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Inhibitory Effect of *Nocardia rubra* Cell Wall Skeleton on Carcinogen-Induced Mammary Tumorigenesis in Rats*

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Abstract—*The inhibitory effects of the cell wall skeleton (CWS) of* Nocardia rubra, *a potent immunopotentiator, on carcinogen-induced mammary tumorigenesis and serum prolactin levels in rats were studied. Sprague–Dawley female rats were divided into four groups at* 4 weeks *of age; the first group served as the control and the second group received* 150 μg *CWS of* N. rubra *every other week throughout the experiment. The third and the fourth groups were given CWS every other week only before and after the administration of* 7,12-*dimethylbenz(a)anthracene (DMBA), respectively. CWS was injected intravenously (i.v.) for the first three injections and subcutaneously (s.c.) thereafter. All rats were given single i.v. injections of fat emulsion of* 5 mg *DMBA at* 8 weeks *of age. Both incidence and progression of mammary tumors were significantly inhibited in rats treated with CWS throughout the experiment and in rats given CWS only after DMBA administration. On the other hand, treatment with CWS only before DMBA injection had little effects. CWS of* N. rubra *reduced serum level of prolactin, an important hormone for mammary tumorigenesis.*

INTRODUCTION

FROM the view point of importance of immune mechanism in tumorigenesis, several works have been done on the effects of cell wall skeleton (CWS) of Bacillus Camette–Guérin and other species of bacteria on tumorigenesis. While the possible efficiency of immunotherapy of human breast cancer has been stressed [1–3], fundamental studies on this problem are scanty. We previously demonstrated the prophylaxis by CWS of *N. rubra* of spontaneous mammary tumorigenesis in mice [4].

In this paper, the effects of CWS of *N. rubra* on development and progression of carcinogen-induced mammary tumors in rats, which are tumors of quite different type from spontaneous mammary tumors in mice and other representative animal model for human breast cancer, were studied.

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MATERIALS AND METHODS

CWS of N. rubra

Preparation of CWS of *N. rubra* was the same as described previously [5]. CWS mixed with mineral oil was suspended in 0.85% NaCl solution containing 0.2% Tween 80 at the concentration of 150 μg/0.1 ml [6, 7].

Animals

Sprague–Dawley female rats were divided into 4 groups. The control and group NR were injected with vehicle only and 150 μg CWS of *N. rubra*, respectively, every other week throughout the experiment beginning 4 weeks of age. Group NR_i was given CWS three times at 4, 6 and 8 weeks of age. Group NR_p received CWS every other week beginning immediately after DMBA administration. CWS was injected i.v. at the first three times and s.c. thereafter. All rats were given single i.v. injections of 5 mg 7,12-dimethylbenz(a)anthracene (DMBA: The Upjohn Co., Kalamazoo, MI, U.S.A.) on the afternoon of proestrus at 8 weeks of age;

DMBA-induced mammary tumorigenesis depends largely upon the estrous phases when DMBA is injected and the administration on the afternoon of proestrus is most effective [8]. Blood samples for assay of prolactin were collected from the tail vein in some animals in each group under the light ether anesthesia [9] on the afternoon of proestrus prior to DMBA injection.

Rats were kept three or four per cage, maintained in an animal room that was air-conditioned (24±0.5°C and 65–70% r.h.) and artificially illuminated (14 hr of light from 5 a.m. to 7 p.m.) and provided with a commercial diet (CA-1: CLEA Japan Inc., Tokyo, Japan) and tap water *ad libitum*.

Mammary tumorigenesis

Each rat was checked for palpable mammary tumors every 7 days beginning 3 weeks after DMBA injection until the 3rd week after the first tumor appearance or the 26th week after DMBA injection when all control rats developed tumors. The number and size of mammary tumors expressed in terms of the geometric mean of the major two diameters were recorded. The changes in mammary tumor size during 2 weeks after appearance were also checked. Category of changes in size was defined as follows: progressive, >50% increase; regressed, >50% decrease; and static, <50% change in size.

At killing by decapitation, blood was collected from the trunk and serum was stored at −20°C for assay of prolactin.

Serum prolactin level

Serum prolactin was assayed by radioimmunoassay as an index of the rate of pituitary secretion. At 8 weeks of age, the control and group NR_p and groups NR and NR_i were under the same conditions concerning CWS; the former two groups were never exposed to CWS and the latter two groups received three times injections of CWS. Thus, data of each two groups were pooled in 'Results'. At autopsy, blood was collected in the morning and there were no considerable differences in the prolactin values between estrous phases; the data were also pooled in 'Results' in each group.

Statistics

Significant differences in mammary tumor incidence and the ratio of tumors in each category were evaluated by χ^2-test and those of the other parameters were evaluated by the Student *t*-test.

RESULTS

Body weight change

There were little influences of CWS on body weight changes; the body weight at the start of experiment (4 weeks of age) was about 80 g in all rats, and carcass weights (body weights − tumor weights) at autopsy were 292±10 g, 296±9 g, 279±8 g and 270 ±7 g in the control; groups NR, NR_i and NR_p, respectively.

Mammary tumorigenesis

Mammary tumor incidence and latency period in each group are illustrated in Table 1. There were no significant differences between groups in the incidence at 10 and 20 weeks after DMBA administration. Meanwhile, all rats developed mammary tumors at 26 weeks in the control, when mammary tumor incidences in groups NR, NR_i and NR_p were 73%, 86% and 64%, respectively. The difference between the control and group NR or group NR_p was statistically highly significant ($P<0.01$).

Both mean and/or range of the onset age of mammary tumors were apparently larger in the control than in any experimental group, although the differences were not statistically significant.

As shown in Table 2, the percentage change in mammary tumor size during 2 weeks after appearance was significantly lower in groups NR and NR_p than in the control and group NR_i ($P<0.01$).

Groups NR and NR_p were also significantly smaller than the control and group NR_i in the ratio of progressive tumors ($P<0.05$, 0.02 or 0.01). The ratio of static tumors in group NR was higher than those in the control and group NR_i ($P<0.05$ or 0.02). There were no significant differences between groups in the ratio of regressed tumors and the number of tumors per rat at autopsy.

Serum prolactin level

Results are presented in Table 3. Serum prolactin level on the afternoon of proestrus at 8 weeks of age was significantly higher in the control and group NR_p which were never exposed to CWS than in groups NR and NR_i which received i.v. injections of CWS three times ($P<0.01$).

Table 1. Incidence and onset age of DMBA-induced mammary tumors in each group

Group and treatment*	No. of rats	Cumulative mammary tumor incidence (%) Weeks after DMBA administration 10	20	26	Mean and range of latency period (weeks)
Control	26	42 (11)†	73 (19)	100 (26)[a]	13.7 (5.6–25.7)
NR	22	32 (7)	59 (13)	73 (16)[b]	12.2 (5.6–21.3)
NR_i	29	45 (13)	83 (24)	86 (25)	10.9 (4.6–23.4)
NR_p	25	44 (11)	60 (15)	64 (16)[c]	10.0 (6.6–21.0)

*The control or group NR was injected with vehicle only or 150 μg cell wall skeleton (CWS) of *Nocardia rubra* every other week throughout the experiment beginning 4 weeks of age. Group NR_i received CWS three times at 4, 6 and 8 weeks of age only before DMBA injection. Group NR_p was given CWS every other week beginning immediately after DMBA injection. CWS was administered i.v. for the first three injections and s.c. thereafter. All rats were given single i.v. injections of 5 mg DMBA on the afternoon at 8 weeks of age and checked for palpable mammary tumors until the 3rd week after the first tumor appearance or the 26th week after DMBA.

†Number in parenthesis is number of rats with mammary tumors.

Significance of difference: a/b, c: $P<0.01$.

Table 2. Change in mammary tumor size and number of tumors at autopsy in each group

Group and treatment*	Change in mammary tumor size: No. of tumors examined†	Change in size during 2 weeks after appearance (%)	Per cent and No. of tumors in each category‡: Progressive	Static	Regressed	No. of mammary tumors per tumor-bearing rat at autopsy
Control	25	90.0±14.0[a]	64 (16)[e]	36 (9)[i]	0 (0)	1.9±0.2 (26)§
NR	21	21.4±14.0[b]	24 (5)[f]	71 (15)[j]	5 (1)	2.3±0.5 (16)
NR_i	28	80.8±13.3[c]	57 (16)[g]	43 (12)[k]	0 (0)	2.4±0.3 (25)
NR_p	19	18.0±14.6[d]	26 (5)[h]	63 (12)	11 (2)	1.7±0.2 (16)

*See Table 1 for details of treatment.

†Only mammary tumors of which sizes were measured three or four times (the first and the second tumors).

‡Category of mammary tumor progression: progressive, >50% increase; regressed, >50% decrease and static, <50% change in size during 2 weeks after appearance, respectively.

§Number of rats examined.

Significance of difference: g/h; j/k: $P<0.05$, e/h; f/g; i/j: $P<0.02$, a,c/b,d; e/f: $P<0.01$. a and c are significantly different from zero at $P<0.01$.

Table 3. Effects of cell wall skeleton of Nocardia rubra *(NR) on serum prolactin levels*

On the afternoon of proestrus (8 weeks of age)			On the morning of various estrous phases (12–34 weeks of age)		
Group and treatment*†	No. of estimates	Serum prolactin levels (ng/ml)	Group and treatment	No. of estimates	Serum prolactin levels (ng/ml)
Control, NR_p	14	180±23‡[a]	Control	19	15±2
			NR	14	9±1
NR, NR_i	18	123±15[b]	NR_i	24	13±3
			NR_p	23	19±6

*See Table 1 for details of treatments.
†By this age, the control and group NR_p and groups NR and NR_i were under the same conditions concerning cell wall skeleton (CWS) of *N. rubra*; the former two groups were not exposed to CWS and the latter two groups received three times injections of CWS. Thus, the data in each two groups were pooled.
‡Means±S.E.M.
Significance of difference: a/b: $P<0.01$.

On the other hand, there were no significant differences between groups in the level on the morning at 12–34 weeks of age irrespective of exposure to CWS.

DISCUSSION

The present study shows that both incidence and progression of DMBA-induced mammary tumors in rats were markedly suppressed by every other week-injections of CWS of *N. rubra*. Immunopotentiation by CWS of *N. rubra* has repeatedly been confirmed [5, 10–15]. Thus, in this study, immunopotentiation by CWS of *N. rubra* could primarily contribute to the inhibition of DMBA-induced mammary tumorigenesis.

Treatment with CWS only after DMBA administration had inhibitory effects on mammary tumorigenesis to the extent similar to the treatment throughout the experiment. On the other hand, treatment only before DMBA administration had no effects. These findings suggest that CWS of *N. rubra* mainly acts on the process of mammary tumor progression rather than its initiation under the present experimental conditions.

This study also shows that the inhibition by CWS of mammary tumor appearance was statistically significant only at 26 weeks after DMBA administration, but not at 10 and 20 weeks. These data infer that CWS is effective on mammary tumors which appeared later, i.e., tumors with low growth potential, but less so on tumors which developed earlier, i.e., had a high growth potential. This is partially reflected by the larger mean and/or range of the age of onset of mammary tumors in the control, than in rats receiving CWS. The results also imply the limit of inhibitory activity of CWS of *N. rubra* on carcinogen-induced mammary tumors in rats.

Whereas CWS of *N. rubra* had little effect on serum prolactin level on the morning on which it is generally low, irrespective of estrous phases, it significantly reduced the level on the afternoon of proestrus that is the highest during the cycle. This agrees with the results in mice [4] and this declined secretion of pituitary prolactin, a primary hormone for carcinogen-induced mammary tumorigenesis [16], may partially contribute to the inhibition by CWS of *N. rubra* of progression of this type of mammary tumors. On the other hand, little inhibitory effect of CWS treatment prior to DMBA was observed, despite the significant decline in serum prolactin. This would be ascribed to the insufficient decline (32%, from 180 ng to 123 ng) to counteract the potent carcinogenicity of DMBA, which may also be the case for immunopotentiation, since CWS was given only three times in this group.

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