Marked Enhancement In Vivo of Adjuvant Activity of Muramyl Dipeptide to Protein Antigens and to Synthetic Weak Immunogens with Monoclonal Anti-Muramyl Dipeptide Antibodies

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Priming of mice with complexes of antigen coupled to muramyl dipeptide and monoclonal anti-muramyl dipeptide antibodies enhanced the adjuvant activity of muramyl dipeptide on the humoral response to the antigen. The enhancement did not occur with free (uncoupled) muramyl dipeptide and required the presence of an adjuvant-active hapten within the complex as well as the Fc fragment of the monoclonal antibody. This system proved highly effective in eliciting antibodies to synthetic weak immunogens whereas muramyl dipeptide, on its own, exerted very little or no adjuvant activity. The effect was not due to a general polyclonal activation and was restricted to the antigen coupled to the synthetic adjuvant. Possible pathways involved in this phenomenon are discussed.

The regulation of cellular and humoral immune responses by antibodies has been extensively investigated in the last decade (reviewed in references 37 and 38). The mechanisms involved in such regulation are quite complex and depend on the system used. In none of these studies was the antigen also an adjuvant. Moreover, antibodies or some of their fragments possess adjuvant or immunomodulating properties in their own right (14, 20, 27).

Muramyl dipeptide (MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine) has been shown to be the minimal structure of a bacterial cell wall peptidoglycan that can substitute for mycobacteria in Freund complete adjuvant (10). The 492-dalton synthetic copy of this adjuvant has been successfully utilized in the preparation of certain synthetic vaccines (3, 8). Besides its adjuvant activity, MDP was found to exert a variety of immunomodulatory and neuromodulatory effects in several mammalian hosts (2, 12, 19). When monoclonal antibodies to MDP were produced, it seemed appropriate to study their effects on certain biological activities of MDP. In this respect anti-MDP antibodies were found previously to enhance by 100- to 1,000-fold MDP-induced macrophage activation in vitro (22).

In this study, we report the modulation of the in vivo adjuvant activity of MDP with monoclonal antibodies to this synthetic glycopeptide. Enhancement of the kinetics and the magnitude of the MDP adjuvant effect on the response to protein antigens was observed upon priming of mice with complexes of MDP coupled to an antigen and anti-MDP antibodies. This system proved highly effective in eliciting antibodies to weak immunogens and even to neutral synthetic antigens, whereas MDP on its own exerted very little or no adjuvant effect. Some aspects of the mechanism of this enhancement will be presented.

MATERIALS AND METHODS

Antigens. Human albumin (HA) was purchased from Calbiochem (La Jolla, Calif.). The polypeptide fragment of type 2 streptococcal M protein (M24) and the synthetic 35-amino-acid subpeptide fragment of M24 (S-CB7) were prepared as described elsewhere (7, 16).

Haptens, synthetic adjuvants, and analogs. 2,4-Dinitrophenol-lysine (DNP-Lys) was purchased from Serva, Heidelberg, West Germany. MDP was obtained from the Pasteur Institute (Paris, France). Derivatives of MDP, namely, N-acetylmuramyl-L-alanyl-D-isoglutamine [MDP(D-D)], N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysine (MDP-Lys), MDP(D-D)-L-Lys, and multi-poly(MDP)-poly(DL-alanyl)-poly(L-lysine) (MDP-A—L) and the neutral A—L chain were kindly provided by P. Lefrancier at the Choay Institute (Paris, France) and were described elsewhere (9, 24, 26, 35).

Preparation of antigen-hapten conjugates. Conjugates of HA with MDP-Lys, MDP(D-D)-L-Lys, or DNP-Lys were prepared by using glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) as the coupling reagent. Briefly, 5 mg of HA and 20 mg of the hapten were dissolved in 2.4 ml of phosphate-buffered saline (PBS) at pH 7.4. To the mixture 50 or 100 µl of 8% glutaraldehyde was then added under constant stirring, and coupling occurred over a 30- to 60-min period at 4°C. The conjugate was then extensively dialyzed against PBS, centrifuged at 1,000 × g for 30 min, filtered through a 0.22-µm membrane filter (Millipore Corp., Bedford, Mass.), and stored in working samples at −20°C. The MDP or DNP content in each conjugate was determined as previously described (33, 34), and the molar ratio of hapten to antigen was calculated. Similarly, HA on its own was treated with glutaraldehyde and used as the antigen control.

M24—MDP-Lys conjugates were prepared as described above. The S-CB7—MDP conjugate was kindly provided by M. Jolivet and was prepared as previously described (21).

Conjugates of MDP-Lys or DNP-Lys with horseradish peroxidase (HRPO) were prepared by the periodate technique of Nakane and Kawaoi (30). Two such conjugates, HRPO—MDP-Lys2 and HRPO—DNP-Lys80, were used to assess the avidity of the monoclonal anti-MDP and anti-DNP antibodies described below.

Murine monoclonal antibodies. The preparation and characterization of monoclonal anti-MDP antibodies have been

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TABLE 1. Fold increase in antibody levels to HA on various days after immunization with complexes of HA-MDP-Lys and monoclonal anti-MDP antibody

<table>
<thead>
<tr>
<th>Expt</th>
<th>Primed with:</th>
<th>Boosted with antigen (50 μg)</th>
<th>Fold increase in anti-HA levels on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen (50 μg)</td>
<td>Antibody (100 μg)</td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>HA</td>
<td>None</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys66</td>
<td>Anti-MDP (m-52-11)</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys66</td>
<td>None</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys66</td>
<td>Anti-MDP (M-52-11)</td>
<td>HA</td>
</tr>
<tr>
<td>2</td>
<td>HA</td>
<td>None</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys16</td>
<td>Anti-MDP (M-52-11)</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys16</td>
<td>None</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys16</td>
<td>Anti-MDP (M-52-11)</td>
<td>HA</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys16</td>
<td>Anti-MDP (M-5-5)</td>
<td>HA</td>
</tr>
<tr>
<td>3</td>
<td>HA-MDP-Lys32</td>
<td>None</td>
<td>HA-MDP-Lys32</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys32</td>
<td>None</td>
<td>HA-MDP-Lys32</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys32</td>
<td>Anti-DNP (U-7-27)</td>
<td>HA-MDP-Lys32</td>
</tr>
<tr>
<td></td>
<td>HA-MDP-Lys32</td>
<td>Anti-MDP (M-52-11)</td>
<td>HA-MDP-Lys32</td>
</tr>
</tbody>
</table>

described before (5). In this study, two such monoclonal antibodies pertaining to different immunoglobulin G (IgG) subclasses were used. The clones were M-52-11 (IgG2a) and M-5-5 (IgG2b). The avidity of binding of the monoclonal antibodies to MDP and to its various analogs was determined by an inhibition assay of the binding of HRPO-MDP-Lys to the monoclonal antibodies as described elsewhere (4, 5).

Control monoclonal antibodies with anti-DNP specificity, namely, U-7-27 (IgG2a) and U-12-5 (IgG2b), were kindly provided by Z. Eshhar of the Weizmann Institute, Rehovot, Israel, and were prepared as previously described (11).

An ammonium sulfate precipitate (50%) of ascitic fluid from mice immunized with each clone, referred to as ascitic immunoglobulin, was prepared and used in most experiments. An IgG fraction of the antibodies, used in some experiments, was prepared by passing the ascitic immunoglobulin over an S-200 column (Pharmacia Fine Chemicals, Piscataway, N.J.) and collecting the 160,000-dalton peak recognizing the corresponding hapten.

Preparation of Fab'. An IgG fraction of the monoclonal anti-MDP (clone M-52-11) was dialyzed against 0.1 M sodium acetate buffer (pH 4.5). To 25 mg of IgG in 3 ml, 1 mg of solid pepsin (Sigma) was added and incubated at 37°C for 18 h. The pH was then adjusted to 8.0 with 1 M Tris, and the digested IgG was dialyzed against PBS (pH 7.4). Removal of Fc fragments and undigested IgG was accomplished by passing the preparation twice over a 10-ml Sepharose-protein A column (Pharmacia). The unbound F(ab')2 fragment was collected and tested for its purity by an enzyme-linked immunosorbent assay (ELISA) technique (described below) employing rabbit anti-mouse subclass-specific antisera (Litton Bionetics, Inc., Charleston, S. C.). The pure F(ab')2 preparation of M-52-11 had the same activity in binding to HRPO-MDP-Lys as undigested IgG of the same antibody.

Immunization. Female BALB/c mice 6 to 8 weeks old were purchased from Iffa-Credo (L'Arbresle, France) and were immunized by the following protocol, unless otherwise mentioned. Groups of mice (five to seven per group) were primed intraperitoneally (i.p.) with either 50 μg of antigen, 50 μg of antigen-hapten conjugate (50 μg of antigen in the conjugate), or 50 μg of the conjugate mixed with monoclonal antihapten antibody (0.8 to 500 μg). Mice were bled from the orbital plexus veins 7, 14, and 28 days after priming, boosted (i.p.) on day 28 with 50 μg of antigen, and bled again 7 days after the boost (35-day response). Sera were separated and stored at -20°C until tested. Preliminary experiments for immunization with complexes showed that a ratio (weight/weight) of hapten to antihapten in the range of 1/5 to 1/50 produced a marked effect whenever the effect existed. Thus, in all experiments, unless otherwise mentioned, 100 μg of ascitic immunoglobulin of monoclonal antihapten antibody was mixed with 50 μg of the conjugate (containing between 2.6 and 19 μg of hapten) and injected into one mouse.

ELISA. The ELISA technique has been described in detail elsewhere (6). Briefly, 10 μg of antigen (untreated) per ml was coated onto wells of flat-bottomed micro-ELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.) for 3 h at 4°C. After the plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween), fourfold dilutions of test sera ranging from 1/400 to 1/409,600 were added in duplicate or triplicate to the wells and incubated for 2 h at room temperature. After further washing with PBS-Tween, rabbit anti-mouse immunoglobulin (total class or subclass specific; Litton Bionetics) conjugated to HRPO (30) was added to the wells and incubated overnight at 4°C. The anti-mouse immunoglobulin-HRPO conjugate was used at 1/10,000, and the anti-mouse class- or subclass-specific-HRPO conjugates were used at 1/1,000 dilution. The unbound conjugate in the wells was then washed off with PBS-Tween, and the bound enzyme was assayed with O-phenylenediamine and H2O2 as the substrate. The reaction was stopped after 30 min by adding 12.5% H2SO4, and the optical density (OD) was read at 492 nm in a Titertek Multiskan ELISA reader (Flow Laboratories, Inc., Rockville, Md.).

Presentation of data. The conditions for antibody measurement in the ELISA (concentration of antigen coated, dilution of conjugate, time of assay) were kept constant for all experiments. The results are thus presented either as OD values or as the fold increase in antibody levels. The latter was calculated as the ratio of the OD after immunization to the OD before immunization at a dilution of 1/400 of the serum. Under the conditions used, the sera before immunization usually gave OD values between 0.05 and 0.1. In certain cases (mostly in the secondary response) some immune sera had to be diluted more than 1/400 to give OD values contained in the linear phase of antibody binding in
TABLE 2. Fold increase in antibody levels to HA after immunization with complexes containing non-adjuvant-active hapten

<table>
<thead>
<tr>
<th>Antigen (50 µg)</th>
<th>Antibody (100 µg)</th>
<th>Fold increase in anti-HA level (±SD) on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>None</td>
<td>11.5 ± 0.5, 1,433 ± 144</td>
</tr>
<tr>
<td>HA-MDP(p-d)-Lys16</td>
<td>None</td>
<td>6.6 ± 0.0, 812 ± 99</td>
</tr>
<tr>
<td>HA-MDP(p-d)-Lys16</td>
<td>Anti-MDP</td>
<td>5.0 ± 1, 691 ± 72</td>
</tr>
<tr>
<td>HA-DNP-Lys16</td>
<td>None</td>
<td>12.5 ± 0.5, 1,603 ± 95</td>
</tr>
<tr>
<td>HA-DNP-Lys16</td>
<td>Anti-MDP</td>
<td>13 ± 1, 1,638 ± 160</td>
</tr>
<tr>
<td>HA-DNP-Lys16</td>
<td>Anti-DNP</td>
<td>12.5 ± 0.5, 1,580 ± 45</td>
</tr>
</tbody>
</table>

* All groups received 50 µg of HA (i.p.) on day 28.

Higher levels of anti-HA antibodies were detected in both the primary and the secondary responses in mice primed with any of the conjugates (HA-MDP-Lys) as compared to mice primed with HA on its own, thus confirming the adjuvant activity of MDP. Moreover, priming with complexes of HA-MDP-Lys and anti-MDP antibodies enhanced the adjuvanticity of MDP and induced a further increase in the anti-HA response (Table 1). This enhancement of anti-HA antibodies was not class specific and was observed with all four subclasses of IgG (data not shown). The enhancing effect was shown to be MDP-anti-MDP specific, since (i) priming with HA (without MDP) and anti-MDP antibody did not enhance the response to the antigen, and (ii) priming with HA-MDP-Lys mixed with a monoclonal anti-DNP antibody did not modify the anti-HA response (Table 1).

Experiments performed with HA mixed with free MDP and monoclonal anti-MDP antibodies did not show any effect (enhancing or suppressive) of the monoclonal antibody on the adjuvanticity of MDP (data not shown). Thus in all the following experiments, conjugates of MDP with antigens were used.

**Ensemble of the anti-HA response upon priming with complexes of HA-hapten and antihapten antibodies requires the presence of an adjuvant-active hapten.** Experiments were conducted to test whether priming with complexes that do not contain an adjuvant structure can enhance the response to the antigen. Thus, two types of complexes were used: (i) monoclonal anti-MDP antibody (M-52-11) mixed with HA coupled to MDP(p-d)-Lys (a nonadjuvant stereoisomer of MDP known to bind to anti-MDP in the ELISA) and (ii) monoclonal anti-DNP antibody (U-7-27) mixed with HA coupled to DNP-Lys (Table 2). It can be noted that priming with either HA-MDP(p-d)-Lys or HA-DNP-Lys16 did not result in enhancing the level of antibodies to HA over that induced by the antigen alone. This confirms the lack of adjuvant activity of such hapten and even reveals a suppressive effect of MDP(p-d)-Lys. Moreover, priming with complexes of either one of the two conjugates and the corresponding antihapten antibody did not modify the response to the antigen. Similar results were observed with other conjugates of different hapten/antigen molar ratios.

To further understand the mechanism of the synergistic effect between adjuvants and complexes on antibody pro-

**TABLE 3. Antibody levels to HA after immunization with HA-DNP conjugates in the presence or absence of adjuvant and specific anti-DNP monoclonal antibodies**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Primed with:</th>
<th>Boosted* with antigen (50 µg)</th>
<th>Fold increase in antibody levels to HA on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen (50 µg)</td>
<td>Adjuvant (100 µg)</td>
<td>Anti-DNP antibody (100 µg)</td>
</tr>
<tr>
<td>1</td>
<td>HA-DNP-Lys28</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-7-27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-12-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MDP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-7-27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-12-5</td>
</tr>
<tr>
<td>2</td>
<td>HA-DNP-Lys19</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-7-27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-12-5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MDP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>U-7-27</td>
</tr>
</tbody>
</table>

* Boosted i.p. on day 28.
duction, the following experiments were performed. Mice were primed with complexes of HA–DNP-Lys and anti-DNP antibodies in the absence or presence of 100 μg of free MDP as an adjuvant. The results of two experiments with two HA–DNP-Lys conjugates and two monoclonal anti-DNP antibodies (U-7-27 and U-12-5) are presented in Table 3. Similar to the previous results and in the absence of MDP, priming with complexes of HA–DNP-Lys and anti-DNP antibodies did not enhance the response to HA over that induced by the conjugate alone. However, when MDP was injected into mice together with the complexes, an enhancement of the adjuvant effect of MDP on the anti-HA response was noted early in the primary response (day 14). This effect was not maintained either in the late primary response (day 28; data not shown) or in the secondary response, indicating that the observed enhancement was only on the kinetics and not on the magnitude of the antibody response to HA.

Differences in the avidity of the antibodies to their haptons could account for the lack of enhancement of the response to the carrier after immunization with carrier-hapten-antihapten complexes. Consequently, the avidities of the four monoclonal antibodies to their respective free or conjugated haptons were measured in an ELISA by using an inhibition of binding of a known concentration of HRPO-labeled hapten to the anti-hapten antibody (Table 4). It was noted that the amount of free DNP-Lys required to inhibit 50% of the binding of a 1/500 dilution of HRPO–MDP–Lys₂ (64 ng of MDP-Lys) to M-52-11 or to M-5-5 (20 μg/ml coated onto the wells) was $1.8 \times 10^7$ or $5.4 \times 10^7$ pmol. These values dropped to 11 or 20 pmol, respectively, when MDP, as an inhibitor, was present in a multivalent form on HA (HA–MDP–Lys₅₅). Similarly, the amount of free DNP-Lys required for 50% inhibition of binding of a 1/10₃ dilution of HRPO–DNP–Lys₅₅ (69 ng of DNP-Lys) to U-7-27 or U-12-5 was $4 \times 10^7$ or $4 \times 10^7$ pmol. When multivalent DNP was used as the inhibitor (HA–DNP–Lys₅₅), only 97 or 12 pmol, respectively, gave 50% inhibition. These values indicated that there were no major differences between the avidity of the anti-MDP and that of the anti-DNP antibodies (especially M-5-5 versus U-12-5) to their respective haptons (free or conjugated). Thus the lack of enhancement of the anti-HA response after priming with complexes of HA–DNP–anti-DNP cannot be attributed to the nature of the avidity of the monoclonal anti-DNP antibodies used. It is worthwhile to add that the OD values for the direct binding of the anti-DNP monoclonal antibodies to HRPO–DNP–Lys₅₅ were comparable to those of the anti-MDP monoclonal antibodies to HRPO–MDP–Lys₅₅ (data not shown).

Enhancement of the adjuvanticity of MDP with monoclonal anti-MDP antibody is Fc dependent. To determine whether the enhancement of the adjuvanticity of MDP was dependent on the Fc fragment of the monoclonal anti-MDP antibody, mice were primed with the conjugate HA–MDP–Lys₅₅ with or without either an IgG preparation of anti-MDP (M-52-11) or an F(ab')₂ fragment of the same antibody. The results of the anti-HA response on day 7 (mean of two experiments) demonstrated that the enhancement of the MDP adjuvant effect could only be achieved with an intact anti-MDP molecule (Fig. 1). Similar effects were observed later after primary or secondary immunization. This indicated that the Fc part of the anti-MDP antibody was an essential constituent of the complex for the enhancement of the anti-HA response.

**Effect of boosting with HA–MDP–anti-MDP complexes on an ongoing response to HA.** To investigate whether the enhancement of the adjuvanticity of MDP with the monoclonal anti-MDP antibody was restricted to the priming step, the following experiments were performed. Mice (seven per group) were primed with HA and were boosted 3 weeks later with either HA, HA plus anti-MDP, HA–MDP–Lys₅₅, or HA–MDP–Lys₃₂ plus anti-MDP. Other groups of mice were primed with HA–MDP–Lys₃₂ and were boosted 3 weeks later with either the conjugate alone or with a complex of the conjugate and monoclonal anti-MDP antibody. The response to HA was measured 7 days after the boost. The results of a pool of two experiments are presented in Fig. 2. In mice primed with HA, a boost with complexes of HA–MDP–Lys–anti-MDP did not enhance the anti-HA response over a boost with the conjugate alone (Fig. 2). However, in mice primed with HA–MDP–Lys₃₂, a boost with the complexes induced a significantly higher ($P < 0.01$) anti-HA secondary response compared with a boost with the conjugate on its own (Fig. 2). The enhancement by complexes of the MDP adjuvant effect in a strict secondary

response occurred only when the adjuvant was used in the priming step.

Enhancement of the adjuvant activity of MDP to a bacterial cell wall protein or to synthetic peptides with monoclonal anti-MDP antibody. To determine whether the enhancement of the adjuvant activity of MDP by complexes was restricted to the specific antigen chosen (HA), we utilized a similar system employing a streptococcal cell wall protein, M24 (33,500 daltons). When used as antigen, 50 µg of M24 protein, on its own, induced weak primary (10-fold increase) and secondary (28-fold increase) responses. However, when coupled to MDP-Lys and the conjugate (M24-MDP-Lys) used as antigen, the antibody response to M24 was potentiated (Fig. 3a) due to the adjuvant effect of MDP (26- and 532-fold increases in the primary and secondary responses, respectively). This response was not modified when the conjugate was administered mixed with monoclonal anti-DNP antibody (U-7-27). However, the adjuvanticity of MDP on the response to M24 was highly enhanced when mice were primed with complexes of M24-MDP-Lys and anti-MDP antibody (M52-11). The enhancement was still marked in the secondary response after a boost with the conjugate alone (Fig. 3a).

To further investigate the possible use of antigen–MDP–anti-MDP complexes in enhancing antibody responses to small, weakly immunogenic, defined synthetic peptides, we studied the response to the S-CB7 peptide fragment (4,272 daltons) of M24 protein. Immunization of mice with 50 µg of S-CB7 did not induce in our hands detectable antibodies to the peptide, in either a primary or a secondary response.

Priming and boosting with 50 µg of a conjugate of S-CB7–MDP2 elicited no detectable antipeptide antibodies in the primary response and very low levels in the secondary response (Fig. 3b). Similarly, a lack of anti-S-CB7 antibodies was observed in mice primed with the same conjugate mixed with monoclonal anti-DNP antibody. In contrast, the immunogenicity of the S-CB7 peptide was highly potentiated upon priming with a complex of S-CB7–MDP2 and anti-MDP antibody. Boosting on day 28 with the conjugate alone resulted in a very strong secondary response (day 35) to the peptide only in mice that were primed with the complex.

FIG. 3. (a) Fold increase in antibody levels to M24 protein after priming with 50 µg of M24-MDP-Lys and with or without 100 µg of either anti-DNP or anti-MDP monoclonal antibodies. A boost of 50 µg of M24-MDP-Lys was given to all groups 28 days after priming. (b) Fold increase in anti-S-CB7 antibodies after priming with 50 µg of S-CB7–MDP2 with or without 100 µg of either anti-DNP or anti-MDP monoclonal antibodies. All groups were boosted, after 28 days, with 50 µg of the conjugate alone.

Priming with MDP-A–L–anti-MDP complexes induces antibodies to the neutral non-immunogenic A–L chain with no evidence of polyclonal activation. Experiments were performed to answer the following three questions. (i) Does priming with complexes of MDP-A–L–anti-MDP elicit antibody response to the neutral, non-immunogenic A–L chain? (ii) Do MDP and anti-MDP need to be administered as preformed complexes for the enhancing effect to occur? (iii) Does priming with such complexes induce a general polyclonal activation? The answers to these questions are presented as the results of three experiments in Fig. 4. In experiment 1, mice were primed (i.p.) with complexes of MDP-A–L (25 µg containing 5 µg of MDP) and anti-MDP (100 µg) in the presence of 50 µg of HA. In experiments 2 and 3, the monoclonal antibodies were injected, i.p. and intravenously, respectively, 2 h before i.p. administration of a mixture of MDP-A–L and HA. Control groups of mice primed with HA, MDP-A–L, or antibody separately were included. Mice were then bled 7, 14, and 28 days after priming, boosted with 50 µg of HA, and bled again 7 days later. Antibodies to A–L and to HA were measured for each bleeding. Similar profiles of antibody responses were observed on all days tested (with differences in the magnitude of the response), and thus only the results for the day 14 primary response are shown. Priming with either MDP-A–L alone nor MDP-A–L mixed with anti-DNP antibodies

FIG. 2. Effect of boosting with complexes of HA-MDP and anti-MDP antibodies on an ongoing response to HA. Arrows indicate standard deviations.
elicit a measurable anti-A-L response (Fig. 4a). In contrast, priming with MDP-A-L and anti-MDP, either as preformed complexes or when the antibody was injected 2 h before MDP-A-L, did elicit the induction of antibodies to A-L (Fig. 4a). Moreover, priming with a mixture of HA and MDP-A-L together with anti-MDP antibody (M-52-11) did not enhance the response to HA as compared with priming with the mixture in the absence of the monoclonal antibody (M-52-11) (Fig. 4b). This indicated that MDP-anti-MDP complexes modulate specifically the response to the coupled antigen in the complex with no effect on the responses to another noncoupled antigen.

Finally, it is worthwhile to mention that in all experiments presented in this work, immunization with conjugates or complexes containing MDP never elicited antibodies to MDP in the immunized mice. On the other hand, mice immunized with conjugates containing DNP produced antibodies to the hapten that were mainly of the IgG1 isotype (data not shown). Passively administered monoclonal antibodies were still detected in minute quantities in the sera of mice for a period of 10 days.

DISCUSSION

Passively administered antibodies complexed with an antigen have been reported to modulate the immune response to the latter. Enhancement as well as suppression of the induced humoral response has been observed post-immunization with such complexes (37, 38). This regulatory effect was found to be dependent on several criteria such as class (13, 29, 32), affinity (39), and Fc fragment of the passive antibody (1, 13) as well as on the antigen/antibody ratio and the amount of the immune complex injected (28).

The data presented herein demonstrate an enhancement of the adjuvant activity of MDP upon complexing with monoclonal anti-MDP antibodies. The phenomenon was restricted to MDP coupled to an antigen and did not occur when free MDP was mixed with the antigen. This could be explained by a requirement for the presence of antigen within the complex or by the relative low avidity of the monoclonal antibodies to monovalent free MDP or by both. Enhancement of the adjuvant activity of MDP by the antibody was seen very early (7 days) in the primary response and continued throughout the secondary response. The effect was not restricted to one isotype of the induced antibody and was dependent on the Fc fragment of the monoclonal anti-MDP antibody.

The presence of an adjuvant-active hapten within the complex was essential for enhancing the antibody response to the carrier. This was demonstrated by (i) priming with complexes of HA-DNP and anti-DNP antibodies and (ii) priming with HA coupled to the non adjuvant-active stereoisomer MDP-D and complexed with monoclonal anti-MDP antibody [known to bind to MDP-D in the ELISA]. In both cases priming with complexes did not induce an enhancement of the anti-HA response over priming with either the antigen or the conjugate alone (Table 2). Moreover, when complexes of HA-DNP and anti-DNP antibodies were administered together with an adjuvant (MDP), an enhancement in the kinetics of the response to the carrier (HA) was observed (Table 3). Thus, it appears likely that the presence of an adjuvant in the priming step plays an important role in dictating the outcome of the response to antigen-antibody complexes. This is documented further by the results in Fig. 2 whereby mice primed with HA-MDP, and not those primed with HA alone, showed a unique enhancement of the secondary response to the antigen upon boosting with complexes of HA-MDP and anti-MDP antibody. These results suggested that a certain type of helper cell is primed by the adjuvant and is essential for enhancing the antibody response. Such a cell has been described by Maillard and Bloom (25) as a T-cell which, in the presence of the adjuvant, produces soluble factors that mediate the enhancing effect. The requirement for an adjuvant in our study may also explain the findings of other workers using the non-adjuvant-active hapten (DNP) and reporting an enhanced response to the carrier after priming with complexes of carrier hapten and anti-DNP antibody (15, 31, 32). In these reports, an adjuvant such as Freund complete adjuvant or Bordetella pertussis was always used either at the priming step or for preparing helper T-cells in transfer experiments.

The most striking aspect of priming with complexes of antigen-MDP and anti-MDP antibody is the ability to induce strong humoral responses to very weak immunogens. Immunization with conjugates of MDP coupled to either the synthetic peptide S-CB7 or to the synthetic neutral A-L chain did not induce a detectable primary antibody response to the carrier. In contrast, when anti-MDP antibodies were mixed with either conjugate and used for priming mice, a detectable antitcarrier response was induced within 7 days (Fig. 3 and 4). This effect was restricted to the antigen within the complex and occurred even when the anti-MDP antibody was passively administered 2 h before the conjugate (Fig. 4). These findings are of potential importance for the field of synthetic vaccines, especially where synthetic fragments of antigens are known to be weak immunogens.

The mechanism of action of MDP as an adjuvant is not fully understood. However, taking the above-mentioned facts into consideration, one can predict several pathways for the enhancement of the adjuvanticity of MDP with
anti-MDP antibody. The simplest explanation would be that priming with MDP-containing complexes facilitates the uptake of the antigen-MDP conjugate, through the Fc fragment of the bound antibody, by antigen processing and presenting cells. This can serve two purposes: (i) concentrating the antigen in an immunogenic form at strategic points in the lymphoid system and (ii) delivering MDP to the macrophage, which is known as one of the major target cells for this synthetic adjuvant (23). Several approaches to improve MDP delivery, such as micropumps (17) or liposomes (18, 36), have been utilized. Complexing with antibody could be another approach that has been shown to enhance by 1,000-fold the MDP-induced macrophage activation in vitro (22).

Another pathway by which anti-MDP antibody could enhance the adjuvanticity of MDP is the generation of helper T-cells. Our results showing enhancement of the secondary response to HA, on boosting with complexes (HA-MDP and anti-MDP) only in mice that were primed with the adjuvant, argue in favor of this pathway. These results also eliminate the possibility of a direct effect on B-cells by complexes as the mechanism of enhancement. Further experiments are being pursued to establish the exact mechanism at the cellular level of this enhanced adjuvant activity by antibody. However, it is most likely that a combination of pathways such as enhanced antigen processing and localization, enhanced macrophage activation through better delivery of MDP and through an Fc signal, and enhanced priming of helper T-cells, will account for the described phenomenon.

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LITERATURE CITED


