CHEMO-IMMUNOTHERAPY OF METHYLCHORANTHRENE-INDUCED FIBROSARCOMA BY CONCAVALIN A-BOUND TUMOR VACCINE, LEVAMISOLE AND MITOMYCIN C

MASAMI OKABE, MAKOTO MORIMOTO AND HIROFUTO MARUMO

Pharmaceuticals Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., 1188 Nagaizumi-cho, Sunto-gun, Shizuoka 411, Japan

(Received September 3, 1981)

Concanavalin A (ConA)-bound tumor cell vaccine of methylchoranthrene-induced fibrosarcoma (Meth 1) induced tumor-specific immunoprophylactic and immunotherapeutic response against an inoculum of live Meth 1 cells in histocompatible animals. ConA-free Meth 1 vaccine induced much less response under the identical experimental conditions. Immunotherapeutic potency of ConA-bound Meth 1 vaccine was enhanced by levamisole, and 37% of the animals inoculated with 10^4 live Meth 1 cells at day 0 were cured when they were administered 10^6 cells of ConA-bound Meth 1 vaccine at days 1 and 8 and 0.63 mg/kg levamisole at days 1, 2 and 3. Delayed administration of levamisole at days 8, 9 and 10 was less effective than the earlier administration, but still produced a 17% cure of Meth 1-bearing animals when combined with ConA-bound Meth 1 vaccine. Immunotherapeutic response under these regimens was further enhanced by mitomycin C, and approximately 60% of the animals inoculated with 10^5 Meth 1 cells were cured when three agents were administered at the defined intervals. These results suggest the feasibility of the regimen in which the therapeutic response induced by immunotherapeutic agents is further enhanced by the selected chemotherapeutic agents.

Keywords—chemo-immunotherapy; tumor vaccine; levamisole; mitomycin C

INTRODUCTION

Levamisole is potent in modulating host immunity in different ways (refer to references 1, 2 and 3). One of the activity of levamisole associated with the induction of anti-tumor immunity is the potentiation of several types of tumor vaccines. However, the results reported in the past regarding the effectiveness of levamisole differ according to the experiments. Levamisole did not potentiate tumor vaccines of B16 melanoma and of adenocarcinoma 15901,⁴⁾ whereas it potentiated tumor vaccine of L1210 leukemia^{5,6)} and 3-methylchoranthrene-induced fibrosarcoma (Meth 1).⁷⁾ Furthermore, it is not clear whether the observed immunoprophylactic activity of levamisole-potentiating tumor vaccines might lead to beneficial effects in the therapeutic regimen.

The present study confirmed the activity of

levamisole in potentiating Meth 1 tumor vaccine, and showed that the anti-tumor immunity induced by tumor vaccine and enhanced by levamisole as found in the immunoprophylactic regimen was valid in the therapeutic regimen, since more than half of the Meth 1-bearing animals were cured when administered tumor vaccine and levamisole. Cure incidence in the combined regimen was higher than that in the regimen utilizing either one of the two agents. The observed therapeutic effect was not negated, but rather enhanced by mitomycin C, suggesting the feasibility of combined immunotherapy by tumor vaccine and levamisole with anti-tumor chemotherapeutic agents.

MATERIALS AND METHODS

Tumors and Animals — An ascites of fibrosarcoma (Meth 1), originally induced in BALB/c

mice with 3-methylchoranthrene, has been passaged in the same strain of mice.

Male (BALB/c×DBA/2) F₁ mice (CD2F₁), 7–8 weeks old, were utilized for immunoprophylactic and immunotherapeutic experiments. These animals were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, JAPAN.

L1210 and P388 murine leukemia cells donated by the Cancer Chemotherapy Center, JAPAN, were also utilized for the experiments.

Drugs — Levamisole hydrochloride (Kyowa Hakko Kogyo Co., Ltd., JAPAN) was dissolved in sterile phosphate-buffered saline (PBS), and was administered intraperitoneally (*i.p.*) in a volume of 0.2 ml/mouse, at the indicated dosages and timings.

Vaccines — Meth 1 fibrosarcoma cell vaccines were prepared according to the modified method of Kataoka.⁸⁾ Briefly, Meth 1 cells were incubated with 100 µg/ml of mitomycin C (MMC: Kyowa Hakko Kogyo Co., Ltd., JAPAN) for 30 minutes at 37°C (MMC-Meth 1 vaccine), and were incubated with 165 µg/ml of Concanavalin A (ConA: Miles-Yeda, U.S.A.) for one hour in an ice bath.

After being washed three times with PBS, the collected cells (referred to as MMC-ConA-Meth 1 vaccine) were utilized for the following experiments.

Evaluation of Immunity — For the immunoprophylaxis experiment, animals were immunized intraperitoneally with 10⁶ cells of MMC-ConA-Meth 1 vaccine or MMC-Meth 1 vaccine, twice at one-week intervals. Mice were challenged with a different cell number of intact Meth 1 tumor or leukemias, seven days after the second vaccination. The number of surviving mice was counted forty days after the tumor challenge, and the mean survival time (MST) of each group was calculated for mice which died within forty days.

For the immunotherapy experiments, animals were initially inoculated intraperitoneally with Meth 1 cells (10⁴ or 10⁵ cells/mouse), and these were administered with the vaccine twice on the first day and on the eighth day after the first

tumor inoculation.

In another chemo-immunotherapeutic experiment, mice were administered with 1.0mg/kg of mitomycin C five days after the inoculation of 10⁵ Meth 1 cells in addition to the above immunotherapeutic regimen. Mice which survived for forty days were re-challenged with the same number of live tumor cells, and those surviving for another forty days after the re-challenge were counted for “eighty-day survivals” or “cures”. MSTs were calculated for mice which died within eighty days, and for those which survived for at least eighty days, although they did bear tumors.

RESULTS

1. Immunoprophylactic and Immunotherapeutic Potencies of ConA-Bound Tumor Vaccine

Animals were administered 10⁶ vaccine cells of either MMC-ConA-Meth 1 or MMC-Meth 1, and were subsequently inoculated *i.p.* with any of the indicated live tumor cells for examining the anti-tumor immunity. ConA-bound Meth 1 tumor vaccine had a immunoprophylactic potency showing perfect resistance to the Meth 1 challenge of 10⁵ cells, while ConA-free MMC-Meth 1 vaccine was much less potent than ConA-bound vaccine, as indicated by the number of forty-day survivors (Table I). This ConA-bound vaccine-induced immunoprophylaxis was tumor-specific to Meth 1 cells, because mice treated with MMC-ConA-Meth 1 vaccine did not show any resistance against the challenge of P388 and L1210 murine leukemias.

Immunotherapy by ConA-bound vaccine administered on day 1 and on day 8 after the inoculation of 10⁴ cells of Meth 1 resulted in a significant increase in the mean survival time (MST) of tumor-bearing animals over that of the untreated control group ($p < 0.05$ by Mann-Whitney's U Test). ConA-free vaccine was also much less potent than ConA-bound vaccine in the immunotherapeutic regimen (Table II).

Table III shows the effect of vaccine size on immunotherapeutic potency of ConA-bound Meth 1 vaccine. The size of 10⁵ cells of vaccine

was the most effective, and that of 10^4 cells was not effective. Both the sizes of 10^6 and 10^7 cells were somewhat less effective than the 10^5 cells.

2. Immunotherapy by Vaccine in Concert with Levamisole

The effect of levamisole on the immunotherapeutic response by ConA-bound Meth 1 vaccine was examined and the results of three experiments against an inoculum of 10^4 Meth 1 cells are summarized in Table IV. Vaccine (sub-optimal size of 10^6 cells/mouse) alone could increase the MSTs beyond those of the control group in all three experiments, and only one eighty-day survivor was obtained. Levamisole combined with

the vaccine augmented the therapeutic effect of the vaccine at all indicated doses. Thirty seven percent (37%) of the cured mice (eighty-day survivors) were obtained in the group treated with 0.63 mg/kg/d of levamisole administered on days 1, 2 and 3 ($p < 0.001$ by Fisher's Exact Test). The enhancing effect of levamisole was obtained at broad-ranging doses from 0.16 mg/kg/d up to 10 mg/kg/d ($p < 0.01$). Delayed administration of levamisole from day 8 to day 10 was less effective than earlier administration, but 17% of the mice were still cured at doses of 0.63 mg/kg/d and 10 mg/kg/d ($p < 0.05$).

3. Chemo-Immunotherapy Experiments by Vaccine

TABLE I. Immuno-Prophylactic Effect of MMC-ConA-Meth 1 Vaccine and MMC-Meth 1 Vaccine ^{a)}

Challenging Tumor and size		Number of 40-day survivors (MST) ^{b)}					
		MMC-ConA-Meth 1		Vaccinated with MMC-Meth 1		Not vaccinated	
Meth 1	10^2	5/ 5	(—)	5/5	(—)	1/5	(28.8)
	10^3	5/ 5	(—)	2/5	(29.7)	1/5	(24.0)
	10^4	5/ 5	(—)	1/5	(22.7)	1/5	(18.8)
	10^5	5/ 5	(—)	0/5	(18.2)	0/5	(18.2)
	10^6	1/ 5	(13.8)	0/5	(15.0)	0/5	(14.0)
	10^7	0/ 5	(12.2)	0/5	(11.8)	0/5	(12.4)
P388	10^3	0/10	(14.3)	N.T. ^{c)}		0/10	(14.2)
L1210	10^3	0/10	(11.0)	N.T.		0/10	(11.0)

a) $CD\bar{F}_1$ mice were immunized intraperitoneally with or without 10^6 cells of MMC-ConA-Meth 1 vaccine or MMC-Meth 1 vaccine twice at one-week intervals. The different cell number of intact Meth 1 tumors or leukemias were challenged into mice seven days after the last vaccination.

b) Mean survival time (d): Excluding survivors:

c) Not tested.

TABLE II. Immunotherapeutic Potency of ConA-Bound Meth 1 Tumor Vaccine ^{a)}

Vaccines utilized for therapy	Number of 80-day survivors and MST (d)	
None	0/10	26.9
MMC-ConA-Meth 1	1/10	40.1 ^{b)}
MMC-Meth 1	0/10	28.7

a) $CD2F_1$ mice were inoculated with 10^4 cells of Meth 1 tumor on day 0. MMC-ConA-Meth 1 vaccine or MMC-Meth 1 vaccine was administered twice on day 1 and on day 8 at a size of 10^6 cells/ mouse/ dose.

b) $p < 0.05$ by Mann-Whitney's U-test.

and Mitomycin C combined with Levamisole

As mitomycin C has been reported to enhance the immunoprophylactic and immunotherapeutic potency⁸⁾ of the ConA-bound L1210 murine leukemia vaccine, a chemo-immunotherapeutic regimen by ConA-bound Meth 1 vaccine and mitomycin C was settled, and the potentiating effect of levamisole on that regimen was examined. Mitomycin C (1.0mg/kg: Sub-

optimal dose in this regimen) was administered on day 5 after the inoculation of 10^5 live Meth 1 cells, as indicated in the previous report.⁸⁾

The results of four experiments are summarized in Fig. 1. ConA-bound Meth 1 vaccine plus mitomycin C (group 4) produced 27% (8/30) cured mice, and was significantly different from the vaccine-treated group (group 2: 3/40 cures), but it was not significant regarding the

TABLE III. *Immunotherapy of Meth 1 by MMC-ConA-Meth 1 Vaccine*^{a)}

Cell size of vaccine	Number of 80-day survivors and MST (d)						Total survivors
	Exp. 1		Exp. 2		Exp. 3		
—	0/7	16.9	0/7	21.5	0/10	20.5	0/24
10 ⁷	1/7	30.5	1/7	18.6	0/10	20.8	2/24
10 ⁶	1/7	30.2	2/7	29.8	0/10	20.5	3/24
10 ⁵	1/7	32.7 ^{b)}	2/7	38.8 ^{b)}	3/10	51.0 ^{b)}	6/24 ^{c)}
10 ⁴	0/7	22.0	N.T.		0/10	20.4	0/17

a) Meth 1 tumor inoculation: 10^4 cells/mouse.

b) $p < 0.05$ by Mann-Whitney's U-test (vs. No vaccine control).

c) $p < 0.05$ by Fisher's Exact test (vs. No vaccine control).

TABLE IV. *Effect of Levamisole on Immunotherapy of Meth 1 Tumor by Vaccine*^{a)}

Vaccine	Levamisole (mg/kg)	No. of 80-day survivors and MST (d)						Total survivors (%)
		Exp. 1		Exp. 2		Exp. 3		
—	—	0/10	27.6	0/10	24.9	0/10	29.0	0/30 (0)
+	—	1/10	56.9 ^{b)}	0/10	32.7	0/10	42.9 ^{b)}	1/30 (3)
+	D1—D3	10	2/10 44.1	2/10	43.1	3/10	63.7	7/30 (23) ^{c)}
+		2.5	2/10 33.5	1/10	38.2	4/10	39.7	7/30 (23) ^{c)}
+		0.63	4/10 54.2	2/10	40.3	5/10	46.8	11/30 (37) ^{d)}
+		0.16	N.T.	0/10	36.3	6/10	55.3	6/20 (30) ^{c)}
+	D8—D10	10	1/10 37.4	0/10	23.8	4/10	55.2	5/30 (17) ^{e)}
+		2.5	1/10 45.8	1/10	37.8	2/10	27.3	4/30 (13)
+		0.63	3/10 49.5	0/10	33.7	2/10	47.6	5/30 (17) ^{e)}
+		0.16	N.T.	1/10	32.3	3/10	34.7	4/30 (13)

a) Meth 1 tumor inoculation: 10^4 cells/mouse (D0), Vaccine: MMC-ConA-Meth 1; 10^6 cells/mouse/dose, D1 and D8

b) $p < 0.05$ vs. not treated control (by Mann-Whitney's U-test).

c) $p < 0.01$ vs. vaccine alone group (by Fisher's Exact test).

d) $p < 0.001$ same as above (by Fisher's Exact test).

e) $p < 0.005$ same as above (by Fisher's Exact test).

mitomycin C-treated group (group 3: 6/30 cures). On the other hand, 62% (18/29) of the animals were cured when 10 mg/kg/d of levamisole was administered on days 1, 2 and 3 in concert with the above chemo-immunotherapy regimen of vaccine and mitomycin C (group 5), which was significantly different ($p < 0.01$ by Fisher's Exact Test) from the chemo-immunotherapy group (group 4).

Such an enhancing effect of levamisole was also obtained in another protocol of delayed administration on days 8, 9 and 10 (group 6). Sixty percent (60%) [18/30] of the animals were cured at the dose of 2.5 mg/kg/d levamisole, which was also significantly different ($p < 0.01$) from the chemo-immunotherapy group (group

4). A cure ratio from 45% to 63% for the mice was obtained in both protocols at various doses from 0.16 mg/kg/d to 10 mg/kg/d of levamisole. On the other hand, no enhancing effect was obtained when levamisole was administered before or three days after chemotherapy (group 7 and group 8). The potency of levamisole seems to be induced efficiently in the presence of the tumor vaccine. As a summary, the most beneficial therapeutic regimen was a chemo-immunotherapy by vaccine and mitomycin C combined with levamisole, just after the vaccination.

DISCUSSION

In the present study, it was demonstrated that ConA-bound Meth 1 vaccine showed immuno-

FIG. 1. Effect of Levamisole on the Chemo-Immunotherapy in Combination with the Vaccine and/or Mitomycin C Against Meth 1 Tumor

Groups of animals inoculated i.p. with live Meth 1 cells (↓ : 10⁵ cells/mouse) were given i.p. MMC (◆ : 1 mg/kg) on day 5 with or without 10⁶ MMC-ConA-Meth 1 vaccine (▽) on days 1 and 8.

Levamisole (●) was administered on days 1, 2 and 3 or day 8, 9 and 10 at a defined dose.

- a) $p < 0.01$ vs. group 1.
- b) $p < 0.01$ vs. group 2.
- c) $p < 0.01$ vs. group 4.
- d) $p < 0.05$ vs. group 4.
- e) $p < 0.02$ vs. group 3.

prophylactic and immunotherapeutic potencies, and that levamisole enhanced the anti-tumor immunity induced by ConA-bound tumor vaccine combined with or without mitomycin C.

The binding of ConA to the vaccine cells proved to be essential for the immunoprophylactic and immunotherapeutic potencies, as shown in Table I and Table II. ConA bound to the vaccine cells, but not free ConA, appears to be effective *in vivo* as well as *in vitro* blastogenic activity.⁹⁾ These properties of Meth 1 tumor vaccine were similar to those of ConA-bound L1210 leukemic vaccine reported by Kataoka.¹⁰⁾ Meth 1 tumor vaccine, however, seems to be more immunogenic than that of L1210 leukemic vaccine, because the marked immunoprophylactic effect was obtained with 10^5 cells of Meth 1 vaccine against the challenge of intact 10^5 Meth 1 cells,⁷⁾ but more than 10^6 cells of L1210 leukemia vaccine was required to obtain the apparent resistance against the challenge of 10^2 L1210 leukemic cells, as reported by Kataoka *et al.*¹⁰⁾

The enhancing effect of levamisole was higher on the immunoprophylactic activity of Meth 1 tumor vaccine⁷⁾ than that of L1210 vaccine.⁵⁾ Thus, the Meth 1 tumor vaccine system seems to be a suitable system for evaluation or screening of some immuno-potentiating agents.

The optimal vaccine size for the immunotherapeutic potency of ConA-bound Meth 1 tumor vaccine was 10^5 cells/mouse, and larger vaccine sizes (*e.g.* 10^6 and 10^7 cells/mouse) somewhat reduced the therapeutic effects (Table III). There is no report to show such a result in the L1210 leukemic vaccine system, even at the size of 10^7 cells/mouse. Although we possess no evidence to explain the above result in the Meth 1 tumor vaccine system, one possible idea is that ConA bound to this Meth 1 vaccine might activate the suppressor cells, as well as the effector cells, at larger sizes.

On the other hand, there has been a report dealing with unsuccessful results utilizing the combination of levamisole and tumor vaccines against B16 melanoma and adenocarcinoma 15901.⁴⁾ Our results, however, show that

levamisole displayed a potentiating effect on the therapeutic activity of ConA-bound Meth 1 tumor vaccine, providing favorable cures in Meth 1 tumor-bearing mice. Cure incidence in the combined regimen was higher than in the regimen utilizing either one of them. The effective dose of levamisole covered a broad range from 0.16 mg/kg/d up to 10 mg/kg/d. The enhancing effect of levamisole on the immunotherapeutic activity of Meth 1 tumor vaccine was obtained in the early administration just after the first vaccine, and produced a 37% cure of Meth 1-bearing mice. The delayed administration of levamisole after the second vaccine was less effective than the earlier administration, although a 17% cure ratio of mice was produced.

Under the latter regimen, the number of tumor cells increases to an amount so large at the timing of the second vaccine that the effector cells associated with vaccine and levamisole could not sufficiently kill them. Indeed, when the inoculum size of Meth 1 cells was as large as 10^5 cells/mouse, the therapeutic effect by vaccine and levamisole decreased (data not shown).

The immuno-therapeutic response of ConA-bound Meth 1 tumor vaccine was enhanced by mitomycin C combined on day 5, although it appeared to additive (Fig. 1, group 4 vs. group 2 and group 3). The therapeutic response under these regimen, however, was further enhanced by levamisole, and approximately 60% of the animals inoculated with 10^5 Meth 1 cells were cured when the three agents were administered at the defined intervals as shown in Fig. 1 (group 5 and group 6). The enhancing effect of levamisole was obtained in the combination with the tumor vaccine, but was not obtained without the vaccine, or rather, negated the effect of mitomycin C. These results suggest that levamisole is effective only if the host immunity system can be sufficiently stimulated by the tumor vaccine or tumor-associated antigens (although we cannot explain the reason why levamisole negated the effect of mitomycin C).

Kataoka⁸⁾ reported that the combined administration of mitomycin C on day 5 and ConA-

bound L1210 leukemic vaccine on day 1 and day 8 was the most effective regimen, and induced an enhanced therapeutic response greater than inducible by either of them, and that mitomycin C potentiated the anti-tumor immunity induced by the vaccine by abrogating suppressors associated with vaccine-bound ConA, and the chemotherapeutic potency of mitomycin C played no major role in the enhancement. Under our experimental conditions, however, mitomycin C showed a weak but apparent chemotherapeutic response, which appeared to be due to its cytoreductive effect (Fig. 1, group 3). We cannot negate the possibility of mitomycin C to enhance the anti-tumor immunity induced by ConA-bound Meth 1 vaccine by abrogating possible suppressors. If mitomycin C does not only reduce most of the tumor cells but also concomitantly abrogates possible suppressors in our chemo-immunotherapeutic regimen, levamisole might selectively stimulate the effector cells induced by ConA-bound Meth 1 tumor vaccine. Indeed, levamisole displayed a markedly-enhancing effect at broad-ranging doses and at both timings of early and delayed administrations under these regimens utilizing three agents.

These results and related ideas allow us to suggest the feasibility of the above chemo-immunotherapeutic regimen, although clarification of the enhancing mechanism of levamisole remains.

Acknowledgements The authors would like to express their appreciation to Dr.T.Kataoka of the Cancer Chemotherapy Center, Tokyo, JAPAN, for his kind and helpful advice, and also to Miss K.Yamamoto for her technical assistance.

REFERENCES

- 1) W.K.Amery, F.Spreafico, A.F.Rojas, E.Denissen, and M.A.Chirigos: Adjuvant treatment with levamisole in cancer, a review of experimental and clinical data, *Cancer Treat., Rev.*, **4**, 167 (1977).
- 2) J.Symoens and M.Rosenthal: Levamisole in the modulation of immuneresponse, the current experimental and clinical state, *J.Reticuloendothel. Soc.*, **21**, 175 (1977).
- 3) M.A.Chirigos and W.K.Amery: "Combined levamisole therapy: an overview of its protective effects, immunotherapy of human cancer: The University of Texas Cancer Center M.D.Anderson Hospital and Tumor Institute, Raven Press New York, 1978.
- 4) I.J.Fidler and L.E.Spitler: Effect of levamisole *in vivo* and *in vitro* murine host response to syngeneic transplantable tumor, *J. Natl. Cancer Inst.*, **55**, 1107 (1975).
- 5) T.Kataoka, S.Tsukagoshi, Y.Sakurai and M.Okabe: Potentiation of L1210 murine leukemia vaccine *in vivo* by levamisole, *Gann*, **70**, 515 (1979).
- 6) F.Spreafico, A.Vecchi, A.Mantovani, A.Foggi, G.Franchi, A.Anaclerio and S.Garattini: Characterization of the immunostimulants levamisole and tetramisole, *Eur. J. Cancer*, **11**, 555, (1975).
- 7) M.Okabe, M.Morimoto, N.Nakamura, K.Mineura and M.Tanaka: Potentiation of immune resistance induced by Concanavalin A-bound tumor vaccine by levamisole, *Gan to Kagakuryoho (Cancer and Chemotherapy)*, Japan, **7**, 835 (1980).
- 8) T.Kataoka, K.Ogihara, and Y.Sakurai: Immunoprophylactic and immunotherapeutic response by Concanavalin A-bound tumor vaccine enhanced by chemotherapeutic agents eliminating possible suppressors, *Cancer Res.*, **40**, 3839 (1980).
- 9) T.Kataoka, F.Ohashi, and Y.Sakurai: Blastogenic potency of Concanavalin A-bound L1210 leukemic vaccine associated with its immunogenic activity, *Gann*, **70**, 155 (1979).
- 10) T.Kataoka, F.Ohashi, S.Tsukagoshi, and Y.Sakurai: Induction of resistance to L1210 leukemia in BALB/c × DBA/2CrF₁ mice, with L1210 cells treated with glutaraldehyde and concanavalin A, *Cancer Res.*, **37**, 964 (1977).