EFFECTS OF MURINE LEPROSY BACILLI ON SPONTANEOUS MAMMARY TUMOR IN MICE

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INTRODUCTION

The antitumor effect of not only BCG and some other mycobacteria including *M. tuberculosis*[1,2], *M. kansasii, M. smegmatis*[3], *M. butyricum*[4-6], but also other organisms such as *Nocardia asteroides, N. rubra, Corynebacterium diphtheriae*[7] or its toxin[8], anaerobic corynebacteria[9-14], *Bordetella pertussis*[15,17], or even some protozoa[18,19] has been reported. The mechanism of the action of these organisms is still a matter of speculation and may be different by agents[8]. But, in most cases, the action seems to reflect a generlaized stimulation of phagocytes and the activation of reticuloendothelial system[1,12,20,21], resulting in the production of serum antibodies and delayed hypersensitivity reactions[22].

However, none seems to have studied the possibility of tumor suppression with *Mycobacterium lepraemurium* which is non-pathogenic to humans; one of the reasons may be that lesions experimentally produced by this organism in animals is analogous to the lepromatous lesions in humans and lepromin reactions with this organism do not appear, which suggests that cellular immunity may not be induced in the hosts.

The present work was undertaken (a) to study the effects of live or heat-killed murine leprosy bacilli on the spontaneous mammary tumors in mice, and (b) to know whether the tumor suppression, if any, would accompany the murine lepromin reaction.

MATERIALS AND METHODS

Organisms used. *Mycobacterium lepraemurium* Hawaii strain was inoculated into C3H mice intraperitoneally. They were sacrificed 4 to 6 months later, and enlarged liver and spleen were resected aseptically. The organs were cut into pieces, added with 5 volumes of 0.2% trypsin phosphate buffer and digested for 2 hrs at 37°C, and homogenized in Teflon glass homogenizer. In order to remove undigested organ debris, the suspension was added with 2 volumes

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of phosphate buffer and centrifuged at 1,000 rpm for 10 minutes. The supernatant was then centrifuged at 4,000 rpm for 30 minutes. The sediment was again added with phosphate buffer and centrifuged. This procedure was repeated more than 6 times. The sediment finally obtained was composed solely of acid-fast bacilli and no organ debris was found by Ziehl-Neelsen stain. The sediment was added with phosphate buffer, and appropriate bacillus concentrations were obtained by counting the number of the bacilli using hemocytometer. These suspensions will be referred to as living *M. lepraemurium* (LMlm). When LMlm was heated at 100°C for 30 minutes, they will be referred to as heat-killed *M. lepraemurium* (HKMlm). Both suspensions were stored at —20°C and used within 4 months after preparation.

In some cases equal volume of Freund's incomplete adjuvant (FIA) was added to LMlm or HKMlm and converted to water-in-oil emulsion by Waring blender (LMlm+FIA, or HKMlm+FIA). One tenth ml of the desired bacillus concentration of the preparation was injected into mice intraperitoneally.

Murine lepromin tests (*MLT*). 10^7 living cells of *M. lepraemurium* suspended in 0.025 ml phosphate buffer were heated at 100°C for 30 minutes. Right footpad of mice was injected with 0.025 ml of this suspension. The thickness of both footpads was measured by dial-gauged calipers (Mitsutoyo, Japan) at 48 hrs (early reaction), 1,2,3,4, and, in one experiment, 5 weeks (late reactions) after injection. The calipers employed can measure up to 0.005 mm. The thickness of right footpad after substracting that of left footpad (control) will be referred to as the value of MLT.

Animals. C3H/He female mice obtained from Animal Center of Kyoto University were used. The mice within two weeks difference in age were divided into groups randomly, including one untreated group, in a series of experiments. CMF pellets (Oriental Yeast Co., LTD) and tap water were given *ad. lib.*

Examination of tumors. All mice were examined tumor incidence by inspection once a week. In cases when LMlm was injected, all the tumors produced were biopsied and the smears were stained with Ziehl-Neelsen and Giemsa-May-Grünwald stain. No tumors ever contained acid-fast bacilli. At death the weight of liver and spleen were measured. The smears of liver and spleen were also stained with Ziehl-Neelsen and Giemsa-May-Grünwald stains.

RESULTS

In the first experiment sixty C3H/He female mice were divided into five groups; ten each of first two groups were given with 10^8 LMlm or 10^8 LMlm+FIA, respectively, at the age of 19 week. The mice of group 3 were injected with HKMlm 3 times at the age of 19, 29, and 36 week in the dose of 10^8, 3×10^7, and 10^8, respectively; and ten mice of group 4 were given the same inocula at the same age as group 3 except that the first injection was 10^8 HKMlm+FIA. The remaining 20 mice were served as the untreated controls. Observation was made until 60 weeks of age. Results are summarized in Table 1.

As seen in the table, when 10^8 LMlm were injected (group 1), four mice died of murine leprosy before producing tumors. Spontaneous mammary tumor was produced in all the

Table. 1 Incidence of spontaneous mammary tumor in C3H/He female mice within 60 weeks of age (Experiment 1)

Group	Treatment	No. of mice Used	FT[1]	DML[2]	T[3]	TTFF[4] (weeks)	MTAT±SD[5] (weeks)
1	LMlm	10	0	4	6	27	36.6± 6.24
2	LMlm+FIA	10	0	1	9	26	40.1± 7.89
3	HKMlm	10	0	0	10	32	41.3± 5.81
4	HKMlm+FIA and HKMlm	10	3	0	7	33	46.7± 9.43
5	Control	20	0	0	20	23	38.3±10.48

1) Free from tumor at 60 weeks of age.
2) Died of murine leprosy before tumor appearance.
3) Produced tumor.
4) Time when tumor was first found.
5) Mean tumor appearance time±Standard deviation.

remaining 6 mice. Tumor was first observed at 27 weeks of age. The mean age of tumor incidence of these 6 mice was 36.6±6.24 weeks which shows little difference from that of the control, 38.3±10.48.

In group 2 where 10^8 LMlm plus FIA was injected, only one mouse died of murine leprosy, and all the remaining 9 mice produced tumors, the first incidence of tumor and the mean age of tumor appearance being 26 and 40.1±7.89 weeks, respectively. This also indicates that no tumor suppression was seen. It should be mentioned here that lepromatous lesions in the liver were not marked and the swelling of spleen was clearly reduced in these 9 mice compared with those infected with LMlm alone.

When HKMlm was injected 3 times (group 3), the first incidence of tumor was at 32 weeks of age which was 9 weeks later than 23 weeks of the control. However, tumor suppression was not observed thereafter; and the mean age of tumor appearance was 41.3±5.81 weeks.

When the first inoculum was replaced by HKMlm plus FIA instead of HKMlm (group 4), a difinite tumor suppression was observed. The first tumor incidence in this group was 33 weeks of age which was 10 weeks later than that of the control, and 3 mice were free from tumor even at 60 weeks of age. Fig. 1 shows the cummulative frequencey of tumor in groups 4 and 5 (control).

MLT was performed in the first four groups at 40 weeks of age. Fig. 2 shows the average MLT values at different times after the test. No clear reaction was observed in LMlm group up to 5 weeks after the test. In LMlm+FIA group slight early and late reactions were observed. On the contrary, both early and late reactions were clearly obtained in groups of both HKMlm and HLMlm+FIA; especially the late reaction was observed clearly as early as 2 weeks after the test in HKMlm+FIA group.

In the second experiment 36 mice were divided equally into three groups. The first group of mice were given with 10^6 LMlm at the age of 6 week, the second group was administered 7 times with HKMlm at the age of 6, 11, 16, 21, 26, 32, and 37 week. The dose of the first

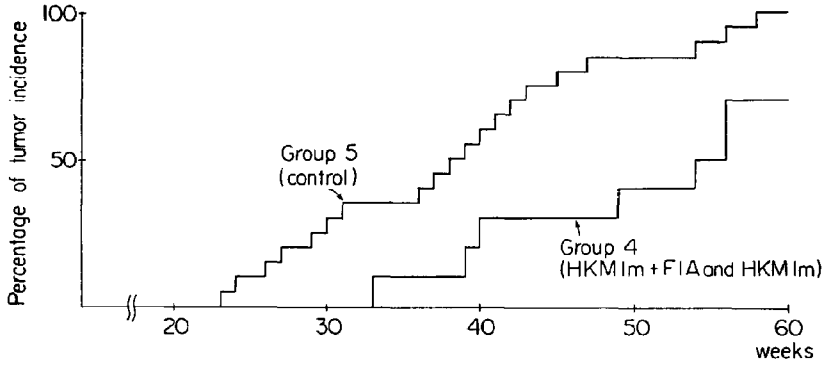

Fig. 1 Cummulative frequency of spontaneous mammary tumor in C3H/He female mice treated with HKM1m+FIA and HKM1m

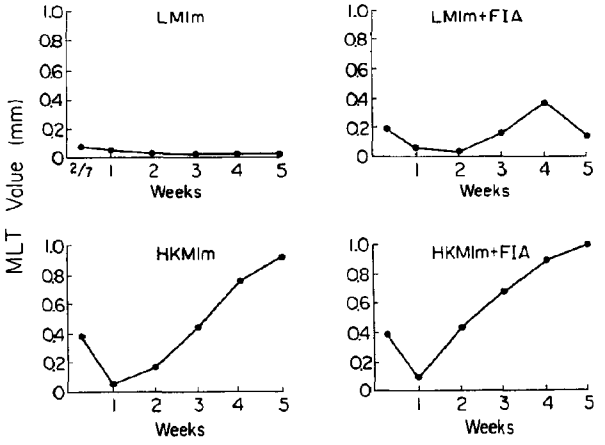

Fig. 2 Murine lepromin test values in Experiment 1

Table. 2 Incidence of spontaneous mammary tumor in C3H/He female mice within 50 weeks of age (Experiment 2)

Group	Treatment	Used	No. of mice FT[1]	DML[2]	T[3]	TTFF[4] (weeks)
1	LM1m	12	0	6	6	27
2	HKM1m	12	8	0	4	32
3	Control	12	3	0	9	25

1) Free from tumor at 50 weeks of age.
2) Died of murine leprosy before tumor appearance.
3) Produced tumor.
4) Time when tumor was first found.

injection was 10^6, but that of the others was 10^7. The third group of mice were served as controls. Observation was made until 50 weeks of age.

The results are shown in Table 2. When LMlm was injected (group 1), six mice (50%) died of murine leprosy before tumor appearance, and all the remaining mice produced tumors. However, when HKMlm was injected 7 times (group 2), tumor was first observed at 32 weeks of age which was 7 weeks later than 25 weeks of the control group, and only four mice (33%)

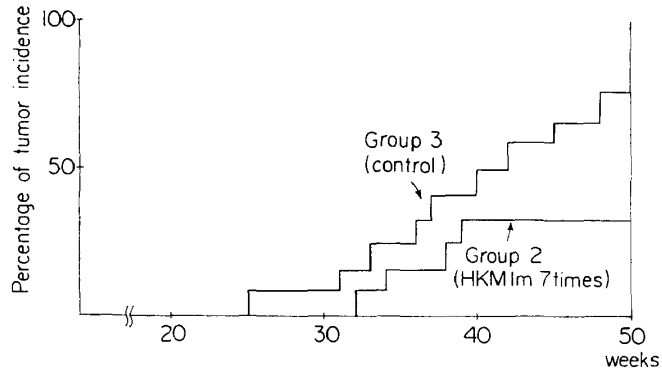

Fig. 3 Cummulative frequency of spontaneous mammary tumor in C3H/He female mice treated with HKM1m 7 times

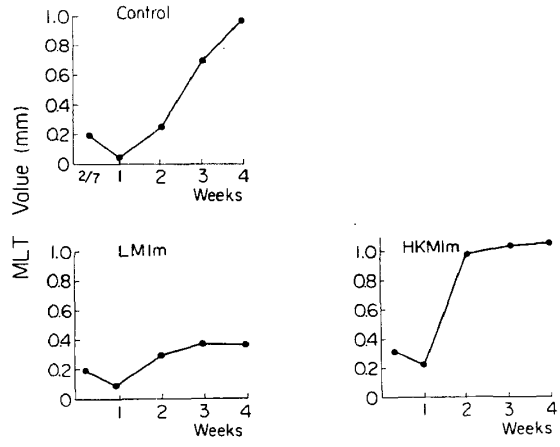

Fig. 4 Murine lepromin test values in Experiment 2

produced tumors in contrast to nine (75%) in the control group. In Fig. 3 cummulative frequency of tumor of this group is compared with that of the control.

Fig. 4 shows the results of MLT of these three groups performed at the age of 27 week. In the untreated control 48 hr value was weak, but 2 weeks and thereafter the reaction became stronger reaching maximum at 4 weeks. In LMlm group the reactions were weak throughout the observation as were the case in the first experiment. On the contrary, the characteristic feature of HKMlm group was that very strong late reaction was obtained as early as 2 weeks which was comparable to the maximum value observed at 4 weeks in the control group. It should also be noted that in this group the difference in values was not observed between tumor-bearing and non-bearing mice.

DISCUSSION

It was shown in the present experiments that LMlm, even though given in different doses at different ages and whether with or without FIA, had no effect in suppressing the spontaneous mammary tumor of mice. Moreover, as the organism is pathogenic for mice, some mice died of murine leprosy before tumor incidence and MLT was faint. The only interesting finding in the case of LMlm was that in LMlm+FIA group fewer mice died of leprosy and the lepro-

matous lesions were less severe than LMlm alone group. This finding recalls us the work of Uyeda *et al.* (23) who found that mortality of mice infected with virulent tubercle bacilli H37Rv (human type) or Ravenel (bovine type) mixed with paraffin oil was much less than that given with water suspensions of these organisms. They explained the fact that the organisms in oil were prevented from ingestion by phagocytes in which the bacilli can multiply. Whether the same is true in the case of murine leprosy bacilli or not remains to be studied.

The most striking feature of the present work was the retardation and decrease of tumor appearance in mice repeatedly treated with HKMlm. In this case the age of mice and frequency of treatment seem to influence the effect of HKMlm. In comparison with the results of group 3 in Table 1 and group 2 in Table 2, it may be said that stronger tumor suppression will be obtained if the treatment begins in younger age and repeats more frequently. Freund's incomplete adjuvant seems to aid the effect of HKMlm.

It should be noted that strong murine lepromin reactions, especially late reactions, were observed by the repeated HKMlm injections. This indicates that the ability to suppress the incidence of spontaneous mammary tumor in mice has some relation to the positive conversion of murine lepromin reaction which is said to be an expression of cellular immunity. However, as there was no difference in MLT values in tumor-bearing and non-bearing mice in HKMlm group in the second experiment, cellular immunity alone seems not enough to suppress mammary tumors.

SUMMARY

When heat-killed *Mycobacterium lepraemurium,* with or without Freund's incomplete adjuvant, was injected intraperitoneally into C3H/He female mice repeatedly, incidence of spontaneous mammary tumor was retarded or suppressed. The murine lepromin reactions, especially late reactions, became stronger by this treatment. Freund's incomplete adjuvant seems to aid the effect of heat-killed bacilli. Live cells of *Mycobacterium lepraemurium,* with or without the adjuvant, had no tumor suppressing effect and the murine lepromin reactions were weak.

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