Rabbit Antipeptide Antibodies against Restricted Domains of the Histocompatibility Complex

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Synthetic Peptides, Antipeptide Antibodies, Histocompatibility Antigens

The work reported here concerns the specificty of four antibodies elicited by synthetic peptides corresponding to three domains of the HLA-B7 glycoprotein, and one of the β_2 microglobulin. One of the four peptides had been previously investigated, but the data obtained by its elicited antibodies were uncomplete.

Of the three HLA-B7 peptides assayed, one was unable to raise an immune response. The other two produced antibodies in good titer that reacted with the antigen peptide, but were unable to recognize antigenic determinants when tested on membrane glycoproteins solubilized from human lymphoblastoid cells. In contract, the antibody elicited by the only fragment from the $\beta_2$microglobulin recognized the native $\beta_2$microglobulin molecule as well as HLA/β_2m complexes, and reacted with intact human cells, as determined by ELISA and by FACS analysis. This peptide, which contains one of the most hydrophylic fragment of the whole molecule, is likely an important antigenic site, since it is also recognized by traditional antisera raised against native $\beta_2$microglobulin.

Introduction

HLA-A, -B, and -C antigens are membrane glycoproteins composed by two non-covalently associated polypeptide chains. The larger component (MW 45000 daltons) is a highly polymorphic glycoprotein encoded by the Mayor Histocompatibility Complex located on chromosome 6, and carries determinants responsible for allospecificity. The small component (MW 12000 daltons) is a serum protein encoded on chromosome 15, and which contains no allotypic determinants [1].

The biggest part of the heavy chain, including an oligosaccharide unit bound to Asn 86, lies on the external membrane of the cell: a small portion enters the lipid bilayer and the cytoplasm [2].

The $\beta_2$microglobulin is entirely extracytoplasmatic, is non-covalently but tightly bound to the heavy chain, and contributes to the folding of this latter molecule. The precise role of the $\beta_2$microglobulin in the immune system is unclear, although the binding of this molecule to the nascent heavy chain appears to be required for the complete cell expression of these membrane glycoproteins [3, 4]. Circular dichroism data [5] indicate that the heavy chain undergoes a conformational change after binding to the small molecule. However, the final 3-D structure of the complex, and the localization of its surface domains, is to day almost completely unknown.

In recent years a number of systems have been developed to predict protein conformations from the amino acid sequence [6, 7] as well as for locating antigenic determinants by analyzing the regions of greatest local hydrophilicity [8]. By this method, taking advantage of the availability of the amino acid sequences for the HLA heavy chains with different specificity [9, 10], as well as for $\beta_2$microglobulins from different sources [11, 12], one could predict the localization of the most probable antigenic sites of the histocompatibility antigens. The direct proof of this theoretical approach has to be obtained by the determination of the binding capacity of the antibodies raised against every particular region, to the native, folded molecule. Preliminary experiments performed with this technique seem to indicate that fragment 39 to 50 of the HLA-B7 glycoprotein is buried or masked [13], while at least a portion of the fragment 61 to 83 must be exposed on the surface of the HLA/β_2m complex, since the antipeptide antibody promptly recognizes the intact

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Abbreviations: KLH, keyhole limpet hemocyanin; MBS, M-maleimidobenzoyl-N-hydrohysuccinimide ester; PBS, phosphate-buffered saline; AG, Agarose; DADPA, diaminodipropylamine; GP, membrane glycoproteins; FCS, fetal calf serum; FACS, fluorescein-activated cell sorter; β_2m, beta-2-microglobulin.

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complex in binding and immunoprecipitation assays [14].

In the present study, we selected new fragments from the HLA/$\beta_2$microglobulin complex, and investigated the antibodies elicited by these peptides in their ability to bind to native membrane glycoproteins from a suitable cell line, with the aim of elucidating the antigenic structure of the histocompatibility antigens, and of selecting these antibodies that could be employed in future functional studies.

Materials and Methods

Cell line and preparation of the glycoproteins

The human homozygous lymphoblastoid cell line GM 3107 (HLA-A3,3: B7,7) was a generous gift of R. Tosi, Istituto di Biologica Cellulare, Rome.

A small amount of membrane glycoproteins were isolated from these cells by affinity chromatography on Lens culinaria lectin-Sepharose, as reported by others [15].

Synthesis of peptides

The sequence of the peptides used in the present investigation were chosen from the HLA-B7 glycoprotein [9] and from the human urinary $\beta_2$microglobulin [11]. Three peptides were prepared by the solid-phase method [16] using a Beckman Synthesizer, Model 990 B, and one, HLA-B7$_{(134-146)}$ by manual synthesis. In all but this last peptide, a cysteine was added to the C-terminal amino acid of each fragment, to facilitate the coupling to the carrier protein and to the Agarose-DADPA resin used as immunoadsorbent.

Coupling of the peptides of the carrier protein

Three peptides were coupled to KLH with the aid of maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) [17], through the cysteine at the COOH-terminal. Coupling efficiency, calculated by the determination of residual free -SH groups in solution after three hours reaction, [18], varied from 27% to 61%.

Peptide HLA-B7$_{(134-146)}$, which lacked a free -SH group, was coupled to KLH by the use of glutaraldehyde.

Production of antisera

Following an initial subcutaneous injection of 300 µg of peptide-carrier complex in complete Freund's adjuvant, each of four rabbits received bimonthly 150 µg of the complex in incomplete adjuvant. The animals were bled the first time after 30 days, and then regularly every second week.

Antiserum 4246 was a generous gift of Dr. William Church, and was obtained by immunization of a rabbit with bovine $\beta_2$microglobulin purified from cow colostrum.

Binding assays

The binding of antisera or purified rabbit antibodies to the antigens was routinely assayed by the Enzyme Linked Immuno Sorbant Assay. The method has been described elsewhere [13].

Preparation and use of the immunoadsorbents

For the purification of antipeptide antibodies from the immune sera, specific immunoadsorbents were prepared coupling each peptide to agarose-diaminodipropylamino resins (AG-DADPA) (Pierce), through the C-terminal cysteine by the aid of MBS. Details of this method will be reported elsewhere (Chersi *et al.*, in preparation). Different subclasses of antibodies from immune serum AC14, directed against different epitopes in the fragment β_2m$_{(32-50)}$, were separated on an immunoadsorbent prepared from Agarose-diaminodipropylamine resins and membrane glycoproteins from GM 3107 cells. For that pourpose, AG-DADPA resin (4 g) was reacted with 2 mg of glycoproteins and 5 ml of a 25% solution of glutaraldehyde in PBS, under stirring. After 90 min, the immunoadsorbent was washed by repeated centrifugations in PBS. The binding of the glycoproteins to the resin was only 27%, as determined by spectrophotometric measurements.

Partially purified antibodies from immune serum AC14 were separated on a 0.8 × 4 cm column of this resin, using an initial washing with the starting buffer (PBS), followed by 0.2 M glycine-HCl buffer pH 2.3.

Preparation of the F (ab')$_2$

2 mg of purified antipeptide IgG were digested with pepsin at 37 °C for 18 hours, in 0.12 M acetate buffer pH 4.0 with an enzyme-to-substrate ration of

3 : 100. Samples were then centrifuged, dialyzed against PBS, and treated for 2 hours at 4 °C with a suspension of Protein A-Sepharose, to remove undigested IgG.

Indirect immunofluorescence

10^6 GM 3107 cells in 0.05 ml PBS containing 10^{-2} M sodium azide were incubated with 100 µl of antibody (0.70 mg/ml) for 45 min at 2 °C, washed by centrifugation through FCS, treated with fluorescein-labeled goat anti-rabbit IgG (45 µg), centrifuged again through FCS, washed with PBS, and finally fixed in 10% formalin. Samples of this preparation were submitted to traditional fluorescence microscopy and to fluorescence-activated cell sorter analysis. Normal rabbit IgG was treated and tested in the same way as a control.

Results

Table I summarizes the sequences of the four peptides used as immunogens in the present investigation. All together they cover approximately 20% of the total amino acid sequence of the HLA-B7 heavy chain, and about the same of the $\beta_2$microglobulin. Their schematic localization in the primary sequences of both proteins is reported in Fig. 1.

The selection of two of the four fragments studied was based on the presence of regions with high local hydrophilicity, according to a recent method for locating the most likely antigenic determinants [8]. The peptide corresponding to pos. 186 to 200 of the heavy chain was selected because of its unusual amino acid composition, since it contained four histidine residues in a total of 15 amino acids, and it was assumed that it would elicit a rabbit antibody unable to show fortuitous crossreactivity with unrelated proteins. The small peptide HLA-B7$_{(134-146)}$ was chosen arbitrarly.

Table I. Amino acid sequences of the peptides selected from the HLA-B7 heavy chain and $\beta_2$microglobulin, and used for immunization.

HLA-B7:		Antiserum
134–146	TAADTAAQITQRK	AC07
186–200	KTHVTHHPISDHEATC	AC10
215–232	LTWQRDGEDNTQDTELVEC	AC11
$\beta_2$microglobulin		
32–50	PSDIEVDLLKDGERIEKVEC	AC14

For each peptide, the six-residue fragment with the highest local hydrophylicity has been underlined. The average hydrophylicity value, calculated according to Hopp [8], is respectively 0.7, 0.8, 2.0 and 2.2.
Antibodies to peptide HLA-B7$_{(215-232)}$ were partially investigated in a previous study [14].

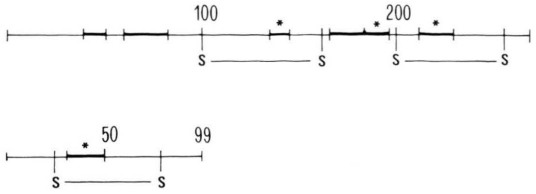

Fig. 1.Schematic localization in the primary structure of HLA-B7 and $\beta_2$microglobulin of the peptides used as immunogens in this (*) and in previous investigations.

The four peptides were coupled to KLH by aid of MBS or glutaraldehyde, and injected into rabbits. The animals were bled the first time after three immunizations, and then regularly every second week. The amount of elicited antipeptide antibody in each immune serum was assayed by ELISA. Three out of four antisera promptly reacted with the inducing peptide, while antiserum AC10, raised against the fragment HLA-B7$_{(186-200)}$ did not recognize its antigen, and therefore was not investigated further.

The three antisera AC07, AC11 and AC14 were then purified on the specific immunoadsorbent, and further characterized in their ability to bind to intact glycoproteins solubilized by nonionic detergents from human lymphoblastoid cell lines, using the ELISA. It was found that antibody AC07 did not react, antibody AC11 reacted poorly, while antibody AC14 against β_2m$_{(32-50)}$ was able to recognize antigenic determinants located on the surface of the native complex. Moreover, it reacted with human urinary $\beta_2$microglobulin as well as with bovine $\beta_2$microglobulin purified from colostrum. On the other hand, a polyclonal antiserum (4246), raised in a rabbit against the bovine $\beta_2$microglobulin, was able to react with this fragment in ELISA, recognizing therefore an antigenic domain resembling the fragment used for immunization (Fig. 2).

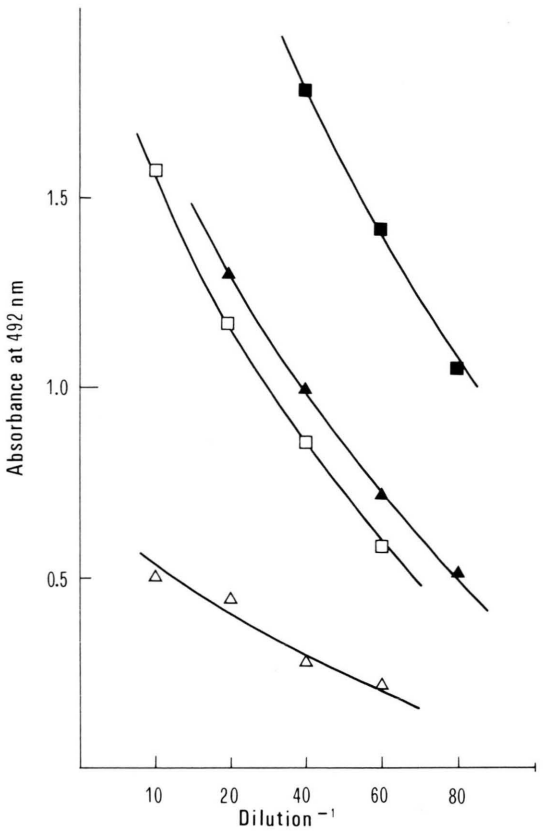

Fig. 2. Binding of purified antipeptide antibody AC14 to human (■) and to bovine $\beta_2$microglobulin (□), and of rabbit immune serum 4246 to human $\beta_2$microglobulin (▲) and to the synthetic fragment $\beta_2m_{(32-50)}$ (△), as evaluated in ELISA. The protein concentration of antibody AC14 was 0.08 mg/ml. Immune serum 4246 was arbitrarly diluted to a protein concentration of 10 mg/ml. 25 µl of each sample were used for the test, at the dilutions indicated.

Inhibition experiments confirmed that the binding of antibody AC14 to these antigens was selective and specific: increasing amounts of the inducing peptide $\beta_2m_{(32-50)}$ inhibited almost completely the reaction of antibody AC14 with membrane glycoproteins from GM 3107 cells, and with human or bovine $\beta_2$microglobulins (Fig. 3). The peptide, however, did not significantly inhibit the reaction of polyclonal antiserum 4246 with human or bovine $\beta_2$microglobulins (data not shown).

However, it can be demonstrated that only a small subclass of the antibody population elicited by peptide $\beta_2m_{(32-50)}$ is effectively directed against antigenic sites of the intact HLA/β_2m complex, the majority of the Ig molecules being directed against the synthetic peptide. A chromatography on a suitable immunoadsorbent (AG-DADPA-GP) allows to separate the antipeptide antibodies into two fractions, one reacting almost exclusively with the synthetic peptide, the other with the peptide as well as with intact membrane glycoproteins. This latter fraction accounts for about 20% of the total antibodies loaded, and this confirms preliminary studies in which the percentage of antipeptide antibodies reacting with native HLA-DR molecules was estimated by inhibition techniques (Chersi *et al.*, unpublished results). A slight increase in the binding of antibody AC14 to intact glycoproteins can be obtained when the antigen is first subjected to full oxidation by performic acid, before being immobilized on microtiter plates: this procedure might cause a partial unfolding of the complex and exposure of burried or masked antigenic sites. The binding of this antibody to intact cells was evaluated by ELISA, using the F(ab')$_2$ fragment of the

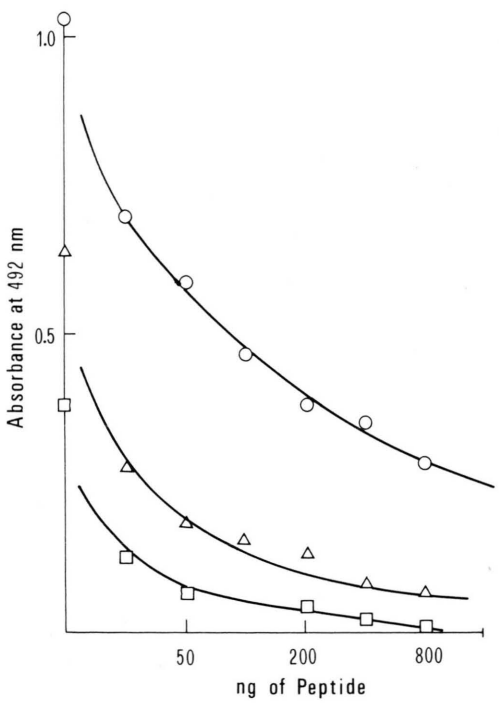

Fig. 3. Inhibition by increasing amounts of peptide $\beta_2m_{(32-50)}$ in the binding of antibody AC14 (1 µg protein) to human (○) and bovine $\beta_2$microglobulins (△), and to membrane glycoproteins solubilized by nonionic detergents from GM 3107 cells (□), as evaluated in ELISA.

purified antibody, to minimize non-specific binding and background activity, and by Cell Sorter Analysis: Using the indirect immunofluorescence assay, from 33% to 39% of 3107 cells resulted stained by fluorescein-labeled goat antirabbit IgG, when previously incubated with antibody AC14, while only 7% of the same cells were labeled when normal rabbit IgG replaced antibody AC14 under the same conditions.

Discussion

Four antipeptide antibodies directed against different regions of the HLA/$\beta_2$microglobulin complex were investigated in the present paper in their ability to bind to the antigen peptide and to native membrane glycoproteins and cells.

One of the four peptides used as immunogen, *i.e.* HLA-B7$_{(186-200)}$ was not able to elicit rabbit antibodies: this result may reflect an identity or a close similarity in sequence between this fragment, and one present in a protein of the animal. The immune response of the animal, in fact, was exclusively directed against the carrier protein, as evaluated by ELISA.

Antipeptide antibody AC07, characterized by a good titer in the binding to the antigen peptide, was not able to react with intact glycoproteins solubilized by non-ionic detergents from the cell line GM 3107. As for antipeptide antibodies described in previous papers [13, 14] the most likely explanation for this result is that the fragment selected for the immunization is buried in the interior of the native molecule, the HLA/$\beta_2$microglobulin complex, where it is not avaible for interreaction with the antibody. An alternative possibility is that the coupling of this fragment to the carrier protein through the N-terminal amino and the C-terminal lysine ε-amino groups by glutaraldehyde prevented the peptide from assuming, for at least part of the time, a configuration that resembled that occurring in the intact protein.

Antibody AC11, raised against peptide HLA-B7$_{(215-232)}$, which is comparable to antibody 6153 partially investigated in a previous study [14], shows low but still measurable activity when assayed against native membrane glycoproteins. Although a partial exposure of this fragment could not be excluded, we think that its partial activity might be better explained in terms of crossreactivity of this antibody with a not-related antigenic site: one possibility is the region 32–50 of the $\beta_2$microglobulin, since the comparison of the amino acid sequences of the HLA-B7$_{(215-232)}$ and β_2m$_{(32-50)}$ peptides reveals fortuitous similarities, as a D G E fragment, a E–K V C terminal piece, and a D– – –D–E section. Crossreactions of antipeptide antibodies have been reported, as a result of identical or closely similar epitopes that may occasionally occur in otherwise unrelated proteins [19]. Partial crossreactivity can in fact be demonstrated between the two peptides and antibodies AC11 and AC14.

Antibody AC14, raised against β_2m$_{(32-50)}$, reacts with human urinary $\beta_2$microglobulin, and is the only one of the four antibodies tested able to bind in Enzyme Linked Immuno Sorbant Assay to intact membrane glycoproteins and to GM 3107 cells. The binding to these cells can be confirmed by conventional fluorescence microscopy and FACS analysis. The antibody reacts also with bovine $\beta_2$microglobulin isolated from cow colostrum, as a result of the 75% homology in amino acid sequence between the human and the bovine proteins. On the other hand, a polyclonal antiserum raised in a rabbit against the intact bovine $\beta_2$microglobulin recognizes the β_2m$_{(32-50)}$ fragment in binding assays. This result is somehow unexpected, since antibodies to an intact protein very seldom react with its fragments, expecially when presenting low molecular weights.

The binding data suggest the following considerations:

1) The domain 32–50 of the $\beta_2$microglobulin is exposed (at least partly) on the surface of the native molecule.

2) This domain is also exposed on the HLA/β_2m complex, is not involved in the binding to the heavy chain, is not sterically masked by neighbouring groups because of this interreaction.

3) This region likely contributes to the antigenicity of the whole molecule. However, the precise role of this domain in the intact complex has still to be elucidated.

The analysis of the binding data of the antipeptide antibodies to the HLA/β_2m complex presented here, and in previous papers [13, 14] indicate that, as far as today, only two regions of the histocompatibility complex are effectively recognized by the antipeptide antibodies investigated: one is the hypervariable region of the heavy chain (res. 61 to

83), one is the fragment 32–50 of the $\beta_2$microglobulin. This scarce ability of the antipeptide antibodies in reacting with native structures seems to be contradicted by recent studies [20] that indicate that the majority of monoclonal antipeptide antibodies recognize native cognate proteins.

Unless one admits that the majority of our eight peptides investigated were unfortunately selected from internal regions of the HLA/β_2m complex, and taking into account the special methodology by which monoclonal antibodies are selected, this result suggests that nost rabbit antipeptide antibodies might contain small, often undetectable populations of immunoglobulins able to react with native cognate structures: the scarce representation of these molecules could be due to the low probability by which a synthetic peptide may adopt in solution a conformation resembling the structure in the intact molecule. Further studies are in progress to evaluate this possibility.

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