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A SYNTHETIC ADJUVANT EFFECTIVE IN INDUCING ANTITUMOR IMMUNITY

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Guinea pigs which were injected repeatedly with a mixture of a 3*M* KCl extract of line 10 tumor and 6-O-(2-tetradecyl-hexadecanoyl)-muramyl-dipeptide (B30-MDP) rejected a tumor graft of line 10. Such an adjuvant effect of B30-MDP was also demonstrated when X-ray-treated line 10 cells were mixed with B30-MDP dissolved in phosphate-buffered saline and inoculated into the animals.

Key words: Adjuvant — Antitumor immunity — Guinea pig tumor — B30-MDP

In the mid-1970s, 2 groups of investigators revealed that N-acetyl-L-alanyl-D-isoglutamine (MDP) is the minimum structural unit responsible for the immunopotentiating ability of various bacterial cell walls.[1,2] However, MDP requires a water-in-mineral oil emulsion as a vehicle to exert its full adjuvant capacity, particularly the ability to induce delayed-type hypersensitivity (DTH).[3] A number of derivatives which are more lipophilic than the parent MDP molecule, 6-O-acyl-MDPs, were synthesized to overcome the problem.[4–6] Among them, the derivative whose muramic acid residue was substituted by α-branched higher fatty acids at the carbon-6 position, 6-O-(2-tetradecyl-hexadecanoyl)-MDP (B30-MDP) was found to exert strong adjuvant activity for induction of DTH and humoral immunity against ovalbumin without an oil vehicle.[7,8]

Recently, Nerome *et al.* developed a new, potent influenza vaccine in which purified viral hemagglutinin (HA) and neuraminidase (NA) were incorporated into liposomes made from B30-MDP and cholesterol (unpublished results). They found that this vaccine induced a marked elevation in anti-HA antibody production, about 20 times higher than that obtained by HA+NA dissolved in phosphate-buffered saline (PBS), when injected into mice, as well as DTH induction in guinea pigs. In the guinea pig tumor system, so-called "tumor vaccine," i.e., X-ray-treated tumor cells mixed with live BCG[9] or a 3*M* KCl extract of tumor cells emulsified in complete Freund's adjuvant[10] has shown a significant prophylactic effect against tumor challenge. In the present study, we attempted to develop a new tumor vaccine which is simple but much more potent with little deleterious effect, using B30-MDP as the adjuvant.

Inbred Sewall Wright strain 2 guinea pigs were purchased from Nisseiken Co., Tokyo. An ascites variant of diethylnitrosamine-induced hepatocarcinoma line 10 (L 10), which is syngeneic with strain 2, was obtained from Dr. B. Zbar, National Cancer Institute, Bethesda, Md., and maintained by intraperitoneal passage in strain 2 guinea pigs.[11,12] A 3*M* KCl extract of L 10 cells was partially purified by precipitation with 2*M* ammonium sulfate (soluble tumor-associated antigen; sTAA). The sTAA elicited a tumor-specific DTH skin reaction in L 10-immunized guinea pigs. About 100 μg of protein of the sTAA elicited the DTH reaction with an intensity equivalent to that elicited with 1×10^6 live L 10 cells. Synthetic MDP and its derivative, B30-MDP, were obtained from Dai-ichi Seiyaku Co. Ltd., Tokyo. Four different kinds of preparations were used. Namely, (1) sTAA solution (2 mg of protein/ml) was mixed with an equal volume of an MDP or B30-MDP suspension in PBS. (2) L 10 cells irradiated with 20,000 R with a ^{60}Co irradiation unit (2×10^7/ml) were mixed with an equal volume of MDP or B30-MDP suspension. (3) Liposomes were prepared by the method described by Nerome *et al.* (unpublished results). Briefly, 4 ml of sTAA containing 40 mg of protein was mixed with 10 ml of PBS containing 10 mg of B30-MDP, 10 mg of cholesterol (Sigma Chemical Co., St. Louis, Mo.)

Table I. Adjuvant Effect of B30-MDP on Induction of Antitumor Immunity by Soluble Antigen

Group	Form of adjuvant	Amount/dose MDP (μg)	Amount/dose Antigen (μg)	Survival of individual animals[a] (days)	No. of animals (tumor-free/tested)
Exp. 1					
1.	Non-treated	—	—	55, 56, 58, 60, 61	0/5
2.	PBS	—	100	56, 58, 59, 59, 61	0/5
3.	Liposome (B30-MDP)	40	100	59, 88, >200, >200, >200	3/5
4.	Mixture (B30-MDP)	40	100	59, 75, >200, >200, >200	3/5
5.	CFA	100[b]	100	56, 58, 58, 61, 62	0/5
Exp. 2					
6.	Non-treated	—	—	52, 53, 58, 59	0/4
7.	PBS	—	100	58, 63, 65, 68	0/4
8.	Mixture (MDP)	50	100	61, 67, 76, 79	0/4
9.	〃 (B30-MDP)	50	100	67, >150, >150, >150	3/4
10.	〃 (〃)	5	100	86, >150, >150, >150	3/4
11.	〃 (〃)[c]	50	100	53, 56, 98, >150	1/4

Strain 2 guinea pigs were immunized by 4 successive id injections of sTAA dissolved in PBS (Gr. 2 and 7), incorporated into liposomes (Gr. 3), mixed with B30-MDP (Gr. 4, 9, 10 and 11) or MDP (Gr. 8) or emulsified with CFA (Gr. 5) at 1-week intervals. Eleven days after the last injection, the animals were challenged id with 1×10^6 live L 10 cells.

a) Day of death after L 10 challenge.

b) Amount of dry bacilli of *Mycobacterium tuberculosis*, strain Aoyama B.

c) Animals of this group were given only 1 injection, 11 days before tumor challenge.

and 600 mg of *n*-octyl-β-D-glucopyranoside (octylglucoside; Sigma). Then the mixture was sonicated for 10 min with an ultrasonicator (Model UD-200; Tomy Seiko Co., Tokyo) and dialyzed thoroughly against PBS to remove octylglucoside. The amount of B30-MDP and sTAA was adjusted by dilution with PBS. (4) sTAA solution (2 mg/ml) was emulsified well with an equal volume of complete Freund's adjuvant (CFA). Strain 2 guinea pigs weighing more than 500 g were immunized by 2 or 4 successive intradermal (id) injections of 0.1 ml of the preparation at 1-week intervals. The animals were challenged with an id inoculation of 1×10^6 live L 10 cells, 7 or 11 days after the last inoculation. The primary tumors at the inoculation sites and metastatic tumors in the regional lymph nodes were measured weekly until the animals died.

As shown in Table I (Exp. 1), the liposome preparation and the mixture were equally effective in inducing strong antitumor immunity against L 10 challenge. On the other hand, CFA was no more effective than sTAA dissolved in PBS. Since the sTAA used in the present study was still crude, it was difficult to control and estimate the amount of sTAA incorporated into the liposome preparation. Only the total amounts of B30-MDP and sTAA present in the preparations were determined and adjusted. Under the above conditions, the results obtained in the individual experiments possibly fluctuated. In the other test, 4 out of 4 animals immunized with liposome preparation were tumor-free. The mixture may be the better one, since it is simple but potent enough. The efficacy of the mixture was confirmed by the next experiment, in which a lower concentration of B30-MDP and a single administration of the mixture were also tested. The results are shown in Table I (Exp. 2). It can be seen that sTAA simply mixed with B30-MDP induced antitumor immunity even when the amount of B30-MDP was decreased to 5 μg/dose. A single injection given 11 days before the L 10 challenge had little effect. The parent MDP molecule without an oil vehicle showed poor adjuvanticity (Gr. 8) as suggested by the experimental results obtained with ovalbumin.[7] As shown in Table II, B30-MDP exhibited a strong ad-

Table II. Adjuvant Effect of B30-MDP on Induction of Antitumor Immunity by X-Ray-irradiated Tumor Cells

Immunogen[a]	Survival of individual animals[b] (days)	No. of animals (tumor-free/tested)
Non-treated	53, 53, 60, 61, 64	0/5
L 10 cells/PBS	49, 50, 57, 58, 63	0/5
L 10 cells/MDP	53, 53, 53, 61, 68	0/5
L 10 cells/B30-MDP	>100, >100, >100, >100, >100, >100, >100, >100	8/8
L 10 cells/BCG[c]	>100, >100, >100, >100, >100	5/5

a) Strain 2 guinea pigs were immunized by 2 successive id injections of X-ray-irradiated L 10 cells (1×10^6) suspended in PBS or mixed with MDP or B30-MDP at a 1-week interval. Seven days after the last injection, the animals were challenged id with 1×10^6 live L 10 cells.
b) Day of death after L 10 challenge.
c) 1×10^6 X-ray-irradiated L 10 cells were mixed with a BCG suspension (10^8 viable units/ml).

juvant effect on the induction of anti-L 10 immunity by X-ray-treated L 10 cells. The effect was comparable to that of BCG. Again, the parent MDP molecule was not effective. Positive DTH skin reactions ($\geq$10 mm in diameter) were elicited with 10^6 L 10 cells inoculated as a challenge in most of the animals immunized with the preparations containing B30-MDP. Another hepatocarcinoma L 1, which is syngeneic with strain 2 but antigenically different from L 10,[12] could not elicit any DTH skin reaction in those immunized animals (data not shown). Taken together, these results strongly suggested that the antitumor effect demonstrated is due to an immune mechanism. However, the number of animals was small, and the relationship between the size of the DTH reaction and the intensity of the immunity was not clear.

At the site of injection of the preparations containing about 50 μg of MDP per dose, although granulomatous reactions about 15 mm in diameter (induration→central necrosis →ulcer→scab) appeared from 1 week after the injection, the damage to the skin healed completely within 1 month. However, 5 μg of B30-MDP per dose elicited only slight induration which disappeared quickly, within 2 weeks after the injection. These findings show that decreasing the amount of B30-MDP resulted in decreasing the deleterious effects without reducing the ability of the mixture to induce antitumor immunity.

The experiments reported here revealed that TAA can induce antitumor immunity when it is simply mixed with a small amount of B30-MDP and administered to guinea pigs. The marked efficacy of this "tumor vaccine" may allow a new approach to the immunotherapy of tumors. For instance, if resected tumor cells are mixed with B30-MDP and administered to the host, concomitant immunity may be induced and work to inhibit the growth of residual metastatic tumor cells. Following this line, a pilot study is now under way and promising results are being obtained.

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