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MODE OF ACTION OF A MYCOBACTERIAL WATER-SOLUBLE ADJUVANT, MAF3*

Yoshio KUMAZAWA[1,2], Kimifusa MIZUNOE[1] and Kimio YASUHIRA[2]

[1] *Department of Immunology, The Kitasato Institute, Tokyo*
[2] *Department of Pathology, Chest Disease Research Institute, Kyoto University, Kyoto*

INTRODUCTION

The antibody response requires the participation of bursa-equivalent organ-derived lymphocytes (B-cells), whose progeny are antibody-forming cells (AFC); thymus-derived lymphocytes (T-cells); and macrophages or their chemical surrogates[6,9,16,28]. In contrast, cell-mediated immunity is based on functions of effector T-cells and macrophages or their chemical mediators[4,5,20,23]. To clarify cellular mechanisms involving in these phenomena, many adjuvants have been used as immunological tools. Recently, it has been clarified that mycobacterial water-soluble fractions can be substituted for heat-killed tubercle bacilli in Freund's complete adjuvant (FCA)[1,12,18,19,22,28]. These water-soluble fractions, which probably share common chemical structure indispensable for the adjubant activity is a structural unit, MurNAc-L-Ala-D-isoGln, of the peptidoglycans[8,17].

The adjuvant activity of water-soluble fractions has been demonstrated by the enhancement of antibody responses, especially IgG2 antibody response and delayed type hypersensitivity (DTH) induction against ovalbumin (OA) antigens in guinea pigs[18]. Little is known, however, about the adjuvant effects of these fractions on immune responses against the hapten-carrier type of antigens[2]. In the present paper, the authors describe the adjuvant effects of a mycobacterial water-soluble adjuvant, MAF3, on the humoral and cellular immune responses of guinea pigs to dinitrophenol (DNP) conjugated with different carriers: bovine serum albumin (BSA), ovalbumin (OA), guinea pig serum albumin (GPA), dextran, and MAF3.

MATERIALS AND METHODS

Animals. Adult Hartley guinea pigs, weighing 300 to 500 g, were used in all experiments.

* Part of this experiment was reported in a short communication elsewhere[30].

They were housed in the same room and fed with laboratory chow, fresh cabbage and water *ad libitum*.

Antigens and adjuvants. OA (INC Pharmaceuticals, Inc., Ohio), BSA (Seikagaku Kogyo Co., Tokyo), Dextran T2000 (Pharmacia Fine Chemicals, Sweden) and ε-N-DNP-Lysine (DNP-Lys) (Tokyo Chemical Industry, Tokyo) were commercially available. GPA was prepared from guinea pig serum as Chon's fraction V. MAF3 was prepared from the delipidated cells of *Mycobacterium tuberculosis strain Aoyama B* by hydrogenolysis and gel filtration as described elsewhere[18]. This was adjuvantogenic in the immunization of guinea pigs with heterologous proteins and a heteropolymer(s) consisting of approximately 76 to 79% mucopeptide. The carrier proteins described above were conjugated with DNP by the method of Eisen[7], and the ratio of DNP to protein in the conjugates was calculated after removal of unconjugated DNP by gel filtration on a Sephadex G-25 column. Protein was measured by Lowry's method, and DNP was estimated by absorption at $E_{360\ nm}$. Dextran was conjugated with DNP-Lys with the use of cyanuric chloride (Wako Pure Chemical Industry, Tokyo)[11]. The amount of DNP coupled to OH-groups of dextran was calculated from its weight and absorption at $E_{360\ nm}$, and the polysaccharide was measured by the anthrone method[25]. Thus, the DNP numbers conjugated to a carrier molecule were estimated as follows: DNP_{13}-BSA, DNP_{15}-OA, DNP_{31}-dextran, DNP_8-GPA and $DNP_{0.1}$-MAF3.

Immunization. An aqueous solution of one of the antigens with or without MAF3 was mixed with an equal volume of Frend's incomplete adjuvant (FIA) (Difco Laboratories, Michigan) and injected into the hind footpads of guinea pigs. Each animal received 10, 100, or 1,000 μg of the antigen with or without 100 μg of MAF3.

Serological test and DTH reaction. At the intervals shown in the figures, blood was drown by heart puncture, and the serum was separated. DNP-OA was conjugated to sheep erythrocytes (SRBC) by the chromium (III) chloride method[10], and the DNP-SRBCs were subjected to passive hemolysis in the presence of immune sera and guinea pig complement. Anti-DNP antibodies were titrated as a function of Log 2. Skin reactions to DTH were tested by the intracutaneous injection of 100 μg (0.1 ml) of an antigen 6 weeks after immunization, and the area of local erythema was measured 48 hours later. The corneal reaction was tested simultaneously as described previously[18]. Briefly, a small amount of DNP-BSA or other antigen solution (20 μg/ml), enough to make a transient opaque disc approximately 5 mm in diameter in the cornea, was carefully injected intracorneally and the opacity was inspected 48 h later. The degree of opacity was graded: strong, 3; moderate, 2; weak, 1; or negative, 0.

RESULTS

Effect of MAF3 on immune response to hapten conjugated with T-cell dependent carrier antigen. The adjuvant activity of MAF3 on anti-hapten antibody production and DTH induction was investigated in guinea pigs immunized with DNP-BSA. As shown in Fig. 1, no anti-DNP antibody was detectable one week after the immunization either with or without the concomitant use of MAF3. However, on and after the second week, the antibody production increased markedly. The response reached a maximum in the third week and continued through the

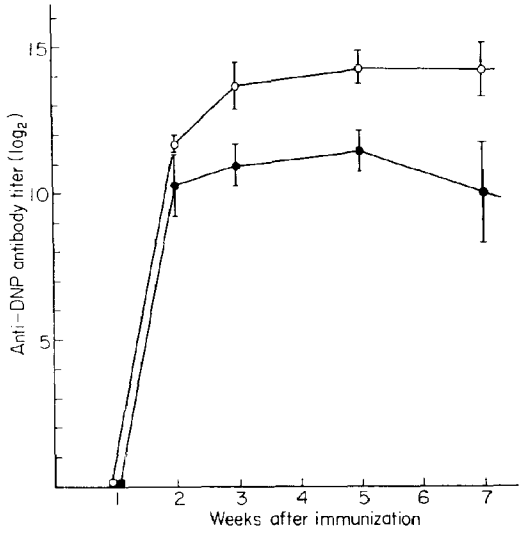

Fig. 1 Adjuvant effect of MAF3 on anti-DNP antibody response of guinea pigs after immunization with 100 μg DNP-BSA in FIA. [○] DNP-BSA+MAF3, [●] DNP-BSA alone.

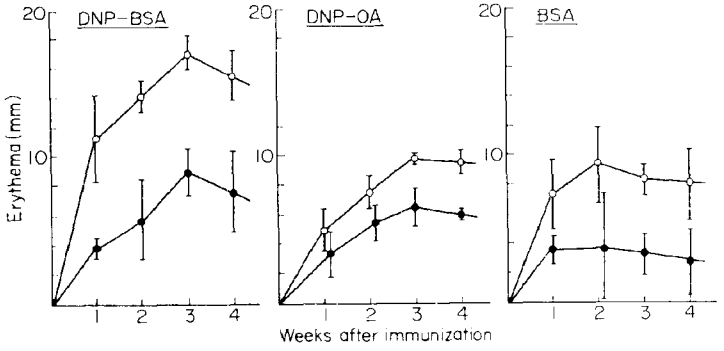

Fig. 2 Adjuvant effect of MAF3 on induction of delayed type skin reaction in guinea pigs after immunization with 100 μg of DNP-BSA. [○] DNP-BSA+MAF3, [●] DNP-BSA alone. Challenging antigens: 100 μg of DNP-BSA, 100 μg of DNP-OA, and 100 μg of BSA. The diameter of erythema was measured 48 h after intracutaneous challenge with these antigens. Each dot shows arithmetic mean±standard deviation (SD) (5 animals/group).

seventh week. The antibody production was always greater in animals immunized with DNP-BSA plus MAF3 than in the immune controls.

DTH reactions in these animals were examined by delayed skin reaction to DNP-BSA, DNP-OA or BSA, as shown in Fig. 2. When conducted with DNP-BSA, the reaction appeared in all the immune animals, and it was markedly stronger in those treated with the antigen plus MAF3 than in those given antigen alone. When DNP-OA was employed as the challenging antigen, the reaction resembled that caused by DNP-BSA, except that the area of erythema was much smaller. When challenged with BSA, no reaction occurred in animals immunized with the antigen alone, and a moderate reaction in those immunized with the antigen plus adjuvant. As a rule, animals immunized with the hapten-carrier antigen plus adjuvant showed stronger

Table. 1 Adjuvant effect of MAF3 on induction of delayed corneal reaction in guinea pigs immunized with DNP-BSA in FIA

Immunized with	Challenged[a] with	Strength of corneal reaction						
		1	2	3	4	6	8	week
DNP-BSA+MAF3	DNP-BSA	0.5[b]	2.4	2.5	2.6	2.4	3.0	
DNP-BSA alone	//	0	0.2	0	0.1	0.2	0	
DNP-BSA+MAF3	BSA	0	1.2	1.0	0.4	0.1	0	
DNP-BSA alone	//	0	0	0	0	0	0	

[a] Guinea pigs were intracorneally injected with the antigen making a transient opaque disc approximately 5 mm in diameter.
[b] Each number shows the arithmetic mean calculated from the results of 5 animals per group. 3, strong; 2, moderate; 1, weak; and 0, negative.

reactions to the hapten or the carrier than did those immunized with the conjugates alone. Furthermore, the adjuvant activity of MAF3 was confirmed by the corneal test, as shown in Table 1. The reaction was negative or minimal in guinea pigs immunized with DNP-BSA alone, while those immunized with DNP-BSA plus MAF3 showed positive reactions. DNP-BSA evoked stronger reactions in these animals than did BSA.

Effect of MAF3 on immune response to hapten conjugated with a T-cell independent carrier antigen. Whether MAF3 was given or not, guinea pigs immunized with 10 or 100 μg of DNP-dextran showed no detectable anti-DNP antibody response during the 5 weeks after immunization. No antibodies were detected after immunization with 1,000 μg of this conjugate either, but the concomitant use of MAF3 evoked an anti-DNP antibody response in these animals, as illustrated in Fig. 3. These findings show that MAF3 is adjuvant-active with T-cell independent antigen in producing anti-hapten anti-bodies when a large amount of the antigen is given at the time of immunization.

DTH reactions did not appear in any of these animals immunized either with or without the adjuvant. Both dermal and corneal reactions were negative. This probably means that effector T-cells triggering DTH reactions could not be generated in guinea pigs immunized with the T-cell independent antigen even if given together with the adjuvant.

Effect of MAF3 on the immune response to hapten conjugated with homologous protein. The kinetics of anti-DNP antibody response in the guinea pig was studied during a 5-week period after immunization with 10, 100 or 1,000 μg of DNP-GPA. The data obtained are shown in Fig. 4. In animals immunized with 10 or 100 μg of DNP-GPA alone, antibody appeared during the first week after immunization. The antibody response reached a maximum in the third week and continued through the fifth week. No definite dose response was noted. The adjuvant activity of MAF3 in the antibody response was recognized. After 1,000 μg of the antigen, however, the antibody response was delayed and suppressed, and there was very little adjuvant effect of MAF3.

When DTH reaction was examined in the skin 6 weeks after the immunization, the adjuvant effect of MAF3 was clearly demonstrated in the DNP-GPA immune animals, as shown in Table

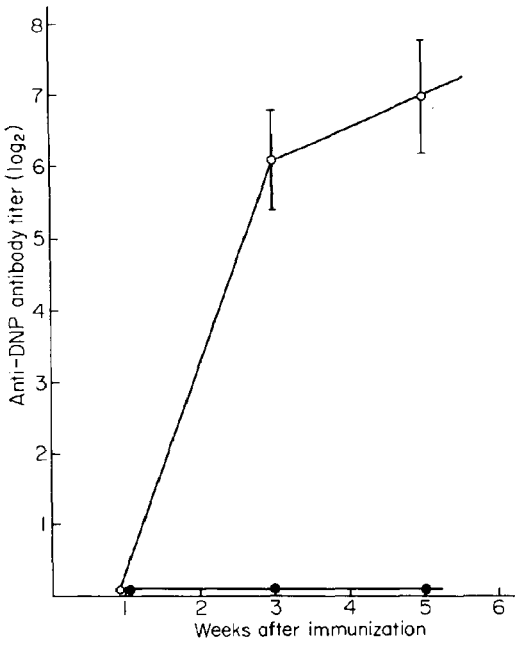

Fig. 3 Adjuvant effect of MAF3 on anti-DNP antibody response of guinea pigs after immunization with 1,000 μg of DNP-dextran in FIA. [○] DNP-dextran + MAF3, [●] DNP-dextran alone.

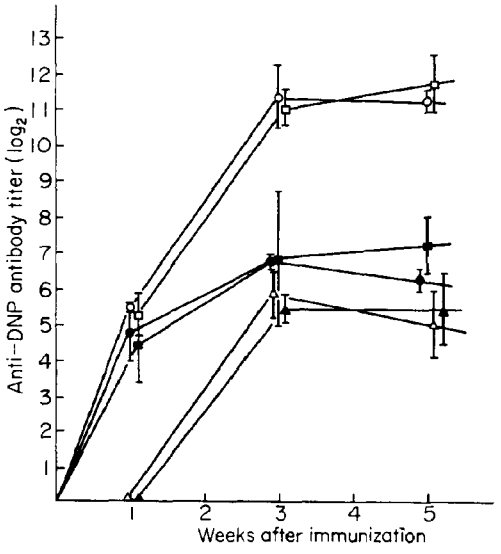

Fig. 4 Adjuvant effect of MAF3 on anti-DNP antibody response of guinea pigs after immunization with DNP-GPA in FIA. [○] 10 μg of DNP-GPA+MAF3, [●] 10 μg of DNP-GPA alone, [□] 100 μg of DNP-GAP+MAF3, [■] 100 μg of DNP-GPA alone, [△] 1,000 μg of DNP-GPA+MAF3, [▲] 1,000 μg of DNP-GPA alone.

Table. 2 Adjuvant effect of MAF3 on induction of delayed skin reaction of guinea pigs immunized with DNP-GPA in FIA

Immunized with	Erythema (mm in diameter)[a]		
	DNP-GPA	DNP-OA	DNP-Lys
10 μg of DNP-GPA+MAF3	9.0±0.7[b]	4.2±4.8	0
10 μg of DNP-GPA alone	5.8±0.7	5.3±4.7	0
100 μg of DNP-GPA+MAF3	21.7±2.0	8.1±1.7	0
100 μg of DNP-GPA alone	8.1±0.4	0	0
1,000 μg of DNP-GPA+MAF3	4.2±4.9	0	0
1,000 μg of DNP-GPA alone	0	0	0

[a] Skin tests were conducted 6 weeks after immunization and erythemas provoked were measured in diameter 48 h after challenging injection.
[b] Arithmetric means ±SD (5 animals/group)

2. However, little or no detectable reaction was induced after a high dose (1,000 μg) of this antigen, despite the concomitant use of the adjuvant. When challenged with DNP-OA, only animals immunized with 100 μg of this antigen fortified by additional MAF3 showed a delayed reaction. The challenge injection with DNP-Lys did not provoke any delayed reaction even when the adjuvant had been used.

Effect of MAF3 on the immune response to hapten conjugated with MAF3. The anti-DNP

Table. 3 Anti-DNP antibody response and DTH reaction in guinea pigs immunized with DNP-MAF3 in FIA

Immunized with		Anti-DNP titer[a]		Erythema (mm in diameter)[b]				
		2 week	5 week	DNP-Lys[c]	DNP-OA	DNP-GPA	DNP-MAF3	MAF3
DNP-MAF3	20 μg	0	0.8±1.8	0	0	0	0	ND[d]
//	100	2.1±1.8	5.2±0.8	0	0	trace	12.5±2.3	ND
//	500	7.1±1.1	7.0±0	0	0	trace	15.1±2.5	ND
MAF3	100	0	0	0	0	0	0	13.3±2.3

[a] Passive hemolysis titer in Log 2. Arithmetric means±SD (% or 10 animals/group).
[b] Skin erythema size measured in diameter 48 h after challenging injection. Arithmetric means ± SD (5 animals/group).
[c] Challenging antigens (100 μg, each) was injected intracutaneously into the flank of guinea pigs 6 weeks after immunization.
[d] Not done

antibody response of guinea pigs was studied on the second and fifth week after an injection of 20, 100 or 500 μg of DNP-MAF3 (Table 3). Immunization with 20 μg of this antigen complex produced no detectable anti-DNP antibody in the second week and a low antibody titer in the fifth week. After 100 or 500 μg, animals showed obvious anti-DNP antibody response in both the second and fifth weeks. These findings indicate that MAF3 coupled to DNP is capable of playing the roles of both carrier and adjuvant.

Table 3 also shows the dermal DTH reaction in these animals after an injection with one of the antigens, DNP-Lys, DNP-OA, DNP-GPA, or DNP-MAF3. After challenge with the former three antigens, no positive reaction was demonstrated. When immunized with a large amount (100 μg or over) of DNP-MAF3 and challenged with the same antigen complex, obvious DTH reactions were observed. The reactions are likely to be considered to be due to the antigenicity of the carrier, because similar reactions were evoked by challenging injections of animals immunized with the carrier alone, as is also shown in the table. One may note the insufficient substitution of the antigenic sites of the acrrier with the hapten, as far as DNP-MAF3 is concerned.

DISCUSSION

When guinea pigs were immunized with a hapten-carrier type of antigen, DNP-BSA, in FIA, anti-hapten antibodies were demonstrable in immune sera 2 weeks after the treatment. Concomitant use of MAF3 enhanced the antibody response strikingly. This means that MAF3 is capable of stimulating the generation of hapten-specific B-cells and/or carrier-specific helper T-cells in the guinea pig. On the other hand, immunization with any amount of DNP-dextran alone could not raise the antibody response, while the response was seen only when 1,000 μg of this antigen plus 100 μg of MAF3 was used. DNP-dextran is known as one of the T-cell independent antigens in mice[23]. Although the thymic independence of this antigen has not been clearly demonstrated in the guinea pig as yet, this may be possible as no cellular immunity to this

antigen could be induced, as noted above. Thus the adjuvant activity of MAF3 in the induction of anti-hapten antibodies in the guinea pig works on B-cells, without any help of T-cell activity, directly through B-cell stimulation[19] or indirectly through macrophage activation[23,24,26].

Guinea pigs immunized with DNP-GPA could produce anti-DNP antibodies. This is because carrier-specific T-cells could recognize new antigenic determinants formed by coupling DNP with homologous proteins[28]. MAF3 was capable of enhancing the anti-hapten antibody response, when given with a low does (10 or 100 μg) of this antigen complex. However, treatment with a high does (1,000 μg) seemed to induce partial tolerance to the antigen, and the concomitant use of MAF3 could not destroy this tolerance.

The adjuvant effect of MAF3 on T-cells could be detected in DTH reactions. The animals immunized with DNP-BSA or -GPA plus MAF3 showed markedly stronger DTH reactions in the skin and/or cornea than those immunized with the antigen alone. Levine[21] has reported that if immunized animals are challenged with the carrier itself or hapten coupled to different carrier proteins, the DTH reactions appear weaker than those induced by the sensitizing antigen. This finding is supported by the data obtained in the present experiments. Delayed skin reactions caused by BSA or DNP-OA in DNP-BSA immunized animals and by DNP-OA and DNP-Lys in DNP-GPA immunized animals were negligible and were enhanced by the concomitant use of MAF3 at the time of immunization. Moreover, no response was seen to a challenging injection of DNP-Lys, the lysine moiety being a poor carrier under these conditions. These results indicate that in the development of DTH, MAF3 is capable of enhancing the generation of the effector T-cells and that the effectors can repond not only to the hapten but also to the carrier.

Immunization with DNP-MAF3 raised the anti-hapten antibody response in guinea pigs, but could not generate hapten-reactive effector T-cells. Positive skin reactions to DNP-MAF3 or MAF3 alone in animals immunized with DNP-MAF3 seemed to be due to the establishment of immunity to the carrier antigen. Janeway et al[13,14,15]. have reported that immunization with DNP-H37 can generate hapten-reactive effector T-cells which are capable of reacting with the hapten coupling to different carriers. The reason for the discrepancy between our results and those of Janeway et al. is not clear as yet, but it is possible that it depends on the difference of substitution rates of antigenic sites on carrier antigens with the hapten, DNP.

SUMMARY

The concomitant use of a mycobacterial water-soluble adjuvant, MAF3, at the time of immunization, enhanced anti-hapten antibody production in guinea pigs treated with either heterologous or homologous dinitrophenylated proteins. This may be due to stimulation by the adjuvant of B-cells and/or helper T-cells. The adjuvant activity of MAF3 in anti-hapten antibody production was seen in immunization with a T-independent antigen, DPN-dextran, although it appeared only when a large amount of the conjugated antigen was introduced as the immunizing antigen. Delayed hypersensitivity reactions which require the participation of effector T-cells were also increased by MAF3. When challenged with the same dinitrophenylated protein antigen as the immunizing antigen, the delayed reactions in the skin or cornea were much stronger in animals treated with the antigen plus adjuvant than in control immune animals. The enhance-

ment was seen when T-dependent but not T-independent antigen was used together with the adjuvant at immunization. No delayed hypersensitivity reaction was demonstrated after immunization with DNP-dextran either with or without MAF3.

Conjugates of DNP with MAF3 were immunogenic in the production of anti-DNP antibodies in guinea pigs. However, delayed skin reaction in the these animals were induced by challenging injections of the same conjugates as the immunizing antigen or MAF3, the carrier, but not of DNP-GAP, DNP-OA or DNP-Lys. Similar skin reactions were observed in guinea pigs immunized with MAF3 and challenged with the same antigen. Therefore, DNP-MAF3 is defective as a hapten-carrier antigen presumably because of insufficient substitution of antigenic sites of the carrier with the hapten.

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Request for reprints should be addressed to Dr. K. Yasuhira, Department of Pathology, Chest Dis. Res. Inst., Kyoto University, Kyoto 606.

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