Antibodies to Hepatitis B Surface Antigen (HBsAg) Elicited by Immunization with a Synthetic Peptide Covalently Linked to Liposomes

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SUMMARY

Peptides synthesized for potential application as antiviral vaccines have been mostly tested in the form of conjugates with carrier proteins. The possible use of several distinct synthetic vaccines in prophylaxis would be facilitated by the availability of fully synthetic immunogens. A synthetic peptide corresponding to residues 135 to 155 (P135–155) of hepatitis B surface antigen (HBsAg) failed to elicit in free form anti-peptide antibodies or anti-HBs. However, polymers of P135–155 (prepared by linking to diaminoalkanes) and synthetic conjugates prepared by binding P135–155 to liposomes or polylysine were immunogenic. A poor correlation was observed between anti-peptide and anti-HBs responses elicited by these conjugates. Glutaraldehyde-fixed liposomes appeared to be the carriers of choice for inducing anti-HBs.

The generation of antibodies to proteins by immunization with short peptides having the amino acid sequence corresponding to the sequence of preselected protein fragments appears to be a frequent event (Niman et al., 1983). Nevertheless, the elicitation of antibodies with a high binding constant for the native protein may depend on the appropriate stable conformation of the synthetic peptide immunogen (Pfaff et al., 1982; Neurath et al., 1982; Ionescu-Matiu et al., 1983; Kennedy et al., 1983). For this reason, immunization with synthetic peptide analogues of various virus proteins only rarely results in production of virus-neutralizing antisera comparable to those elicited by the viruses (virus proteins) themselves (Pfaff et al., 1982). Thus, the preparation of synthetic immunogens optimally mimicking antigenic determinants on intact viruses remains a challenge. Replacement of commonly used protein carriers [keyhole limpet haemocyanin (KLH), albumin etc.] by synthetic carriers, successfully used with some synthetic peptides (Haber et al., 1965; Stason et al., 1967; Arnon et al., 1980; Audibert et al., 1982), represents part of such a challenge. Although recent reports indicate that free synthetic peptides can be immunogenic (Dreesman et al., 1982; Schmitz et al., 1983), even in these cases the antibody response is enhanced by linking the peptides to a protein carrier (Sanchez et al., 1982).

A synthetic peptide [linked to KLH or Limulus polyphemus haemocyanin (LPH)] corresponding to residues 135 to 155 (P135–155) of hepatitis B surface antigen (HBsAg) elicits antibodies to HBsAg (anti-HBs; Neurath et al., 1982). We present here evidence that a protein carrier is not required for an anti-HBs response evoked by this peptide.

After it was established in preliminary experiments that free P135–155 synthesized as described (Neurath et al., 1982) failed to induce anti-P135–155 and anti-HBs, the following approaches were used to prepare immunogenic derivatives. (i) Polymerization of the peptide by linkage to diaminoalkanes and polylysine, respectively. (ii) Linkage to polyglutaraldehyde microspheres. (iii) Covalent linkage to liposomes, known to provide a substitute for protein carriers in eliciting antibodies to haptons (Yasuda et al., 1977; Dancey et al., 1977, 1978; Tadakuma et al., 1980). (iv) Incorporation of L-tyrosine azobenzene-p-arsonate (RAT) into the above conjugates [(ii) and (iii)]. RAT induces cellular immune responses but no antibody response against itself or elicits or enhances antibody responses to haptons it is linked to.
Table 1. List of crosslinkers and carriers used for the preparation of P135–155 conjugates

<table>
<thead>
<tr>
<th>No.</th>
<th>Conjugate preparation</th>
<th>No.</th>
<th>Conjugate preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Poly-D-lysine (mol. wt. $3 \times 10^4 - 7 \times 10^4$)</td>
<td>15a</td>
<td>11 + lipid A</td>
</tr>
<tr>
<td>2</td>
<td>1 + N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)</td>
<td>15</td>
<td>15a + RAT</td>
</tr>
<tr>
<td>3</td>
<td>1 + MDP</td>
<td>16</td>
<td>13 + lipid A</td>
</tr>
<tr>
<td>4</td>
<td>1,4-Diaminobutane</td>
<td>17</td>
<td>16 + RAT</td>
</tr>
<tr>
<td>5</td>
<td>1,6-Diaminohexane</td>
<td>18</td>
<td>11 treated with ethylenediamine</td>
</tr>
<tr>
<td>6</td>
<td>1,8-Diaminooctane</td>
<td>19</td>
<td>18 + RAT</td>
</tr>
<tr>
<td>7</td>
<td>1,10-Diaminodecane</td>
<td>20</td>
<td>Liposomes containing stearylamine</td>
</tr>
<tr>
<td>8</td>
<td>1,12-Diaminododecane</td>
<td></td>
<td>with oxidized P135–155 (see 9)</td>
</tr>
<tr>
<td>9</td>
<td>Oxidized P135–155 linked to LPH</td>
<td>21</td>
<td>20 except P135–155 was oxidized after</td>
</tr>
<tr>
<td>10</td>
<td>Oxidized P135–155 linked to poly-L-valine</td>
<td></td>
<td>attachment to liposomes</td>
</tr>
<tr>
<td>11</td>
<td>Liposomes containing stearylamine, &amp; treated with glutaraldehyde</td>
<td>22</td>
<td>Stearic acid-containing liposomes</td>
</tr>
<tr>
<td>12</td>
<td>11 + L-tyrosine azobenzene-p-arsonate (RAT)</td>
<td>23</td>
<td>Polyglutaraldehyde microspheres</td>
</tr>
<tr>
<td>13</td>
<td>11 + sphingomyelin (from bovine brain)</td>
<td>24</td>
<td>23 + RAT</td>
</tr>
<tr>
<td>14</td>
<td>13 + RAT</td>
<td>25</td>
<td>23 treated with L-aminocaproic acid</td>
</tr>
<tr>
<td>15a</td>
<td>1 + lipid A</td>
<td>26</td>
<td>23 treated with L-cysteine</td>
</tr>
</tbody>
</table>


The experimental conditions for synthesizing and testing the P135–155 derivatives were as follows. Each of the conjugates of P135–155 listed in Table 1, except conjugate 3, was mixed 1:1 with complete Freund’s adjuvant and injected into two New Zealand White rabbits (65 to 160 µg of peptide per rabbit). The rabbits were further injected at bi-weekly intervals with equal doses of conjugate in incomplete Freund’s adjuvant (not used for conjugate 3). Blood specimens were taken 2 weeks after each injection.

To prepare conjugates 1 and 4 to 8 (Table 1), 1 mg quantities of P135–155 were activated with a 2 molar excess of N-ethyl-N’-(dimethylaminopropyl)carbodiimide (EDAC) and N-hydroxybenzotriazole (NHBTA) and subsequently linked to equimolar quantities of poly-D-lysine and diaminoalkanes (Fluka, Buchs, Switzerland), respectively, as described by Arnon et al. (1980). To prepare conjugates 2 and 3, 1 mg quantities of each EDAC-activated P135–155 and MDP (Calbiochem) were linked to 10 mg poly-D-lysine. P135–155 (800 µg) was oxidized with ferricyanide (Dreseman et al., 1982), activated with EDAC as above and linked to 4 mg LPH. Chromatography on Sephadex G-25 performed under conditions given for Fig. 1 indicated complete linkage of the peptide to LPH (conjugate 9). The oxidized, EDAC-activated peptide (1 mg) was also conjugated to 1 mg polyvaline in a suspension of 2.5 ml 1 M-NaHCO₃ pH 8.5 and 10 ml CHCl₃. The interphase and aqueous phase after centrifugation was used for immunization (conjugate 10).

Liposomes were prepared by the method of Oku et al. (1982). Stearylamine, dilauroyl lecithin and cholesterol were dissolved in glucose-saturated ethanol at final concentrations of 10, 23 and 1-43 mg/ml, respectively. For some liposome preparations, the concentration of dilauroyl lecithin was decreased to 17-5 mg/ml and sphingomyelin was added (10 mg/ml). Other preparations contained lipid A also (420 µg/ml; Calbiochem). The solutions were dialysed against 0-1 m-NaHCO₃ pH 8-5 in dialysis bags with a molecular weight cut-off of 10³ for at least 16 h. The liposomes were treated for about 6 h with glutaraldehyde (final concn. 30 mg/ml), mixed with 0-5 vol. 33-9% (w/w) sodium diatrizoate, floated four times into 1 m-NaHCO₃ by centrifugation for 10 min at 10000 r.p.m. and reacted with 0-84 to 1 mg of P135–155 per 10 mg stearylamine overnight at 20 °C. The linking of P135–155 to liposomes under these conditions was complete. Some preparations were reacted additionally with 7-5 mg RAT (Biosearch, San Rafael, Ca., U.S.A.) per 10 mg stearylamine for 6 h at 20 °C. The liposomes were floated three times into 0-14 m-NaCl, 0-01 m-Tris–HCl, 0-02% NaN₃ (TS) and dialysed against 10⁻⁴ M oxidized glutathione in TS for at least 16 h.

In some cases (conjugates 20 and 21) the stearylamine-containing liposomes were not derivatized with glutaraldehyde but instead directly reacted with EDAC-activated P135–155. Alternatively (conjugates 18 and 19) the activated P135–155 was linked to glutaraldehyde-
treated liposomes further derivatized by reaction with 0.2 M-ethylenediamine at pH 8.5 overnight at 20 °C followed by floating twice into 0.1 M-NaHCO₃ pH 8.5, reduction with 10 μM-sodium dithionite for 1 h at 20 °C and repeated floating into the same buffer. An aliquot of these liposomes was additionally reacted with EDAC-activated RAT. The liposomes were finally dialysed against 10⁻⁴ M oxidized glutathione in TS.

In one preparation (conjugate 22), stearic acid was used instead of stearylamine for the preparation of liposomes. These were dialysed against 0.01 M-NaCl, activated with EDAC (50 mg/ml for 2 h + additional 25 mg/ml for 1 h) at pH 5.5 and 20 °C, floated twice into 0.01 M-NaCl and reacted with P135-155 in 1 M-NaHCO₃ pH 8.5 overnight.

Polyglutaraldehyde microspheres were prepared as described by Margel et al. (1979) using Polysurf 10-36 B (Bartig Industries Inc., New Canaan, Conn., U.S.A.; Margel & Offarim, 1983). One mg of P135–155 was linked to about 50 mg of microspheres under conditions similar to those described for glutaraldehyde-treated liposomes. Conjugate 25 was prepared by treating the microspheres with 5 ml 0·1 M-ε-aminocaproic acid at pH 8·5 overnight. After centrifugation, the microspheres were suspended in dimethylformamide (2 ml) and reacted with 2 mg EDAC + 670 μg NHBTA for 1 h at 20 °C. After centrifugation, the microspheres were resuspended in 2 ml 0·1 M-NaHCO₃ pH 8·5, containing 1 mg of P135–155.

All reagents listed above were of analytical grade and obtained from Sigma, unless indicated otherwise.

The results of all experiments are summarized in Fig. 1 to 3. Free P135–155 (mol. wt. 2664) containing five cysteine residues was in a predominantly monomeric form, since it was eluted after molecular exclusion chromatography in about the same fractions as insulin A chain (Fig. 1 b). Linkage to diaminobutane (Fig. 1 a) and to other diaminoalkanes (data not shown) resulted in formation of P135–155 polymers which were immunogenic and induced homologous antibodies. Conjugate preparations 4, 5 and 7 also induced anti-HBs, while polymers with diaminooctane or dianinododecane linkers (conjugates 6 and 8) failed to do so (Fig. 2) for
Fig. 2. Compilation of antibody responses of individual rabbits to conjugates of P135–155, numbers referring to designations given in Table 1. Antibodies in sera obtained 2 weeks after the third immunization were assayed using a P135–155–β-galactosidase conjugate and Pansorbin (Neurath et al., 1982). The relative titre is given in comparison with antibody levels induced by a P135–155–KLH conjugate. Results of anti-HBs assays by RIA (AUSRIA test, Abbott Laboratories) are given in international milliunits (mIU/ml; Neurath et al., 1982). The line corresponds to the calculated linear regression that best fits the set of all data concerning rabbits with an anti-HBs response. The calculated correlation coefficient (= 0.55) indicates a poor correlation between anti-HBs and anti-P135–155 responses.

reasons not known. Oxidation of P135–155 resulted in polymerization (data not shown). The polymer linked to LPH (conjugate 9) induced high levels of anti-P135–155 but no anti-HBs, unlike P135–155 linked to KLH or LPH in its reduced form (Neurath et al., 1982). This finding again emphasizes the role of peptide conformation in inducing antibodies to the native protein. Linkage of the oxidized peptide to highly hydrophobic poly-L-valine resulted in a conjugate (number 10) of low immunogenicity. P135–155 linked to poly-D-lysine administered with Freund’s adjuvant (conjugate 1) or having covalently linked MDP and given without adjuvant (conjugate 3) induced both anti-P135–155 and anti-HBs. The latter conjugate administered with Freund’s adjuvant (conjugate 2) appeared poorly immunogenic. P135–155 linked to glutaraldehyde-treated liposomes containing stearylamine (conjugate 11) induced levels of anti-HBs comparable to those elicited by conjugates with KLH or LPH (Neurath et al., 1982). Incorporation of sphingomyelin and/or lipid A, components reported to enhance the antigenicity of haptens inserted into liposomal membranes (Yasuda et al., 1977), into the liposomes (conjugates 13, 15a and 16) failed to enhance anti-HBs responses.

Conjugates (18 and 19) prepared by linking P135–155 to glutaraldehyde-treated liposomes through an ethylenediamine bridge rather than directly, had the capacity to induce anti-HBs, but a considerable variability in response between individual rabbits was observed.
Short communication

Fig. 3. Examples of time courses of antibody responses in rabbits immunized with distinct P135–155 conjugates as given in Table 1. (a) Conjugate no. 5; (b) no. 11; (c) no. 12; (d) no. 19. Anti-HBs (■) and anti-P135–155 (□) were assayed as described for Fig. 2.

P135–155 before or after oxidation and subsequently linked to stearylamine-containing liposomes (not fixed with glutaraldehyde; preparations 20 and 21) or to stearic acid-containing liposomes (conjugate 22) induced low levels of anti-P135–155 and no measurable anti-HBs.

P135–155 linked directly to microspheres of polyglutaraldehyde (preparations 23 and 24) induced a primary anti-HBs response. However, the level of anti-HBs decreased in the course of immunization. Anti-HBs was undetectable in sera collected 2 weeks after the third immunization. P135–155 linked to these microspheres through e-aminocaproic acid (conjugate 25) and L-cysteine (conjugate 26) bridges, respectively, either failed (conjugate 25) or was marginally efficient (conjugate 26) in eliciting anti-HBs.

P135–155–KLH or –LPH conjugates elicited a primary anti-HBs response but the level of anti-HBs failed to increase in sera of rabbits after additional antigen doses (Neurath et al., 1982). With the conjugates described above, generally, a decrease of anti-HBs levels was observed 4 or 6 weeks after primary immunization (Fig. 3b) but exceptions were observed in a minority of rabbits (Fig. 3a). This declining trend was uniformly reversed when RAT was inserted into liposomal membranes together with P135–155 (for example, Fig. 3c, d).

The immunogenicity of haptens inserted into liposomal membranes depends on the phospholipid composition of the liposomes and seems inversely related to the fluidity of these membranes (Yasuda et al., 1977; Dancey et al., 1978). Treatment of stearylamine-containing liposomes with glutaraldehyde provides reactive groups suitable for linkage of synthetic peptides and at the same time increases the rigidity of the lipid membranes. Such liposomes, especially when containing carrier function-enhancing RAT sites (Alkan et al., 1971, 1972), appear to be a promising tool for preparing fully synthetic immunogens for eliciting antiviral antibodies.

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