INFECTION AND IMMUNITY, July 1981, p. 312-314
0019-9567/81/070312-03$02.00/0

Vol. 33, No. 1

Persistent Enhancement of Cell-Mediated and Antibody Immune Responses After Administration of Muramyl Dipeptide Derivatives with Antigen in Metabolizable Oil

CLAUDE CARELLI, FRANÇOISE AUDIBERT, AND LOUIS CHEDID*

Immunothérapie Expérimentale, Institut Pasteur, 75724 Paris Cedex 15, France

Received 16 March 1981/Accepted 21 April 1981

Circulating antibody titers can be increased when the antigen is administered in an aqueous medium with *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide). Results reported here show that cell-mediated immunity can be demonstrated when this synthetic adjuvant or an active analog is injected with an antigen (ovalbumin) in metabolizable squalane emulsion. Under these conditions a lipophilic derivative of muramyl dipeptide was shown to be even more active and to enhance long-lasting immune responses.

N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]) has been shown to be the minimal structure capable of replacing mycobacteria in Freund adjuvant (3, 5, 7), i.e., enhancement of circulating antibodies and production of delayed-type hypersensitivity (DTH). It was later demonstrated that even in the absence of mineral oil muramyl peptides can be powerful adjuvants of humoral immunity only (1, 2, 4). More recently, a lipophilic derivative of MDP, 1,*O*-(*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-alanyl)glycerol-3-mycolate, was shown to be more active than MDP since DTH could be induced even after a single injection of the adjuvant and of the antigen in an aqueous medium (9).

The present investigation was undertaken to study the effects on specific immunity of certain MDP derivatives administered with a metabolizable oil, squalane. The nonspecific immunostimulating properties of glycopeptides emulsified in squalane (perhydrogenated squalene) have been previously reported in other models (11, 12, 14). In the following experiments, MDP, *N*-acetylmuramyl-L-alanyl-D-glutamine-α-*n*-butyl ester, and l, *O*-(*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-alanyl)glycerol-3-mycolate were tested; certain groups received MDP with trehalose dimycolate, a mycobacterial cell wall component which is an immunostimulant by itself (8) and to which 1,*O*-(*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-alanyl)glycerol-3-mycolate is structurally related. Synergistic effects between trehalose dimylcolate and MDP have been observed in other systems (10, 11, 13). Our data show that MDP, when given with antigen in squalane, is a potent inducer of DTH and that the lipophilic derivative is the most effective. It has been shown repeatedly that MDP in saline does not induce DTH to antigens.

Glycopeptides and trehalose dimycolate were solubilized in the oily phase (IFA purchased from Difco Laboratories and squalane from Laserson & Sabetay) by sonication (10 min, Bransonic 32 sonicator), and Tween 80 (0.2%, Sigma Chemical Co.) was added to squalane as an emulsifier. Emulsifications were performed after addition of squalane (10 or 50%) or of IFA (50%) to the antigen in saline. The other experimental conditions were indicated in the footnotes of Table 1.

The results reported in Table 1 can be summarized as follows: (i) administration of ovalbumin with IFA or squalane but without the synthetic adjuvants never produced DTH, and skin tests were uniformally negative even after a secondary injection; (ii) when MDP or *N*-acetylmuramyl-L-alanyl-D-glutamine-α-*n*-butyl ester was added, enhanced DTH secondary responses could be observed, although primary DTH was only obtained in IFA; (iii) in contrast, when the lipophilic derivative was added to 50% or even 10% of squalane, primary DTH responses were established and maintained until the end of the 8-week experiment. Indeed, this derivative in squalane induced effects that were at least as strong as those produced by MDP in IFA and stronger than those observed in groups receiving trehalose dimycolate added to MDP in squalane.

Parallel experiments in which 1,*O*-(*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-alanyl)-glycerol-3-mycolate was injected with ovalbumin in phosphate-buffered saline followed 4 weeks later by a boost confirmed previous results in which the antigen was administered only

TABLE 1. *Adjuvant activity of MDP derivatives administered with an antigen in water-in-oil emulsions containing 10 or 50% squalane*

Treatment[a]	Oil (%)	Primary responses at 3 wk		Secondary responses at:			
				6 wk		8 wk	
		DTH[b]	Antibodies[c]	DTH[b]	Antibodies[c]	DTH[b]	Antibodies[c]
IFA	50	0, 0, 0, 0	7.6 ± 0.7	0, 0, 0, 0	11.6 ± 1.2	0, 0, 0, 0,	11.8 ± 0.4
IFA, MDP	50	12, 7, 5, 5	8.9 ± 0.5[d]	7, 5, 7, 6	12.6 ± 0	4, 0, 4, 0	13.1 ± 0.5[d]
Squalane	50	0, 0, 0, 0, 0, 0	5.6 ± 0.6	0, 0, 0, 0, 0, 0	11.6 ± 1.0	0, 0, 0, 0, 0, 0	12.2 ± 0.5
Squalane, MDP	50	0	5.9 ± 0.8	7, 7, 6, 3, 5	11.6 ± 0.7	0, 0, 0, 4, 3	12.4 ± 0.4
Squalane, MDPBUT	50	0, 0, 0, 0, 0, 0	6.3 ± 0.8	4, 0, 4, 3, 7, 10	12.1 ± 0.5	0, 0, 3, 0, 0, 4	13.2 ± 0.5[d]
Squalane, MDP, TDM	50	0, 3, 13, 10, 0, 3	6.3 ± 0.5	7, 5, 7, 8, 9, 7	12.6 ± 0.7	0, 0, 0, 3, 0, 4	12.8 ± 0.4
Squalane, MDP, TDM	10	0, 0, 0, 0	6.3 ± 0.5[d]	10, 12, 13, 11	10.6 ± 0	3, 4, 8, 5	12.6 ± 0
Squalane, MDP AGM	50	0, 10, 0, 3, 6, 10	7.9 ± 1.2[d]	8, 5, 5, 10, 5, 4	11.9 ± 1.1	0, 7, 3, 3, 3, 5	14.4 ± 0.4[d]
Squalane, MDP AGM	10	5, 4, 4, 4, 6, 0	8.0 ± 0.5[d]	12, 12, 10, 7, 13, 9	12.4 ± 0.4[d]	6, 5, 6, 5, 7, 6	13.6 ± 1.0[d]

[a] Four to six guinea pigs per group (450 g, female Hartley, Coblanbel) received on day 1 in each posterior footpad 0.1 ml of emulsion containing 1 mg of ovalbumin (5× crystallized, Sigma) with or without 0.1 mg of the various analogs. After 30 days a booster injection of 200 μg of antigen in saline was given subcutaneously in the nuchal region. Abbreviations: MDPBUT, *N*-acetylmuramyl-L-alanyl-D-glutamine-α-*n*-butyl ester; TDM, trehalose dimycolate; MDPAGM, 1,O-(*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-alanyl)glycerol-3-mycolate.

[b] Skin reaction read after 48 h and expressed in millimeters of induration.

[c] Anti-ovalbumin antibody titers expressed as the $\log_2 \pm$ standard deviation of the inverse of the maximal dilution of serum agglutinating ovalbumin-sensitized sheep erythrocytes.

[d] The increase in response is highly significant ($P < 0.01$) as compared with the respective (IFA or squalane) control by Student's *t* test.

once (2). In these experiments DTH and a strong antibody response could also be observed after 8 weeks, although both responses were less marked than when this derivative was administered in squalane.

Squalane administered with toxoids has already been shown to increase antibody titers (6). Our data demonstrate that, as in the case of IFA, specific cell-mediated immunity cannot be enhanced by this oil unless synthetic glycopeptides are added to the emulsion. Moreover, when a lipophilic derivative was used, strong and long-lasting responses were observed. Results which will be published separately also indicate that under optimal conditions strong adjuvant activity can be observed in the absence of the inflammatory and arthritogenic effects inherent to the use of Freund adjuvants.

MDP derivatives were kindly provided by J. Choay and P. Lefrancier. Trehalose dimycolate was isolated from *Mycobacterium bovis* AN5 by A. Escaut at the pilot plant of the Institut de Chimie des Substances Naturelles du Centre National de la Recherche Scientifique (Gif-sur-Yvette, France) and was a gift from E. Lederer. The skillfull technical assistance of Lydie Caille and Nicole Vachet is greatly acknowledged.

This work has been supported by grant CRL 80-10-02 from the Institut National de la Santé et de la Recherche Médicale.

LITERATURE CITED

1. **Audibert, F., L. Chedid, P. Lefrancier, and J. Choay.** 1976. Distinctive adjuvanticity of synthetic analogs of mycobacterial water-soluble components. Cell. Immunol. **21**:243–249.
2. **Audibert, F., L. Chedid, P. Lefrancier, J. Choay, and E. Lederer.** 1977. Relationship between chemical structure and adjuvant activity of some synthetic analogues of N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP). Ann. Inst. Pasteur (Paris) **128C**:653–661.
3. **Azuma, I., K. Sugimura, T. Taniyama, M. Yamawaki, Y. Yamamura, S. Kusumoto, S. Okada, and T. Shiba.** 1976. Adjuvant activity of mycobacterial fractions: adjuvant activity of synthetic *N*-acetylmuramyl-dipeptide and the related compounds. Infect. Immun. **14**:18–27.
4. **Bomford, R.** 1980. Comparative selectivity of adjuvants for humoral and cell-mediated immunity. I. Effect on the antibody response to bovine serum albumin and sheep red blood cells of Freund's incomplete and complete adjuvants, alhydrogel, *Corynebacterium parvum*, *Bordetella pertussis*, muramyl dipeptide and saponin. Clin. Exp. Immunol. **39**:426–434.
5. **Ellouz, F., A. Adam, R. Ciorbaru, and E. Lederer.** 1974. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Comm. **59**:1317–1325.
6. **Holt, L. B.** 1967. Oily adjuvants, p. 131–136. *In* Symposium Series on Immunobiological Standards, vol. 6. Karger, Basel.
7. **Kotani, S., Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka.** 1975. Immunoadjuvant activities of synthetic N-acetylmuramyl-peptides or -amino-acids. Biken J. **18**:105–111.
8. **Lederer, E.** 1980. Synthetic immunostimulants derived from the bacterial cell wall. J. Med. Chem. **23**:819–825.
9. **Parant, M. A., F. M. Audibert, L. A. Chedid, M. R. Level, P. L. Lefrancier, J. P. Choay, and E. Lederer.** 1980. Immunostimulant activities of a lipophilic muramyl dipeptide derivative and of desmuramyl peptidolipid analogs. Infect. Immun. **27**:826–831.
10. **Ribi, E., R. Parker, S. M. Strain, Y. Mizuno, A. Nowotny, K. B. Von Eschen, J. L. Cantrell, C. A. McLaughlin, K. M. Hwang, and M. B. Goren.** 1979. Peptides as requirement for immunotherapy of the guinea pig line-10 tumor with endotoxins. Cancer Immunol. Immunother. **7**:43–58.
11. **Tenu, J. P., E. Lederer, and J. F. Petit.** 1980. Stimulation of thymocyte mitogen protein secretion and of cytostatic activity of mouse peritoneal macrophages by trehalose dimycolate and muramyl dipeptide. Eur. J. Immunol. **10**:647–653.

12. **Whitehouse, M. W., K. J. Orr, F. W. J. Beck, and C. M. Pearson.** 1973. Freund's adjuvants: relationship of arthritogenicity and adjuvanticity in rats to vehicle composition. Immunology **27**:311–330.
13. **Yarkoni, E., E. Lederer, and H. J. Rapp.** 1981. Immunotherapy of experimental cancer with a mixture of synthetic muramyl dipeptide and trehalose dimycolate. Infect. Immun. **32**:273–276.
14. **Yarkoni, E., and H. J. Rapp.** 1979. Tumor regression after intralesional injection of mycobacterial components emulsified in 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene (Squalane), 2,6,10,15,19,23-hexamethyltetracosene (Squalane), peanut oil, or mineral oil. Cancer Res. **39**:1518–1520.