Recognition of HLA Class II Molecules by Antipeptide Antibodies Elicited by Synthetic Peptides Selected from Regions of HLA-DP Antigens

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Synthetic Peptides, Histocompatibility Antigens

Repeated immunizations of rabbits with chemically synthesized peptides from selected regions of HLA-DP histocompatibility antigens resulted in the production of specific antibodies that were then isolated from the immune sera by chromatography on Sepharose-peptide immunoadsorbents.

The purified antibodies, when tested with an enzyme-linked immunosorbant assay, specifically bound to the inciting fragments; moreover, two of them recognized glycoproteins extracted by nonionic detergents from human chronic lymphocytic leukemia cells, as revealed by binding assays.

The results suggest that amino acid stretches 51–61 of the alpha chain and 80–90 of the beta chain of HLA-DP histocompatibility antigens are likely exposed on the surface of the protein molecule.

The specific recognition of DP regions is strongly suggested by the difference in the binding of those antibodies to soluble membrane proteins, as compared to the binding of monomorphic anti-Class II monoclonal antibodies to the same antigens.

Introduction

Immunization of rabbits with synthetic peptides from suitable regions of a protein one wishes to study has been used effectively as a method of producing and examining antibodies specifically interreactive with that protein.

Thereby, antibodies directed against selected regions of particular proteins have served to determine the precise structural mapping of antigenic sites and surface domains of complex molecules [1–3].

Recently, a group of synthetic peptides, corresponding to amino acid stretches of DR and DQ histocompatibility antigens, have been used for the generation of protein-reactive antibodies, and for localizing exposed regions in those membrane glycoprotein [4, 5]. Thus, the sequences 61 to 73 of DR beta chain, and 63 to 96 of DQ beta chain, appear to be located on the surface of the protein molecule. Those regions are highly polymorphic in DR and DQ alloantigens: this confirms previous observations [4] that regions with high structural variability are likely to be exposed on the surface of membrane glycoproteins.

Polymorphism might be therefore a criterium for predicting and selecting surface domains in HLA alloantigens; unfortunately, the HLA-DP molecules do not offer many possibilities for this type of selection, since the amino acid sequences of the DP alloantigens known till today are surprisingly constant: apart from rare single amino acid substitutions occurring along the chains, there is only a small 4-residue fragment (pos. 84–87) that differs between HLA-DP2 and HLA-DP3. This region might correspond to a cellular recognition site, and has a good probability to be expressed on the surface of the protein [6]. In this investigation, we attempted to produce rabbit antibodies against synthetic peptides corresponding to selected amino acid stretches of DP histocompatibility antigens, and tested their ability to react with human glycoproteins; the binding of the antipeptides antibodies to those antigens was interpreted as evidence for the localization of the corresponding sequence on the exterior of the molecule.

The selection of the peptides to be synthesized was based on comparison of the amino acid sequences of

Abbreviations: CLL, chronic lymphocytic leukemia; GP, glycoproteins; MBS, *m*-maleimidobenzoyl-N-hydroxy succinimide ester; KLH, Keyhole Limpet hemocyanin; NZW, New Zealand White.

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DP, DQ and DR glycoproteins [7–13]: in order to avoid cross-reactivities, we choose for investigation amino acid stretches in DP antigens that presented low sequence homology with either DQ molecules, or DR, or both. One of the stretches selected for synthesis (R: β chain 80–90) included the four residue fragment 84–87, known to be polymorphic in DP alloantigens [6].

The others three fragments were S (β chain, 63–77), F (β chain, 94–108) and D (α chain, 51–61).

Materials and Methods

Peptides

The four peptides to be used for eliciting antipeptide antibodies were selected one from the alpha chain, three from the beta chain of DP histocompatibility antigens [8, 10].

Peptides S (DP β chain, 63–77), F (DP β chain, 94–108), R (DP β chain 80–90) were synthesized by the use of a fully authomatic Beckman peptide Synthesizer, model 990B. Peptide D (DP α chain, 51–61) by manual synthesis. The amino acid composition of all peptides was controlled by amino acid analysis after acid hydrolysis. Four additional peptides were used for evaluating the degree of cross-reactivity of the antipeptide antisera; those were P (DQ2, 3 β chain, 80–90), Q (DQ1 β chain 80–90), G (DQ1, 2, 3 β chain, 94–108) and E (DQ1 α chain, 49–58). All those peptides, available from previous investigations [5], were related in amino acid sequence to the DP peptides used for immunization. The sequence, localization, and the degree of homology of all those fragments is reported in Table I.

Antibodies

Rabbit antipeptide antibodies 203 and 207, raised against peptides 60–77 of β chain of HLA-DQ1 and HLA-DQ2, respectively, and antipeptide antibody 6148, directed against peptide 59–71 of HLA-DR2 β chain, were available from previous investigations [4, 5].

Monoclonal antibodies

The monomorphic anti-Class II monoclonal antibodies 13A/B6, 11A/B5 and PTF 29–12 were kindly provided by Drs. Roberto Tosi and Giorgio Corte.

Production of antisera

Three peptides (R, F, D) were used for immunization: those fragments were first coupled to a carrier protein (KLH or ovalbumin) by the aid of MBS or glutaraldehyde [14]. Three NZW rabbits received an initial subcutaneous injection of 200 μg of peptide-KLH complex in incomplete Freud's adjuvant, and subsequently, bimonthly subcutaneous injection of 100 μg in incomplete adjuvant. The animals were first bled after three injection and then every 14 days. The blood was centrifuged for 15 min at $1600 \times g$ after standing at room temperature for 4 h. The supernatant was stored at −20 °C until used.

Preparation of the immunoadsorbent and purification of the rabbit antibodies

The peptides were bound to Sepharose-AH resin through their C-terminal cysteine by the aid of MBS, or through their N-terminal amino groups, by the aid of glutaraldehyde.

Details were similar to those described for other Sepharose-peptide immunoadsorbents [14]. The resin was washed and equilibrated with PBS, then packed in 1×6 cm columns.

Antibodies precipitated from the immune sera by ammonium sulfate at 32% saturation were then loaded onto the immunoadsorbent. Chromatography was performed in PBS; adsorbed IgG were eluted with 0.2 M glycine-HCl buffer pH 2.6, immediately neutralized, dialyzed in the cold against PBS, and stored at −20 °C until used.

Enzyme-linked immunosorbant assay

For this assay, wells of microtiter plates were precoated with 1 μg of antigen peptide, or with 2 μg of glycoproteins solubilized by nonionic detergents from the cell lines specified later. The antibody was used in serial dilutions. The antigen-antibody complexes were reacted with peroxidase-labelled goat antirabbit IgG, and detected by addition of *p*-phenylendiamine and hydrogen peroxide. Finally the plates were read in a GDV "Immunella" at 492 nm.

Membrane glycoproteins

Human chronic lymphocytic leukemia (CLL) cells were used as a source of soluble membrane glycoproteins. The phenotypes of the cells, as defined by RIA typing [15], were:

CLL 51: DRw6,7; DRw52; DRw53; DQw1w2
CLL 52: DR1,7; DRw53; DQw1w2
CLL 53: DR1,5; DRw52; DQw3
CLL 54: DR1,5; DRw52; DQw1w3
CLL 55: DR2,7; DQw1w3.

Membrane glycoproteins were extracted from those cells by the use of nonionic detergents, then isolated by affinity chromatography on Lens Culinaris Lectin-Sepharose [16].

Results

Before being used for coupling and rabbit immunization, peptide S (DP β, 63–77) was first tested in ELISA against antipeptide antibodies previously produced against three synthetic peptides of HLA-DQ and HLQ-DR glycoproteins, respectively B (DQ1, 60–77), C (DQ2, 60–77), and W (DR2, 59–71); those fragments corresponded approximately, in size and localization, to peptide S. Homology of S to B was 53%, that of S to C, 66%, that of S to W, 78%.

In binding assays, using peptide B and C as positive controls, anti-B, anti-C and anti-W antibodies cross-reacted extensively with peptide S. We concluded therefore that the selection of peptides from this region, where HLA-DQ, -DR, -DP exhibit high sequence homology, might not be suitable for the production of specific antipeptide antibodies. Peptide S, therefore, was not processed further.

Peptides D, F and R were linked to a carrier protein (KLH or ovalbumin) by the use of MBS or glutaraldehyde, and used to inject three NZW rabbits.

After three immunizations, the rabbits were bled from the ear, and the immune sera tested in ELISA for their affinity for the immunogen. All three sera reacted with the inciting peptide. The IgG were then precipitated with ammonium sulfate at 32% saturation, collected by centrifugation, dissolved in water, dialyzed against PBS, and loaded on 1×6 cm columns packed with the corresponding Sepharose-peptide immunoadsorbent, equilibrated in PBS. The specific antipeptide antibodies were then eluted from the resin by 0.2 M glycine-HCl buffer pH 2.6, neutralized with ammonium bicarbonate, dialyzed against PBS, and stored at −20 °C until used. Details of similar procedures were described elsewhere [4].

Those affinity-purified antibodies were then tested in ELISA against all synthetic peptides available, in order to evaluate cross-reactivities. Results are reported in Fig. 1: antibody 544 (anti-R) reacts extensively also with peptide P (DQ2,3, β chain, 80–90) and to a lesser extent, with Q (DQ1 β chain, 80–90). Sequence homology between R and P is 54%, that between R and Q, 45%. The homology of R to the same stretch in DR glycoproteins is considerably lower, so it may be predicted that this antibody will not react with HLA-DR antigens.

Antibody 211 (anti-F) cross-reacts poorly with peptide G (DQ β chain, 94–108: homology 40%). On the other hand, the homology of F to the corresponding stretch in DR proteins is over 80%, and extensive cross-reactivity might be expected.

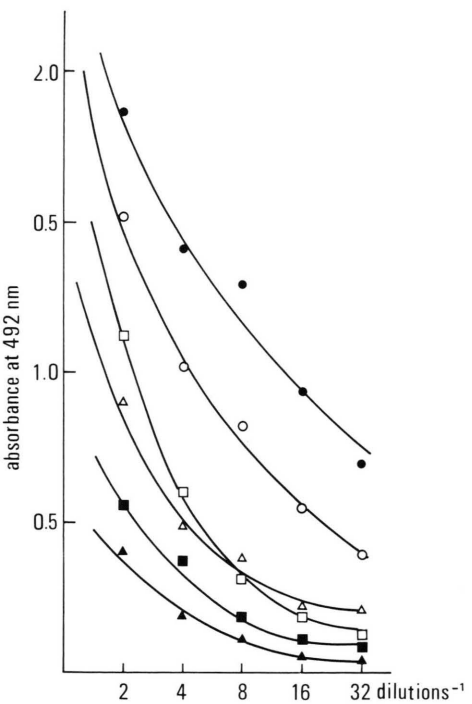

Fig. 1. Binding of affinity-purified antipeptide antibodies to the inducing fragment, and to related peptides, as evaluated in ELISA. Wells of microtiter plates were coated with 1 μg of synthetic peptide. The antibodies (initial concn. 6 μg/ml) were used in the amount of 50 μl, with four serial dilutions. The values obtained with preimmune rabbit IgG, at the same concentrations, were subtracted.
●——●: Ab 208 (anti-D) on peptide D,
○——○: Ab 544 (anti-R) on peptide R,
□——□: Ab 544 (anti-R) on peptide P,
■——■: Ab 544 (anti-R) on peptide Q,
△——△: Ab 211 (anti-F) on peptide F,
▲——▲: Ab 211 (anti-F) on peptide G.

Finally, antibody 208 (anti-D) does not bind to peptide E: the homology between the two fragments is 37%. However, this antibody is expected to react with the related fragment in DR α chain, because of 73% homology.

In order to increase the selectivity of antibody 544 for peptide R and HLA-DP proteins, and to eliminate cross-reactivity with related fragments P and Q, antibody 544 (1.5 mg in 2 ml PBS) was allowed to react for 45 min at 4 °C under gentle stirring, with a small aliquot of Sepharose-peptide P immunoadsorbent (0.2 ml packed gel). The gel was discarded by centrifugation, and the immunodepleted antibody 544 (544 I.D.) tested again with an ELISA on the three peptides R, P, and Q. The binding data (Fig. 2) indicate a sharp drop of antibody affinity for peptides P and Q. This depleted antibody preparation, therefore, is expected to discriminate HLA-DP from HLA-DQ molecules.

The attempt to purify antibody 208 (anti-D) by immunodepletion, in order to avoid cross-reactivity with HLA-DR molecules, was not undertaken: previous observations [14] indicate that antipeptide antibodies elicited by related peptides with high sequence homology are completely adsorbed by

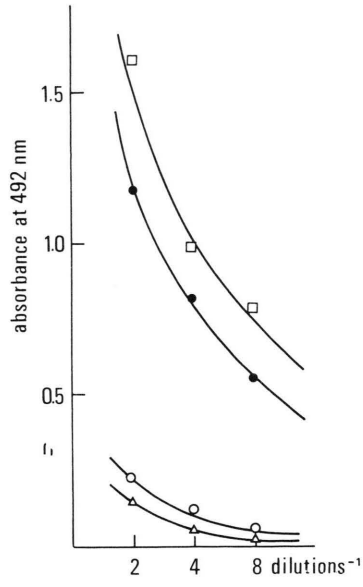

Fig. 2. Binding of antibody 544 I.D. (anti-R) to peptides R, P and Q, after immunodepletion by Sepharose-peptide P immunoadsorbent (Seph-P), as evaluated in ELISA. The binding of the antibody to peptide R before immunodepletion is also reported (□——□).
●——●: Ab 544 I.D. on peptide R,
△——△: Ab 544 I.D. on peptide P,
○——○: Ab 544 I.D. on peptide Q.

Table I. Partial amino acid sequences of α and β chains of HLA-DP, -DR, and -DQ glycoproteins and localization of peptides selected for immunization or for evaluating the degree of cross-reactivity.

	Alpha chains	Peptides
	40 50 60 70 80	
DP	DLDKKETVWETVWHLEEFGQAFSFEAQGGLANIAILNNNNLNTLIQRSNHT	D
DR	-MA----------R-----RFA------A-----VDKA--EIMTK---Y-	
DQ	--ER-------A-RWP--SKFGG-DP--A-R-M-VAKH---IM-K-Y-S-	E
	Beta chains	**Peptides**
	60 70 80 90 100 110	
DP2	DEEYWNSQKDILEEERAVPDRNCRHNYELGGPMTLQRRVQPRVNVSPSKKGPLQHHNLLVCH	S,R,F
DR2	-A----------QA--AV-TY-----GVVESF-V----H-Q-T----------S	W
DQ2	AA----------RK--AV--V-----Q-ELRT------E-T-TI---RTEA-N-------S	C,P
DQ3	AA-------EV--RT--EL-TV-----Q-ELRT------E-T-TI---RTEA-N-------S	
DQ1	VA-------EV--GA--SV--V------VAYRGI-----E-T-TI---RTEA-N-------S	B,Q,G

Homologies:
D with E: 37%
S with W: 78%
S with C: 66%
S with B: 53%
R with P: 54%
R with Q: 45%
F with G: 40%

DR and DQ sequences were aligned in order to give maximum homology with DP sequences.

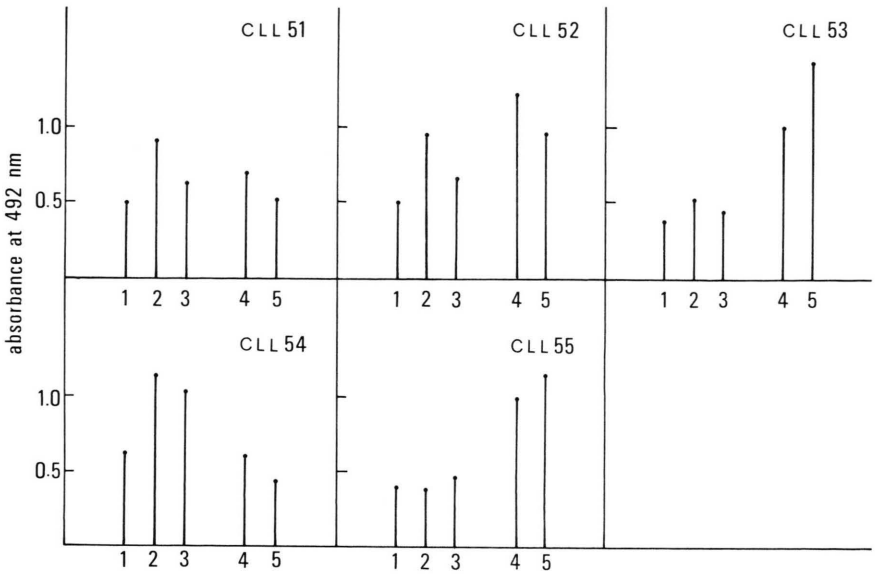

Fig. 3. Binding of antibody 208 (anti-D) and 544 I.D. (anti-R) to membrane glycoproteins extracted by nonionic detergents from 5 different CLL cells bearing different HLA-DQ and HLA-DR specificites. Three monomorphic mouse anti-Class II monoclonal antibodies were used as positive controls. The plates were coated with 2 μg of soluble glycoproteins. Rabbit antibodies had a concentration of 50 μg/ml, mouse MoAbs were diluted to 8 μg/ml. 50 μl were used for the assay, that was performed in duplicate. Ab 211 (anti-F) did not react with the antigens: the values are not reported in the figure. 1: MoAb 11A/B5, 2: MoAb 13A/B6, 3: MoAb PTF 29-12, 4: Ab 208 (anti-D), 5: Ab 544 I.D. (anti-R).

immunoadsorbents prepared from any of the related fragments.

In the last experiment, the three antibody preparations 211, 208 and 544 I.D. were tested with an ELISA on membrane glycoproteins extracted by nonionic detergents from different chronic lymphocytic leukemia (CLL) cells. As positive controls, we used three monomorphic anti-Class II mouse monoclonal antibodies, as negative controls, preimmune rabbit IgG. Rabbit antibodies were diluted to a concn. of 50 μg/ml, mouse antibodies to 8 μg/ml. 50 μl of those solutions were used.

In Fig. 3, the binding of the antibodies to the antigens is reported. Antibody 211 (anti-F) failed to react, while antibodies 208 and 544 I.D. reacted with every soluble glycoprotein preparation. Comparison of those binding data with these obtained by using the three anti-Class II monoclonal antibodies, indicates that the antigenic sites recognized by the two antipeptide antibodies do not correspond to these seen by the mouse antibodies. In fact, rabbit antibodies show high affinity for glycoproteins CLL 53 and 55, mouse antibodies for CLL 54 and CLL 52. Since those latter antibodies are essentially directed against antigenic sites expressed on both DQ and DR molecules, and possibly DP, the difference in reactivity between antipeptide antibodies and the group of monoclonal antibodies might be ascribed to the fact that antibody 544 I.D., and possibly 208, recognized antigenic determinants on a restricted number of glycoproteins, likely DP histocompatibility antigens.

Discussion

Three antipeptide antibodies against different regions of HLA-DP histocompatibility antigens show striking differences in their reactivities when tested against membrane glycoproteins of cells expressing Class II antigens. In fact, antibody 211 against stretch 94–108 of DP beta chain did not bind to soluble membrane glycoproteins, nor to lymphoblastoid cells. On the contrary, antibody 208 elicited by fragment 51–61 of DP alpha chain, and antibody 544 against peptide 80–90 of the beta chain, were both able to recognize antigenic determinants in all glycoprotein preparations.

While the lack of reactivity of the first antibody may be ascribed to an internal localization of the peptide F used for immunization, the two latter ap-

parently localize two exposed regions in the DP molecule, one corresponding to pos. 51–61 of the alpha chain, the other to pos. 80–90 of the beta chain. This last sequence includes the four-residue fragment 84–87 that differs between DP2 and DP3 molecules [6]. It is interesting that this regions corresponds to a stretch of high structural variability in all Class II antigens.

As far as the specificity of those two antibodies is concerned, it is likely that antibody 208 (anti-D) recognizes DP as well as DR molecules, because of the high sequence homology in the 51–61 region of alpha chain of the two proteins. On the contrary, antibody 544 I.D., that originally cross-reacted with fragment P of HLA-DQ2,3 antigens, appears to be quite specific for peptide R after immunodepletion, and might be able to discriminate DP molecules from DQ. Cross-reactivity to DR molecules is unlikely because of low sequence homology.

The specificity of antibody 544 I.D. for a quite restricted group of antigenic sites seems to be confirmed by the comparison of the binding affinity of this antibody for soluble membrane glycoproteins, with these obtained by testing on the same preparations, a group of monomorphic anti-Class II mouse monoclonal antibodies. Independently from the MoAb assayed, the reactivity for the five glycoprotein preparations decreases in the order: CLL 54, CLL 52, CLL 51, CLL 53, CLL 55.

With oversimplification, the difference in affinity of a monoclonal antibody for the glycoprotein preparations reflects different amounts of Class II antigens in the samples. On the other hand, antibody 544 I.D. reacts mostly with CLL 53 and CLL 55, and scarsely with CLL 51 and CLL 54. This completely different reactivity pattern might indicate that this antipeptide antibody recognizes a selected group of specific antigenic sites on a restricted family of Class II antigens, likely on DP molecules.

Under those considerations, a ratio: *binding of 544 I.D./binding of monoclonal antibody* can be calculated for every CLL sample, and this value might represent the relative expression of DP antigens on the cell line under investigation. A low ratio, as for instance for CLL 54, might be ascribed to a low representation of DP molecules on those cells, or to the occurrence of the DP3 alloantigen, that is likely not recognized by antibody 544 I.D.

The utilization of those reagents directed against well defined structures of HLA-DP antigens might be of considerable use for the mapping of surface regions of the native protein, and for the evaluation of the expression of those glycoproteins on a given cell line. The definition of the involvement of exposed regions in the immunologic and functional properties of those scarsely known antigens is actually under investigation in our laboratory.

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