NECROTIC INFLAMMATORY REACTION INDUCED BY MURAMYL DIPEPTIDE IN GUINEA PIGS SENSITIZED BY TUBERCLE BACILLI

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N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP)[1] was shown (1) to be the minimal and essential structure required for adjuvant activity of bacterial cell wall peptidoglycans. Further, MDP has been found to possess various biological activities, other than adjuvant activity, that are exerted by bacterial cell wall peptidoglycans. Since MDP is such a potent immunomodulator, efforts are being made to apply it to a clinical use. MDP should be carefully used, however, because it can induce some detrimental side effects; MDP induced adjuvant arthritis in rats (2), enhanced endotoxic shock in guinea pigs (3), caused pyrexia and leucopenia in rabbits (4–6), and induced the lethargy in cats, rabbits, and guinea pigs (7, 8).

We report here another kind of undesired effect of MDP. In the course of study of antigenicity of MDP, we found that MDP could cause severe necrotic inflammation. Heat-killed tubercle bacilli incorporated into Freund-type water-oil emulsion injected into guinea pig footpads induced extensive necrosis in the footpads at the site of tubercle bacilli injection when, 3–5 wk later, MDP dissolved in Dulbecco's phosphate-buffered saline (PBS) was injected intracutaneously at the flank, and some animals died from generalized shock.

Materials and Methods

Substances Tested for Induction of Necrotic Reaction. Heat-killed tubercle bacilli, strain H37Rv, which were cultured on Sauton medium for 4 wk, were used. Tuberculous protein, PPD, was prepared from the supernatants of the culture of the tubercle bacilli, strain H37Rv, according to the method of Seibert et al. (9). MDP was kindly supplied by Drs. A. Inoue and S. Yokoyama, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. MDP derivatives, L18-MDP, L30-MDP, and B30-MDP, were synthesized as previously described (10), and kindly supplied by Drs. S. Kotani, and T. Shiba, Osaka University, Osaka, Japan. They are conjugates of MDP with linear fatty acids containing 18 or 30 carbon atoms, or a branched fatty acid containing 30 carbon atoms in an ester linkage. A peptidoglycan fragment isolated from an enzyme digest of *Staphylococcus epidermidis* (ATCC 155) cell walls, previously reported (11) and designated SEPS (average chain length of eight disaccharide units) was kindly provided by Dr. S. Kotani.

Lipopolysaccharide (LPS) from *Escherichia coli* (Serotype No. 0,127:B8 Westphal type) and Freund's incomplete adjuvant (lot 636671) were purchased from Difco Laboratories, Detroit, Michigan; carrageenan was purchased from Nitto Kaiso Co., Tokyo, Japan;

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; LPS, lipopolysaccharide; MDP, muramyl dipeptide; OVA, ovalbumin; PBS, phosphate-buffered saline; PPD, tuberculous protein; SEPS, *Staphylococcus epidermidis* peptidoglycan.

brewer's yeast from Nutritional Biochemicals Corp., Cleveland, Ohio; dextran (M.W. 204,000), serotonin, ovalbumin (OVA), and Bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo., and heparin sodium salt, 165 U/mg, was from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Animals. Female Hartley guinea pigs, weighing ~450 g; albino rabbits, weighing ~2.0 kg, WKA and PVG/c rats, weighing ~150 g; and BALB/c and C57BL/6J mice, weighing ~25 g were used.

Injection Schedule for Necrotic Footpad Reaction. Two injections were necessary for the induction of the necrotic reaction. The first (preparatory) injection was made at the left hind footpad, usually with 100 μg of heat-killed tubercle bacilli (H37Rv), or occasionally, with other substances incorporated into 0.2 ml of Freund-type water-oil emulsion. 4–5 wk later, the second (provocative) injection was made intracutaneously at the flank, or occasionally, intravenously with 100–1,000 μg of either MDP or other substances dissolved in PBS.

Results

Induction of Necrotic Footpad Reaction. To determine whether MDP was antigenic or not, we have injected heat-killed tubercle bacilli in water-oil emulsion into the footpads of guinea pigs, then 4 wk later, injected MDP dissolved in PBS intracutaneously at the flank. 24 h after the intracutaneous injection of MDP, animals showed no skin reaction to MDP. However, they showed severe, extensive necrotic inflammation with exudation and hemorrhage in the footpads, the site of previous injection of tubercle bacilli (Fig. 1 and Table I). A number of similar experiments showed that the occurrence of this necrotic reaction was highly reproducible. Since two separate injections of tubercle bacilli and MDP were necessary for this reaction to occur, the first injection will be referred to as a preparatory injection and the second one as a provocative injection.

Guinea pigs given the preparatory and provocative injections also showed, before the development of the conspicuous footpad necrotic reaction, a consistent and reproducible set of general symptoms. The guinea pigs became lethargic and anorexic, and huddled together. Their body weights decreased (Table I)

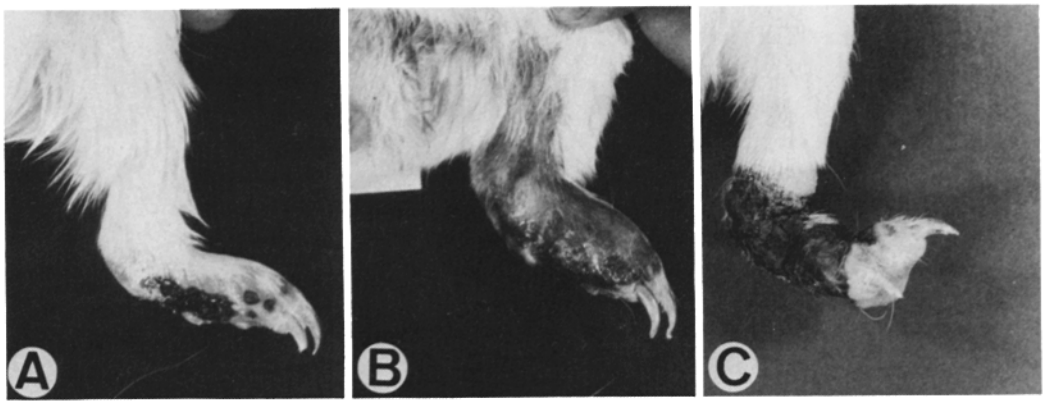

FIGURE 1. Heat-killed tubercle bacilli (100 μg) in water-oil emulsion were injected into the footpads of guinea pigs as the preparatory injection. 4 wk later, the provocative injection was made intracutaneously at the flank with 100 μg MDP in PBS. These photographs show the state of the footpads (*A*) immediately before, (*B*) 24 h after, and (*C*) 30 d after the provocative injections of MDP.

TABLE I

Necrotic Reaction in Footpads and Draining Lymph Nodes Caused by the Provocative Injection of MDP

Time after provocative injection	Material used for provocative injection	Footpad response		Weight of draining lymph nodes	Body weight
		Increase of thickness*	Necrotic reaction‡		
h		%		*mg*	*g*
3	PBS	0	−	1,095 ± 405	ND
	MDP	4	−	1,141 ± 209	ND
	LPS	3	−	1,124 ± 363	ND
24	PBS	−4	−	1,080 ± 166	561 ± 56
	MDP	25	++	2,980 ± 661	534 ± 35
	LPS	−13	−	1,130 ± 458	520 ± 45

Heat-killed tubercle bacilli (100 μg) in water-oil emulsion were injected into the footpads of guinea pigs as the perparatory injection. 4 wk later, the animals received the provocative injection of either PBS, MDP (400 μg), or LPS (200 μg) intracutaneously. After the estimation of footpads, body weights were measured and draining lymph nodes were excised. Each value represents the mean ± SD of four animals. ND, not done.

* Thickness of footpads at the site of preparatory injection of tubercle bacilli was measured by a caliper before, then 3 and 24 h after the provocative injection.

‡ Necrotic footpad reaction was evaluated 3 and 24 h after the provocative injection, and classified as follows: ++, an extensive necrotic inflammation with a marked swelling, exudation, hemorrhage, and ulceration as shown in Fig. 1; +, a moderate necrotic inflammation; ±, a slight necrotic inflammation; −, no change.

and their fur became ruffled. Feces were small, coated by mucous substances, and linked together. When the necrotic inflammatory reaction was very severe, which was usually induced with large amounts of tubercle bacilli and MDP, the animals gradually developed a generalized shock, and died within 19–24 h. The draining lymph nodes were also enlarged (Table I). Although MDP was usually injected intracutaneously as the provocative injection, intravenous injections caused identical local and systemic reactions.

Though tubercle bacilli used for the preparatory injection were usually incorporated into the oil phase of the emulsion, they could prepare animals for necrotic reaction provoked by MDP even when in oil alone (data not shown).

Time Course of Development of Necrotic Reaction. To know the time intervals between the preparatory and provocative injections required for development of necrotic reaction, guinea pigs were injected with tubercle bacilli in water-oil emulsion, divided into two groups, and at different time intervals, each group was intracutaneously injected at the flank with either MDP or PPD dissolved in PBS. One group injected with PPD began to show positive skin reactions at 2 wk, and continued to show them until 20 wk, but showed no necrotic reaction at the footpads throughout the whole experimental period (Table II). The other group injected with MDP showed no skin reaction to MDP throughout the whole experimental period, but began to show footpad necrotic reaction at 3 wk (Table II). The necrotic reaction reached a peak at 4–5 wk, and was no longer observed after 8 wk.

The time course of the degree of necrotic inflammatory footpad reaction after the provocative injection of MDP is shown in Table III. In this experiment, the

TABLE II

Time Course of Appearance of Footpad Necrotic Reaction after Preparatory Injection of Tubercle Bacilli*

Time after preparatory injection	PPD-injected group		MDP-injected group	
	Skin reaction to PPD	Footpad necrotic reaction	Skin reaction to MDP	Footpad necrotic reaction
wk				
1	−	−	−	−
2	+	−	−	−
3	++	−	−	+
4	++	−	−	++
5	++	−	−	++
6	++	−	−	+
7	+	−	−	±
8	+	−	−	−
10	+	−	−	−
12	+	−	−	−
20	+	−	−	−

Heat-killed tubercle bacilli (100 μg) in 0.2 ml of water-oil emulsion were injected into the footpads of 88 guinea pigs. The guinea pigs were separated into two equal groups. At times shown in the table, each group was injected intracutaneously at the flank with 100 μg of either MDP or PPD dissolved in PBS, and skin reaction was measured 24 h later.

* Footpad necrotic reaction was estimated 24 h after provocative injection, and classified as described in Table I.

TABLE III

Kinetics of Development of Necrotic Reaction after Provocative Injection of MDP

Time after provocative injection	Footpad thickness	Footpad necrotic reaction
h	*mm*	
0	11.3 ± 1.2	−
1	11.3 ± 1.3	−
6	11.8 ± 1.3	−
12	14.0 ± 1.0	+
24	13.0 ± 0.8	++

100 μg of heat-killed tubercle bacilli in water-oil emulsion were injected into the footpads of five guinea pigs as the preparatory injection. 4 wk later, the provocative injection was made intracutaneously at the flank, with 100 μg of MDP in PBS. 24 h later, footpad thickness and the necrotic reaction were estimated as described in Table I. Each value represents the mean ± SD of five guinea pigs.

provocative injection of MDP was made 4 wk after the preparatory injection of tubercle bacilli. The necrotic reaction, as measured by the footpad thickness, developed gradually, and the extensive inflammation (Fig. 1) reached a maximum at 12–18 h.

Dose-response Relationship for Development of Necrotic Reaction. Guinea pigs were injected in the footpads with different amounts of tubercle bacilli as the preparatory injection, skin-tested with PPD 3 wk later, and 5 wk later, a fixed amount of MDP was injected intracutaneously as the provocative injection. The

TABLE IV
Effect of Varying Amounts of Tubercle Bacilli on Skin and Necrotic Reactions

Tubercle bacilli	Skin reaction to PPD	Necrotic reaction
μg		
1,000	++	++
100	+	+
10	+	+
1	−	−
0.1	−	−
0.01	−	−
0	−	−

Varying amounts of tubercle bacilli in water-oil emulsion were injected into the footpads of guinea pigs. The guinea pigs were skin-tested with 10 μg of PPD at 3 wk, and injected intracutaneously with 400 μg of MDP at 5 wk. The necrotic reaction was estimated 24 h later, as described in Table I. Four guinea pigs were used for each group.

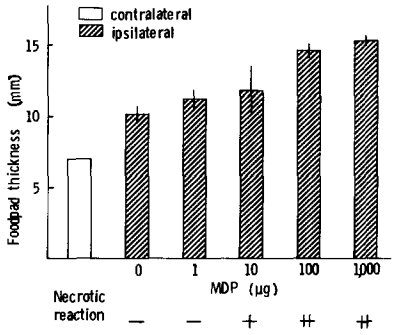

FIGURE 2. 100 μg of heat-killed tubercle bacilli in water-oil emulsion were injected into the footpads of guinea pigs. 4 wk later, the provocative injection was made intracutaneously at the flank with different amounts of MDP. 24 h later, thickness of the footpad at the site of preparatory injection was measured with a caliper. Each column represents the mean ± SD of five guinea pigs. An open column represents the thickness of uninjected footpad controls.

degree of the footpad necrotic reaction induced by the provocative injection of MDP was related to the amount of tubercle bacilli used, 10 μg being the minimum amount necessary for the induction of a significant reaction (Table IV). Data shown in Table IV indicate a parallel between the positive skin reaction to PPD and the development of the necrotic reaction.

When different amounts of MDP were injected for provocation at the flank of guinea pigs that had been prepared 4 wk previously by a fixed amount of tubercle bacilli, the extent of the resulting necrotic reaction was found to depend on the amounts of MDP injected, as shown in Fig. 2.

Substances Required for Preparation or Provocation of Necrotic Reaction. Several substances were tested for their ability either to prepare animals for necrotic reaction provoked by MDP, or to provoke the necrotic reaction in animals that had been prepared by tubercle bacilli. For this purpose, guinea pigs were injected either with tubercle bacilli, SEPS, MDP, or LPS in water-oil emulsion, and 30 d

TABLE V

Attempts to Induce Necrotic Reaction by Various Substances Given as Preparatory or Provocative Injection

Injections		Footpad thickness			Footpad necrotic reaction
Preparatory	Provocative	Before provocative injection	After provocative injection	Increase	
		mm		%	
Tubercle bacilli	MDP	12.3 ± 0.6	14.0 ± 0.5	14	++
	SEPS	12.7 ± 0.6	12.7 ± 0.6	0	−
	LPS	12.7 ± 1.2	11.2 ± 0.3	−12	−
	PPD	12.5 ± 0.5	12.6 ± 0.8	0	−
SEPS	MDP	10.5 ± 0.3	10.5 ± 0.5	0	−
	SEPS	10.3 ± 0.7	10.5 ± 0.6	2	−
	LPS	10.7 ± 0.5	10.2 ± 0.7	−5	−
MDP					
	MDP	9.5 ± 0.5	9.5 ± 0.5	0	−
LPS	SEPS	9.0 ± 0.7	9.3 ± 0.7	3	−
	LPS	9.5 ± 0.7	9.0 ± 0.5	−5	−
	MDP	10.7 ± 0.5	10.7 ± 0.7	0	−
	LPS	11.0 ± 0.7	10.7 ± 0.9	−3	−

100 μg of heat-killed tubercle bacilli, SEPS, MDP, or LPS in water-oil emulsion were injected into the footpads of guinea pigs as preparatory injections. 30 d later, each group of guinea pigs was separated further into groups of four guinea pigs, and the animals of each group were injected intracutaneously at the flank with 400 μg of either MDP, SEPS, LPS, or PPD. Footpad thickness and necrotic reaction were estimated as described in Table I. Each value represents the mean ± SD of four guinea pigs.

later they were intracutaneously injected with either MDP, SEPS, LPS, or PPD dissolved in PBS. No necrotic reaction was induced by any combination of injections of these substances except that of tubercle bacilli as the preparatory injection and MDP as the provocative injection (Table V).

This result was unexpected. We had expected that MDP or SEPS in emulsion could prepare animals because, as reported previously (14), they evoked granulomas indistinguishable from those caused by tubercle bacilli, which we thought might have been essential for the provocation of the necrotic reaction. We wanted to confirm that MDP could not prepare guinea pigs for necrotic reaction provoked later by MDP. For this purpose, a large amount (1 mg) of MDP or B30-MDP in water-oil emulsion was injected into the footpads, and 10, 20, and 30 d later, a large amount (400 μg) of MDP in PBS was injected intravenously. As shown in Table VI, neither MDP nor B30-MDP could prepare the animals for development of necrotic reaction.

We further tested whether the necrotic reaction could be provoked by MDP injected in the site of usual acute inflammations. Carrageenan (4 μg), yeast (20 mg), dextran (4 mg) or serotonin (40 μg), all known to cause acute inflammations, was injected into footpads and, 6 h later, MDP in PBS was injected intracutaneously at the flank. No necrotic reaction occurred in the footpads of these guinea pigs (data not shown).

Close Association Between Development of Delayed Hypersensitivity to Protein Anti-

TABLE VI
Inadequacy of MDP or B30-MDP for Preparatory Injection

Preparatory injection	Interval between preparatory and provocative injections	Footpad thickness			Footpad necrotic reaction
		Before provocative injection	After provocative injection	Increase	
	d	*mm*		%	
MDP (1 mg)	10	9.0 ± 0.5	9.0 ± 0.7	0	–
	20	10.0 ± 1.0	10.0 ± 0.5	0	–
	30	9.0 ± 1.0	9.5 ± 0.8	6	–
B30MDP (1 mg)					
	10	9.0 ± 1.0	9.0 ± 0.7	0	–
	20	10.5 ± 0	10.0 ± 0.5	−5	–
TB (100 μg)	30	9.5 ± 0.5	9.5 ± 0.7	0	–
	10	8.5 ± 0.7	8.5 ± 0.5	0	–
	20	11.5 ± 1.0	13.5 ± 0.7	17	++
	30	12.5 ± 0.5	15.5 ± 1.0	24	++

Inoculum for the preparatory injection consisted of either 1 mg of MDP, 1 mg of B30MDP, or 100 μg of tubercle bacilli incorporated into water-oil emulsion. 10, 20, or 30 d later, the provocative injection was made with 400 μg of MDP in PBS. 24 h later, footpad thickness and necrotic reaction were estimated as described in Table I. Each value represents the mean ± SD of four guinea pigs.

gens and Induction of Necrotic Reaction. The data shown in Table IV indicate a close parallelism between the development of delayed hypersensitivity to PPD and the induction of the necrotic reaction, both of which depended on the amount of tubercle bacilli used as the preparatory injection. Therefore, we studied whether the induction of delayed hypersensitivity was necessary for the development of the necrotic reaction. Guinea pigs were injected with MDP and/or protein antigen (OVA or BSA) in emulsion into the footpad as the preparatory injection, then skin-tested with the protein antigen 2 wk later. 4 wk later, MDP was injected intracutaneously as the provocative injection. As shown in Table VII, when MDP or protein antigen, alone in water-oil emulsion, had been injected into the footpad, neither the delayed hypersensitivity to the antigen nor the necrotic reaction was induced. On the other hand, when MDP mixed with antigen in emulsion had been injected, both the delayed hypersensitivity and the necrotic reaction were induced.

Attempts to Induce Necrotic Reaction in Animals Other Than Guinea Pigs. We wished to determine whether mice, rats, or rabbits also develop this necrotic reaction. In the same way as for guinea pigs, the preparatory injection of tubercle bacilli (500 μg for mice, 200 μg for rats, 1 mg for rabbits) in emulsion was made into the footpads of these animals, and 4 wk later, the provocative injection of MDP (400 μg for mice, 1 mg for rats, 2 mg for rabbits) was given intracutaneously. No necrotic reaction was induced in these animal species (data not shown).

Attempts to Induce the Shwartzman Phenomenon with MDP or Its Derivatives. That this reaction was necrotic and hemorrhagic in nature, and that two injections were necessary for it to occur reminded us of the Shwartzman phenomenon. Whether MDP or its derivatives can cause the Shwartzman phenomenon was thus tested. The preparatory injections for the Shwartzman reaction

TABLE VII

Close Association Between Development of Delayed Hypersensitivity and Induction of Necrotic Reaction

Antigen	Skin test (after 48 h)		Footpad thickness		Necrotic reaction
	OVA	BSA	Before provocative injection	After provocative injection	
			mm		
MDP	−	−	10.1 ± 0.6	10.5 ± 1.0	−
OVA	−	−	8.9 ± 0.5	9.2 ± 0.3	−
BSA	−	−	9.2 ± 0.2	9.0 ± 0.4	−
OVA + MDP	+	−	12.0 ± 0.7	15.2 ± 0.8	+
BSA + MDP	−	+	11.5 ± 0.8	14.8 ± 0.9	+

100 μg of MDP or antigens (OVA or BSA) alone, or a mixture of MDP and antigens in water-oil emulsion were injected into the footpad of guinea pigs. The guinea pigs were skin-tested with 10 μg of protein antigen at 2 wk, and injected intracutaneously with 400 μg of MDP at 4 wk. The necrotic reaction was estimated as described in Table I. Each value presents the mean ± SD of four guinea pigs.

TABLE VIII

Shwartzman Phenomenon by Various Substances Given as Preparatory or Provocative Injection

Provocative injection	Number of rabbits	Preparatory injection			
		LPS (10 μg)	MDP (100 μg)	L18-MDP (100 μg)	B30-MDP (100 μg)
LPS (500 μg)	3	11*	0	0	0
MDP (1 mg)	4	0	0	0	0
L18-MDP (1 mg)	4	0	0	0	0

Four preparatory injections were given intracutaneously at the flank of the 11 rabbits with 10 μg LPS, 100 μg MDP, L18-MDP, and B30-MDP at four different sites. Rabbits were then separated into the three groups, and 24 h later, each group was injected intravenously with either 500 μg LPS, 1 mg MDP, or 1 mg L18-MDP, respectively, and Shwartzman reaction was measured after 24 h.

* Mean of the diameter of hemorrhagic necrosis.

were made intracutaneously at different sites of the flank of 11 rabbits, using LPS (10 μg), MDP (100 μg), L18-MDP (100 μg), and B30-MDP (100 μg), dissolved or suspended in 0.1 ml of PBS. The rabbits thus injected were then divided into three groups (of 3, 4, and 4 animals each), and 24 h later, each group was injected intravenously either with 500 μg LPS, 1 mg MDP, or 1 mg L18-MDP. No group developed the Shwartzman phenomenon except that receiving LPS as provocative injection. (Table VIII).

The occurrence of the local Shwartzman reaction was reported (12) to be inhibited completely by frequent administrations of large doses of heparin. Therefore, we tested whether the necrotic reaction could be inhibited by administration of heparin. The preparatory injection was made with tubercle bacilli. 30 d later, heparin was injected subcutaneously at the flank three times, 2 h before, at the same time with, and 6 h after the provocative injection. As shown

TABLE IX
Necrotic Reaction Was Not Inhibited by Heparin

Total dose of heparin	Footpad thickness		Necrotic reaction
	Before provocative injection	After provocative injection	
U	*mm*		
	With MDP		
—	13.0 ± 1.0	15.3 ± 1.2	+
30	12.3 ± 0.6	15.3 ± 1.2	+
300	12.0 ± 0.8	15.0 ± 0.5	++
3,000	12.3 ± 0.1	14.8 ± 1.0	+++
	Without MDP		
3,000	12.0 ± 0.6	12.0 ± 1.0	−

Preparatory injection was made with heat-killed tubercle bacilli. 30 d later, provocative injection was made with 400 μg MDP. Heparin was injected subcutaneously at the flank 2 h before, at the same time as, and 6 h after the provocative injection. Footpad thickness was measured after 24 h. Each value represents the mean ± SD of four guinea pigs.

in Table IX, heparin had no inhibiting effect on the induction of necrotic reaction. On the contrary, augmentation of the necrotic reaction was observed with heparin administration. To further study the possible relationship between the Shwartzman reaction and the occurrence of the necrotic reaction, we investigated whether the local Shwartzman reaction usually observable in rabbits could also be induced in guinea pigs. For this purpose, the preparatory injection was made with 10 or 100 μg of LPS intracutaneously at the flank of guinea pigs. 24 h later, the animals were injected intravenously with 500 μg of LPS. We did not observe a local Shwartzman reaction as was seen in rabbits (data not shown).

These results strongly suggest that the necrotic reaction induced by MDP is not related to the usual Shwartzman reaction.

Discussion

In the course of study aimed at determining whether MDP is antigenic or not, we observed a hitherto unreported phenomenon; the injection of MDP dissolved in PBS provoked a severe necrotic inflammation with exudation and hemorrhage at the footpad of guinea pigs where killed tubercle bacilli in water-oil emulsion had been injected 3–8 wk previously. The necrotic inflammatory reaction required two injections, preparatory and provocative, for its development.

As reported previously (13, 14) MDP or SEPS in emulsion could evoke massive epithelioid granulomas indistinguishable from those induced by tubercle bacilli. These substances, however, could not prepare guinea pigs for necrotic reaction. This implies that mere granuloma formation is not sufficient for priming. Among several substances so far tested, only tubercle bacilli and MDP plus protein antigens could prepare guinea pigs for necrotic reaction. A close parallelism was noted between the development of skin reaction to PPD and induction of the necrotic reaction (Table IV). MDP plus proteins, but not MDP alone in emulsion prepared the guinea pigs. These results suggest that the development of delayed

hypersensitivity to protein antigens is important for preparation (Table VII). These data may explain why MDP alone, which cannot sensitize guinea pigs (13, 14), failed to prepare the animals.

Among several substances tested, only MDP could provoke the necrotic reaction in the guinea pigs given preparatory injections. That neither SEPS nor LPS could provoke the necrotic reaction in prepared guinea pigs suggests that the activation of macrophages in tuberculous granulomas may not be relevant to or sufficient for provoking the reaction, because both SEPS and LPS are very potent macrophage activators. It also seems unlikely that the necrotic reaction is induced by an immunologic reaction to MDP, because MDP caused the necrotic reaction, but elicited no skin reaction in guinea pigs prepared by tubercle bacilli or MDP, while PPD caused no necrotic reaction but elicited a strong skin reaction (Table II). Also, MDP but not SEPS provoked this necrotic reaction, while MDP-L-lysine-D-alanine, but not MDP, bound to antipeptidoglycan antibodies (15). Thus, although the state of delayed hypersensitivity seems to be required for the preparedness, it is not the autologous protein antigen, but nonantigenic MDP that provokes the necrotic reaction. However, the mechanism for this necrotic reaction still remains obscure.

The necrotic reaction was first thought to be similar to the Shwartzman reaction, since both reactions were necrotic and hemorrhagic in nature and required two separate injections, preparatory and provocative, and because MDP shares many biological activities with LPS. However, we found that they differed from each other in several points. (*a*) Only tubercle bacilli prepared animals, and only MDP provoked this necrotic reaction, whereas only LPS prepared and provoked the Shwartzman reaction. (*b*) >3 wk intervals were necessary between the preparatory and provocative injections for the induction of this necrotic reaction, whereas 24 h intervals were sufficient for that of the Shwartzman reaction. (*c*) The necrotic reaction is observable so far only in guinea pigs, whereas the Shwartzman reaction occurs in rabbits. (*d*) This reaction was worsened with the use of heparin (30–3,000 U heparin injected 2 h before, at the same time with, and 6 h after injection of MDP) (Table IX), whereas the Shwartzman reaction was inhibited by the agent (12). Thus, although this reaction appears similar to the Shwartzman reaction, it actually differs from the Shwartzman reaction in many important respects. Therefore, the mechanisms of these two reactions are probably different.

The importance of the Shwartzman phenomenon has been recognized in the pathogenesis of disseminated intravascular coagulation (16). Whether the necrotic reaction reported here also reflects phenomena occurring in some diseases remains to be clarified. However, it is conceivable that MDP released from dead bacteria in the sites of bacterial infection significantly modify inflammation through the phenomenon described here.

Another important point emerging from the present study is that MDP should be applied cautiously to clinical use. Attempts are being made to apply it to humans in some laboratories and companies. The necrotic reaction can easily lead guinea pigs to fatal generalized shock. Furthermore, it was recently noted (T. Tokunaga, Department of Cellular Immunology, National Institute of Health, Tokyo, Japan, personal communication) that the necrotic reaction also

occurs in monkeys. However, slight modification of the chemical structure of MDP enhanced or suppressed the provoking ability of MDP (our unpublished data). Thus, development of clinically useful MDP derivatives without this detrimental effect may be possible.

Summary

In the course of studies aimed at determining whether MDP was antigenic or not, a hitherto unreported phenomenon was noticed. Injection (a provocative injection) of muramyl dipeptide (MDP) caused severe inflammation, with hemorrhage and necrosis in the footpads of guinea pigs, where tubercle bacilli in water-oil emulsion (a preparatory injection) had been injected 3–8 wk earlier. Sometimes the reaction was accompanied by generalized and fatal shock. Several related substances were tested, and only a combination of tubercle bacilli, or MDP plus proteins as the preparatory injection, and MDP as the provocative injection was found to induce this inflammatory necrotic reaction. Development of delayed hypersensitivity to protein antigens may be important for priming, but MDP and not the protein antigens provoked the reaction. This reaction was, so far, only observed in guinea pigs. Although this reaction appears to be similar to the Shwartzman reaction, the two reactions were found to differ from each other in several important points.

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