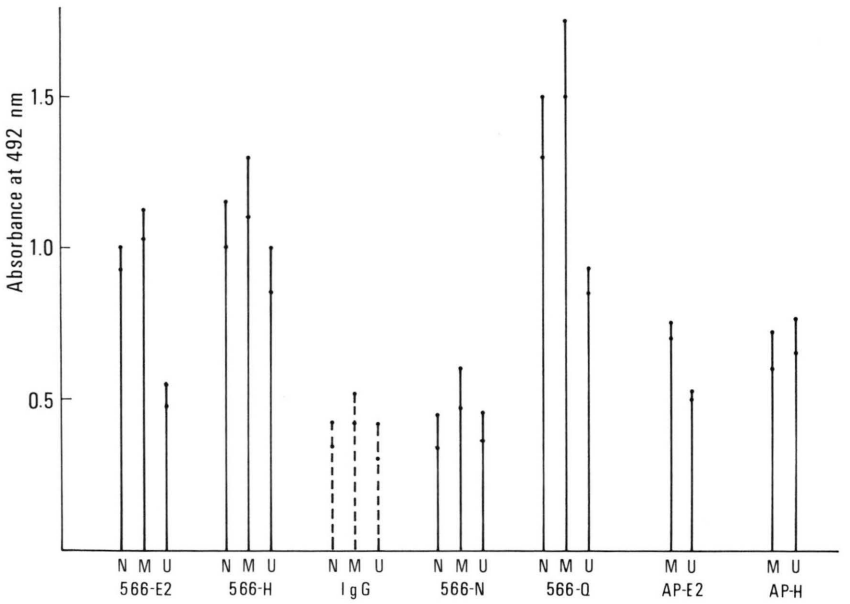

Fig. 2. Binding of antibodies 566-E2 and 566-H, isolated from the serum of rabbit 566 immunized with M14 cells, on Namalwa, M14 and U937 cells, as evaluated in ELISA. Rabbit preimmune IgG, and sample 566-N, were used as negative controls, antibody 566-Q as positive control. Binding of antipeptide antibodies (AP) anti-E2 and anti-H to the same antigens is reported for comparison. N: Namalwa; M: M14; U: U937 cells. All conditions as in Fig. 1.

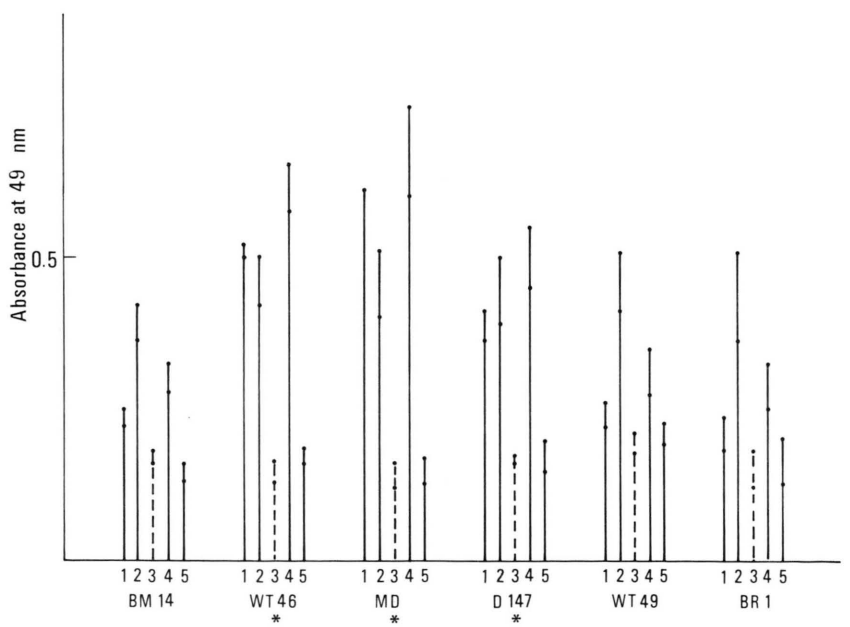

Fig. 3. Specificity of antibodies 566-E2 (1), 566-H (2), and 566-Q (4) for cells expressing different phenotypes, as evaluated by ELISA. Rabbit IgG (3) and sample 566-N (5) were used as negative controls. The specificities of the cell lines used as antigens, as defined by RIA typing [9], were the following: BM14; DR4,4; DQw3, w3; WT46: DR6,6; DQw1, w1; MDV: DR6,6; DQw1, w1; D147: DR1,7; DQw1, w2; WT49: DR3,3; DQw2, w2; BR1: DR5,5; DQw3, w3. Cells expressing the DQ1 phenotype are indicated in the figure by an asterisk. All conditions as in Fig. 1.

from DR2,2; DQ1,1 cells, antibody 566-E2 and 566-H bound 4.1 and 3.3% of the total radioactivity, control antibody 566-Q, 6.6%, while 566-N and preimmune rabbit IgG, 0.36 and 0.25%, respectively. The binding of iodine-labeled glycoproteins by antipeptide antibodies anti-E2, anti-H, and anti-N was remarkably lower, *i.e.* 3.1, 2.4, and 0.29%, respectively.

Discussion

The usefulness of an antipeptide antibody may be dependent on its ability to react with the complex antigen, and to recognize selectively specific amino acid sequences. Of the five peptides tested as source of antibodies, one (peptide M: DQ1 alpha chain, 85–93) was unable to elicit an immune response. This result might be ascribed to the limited size of the fragment used for immunization (9 residues), but seems to be counterdicted by the finding that peptide N, of approximately the same size, was the most effective in producing antipeptide antibodies. The lack of immune response to the peptide might then reflect a close similarity in sequence between the immunogen, and one protein of the rabbit used for immunization.

Two of the four antipeptide antibodies, anti-E2 and anti-H, reacted with cells expressing Class II antigens, thus providing an indication of the surface location of the two fragments in the native glycoproteins. Moreover, since antibody to fragment E1 (54–61) was unable to recognize the complex antigens, only the first 7–8 amino acid residues of peptide E2 might be exposed in the native α, β dimer. The two amino acid stretches 47–61 and 132–144, in addition, appeared to bind subsets of anti-Class II antibodies, as confirmed by the activities of samples 566-E2 and 566-H isolated from the serum of rabbit 566 injected with M14 cells. The titer of these antibodies was presumably low in the complex mixture of Ig produced against the antigenic determinants of the cell membrane, thus they were not detectable by immunoenzymatic methods. However, even peptide Q, which corresponds to a strong antigenic site in several Class II beta chains ([10]; Chersi *et al.*, in preparation), was not recognized in ELISA by anti-M14 antibodies.

The affinities of 566-E2 and 566-H for cells were remarkably higher than those of the corresponding antipeptide antibodies: this might be explained by the fact that, while the formers are directed against the precise conformation that the peptide assumes in the native antigen, antipeptide antibodies can be thought of as a mixture of molecules to the different conformations that the peptide adoptes in solution, with some percentages reactive with the conformations shared by the folded protein [11]; thus, they may poorly bind the fragment in the intact molecule.

As far as the specificities of the two sets of antibodies is concerned, a reasonable assumption supported by the binding data on the panel of cells ex-

Table I. Partial amino acid sequence of alpha chain of HLA-DQ1 histocompatibility antigens (pos. 43–148) and location of peptides selected for synthesis. For peptides selected, corresponding amino acid sequences of DQ2, DQ3 and/or DR are reported for comparison.

```
43                                                                100
 ETAWRWPEFSKFGGFDPQGALRNMAVAKHNLNIMIKRYNSTAATNEVPEVTFSKSPVT
101                                                  148
 LGQPNTLICLVDNIFPPVVNITWLSNGQSVTEDVSETSFLSKSDHSF
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E1	DQ1 (54–61)	F G G F D P Q G
E2	DQ1 (47–61)	R W P E F S K F G G F D P Q G
–	DR	– L E – – G R – A S – E A – –
–	DQ2	K L – L – H R L R · – – – – –
–	DQ3	Q L – L – R R – R P – – – – –
N	DQ1 (77–84)	I K R Y N S T A
–	DR	T – – S – Y – P
M	DQ1 (85–93)	A T N E V P E V T
–	DR	I – – V P – – – –
H	DQ1 (132–144)	E D V S E T S F L S K S D
–	DR	T G – – – – V – – P R E –

pressing different DQ and DR specificites, is that antibodies to E2 appear to be partially specific for DQ1 alpha chains, while antibodies to H bind all DQ but presumably not DR alpha chains.

The binding of both anti-E2 and both anti-H to highly purified, iodine-labeled Class II glycoproteins extracted from cells was relatively low. This might be explained by the fact that DQ molecules, on the cell membrane, are much less represented that DR molecules: apparently, the antibodies were specific enough to bind preferentially, or exclusively, to DQ alpha chains. Sequence homology between positions 47 and 61 of DR and DQ1 alpha chains is 46%, and 53% between positions 132 and 144 (Table I); this low sequence homology between DQ and DR glycoproteins in the stretches considered might result in a low affinity of the two antibodies for DR alpha chains, and explain the low binding to iodine-labeled Class II glycoproteins.

Finally, it should be mentioned that all anti-alpha chain monoclonal antibodies recently described by Cohen [2] were all directed against framework determinants, and did not show allotype restriction. Since DR molecules are much more represented on the cell membrane, than other Class II glycoproteins, it is likely that most antibodies to DQ alpha chains, including those directed to fragments H and E2, were missed in the screening assay.

The present investigation suggests that the new technology based on synthetic peptides might not only allow the mapping of surface regions of HLA-Class II alpha chains, but also produce a panel of anti-alpha chain reagents comparable to that available from hybridoma techniques.

Acknowledgements

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