Determinants of antigenic molecules responsible for genetically controlled regulation of immune responses

(IMMUNOGENETICS/IMMUNE SUPPRESSION/HISTOCOMpatibility complex)

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ABSTRACT The ability of mice bearing the H-2 haplotype to develop helper responses to the random copolymer of Glu,Ala while they developed suppressor responses to the terpolymer of Glu,Ala,Tyr suggested the crucial role of tyrosine in these peptides. On the basis of various considerations, it was postulated that many of the tyrosine residues in Glu,Ala,Tyr would be localized at the NH₂-terminal end of the molecule. To verify this hypothesis, a block terpolymer composed of a short sequence of homopolymer tyrosine covalently bound to the random copolymer of Glu,Ala was synthesized. The present studies, using this block terpolymer, demonstrated that the chemical determinants influencing helper and suppressor responses are distinct and can be present simultaneously in the same molecule. Thus, addition of COOH-terminal tyrosine residues to the Glu,Ala polypeptide converted this immunogenic molecule to an immunosuppressive molecule in mice bearing the H-2haplotype. The mechanism by which these short sequences of tyrosine influence H-2-linked immune responses remains to be determined.

The immune response to thymus-dependent antigens is under the control of genes in the major histocompatibility complex in mice (1), rats (2, 3), guinea pigs (4) and rhesus monkeys (5). Synthetic polypeptide antigens have been very useful in the study of the activity of these genes, which have been termed H-linked Ir genes, and which differ for distinct antigens (6). The responses of mouse strains bearing different H-2 haplotypes to the two related linear copolymers of L-amino acids, poly(Glu₆₀,Ala₄₀) and poly(Glu₆₀,Ala₃₀,Tyr₁₀) (abbreviated to GA and GAT, respectively), are examples of antigens under such distinct genetic control, although the antibodies produced against the two copolymers are very crossreactive. Mice bearing H-2α,β,d,k haplotypes respond, whereas mice bearing H-2β₀,α₀ haplotypes do not respond to both antigens. However, mice bearing the H-2 haplotype, such as the SJL or the congenic resistant B10.S strains, respond to GA but not to GAT. Thus, the presence of 10% tyrosine in the latter copolymer affects critically the immunogenicity of this antigen for H-2 mice (7).

The amino acid copolymers used in these studies are prepared by polymerization of the appropriate N-carboxyamino acid anhydrides, in which the various reactive functions are reversibly blocked. The polymerization reaction starts from an initiation point and is propagated via the free amino group. The distribution of the different amino acid residues along the polymeric chains reflects the rate of polymerization of the respective N-carboxyamino acid anhydrides (8). Since the N-carboxyanhydride of tyrosine polymerizes more slowly than those of γ-benzylglutamate and alanine, it seemed reasonable to assume that poly(Glu₆₀,Ala₃₀,Tyr₁₀) is composed of molecules in which most tyrosine residues are clustered at the amino-termini of the chains. This raised the possibility that a stretch of tyrosine residues is capable of blocking the antibody response to the poly(Glu₆₀,Ala₃₀) portion of the molecules in mice bearing the critical H-2haplotype.

The process controlled by H-linked Ir genes is believed to be concerned with the specific stimulation of helper thymus-derived lymphocytes (T cells) and/or with the process whereby these helper T cells exert their regulatory activity on antibody responses (1, 6, 7). The inability of genetic nonresponder animals to form specific antibody responses to the relevant antigen may indeed be overcome by immunization with the antigen coupled to or complexed with an immunogenic protein carrier such as methylated bovine serum albumin (MeBSA) capable of stimulating helper T cells in these animals (9, 10). Furthermore, our recent studies have demonstrated the stimulation of specific suppressor T cells in mice bearing nonresponder H-2 haplotypes for the copolymer GAT immunized with this antigen (10, 11). These suppressor T cells inhibit specifically the responses of these mice to GAT-MeBSA in vitro and in vitro, to which, as stated earlier, they normally respond. On the basis of these various considerations, the inability of the mice bearing the H-2 haplotype to develop antibody responses to GAT, while they respond to GA, suggests that the stretch of polytyrosine postulated to be present at the NH₂-terminal end of the GAT molecule is capable of suppressing the antibody response to GA determinants in H-2(SJL) mice.

To verify this hypothesis we have prepared a block copolymer composed of a short homopolymer of L-tyrosine covalently bound to a random copolymer of L-glutamic acid and L-alanine, and have investigated the antibody responses to this new copolymer poly(Glu₆₀,Ala₃₀)-poly(Tyr₁₀)(abbreviated to (GA)-T) in mouse strains bearing different H-2 haplotypes. The antibody responses to (GA)-T were compared to those elicited in the same strains by GA and GAT. In addition, we have compared the ability of (GA)-T to suppress the primary plaque-forming cell (PFC) responses of H-2 SJL mice to GAT-MeBSA, (G,A)-T-MeBSA, and GA. The results demonstrate that the newly prepared block copolymer (GA)-T resembles very closely, both in its immunogenic and suppressive properties, the random copolymer poly(Glu₆₀,Ala₃₀,Tyr₁₀). It is, therefore, reasonable to conclude that the inability of mice bearing the H-2 haplotype to produce antibodies against GA in response to GAT or (GA)-T is due to the unique ability of oligo(L-tyrosine) sequences to stimulate suppressor cells capable of suppressing the response to other determinants in this molecule normally immunogenic for these mice. This is, therefore, an example of the demonstration on the same molecules of different determinants capable of stimulating helper and suppressor responses under the control of H-2-linked genes.

Abbreviations: GA, poly(Glu₆₀,Ala₄₀); GAT, poly(Glu₆₀,Ala₃₀,Tyr₁₀)(GA)-T; poly(Glu₆₀,Ala₃₀)-poly(Tyr₁₀); T cells, thymus-derived lymphocytes; MeBSA, methylated bovine serum albumin; PFC, plaque-forming cells.
Fig. 1. (a) Chromatographic separation of oligotyrosine on a Sephadex LH-20 column. (b) Chromatographic separation of (GA)-T on a Sephadex G-50 column. The materials under the first peaks were collected and lyophilized as indicated in Materials and Methods.

MATERIALS AND METHODS

Animals. Mice were obtained from the Experimental Unit of the Weizmann Institute of Science for experiments carried out in Rehovot. The mice studied at Harvard Medical School were either purchased from the Jackson Laboratories, Bar Harbor, Maine, or bred in the animal facility of the Department of Pathology.

Antigens. These polymers were used: poly(Glu\(^{50}\), Ala\(^{30}\), Tyr\(^{10}\)), abbreviated GAT, with an average molecular weight of 47,000; poly(Glu\(^{50}\), Ala\(^{30}\)), abbreviated GA, with an average molecular weight of 45,000, which were purchased from Miles Laboratories, Inc., Miles Research Division, Elkhart, Md. and Pilot Chemicals, Watertown, Mass., respectively.

The synthesis of the block copolymer poly(Glu,Ala)-poly-(Tyr) [abbreviated to (GA)-T] is described below. The block copolymer was prepared by making use of the amino terminus of an oligotyrosine. N-Carboxytyrosine anhydride was dissolved in dioxane, with butylamine (12) as initiator for the polymerization, and kept for 3 days under stirring. The product, oligotyrosine, precipitated out while the excess of the N-carboxytyrosine anhydride remained in the solution. The precipitate was dissolved in dimethylformamide and the solution was passed on a Sephadex LH-20 column, using dimethylformamide as a chromatography solvent. The oligopeptide under the first peak was precipitated with absolute ether (Fig. 1a). Paper electrophoresis at pH 1.9 served as indication for purity. Neutral equivalent, determined by anhydrous titration, was 1490, which means an oligopeptide of five residues.

N-Carboxy-\(\gamma\)-benzyl-L-glutamate anhydride and N-carboxy-L-alanine anhydride were dissolved separately in dioxane, then mixed together, added to a solution of oligotyrosine in dimethylformamide under stirring, and kept for 3 days. The product was precipitated with water and dried over phosphorus pentoxide; the protecting groups were removed with HBr in acetic acid. The reaction product was diazylated using Spectrapor membrane tubing, with molecular weight cutoff 6000–8000 (Spectrum Medical Industries, Los Angeles), lyophilized, and chromatographed on a Sephadex G-50 fine column with 0.01 ammonium bicarbonate as the running buffer (Fig. 1). The material under the first peak was collected and lyophilized. Amino acid analysis of the polymer showed the following amino acid residue ratios: Glu:Ala:Tyr, 65:31:4. The average molecular weight of the final block copolymer was calculated to be 14,000, taking into account the amino acid composition and the fact that the initiator oligotyrosine was on the average a pentamer. Upon sedimentation the polymer gave, in 0.01 M sodium

Table 1. Comparison of immune responses to GA and (G,A)-T (arithmetic mean ± SE) in strains of mice bearing different H-2 haplotypes

<table>
<thead>
<tr>
<th>Strains</th>
<th>H-2</th>
<th>No. of animals</th>
<th>(G,A)-T</th>
<th>No. of animals</th>
<th>GA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H.SW</td>
<td>b</td>
<td>5</td>
<td>26 ± 3</td>
<td>5</td>
<td>26 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>B1O.HTG</td>
<td>g</td>
<td>4</td>
<td>26 ± 6</td>
<td>5</td>
<td>33 ± 5</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>SWR/J</td>
<td>q</td>
<td>5</td>
<td>5 ± 1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SJL/J</td>
<td>s</td>
<td>15</td>
<td>11 ± 2</td>
<td>16</td>
<td>22 ± 3</td>
<td>&lt;0.009</td>
</tr>
</tbody>
</table>

The amount of (G,A)-T was determined by its absorbance at 280 nm after lyophilization and rehydration. The amount of GA-tyramine was determined by the absorbance at 280 nm of the rehydration solution of the lyophilized material.

In most cases, the same antisera were assayed for binding of (G,A)-T and GA-tyramine; 1/5 dilutions of the sera were used. All sera were collected 7 days after secondary immunization with 100 μg of polymer in complete Freund's adjuvant. NS, not significant.
phosphate-0.015 M sodium chloride, pH 7.0, a major peak of 0.975 S, and a minor peak (probably the result of aggregation) of 5.0 S. In 8 M urea only one peak was observed, at 0.22 S.

In order to confirm that the polymer synthesized is indeed a block copolymer composed of a random copolymer of glutamic acid and alanine towards its amino terminus, the polymer was subjected to Edman degradation in a Beckman sequencer, model 890C. Only glutamic acid and alanine, but not tyrosine, were detected after the polymer was subjected to five cycles of degradation.

**Immunizations.** The mice were immunized intraperitoneally with 100 μg of GA, GAT, or (G,A)-T, as indicated. The antigens were emulsified with an equal volume of complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). Secondary immunization was carried out on day 21, and the animals were bled 7 days later. Studies of primary PFC responses were carried out in SJL/J mice. The antigens were administered intraperitoneally in complete Freund’s adjuvant at the dose of 100 μg or 10 μg, as indicated in Table 2.

In order to investigate the ability of GAT or (G,A)-T to suppress the responses of SJL/J mice to GAT-MeBSA, (G,A)-T-MeBSA, or GA, the mice were injected intraperitoneally initially with 100 μg of the antigens in a mixture of 1 mg of magnesium and aluminum hydroxides (Maalox, William H. Rorer, Inc., Fort Washington, Pa.) or with Maalox alone. Three days later, the mice were immunized intraperitoneally with 10 μg of GAT or (G,A)-T, complexed with MeBSA, or with 100 μg of GA, and complete Freund’s adjuvant. Seven days later, the number of GAT-specific IgG PFC per spleen were counted.

**Antigen-Binding Assay.** The antibody responses to (G,A)-T and GA of mouse strains bearing different H-2 haplotypes were measured by antigen binding assay with (G,A)-T-tyramine. These antigens were iodinated with carrier-free 125I (New England Nuclear Corp., Boston, Mass.), and separated from inorganic iodide by passage over Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) Serum samples diluted 1:8 with phosphate-buffered saline were assayed by a modified Farr assay described previously (13).

**Hemolytic Plaque Assay.** The antibody responses to GA, (G,A)-T, GAT-MeBSA, and (G,A)-T-MeBSA were assayed 7 days after immunization by a modification of the Jerne hemolytic plaque technique described by Pierce et al. (14), with sheep red blood cells coupled with GAT. It had been shown earlier that GAT-coupled with sheep red blood cells can be used to detect GA as well as GAT-specific PFC. Since it was noted in our previous studies that the PFC responses in the spleens of the mice immunized with GA, GAT, or GAT-MeBSA are restricted to the IgG class, the plaques were developed using suitable rabbit antiserum against mouse IgG at the appropriate dilution and guinea pig complement as described (15). The specific plaques were determined by subtracting the number of PFC remaining after inhibition by a suitable dilution of GAT from the number of plaques detected on GAT coupled to sheep red blood cells in the absence of the specific inhibitor (15). All assays were done in duplicate, and the number of PFC per spleen was recorded.

**RESULTS**

**H-2-linked differences in antibody responses to GA and (G,A)-T**

Mouse strains can be classified into three groups according to their responsiveness to GAT and GA. A first group, exemplified by mice bearing the H-2o or H-2h haplotypes, C3H.SW and B10.HTG, develops antibody responses to both polymers. A second group of mice with the H-2o or H-2h haplotypes, such as the DBA/1, SWR, or B10.F mice, does not respond to either GAT or GA. The third and more interesting group contains mice bearing the H-2o haplotype, such as the SJL/J or B10.S strains, which respond to GA but not to GAT. The results in Tables 1 and 2 demonstrate that GAT-T has a pattern of response similar to that of GAT but different from that of GA as concerns the distribution of responder strains to these antigens. Mice of the C3H.SW and B10.HTG strains show comparable antibody responses to (G,A)-T and to GA, in contrast to SWR/J and B10.F mice, which do not respond to the two antigens. As predicted, H-2o mice distinguish between GA and (G,A)-T. Stronger secondary antibody responses are elicited by GA than by (G,A)-T in SJL/J and B10.S mice. These differences are highly significant and are detected whether (G,A)-T or GA coupled to tyramine is used as ligand, indicating that (i) the antibodies elicited by immunization with GA or (G,A)-T have similar specificity and (ii) the antibodies elicited by (G,A)-T are directed to determinants on the GA portion of the molecule.

**Table 3.** Suppression of primary PFC response to GAT-MeBSA and (G,A)-T-MeBSA by preimmunization with GAT or (G,A)-T in H-2o SJL/J mice

<table>
<thead>
<tr>
<th>Immunization*</th>
<th>IgG PFC/spleen†</th>
<th>% Suppression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAT-MeBSA</td>
<td>6100 ± 550</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(G,A)-T-MeBSA</td>
<td>7775 ± 1274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAT+ and GAT-MeBSA</td>
<td>1225 ± 1025</td>
<td>80</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>GAT+ and (G,A)-T-MeBSA</td>
<td>375 ± 1175</td>
<td>95</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>(G,A)-T+ and GAT-MeBSA</td>
<td>550 ± 350</td>
<td>93</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(G,A)-T+ and (G,A)-T-MeBSA</td>
<td>1000 ± 703</td>
<td>87</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Number of animals = 4 in each case.
† Arithmetic mean ± SE.
‡ Ten micrograms of GAT or (G,A)-T in Maalox were injected intraperitoneally 3 days before immunization with 10 μg of GAT or (G,A)-T complexed with MeBSA in complete Freund’s adjuvant.
Table 4. Suppression of the primary PFC response to GA by preimmunization with GAT or (G,A)-T in H-2b SJL mice

<table>
<thead>
<tr>
<th>Immunization</th>
<th>No. of animals</th>
<th>IgG PFC/spleen*</th>
<th>% Suppression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>15</td>
<td>8106 ± 1531</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAT* and GA</td>
<td>17</td>
<td>2241 ± 829</td>
<td>73</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>(G,A)-T* and GA</td>
<td>4</td>
<td>275 ± 75</td>
<td>96</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>GA and GAT*</td>
<td>4</td>
<td>6200 ± 951</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

* Arithmetic mean ± SE.
† One hundred micrograms of GAT or (G,A)-T in Maalox were injected intraperitoneally 3 days before immunization with 10 µg of GA in complete Freund's adjuvant.
‡ This group of mice received 10 µg of GA in Maalox followed by 10 µg of GA in complete Freund's adjuvant. NS, not significant.

Primary immunization of SJL/J mice with GAT, (G,A)-T, or GA results in IgG PFC responses only to GA and not to either GAT or (G,A)-T. These differences cannot be attributed to differences in effective immunizing doses, since the animals responded to both 100 µg or 10 µg of GA but not to 100 µg of (G,A)-T in spite of the fact that the L-tyrosine sequences in (G,A)-T constitute only 4% of the amino acid residues.

These results demonstrate that the addition of small sequences of L-tyrosine to an otherwise immunogenic molecule, GA, reduces markedly its immunogenicity for H-2b SJL mice, a strain in which the response to this antigen is under H-2-linked Ir gene control.

Comparable suppressive properties of GAT and (G,A)-T preimmunization on response of SJL/J Mice to GAT-MeBSA, (G,A)-T-MeBSA, and GA

Previous studies from our laboratory have demonstrated the development of specific suppressor T cells after immunization with GAT of strains bearing nonresponder H-2 haplotypes (16). These specific T cells could suppress the primary responses of these mice to GAT-MeBSA in vivo or in vitro. The absence of response to (G,A)-T in SJL/J mice raised the issue of whether this block copolymer is also capable of suppressing primary antibody responses to GAT-MeBSA in this strain. The data in Tables 3 and 4 illustrate that SJL/J mice that are unable to form IgG PFC responses to GAT or (G,A)-T develop these responses after immunization with GAT-MeBSA or (G,A)-T-MeBSA. Further, preimmunization with GAT or (G,A)-T suppresses the responses of SJL/J mice to GA, GAT-MeBSA, or (G,A)-T-MeBSA to a highly significant degree. The conclusion can, therefore, be reached that short sequences of L-tyrosine are seen by SJL/J mice as "suppressor determinants", whereas the GA portion of the molecule is capable of stimulating helper T cells.

DISCUSSION

The antibody responses to the random copolymers GA and GAT are under the control of H-2-linked Ir genes (7, 17). The strain distributions of responsiveness to these two related copolymers, which differ only by the 10% L-tyrosine in GAT, are identical for both antigens with the exception of mice bearing the H-2b haplotype (6, 7). These mice develop antibody responses to GA but not to GAT. In addition, GAT was found to stimulate specific suppressor T cells in mouse strains bearing nonresponder haplotypes to GAT such as H-2b (11). The possibility must, therefore, be considered that, in H-2b mice, oligotyrosine sequences selectively stimulate specific suppressor cells capable of aborting the antibody responses to the GA portion of the molecule.

In the course of synthesis, the different rates of polymerization of the N-carboxyanhydrides of the three amino acids in GAT suggest that the bulk of L-tyrosine residues in GAT should be found at the NH2-terminal end, and that these polytyrosine sequences are indeed the "suppressor determinants" in GAT. This hypothesis is given strong support by the experiments detailed in this paper. A block copolymer was synthesized containing 4% L-tyrosine at the COOH-terminal end coupled to random sequences of L-glutamic acid and L-alanine (G,A)-T. This copolymer is antigenic in selected GAT responder strains. A comparison of the immunogenicity of GA and (G,A)-T for H-2b SJL/J mice revealed that (G,A)-T, in contrast to GA, did not induce a primary response and stimulated significantly weaker secondary antibody responses in this strain. In addition, (G,A)-T suppressed the primary responses of SJL/J mice to GA, GAT-MeBSA, or (G,A)-T-MeBSA. Short sequences of L-tyrosine are specifically suppressive for H-2b SJL/J mice and are recognized by these mice as "suppressor determinants" capable of inhibiting antibody responses to other determinants on the same molecule. These experiments also illustrate that different determinants on the same thymus-dependent antigen may stimulate specific suppression and antibody responses in mouse strains bearing the appropriate H-2 haplotype. Since the immune response to thymus-dependent antigens as well as the stimulation of specific suppressor cells have been shown to be under distinct H-2-linked genetic control (1, 18), these results demonstrate that the determinants capable of stimulating these responses are not necessarily the same. These studies present an example of the specificity of "suppressor determinants," which appears to be as exquisite as the specificity of "helper responses" under H-2-linked Ir gene control (18–20). The critical problem that remains to be explained concerns the manner in which Ir and/or Is genetic control of immune responsiveness and of suppression for specific determinants is achieved. The recent demonstration that thymus-dependent antigens can stimulate T cells specifically only when they are presented by macrophages in close relationship to products of the H-2 complex, and more particularly of the I region, may be considered a clue for this intriguing issue (21–23). A complete understanding of these phenomena, however, will depend upon the identification of the T cell receptor, and of its relationship to Ig V regions and/or to I region products and to our perception of the manner whereby T cells are restricted to antigens perceived in relation to surface molecules coded for by the major histocompatibility complex (22, 24).