Type-Specific Opsonic Antibodies Evoked with a Synthetic Peptide of Streptococcal M Protein Conjugated to Polylysine without Adjuvant

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A chemically synthesized copy (S-CB7) of a fragment (35 amino acid residues) of type 24 streptococcal M protein was covalently linked to polylysine with carbodiimide and injected subcutaneously into rabbits without adjuvant. Although the primary immune responses as measured by enzyme-linked immunosorbent assays at biweekly intervals were weak, the secondary responses as measured by both enzyme-linked immunosorbent assays and opsonophagocytic assays were as high as those obtained previously in rabbits immunized with the peptide conjugate emulsified in complete Freund adjuvant. Injection of murabutide, a synthetic muramyl dipeptide derivative of bacterial peptidoglycan, with the initial immunizing dose of peptide conjugate had no apparent effect on the secondary immune responses. These results indicate that protective immune responses may be raised against polylysine conjugates of chemically synthesized peptide copies of streptococcal M protein without adjuvant.

Recently my co-workers and I established that short synthetic peptide fragments of streptococcal M protein containing as few as 13 amino acid residues evoked protective immunity against the related streptococci if the peptides were covalently linked to polylysine or tetanus toxoid and emulsified in complete Freund adjuvant (1, 2, 5, 10). The need for Freund adjuvant restricts the use of such vaccines to animal studies, because the toxicity of the adjuvant is too great for human use. We have examined more closely the immune response of rabbits to a synthetic peptide (S-CB7) of type 24 M protein linked with carbodiimide to polylysine and injected subcutaneously in phosphate-buffered saline (PBS; 0.15 M NaCl plus 0.02 M phosphate, pH 7.4) alone or with an initial dose of a synthetic adjuvant, butyl derivative of muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-glutamyl-α-n-buty 13 l-lysine) currently undergoing clinical trials (6). The brisk immune response to a single 25-nmol dose of S-CB7 linked to polylysine and emulsified in complete Freund adjuvant was reported previously (2). Without conjugation, the protective immune responses to the synthetic peptide were weak even when the peptide was emulsified in complete Freund adjuvant before injection (2). I now report data to show that when conjugated to polylysine, S-CB7 is highly immunogenic in rabbits without adjuvant.

S-CB7, the chemically synthesized cyanogen bromide-derived fragment 7 of a pepsin-extracted type 24 M protein (pep M24), was prepared as previously described (1, 2, 5, 10) using an automated peptide synthesizer (Beckman Instruments, Inc., Fullerton, Calif.). The synthetic peptide was purified by molecular-sieve chromatography on a column of Sephadex G-50 and further purified by high-pressure liquid chromatography (1, 5). The primary structure of S-CB7 was confirmed by quantitative amino acid analysis and by Edman degradation to the penultimate amino acid residue (5). The sequence of S-CB7 has been reported (2) as follows:

<table>
<thead>
<tr>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys-Ile-Lys-Thr-Leu-Leu-Glu-Glu-Leu-Ala-Asp-Leu-Glu-Leu-Glu-Gly-Ala-Met</td>
</tr>
</tbody>
</table>

The purified peptide was covalently linked to polylysine with carbodiimide (2). The conjugate (25 nmol of peptide) was dissolved in PBS and injected subcutaneously behind the neck of rabbits with or without 100 μg of murabutide. Rabbit sera were collected at 2-week intervals and tested by enzyme-linked immunosorbent assays (ELISA) and opsonophagocytic tests as previously described (1). Each of the rabbits, whether or not it received the synthetic adjuvant, showed only weak primary immune responses as measured by ELISA, but none at all as measured by the opsonization of the related type 24 streptococci during the first 6 weeks after the initial immunizing dose of peptide (Fig. 1). However, a single booster injection of an additional 25 nmol of the conjugated peptide in PBS evoked high titers of antibodies in each of the immunized rabbits in both groups (murabutide treated or untreated) as measured by both ELISA and opsonophagocytic assays (Fig. 1). The antibodies raised against the S-CB7 conjugate reacted specifically with pep M24 and S-CB7, but not with pep M5, pep M6, or pep M19. The protective activities of the antibodies elicited during the secondary immune responses were demonstrated by indirect opsonobactericidal assays (Table 1). These results demonstrate the type specificity and the protective nature of the immune responses to S-CB7.

These studies demonstrate for the first time that opsonic and bactericidal antibodies can be evoked by immunization with a chemically synthesized peptide fragment of streptococcal M protein linked to a synthetic carrier in the absence of adjuvant. Interestingly, the immune responses in the rabbits immunized with the peptide conjugate in PBS alone
TABLE 1. Indirect bactericidal tests of anti-S-CB7-polylysine against type 24 S. pyogenes

<table>
<thead>
<tr>
<th>Rabbit serum</th>
<th>No. of colonies of type 24 streptococci after 3-h growth in test mixtures with fresh blood from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 1 (inoculum 11)</td>
</tr>
<tr>
<td>Preimmune (control)*</td>
<td>7,220</td>
</tr>
<tr>
<td>Anti-S-CB7-polylysine in PBS</td>
<td>1,280</td>
</tr>
<tr>
<td>8227</td>
<td>60</td>
</tr>
<tr>
<td>Anti-S-CB7-polylysine in PBS</td>
<td></td>
</tr>
<tr>
<td>plus murabutide</td>
<td>8231</td>
</tr>
<tr>
<td>8232</td>
<td>1,050</td>
</tr>
<tr>
<td>8233</td>
<td>0</td>
</tr>
</tbody>
</table>

* The test mixtures each consisted of 0.4 ml of fresh, heparinized (10 U/ml) human blood, 0.05 ml of streptococcal inoculum suspended in PBS, and 0.05 ml of preimmune or immune rabbit serum (4). After incubation at 37°C by rotation end-over-end at 8 rpm for 3 h, blood agar pour plates were prepared from each mixture to determine growth of CFU (4).

The means of each of the values obtained with each of the immune sera were significantly different (P < 0.001) from the mean value obtained with the preimmune serum pool as determined by Student's t test.

The preimmune serum consisted of a pool of serum collected from each of the five rabbits (8227, 8228, 8231, 8232, and 8233) before immunization with the peptide conjugate.

were in the same range as those in the rabbits receiving a dose of the synthetic adjuvant, murabutide, in conjunction with the initial immunizing dose of S-CB7. I do not mean to imply that murabutide is not effective as an adjuvant, be-

cause our studies do not allow such a speculation. The purpose of this paper rather is to show that even in the absence of adjuvant one can obtain a protective immune response to a synthetic peptide copy of M protein.

In a previous study (11), we demonstrated that glutaraldehyde-polymerized S-CB7, but not monomeric S-CB7, was capable of evoking secondary immune responses in animals given an initial priming dose of pep M24 in PBS. Pep M24, however, is a large polypeptide fragment of M, 33,500; the intact M24 protein appears to have a molecular weight of ca. 76,000 (unpublished data), while that of S-CB7 is ca. 4,000 (4). The conjugate formed between S-CB7 and polylysine would have a minimum calculated molecular weight of 39,000. The multivalency of the S-CB7 polylysine conjugate in combination with the positive charge conferred by the polylysine probably accounts for its greater immunogenicity as compared with the monomeric form of S-CB7 (2).

The findings reported here may have bearing on the development of protective bacterial vaccines for human use. The ability to evoke protective antistreptococcal antibodies in the absence of adjuvants would circumvent the potential problems arising from the use of these agents. The use of selected peptide fragments, rather than the parent molecule or even the large polypeptide fragment extracted by limited digestion with pepsin, allows disposal of a large part of the M protein molecule which may contain potentially harmful antigenic determinants giving rise to immunological cross-reactions with host tissues (7–9, 12–14). The selection of small peptide regions that contain protective epitopes should shed light not only on the composition of the protective antigenic determinants of the M protein, but also on the mechanisms of acute rheumatic fever and rheumatic heart disease, the pathogenesis of which up to now has remained an enigma.

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LITERATURE CITED


