Partial Characterization of anti-HLA Class II Antibodies Isolated by Aid of Sepharose-Peptide Immunoadsorbents

Alberto Chersi, Maria Cristina Morganti-Kossmann*, Michele Evangelista, and Cristina Cenciarelli

Istituto Regina Elena for Cancer Research, Rome, Italy, and

* Institut für Neurobiologie, Heidelberg, Bundesrepublik Deutschland

Z. Naturforsch. **46c**, 321–323 (1991); received September 10, 1990

Synthetic Peptides, Histocompatibility Antigens, anti-HLA Antibodies

Synthetic peptides selected from HLA-DQ and HLA-DP glycoproteins were coupled to Sepharose, and used for the isolation of anti-HLA Class II antibodies from the immune sera of rabbits immunized with human lymphoblastoid cells expressing Class II antigens.

Antibodies from early and late bleedings displayed remarkable differences in affinity for peptides and for soluble membrane proteins: these differences might be due to an early immune response directed preferentially against surface linear determinants, and to a late response to assembled (discontinuous) sites.

The possibility that such antibodies might be used for the identification of amino acid stretches involved in the formation of the same assembled determinant is considered.

The antigenic sites of proteins are architecturally of two types: they may comprise residues that are on a continuous segment of the protein chain [1], or are distant in sequence but, due to the folding of the polypeptide chain, some into close spatial proximity (assembled or discontinuos sites) [2]. In the immune response against a complex antigen, antibodies to both alternative site architectures should be anticipated [3], although the response is preferentially directed to assembled determinants [4].

Immunization with peptides, on the contrary, results in the production of antibodies to sequential epitopes. An external peptide may elicit protein-reactive antibodies, either because, of the many conformations it adoptes in solution, it occasionally assumes one resembling its cognate structure in the native molecule [5], or because the local mobility of protein segments mimics the flexibility of the peptide in solution and allows the binding of antibody subsets to different conformations [6]. Antibodies to a complex site, and anti-peptide antibodies to the same linear segment, might be different in their specificities and affinities for sequential and discontinuous epitopes.

In order to prove this assumption, two rabbits (266 and 350) were immunized with whole Namalwa cells (HLA-DR 2,4; DQ 1,3), according to the schedule previously reported [7]. Briefly, the rabbits received a first injection of 40×10^6 cells carefully suspended in 500 µl of incomplete Freund's adjuvant, and subsequently, 20×10^6 cells, at ten-day interval. The first blood sample (Bleeding 1) was collected from the ears five days after the third immunization, then regularly every second injection. The serum was recovered 6 h after the bleedings by centrifugation. The anti-Class II activity of the immune sera was controlled by direct binding of highly purified, iodine-labeled Class II molecules (data not shown).

Anti-Class II antibodies were isolated from the immune sera by using immunoadsorbents prepared by coupling suitable supports to synthetic peptides selected from Class II histocompatibility antigens, and known to represent immunogenic surface regions [7, 8]. Peptide A (DQ 3 beta, 63–79), and B (DQ 1 beta, 63–79) were linked to Sepharose-AH by aid of MBS [9], taking advantage of their terminal cysteines, peptide D (DP alpha, 51–61) was coupled to a self-activating support (Affi-Gel 10) by following instructions of the Bio-Rad manual. 5 ml aliquots of the immune sera of each rabbit were then loaded onto columns packed with the immunoadsorbents: columns were washed first with PBS, then with PBS/0.5 M NaCl; adsorbed antibodies were eluted with 0.2 M Gly-HCl buffer, pH 2.6, immediately neutralized, dialyzed in the cold against PBS, and stored at −4 °C until used.

Rabbit 266 resulted to be a poor responder to the immunizations, thus only data concerning rabbit 350 will be reported here. The antibody samples isolated from bleedings 1, 3, and 5 by affinity-chromatography on the 3 absorbents were thus

Abbreviations: PBS, phosphate-buffered saline; MBS, maleimidobenzoyl N-hydroxysuccinimide ester; ELISA, enzyme linked immuno sorbant assay.

Reprint requests to Alberto Chersi, Regina Elena Institute for Cancer Research, Laboratory of Biochemistry, Viale Regina Elena 291, 00161 Rome, Italy.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0300–0321 $ 01.30/0

tested with an ELISA against the peptides and against soluble membrane glycoproteins, using rabbit preimmune IgG as negative control. Antipeptide antibodies against fragments A, B and D, available from previous studies [8, 9], were employed as positive controls.

The ELISA test was performed as previously described [9]. Briefly, wells of microtiter plates were coated with 1 μg of synthetic peptide, or 2 μg of soluble membrane glycoproteins extracted by nonionic detergents from CLL cells expressing Class II antigens; antibodies were adjusted to the same initial concentration (0.010 mg/ml) and used in the amount of 50 μl. Goat anti-rabbit horseradish peroxidase was used as secondary antibody, and *o*-phenylendiamine as substrate. The enzymatic reaction was stopped after 5 min by addition of 25 μl of 4 N sulfuric acid. Wells were then read at 492 nm in a Titertek Multiskan.

The binding data, as derived from ELISA, indicate that antibodies prepared from bleedings 1, 3 and 5 of rabbit 350 exhibit a gradual and constant increase in affinity for membrane glycoproteins, while their binding to the peptides remains substancially constant. Antipeptide antibodies elicited by the respective peptides, on the other hand, greatly increase their affinities for the synthetic fragments, but react always poorly with the complex antigens (Fig. 1).

The high affinity of antibodies 350 for glycoproteins, as compared to those of antipeptide antibodies, may be explained by the fact that such molecules are essentially directed to that precise three-dimensional configuration of the segment, while antipeptide antibodies are directed to different conformations of the synthetic fragment in solution, some of which only may resemble that occurring in the native antigen [10].

For the group of antibodies of rabbit 350, the increase of affinity for membrane glycoproteins with time, associated with the constancy in binding to the peptides, might be due to a continuous modulation and maturation of the immune response, with gradual changes in specificity of antibody subsets produced: antibodies to simple sequential epitopes, as mimicked by the synthetic peptides, might be mainly represented in the early stages of immunization, those directed to complex, discontinuous sites might be the product of a late response.

It should be pointed out that, as suggested by Berzofski [4], if antibodies do bind to linear amino acid stretches, this sequence alone is not necessarily the complete determinant: other residues or groups of residues nearby on the 3-dimensional surface may constitute a complex site and participate in antibody binding.

If this assumption is true, the same antibody might react with different segments of the protein, *i.e.*, with these that participate to the formation of the antigenic site. By testing the affinity-purified antibody on a panel of synthetic fragments from

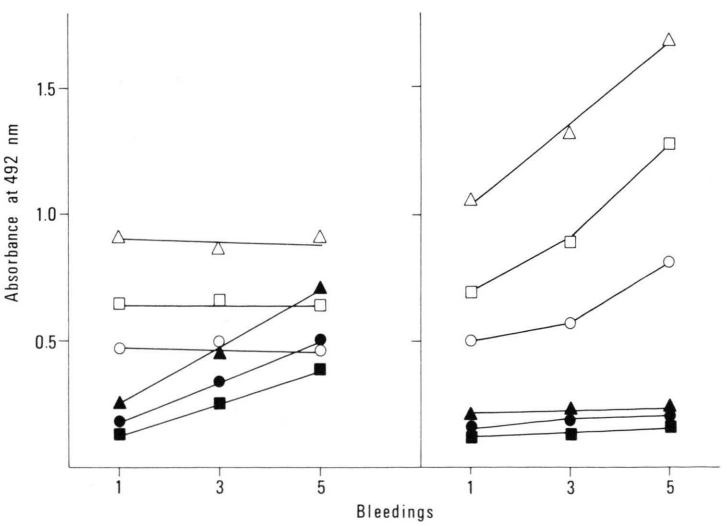

Fig. 1. Binding of affinity-purified anti-Class II antibodies from rabbit 350, bleedings 1, 3 and 5, to synthetic peptides (empty symbols) and to soluble membrane glycoproteins (full symbols), as evaluated by ELISA.
Circles: 350-A; squares: 350-B; triangles: 350-D. The binding of the corresponding antipeptide antibodies to the same antigens is reported on the right side of the figure.
For the test, microtiter plates were coated with 1 μg of synthetic peptide or 2 μg of soluble membrane glycoprotein, and dried at 30 °C. Antibody preparations were adjusted to the same protein concentration (0.010 mg/ml) and used in the amount of 50 μl. The assay was performed in duplicates.

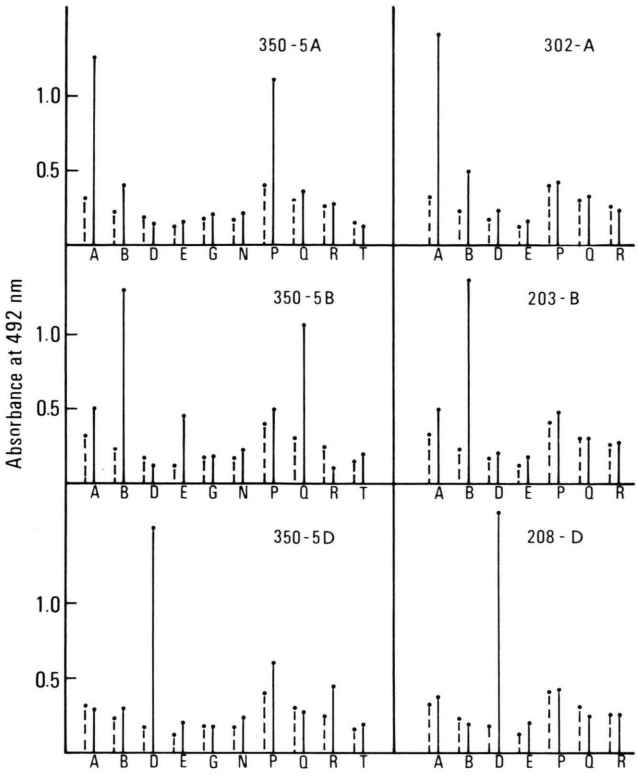

Fig. 2. Binding of antibodies 350-A, 350-B, and 350-D, as determined by ELISA, to a panel of synthetic fragments corresponding to different amino acid stretches of Class II membrane glycoproteins, as follows:
A: DQ3β 63–79; B: DQ1β 63–79; D: DP α 51–61; E: DQ1α 44–58; G: DQ1,2,3β 96–110; N: DQ1α 77–84; P: DQ2,3β 82–93; Q: DQ1β 82–93; R: DPβ 82–93; T: DQ2β 51–59.
Assay conditions as in Fig. 1. Controls (preimmune rabbit IgG) are indicated by dotted lines. 302, 203 and 208 are antipeptide antibodies.

the protein primary structure, it might be possible to individuate some of the segments that constitute an assembled epitope.

When antibodies 350-A, 350-B and 350-D from bleeding 5 were tested in ELISA against a panel of peptides selected from alpha and beta chains of membrane glycoproteins, 350-A and 350-B appeared to react also with peptides P and Q corresponding to positions 82–93 of DQ3 and DQ1 beta chains, respectively. Antipeptide antibodies, tested as a control, did not (Fig. 2). This might be an indication that both fragments 63–79 and 82–93 are close in space and are presumably involved, at least for a segment, in the formation of the same antigenic site. Future data on the structure of Class II antigens [11] will assess whether this hypothesis and this approach might be valid.

Acknowledgements

This study was partly supported by the Associazione Italiana per la Ricerca sul Cancro. The authors thank Mr. L. Dall'Oco for the graphic work.

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Verantwortlich für den Inhalt: A. KLEMM
Satz und Druck: Allgäuer Zeitungsverlag GmbH, Kempten