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Evidence for Host-mediated Antitumor Effects of Lysozyme in Mice Bearing the MCa Mammary Carcinoma

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Abstract—*The host-mediated effects of lysozyme on primary tumor growth and on the formation of pulmonary metastases were investigated in mice bearing the MCa mammary carcinoma. The oral administration of lysozyme to CBA mice for 7 consecutive days before i.v. inoculation of MCa mammary carcinoma cells causes a significant reduction in the formation of lung tumors. The growth of s.c. tumors and the development of lung metastases is also significantly lowered in mice inoculated with tumor cells previously kept at 37°C for 30 min in the presence of peritoneal resident cells or whole plasma samples obtained from normal mice treated with 25–100 mg/kg/day lysozyme for 7 consecutive days. The lysozyme concentration in plasma samples of the treated mice remains undetectable even at daily dosages up to 400 mg/kg, ruling out the hypothesis of a direct effect of the ingested lysozyme. These data seem to suggest a role for host immune reactivity in the antineoplastic effects of lysozyme. The results are consistent with previously reported data and further stress the interesting antitumor properties of the oral administration of lysozyme in mice bearing solid metastasizing tumors.*

INTRODUCTION

THE FORMATION of solid tumor metastases can be significantly reduced by lysozyme treatment in animal models of metastasizing tumors. Lysozyme antimetastatic activity results after either i.v. injection or oral administration of proper dosages of hen egg-white lysozyme [1–3]. The mechanism of the antimetastatic activity, however, remains unknown. It is conceivable that lysozyme can stimulate the effectors of immune surveillance, thereby eliciting host reactivity against the tumor [4]. In favor of such a hypothesis, data exist which indicate lysozyme as a direct activator of immune cells (monocytes and lymphocytes [5–7]) and, after oral administration, as an inducer of immune responses with a potency comparable to that obtained with the broth of *in vitro* digested cell walls of *Bifidobacterium longum*, administered by the same route [8].

Many studies have demonstrated that the products of digested cell walls, namely peptidoglycans of various size, are capable of potentiating host immune mechanisms, as shown in detail for MDP [9, 10], and inhibiting tumor growth in experimental systems of solid metastasizing tumors [11–15]. Thus, after oral administration it could be supposed that lysozyme by an interaction with intestinal bacteria, can liberate peptidoglycans of high and low molecular weight which become the agents responsible for the immunostimulation observed [16], and correspondingly of the antitumor activity observed in lysozyme-treated animals.

Macrophages seem to be the main target of peptidoglycan activity [17, 18]; they are recognized as the cellular effectors of the immune system, responsible for the antitumor and particularly for the antimetastatic effects of MDP [19, 20] and probably also of the other peptidoglycans studied [13]. With the present paper we therefore thought it worthwhile to evaluate the antitumor effects of lysozyme as mediated by resident cells recovered from the peritoneal cavity of lysozyme-treated mice (mainly macrophages and lymphocyte-like cells), using the MCa mammary carcinoma of the CBA mouse. The occurrence of antitumor effects mediated by peritoneal resident cells is determined in terms of the reduction of primary (s.c.) tumor

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growth in mice concomitantly inoculated s.c. with MCa mammary carcinoma cells and peritoneal resident cells. The i.v. inoculation of peritoneal resident cells to mice which previously underwent surgical removal of primary tumor was also performed to evaluate the effects of this treatment on the postsurgical development of lung metastases of MCa mammary carcinoma.

MATERIALS AND METHODS

Lysozyme and animal treatment

The sample of lysozyme presently employed is a pure preparation of hen egg-white lysozyme, obtained from Società Prodotti Antibiotici, Milan, Italy. The CBA mice employed come from a locally established colony, grown according to the standard procedures for inbred strains; only female mice of 20–22 g were used. The animal treatment was performed by oral administration of daily dosages of lysozyme at 25–100 mg/kg/day for 7 consecutive days.

The compound was carefully admixed with the powdered food (Altromin R, Rieper SpA, Bolzano, Italy), for an average daily consumption of 3.5 g/mouse; the amount of powdered food discarded by the animals and the rest at the end of the treatment period were responsible for variations of less than 10% of the administered dose.

Tumor transplantation and evaluation

The MCa mammary carcinoma line employed [21] was originally obtained from the Department of Biology, Rudjer Boskovic Institute, Zagreb, Yugoslavia, and is locally maintained by i.m. passages of 25–100 mm^3 tumor fragments obtained from donors similarly inoculated 2 weeks before. For the reported experiments, single cell suspensions were prepared by mincing 2–3 2-week-old tumors in an equal volume of phosphate-buffered saline (PBS), filtering the poultice through a double layer of sterile gauze, centrifuging it in the cold at 500 ***g*** for 10 min and gently resuspending the pellet in an equal volume of PBS. Cell viability was checked by Trypan blue exclusion: only suspensions having at least 55% viable cells were used.

10^6 viable cells of MCa mammary carcinoma were implanted s.c. or i.m. in CBA mice; primary tumor weight was estimated by caliper measurements, taking the tumor density as equal to 1, as the volume of the rotation ellipsoid having the short and long axes equal to A and B respectively, by the formula:

$$\text{tumor weight} = \frac{\pi}{6} \times A^2 \times B. \qquad (1)$$

Lung metastases were counted on the surface of the lungs, immediately after removal, by means of a low-power stereo microscope; the weight of metastatic tumor of each animal was calculated by applying Eq. (1) to each metastatic nodule counted.

Collection of peritoneal resident cells and preparation of plasma sample

Peritoneal resident cells were collected by washing the peritoneal cavity of CBA mice with 2 ml of sterile PBS. After centrifugation at 500 ***g*** per 5 min, the concentration of viable cells was adjusted to 12×10^6 cells/ml by means of a Coulter Counter, Mod. ZF, with MEM containing antibiotics.

Blood samples, collected from intracardiac puncture in open chested mice with citrated syringes, were centrifuged with a Beckman Microfuge B for 5 min and the undiluted plasma was stored at −20°C until utilization. Before centrifugation, a fraction of each sample was used for counting the number of white blood cells, by means of Coulter Counter determinations in Zaponin lysates (Coulter Scientific SpA).

Smears of blood and of peritoneal fluid, stained with May–Grunwald–Giemsa, were carried out on the preparations employed.

In vitro *incubations*

In vitro incubations of tumor cells with peritoneal macrophages or plasma samples were carried out at 37°C using MEM containing antibiotics; trypan blue exclusion tests were carefully performed before and after the incubation time.

Surgery

Surgical amputations of the whole tumored leg were performed under ketalar anesthesia (125 mg/kg i.p.). After cutting the skin all around the upper thigh, the femoral and circumflex arteries were ligated with a synthetic absorbable suture, and femur muscles and the tumored tissue were cut off and the wound was secured with a silk suture. Blood leaking from the tumor to the surrounding tissue was avoided during intervention.

RESULTS AND DISCUSSION

The i.v. inoculation of MCa mammary carcinoma cells in hosts previously treated orally with lysozyme at 25 and 100 mg/kg/day for 7 consecutive days is followed by the formation of a significantly reduced number and weight of pulmonary tumors (Table 1). This effect is consistent with previous data obtained from studying the formation of spontaneous metastases of Lewis lung carcinoma [2], and, as previously reported for Lewis lung carcinoma, the reduction of the weight of pulmonary tumors is more pronounced than that on their number, as indicated also by the marked reduction of animals having large pulmonary tumors (57% and 47% in the treated groups, respectively, as

Table 1. Effect of host pretreatment with lysozyme on the development of i.v. implanted lung tumors of MCa mammary carcinoma

Pretreatment (mg/kg/day)	Lung metastasis formation*				Animals with large tumors†
	Number	%Var	Weight (mg)	%Var	
0	35.7 ± 4.3	—	23.3 ± 6.6	—	11/12
25	21.7 ± 3.8‡	−39	9.8 ± 3.1‡	−58	8/14
100	22.1 ± 3.6‡	−38	7.5 ± 1.9‡	−68	7/15

*Determined on day 14 from tumor implantation.
†Lung tumor nodules with diameter larger than 1.5 mm.
‡Means significantly different from controls, Newman–Keuls test [25], $P = 0.05$.
%Var: percentage variation as compared with controls.
CBA mice, orally treated with lysozyme at the reported dosages for 7 consecutive days, were inoculated i.v. with 2.5×10^5 MCa mammary carcinoma cells 24 h after the last day of treatment. Each value is reported as the mean ± S.E.

Table 2. Effects of orally administered lysozyme on the number of circulating leucocytes and of peritoneal resident cells in normal CBA mice

Treatment group	Peritoneal resident cells		Circulating leucocytes	
	$\times 10^3/mm^3$	% Var	$\times 10^3/mm^3$	%Var
Controls	3.0 ± 0.3	—	5.3 ± 0.6	—
Lysozyme 25	4.4 ± 0.5*	+47	7.0 ± 0.4*	+32
Lysozyme 100	4.3 ± 0.3*	+43	7.6 ± 0.7*	+43

*Means significantly different from the untreated controls, Newman–Keuls test [25], $P = 0.05$.
%Var: percentage variation as compared to controls.
Each value is the mean ± S.E. of individual samples obtained from groups of five mice, treated daily for 7 consecutive days with lysozyme at 25 mg/kg/day (lysozyme 25) or 100 mg/kg/day (lysozyme 100), which were counted 24 h after the last day of treatment.

compared to 92% of the group of controls).

The oral administration of 25 and 100 mg/kg/day of lysozyme also increases the number of circulating leucocytes and that of the peritoneal resident cells by about 40% as compared to untreated controls (Table 2). This effect is not accompanied by a concomitant increase in the plasma level of lysozyme activity (limit of sensitivity of the biologic test used: 0.5 μg/ml) for daily dosages of lysozyme up to 400 mg/kg/day, or by significant variations of spleen weight in the treated mice (62 or 70 mg, respectively for dosages of lysozyme of 25 and 100 mg/kg/day vs. 57 mg found in the control group). Furthermore, no difference between the treated group and the controls was seen, examining the differential counts of white blood cells in blood, with the exception of monocytes which increase from 2.1± 0.3% (controls) to 2.6 ± 0.04% (+24%; group treated with 25 mg/kg/day lysozyme) or to 2.8 ± 0.3% (+33%; group treated with 100 mg/kg/day lysozyme). No significant modification of the percentages of cell types present in the liquid of peritoneal washing was observed. The cell population in control animals consisted of 30.8 ± 13.4% macrophages, 5.4 ± 2.1% mast cells, 0.5 ± 0.3% eosinophils and 63.3 ± 12.3% lymphocyte-like cells, which was not modified in the treatment groups.

The effects of peritoneal resident cells collected from lysozyme-treated mice on the growth of s.c. primary MCa mammary carcinoma are plotted in Fig. 1. The concomitant s.c. injection of tumor cells and of peritoneal resident cells of lysozyme-treated mice, admixed at 37°C for 30 min before inoculation in a ratio of 10:3, causes a statistically significant reduction of primary tumor growth as compared to a control group in which an equal number of peritoneal resident cells from intact CBA mice of the same sex and body weight was used (Fig. 1, panel B). No reduction of either the viability or the number of tumor cells at the end of the incubation time was detected which could have accounted for the reduced tumor growth, whose overall reduction is comparable to that observed in animals directly treated with lysozyme after s.c. inoculation with MCa mammary carcinoma cells (Fig. 1, panel A).

The similarity of action observed in the groups treated with peritoneal resident cells from lysozyme-

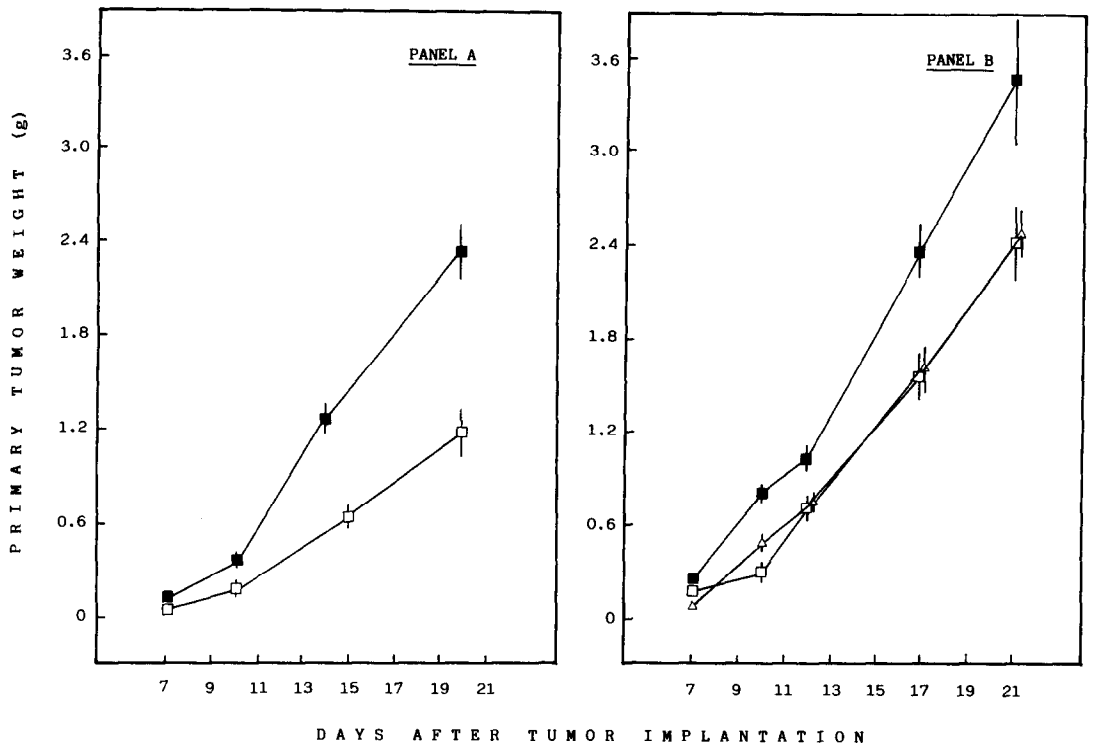

Fig. 1. Effects of the concomitant s.c. inoculation of MCa mammary carcinoma cells and of peritoneal resident cells obtained from lysozyme-treated mice, or of orally administered lysozyme, on the growth of the primary subcutaneous tumor in CBA mice. Groups of 10 *CBA mice received subcutaneously on day* 0 10^6 *MCa mammary carcinoma cells and* 3.5 ± 10^5 *peritoneal resident cells obtained from normal mice* (■—■), *from mice treated with lysozyme at* 25 mg/kg/day (△—△) *or with* 100 mg/kg/day (□—□) (*Panel B*). *Peritoneal resident cells and tumor cells were admixed* in vitro *at* 37°C *for* 30 *min before inoculation in the recipient hosts. A group of* 10 *CBA* (*Panel A*), *inoculated only with tumor cells, was given daily, on days* 1–15 *after tumor implantation, an oral treatment with* 50 mg/kg/day *of lysozyme* (□—□) (*corresponding value in controls:* ■—■). *Each mean value of treated groups is significantly different from the corresponding control value, Newman–Keuls test* [25], P = 0.05.

treated mice and the group treated *in vivo* with lysozyme, together with the modifications of the number of leucocytes and of peritoneal resident cells reported in Table 2, and the apparent absence of variations of the concentration of plasmatic lysozyme after oral treatment, seem to correlate with the hypothesis that the antitumor activity of lysozyme in mice bearing the MCa mammary carcinoma could be the result of the activation of immune responses in the treated mouse [16]. The possibility that the observed effects could be due to a direct interaction of lysozyme with tumor cells, at least as far as the experimental conditions presently described are concerned, should be ruled out. Data cited in previous papers indicate the absence of antitumor activity of lysozyme after *in vivo* inoculation of tumor cells of Lewis lung carcinoma previously kept *in vitro* with a number of lysozyme concentrations [2, 3]. As far as the MCa mammary carcinoma is concerned, it seems that contact between lysozyme and tumor cells is responsible even for the enhancement of lung metastasis formation. The number of lung metastases obtained by i.v. inoculation of MCa mammary carcinoma cells previously kept at 37°C for 30 min with lysozyme is significantly increased from 15.5 (average of controls) to 29.5 and 21.0, respectively for lysozyme concentrations of 50 and 200 μg/ml. This result, probably not simply related to complexation of lysozyme with anionic glycoproteins or proteoglycans on the surface of the tumor cells [22, 23], further stresses the absence of the need for direct contact of lysozyme with tumor cells to determine the reduction of metastatic growth.

Data presently reported suggest that macrophages and lymphocytes (the two major cell types present in the fluid recovered from the peritoneal cavity) could play an important role in the antitumor activity of lysozyme in the MCa mammary carcinoma model, being responsible for a reduction of tumor growth similar to that observed in lysozyme-treated mice, which is of the same magnitude of that reported by Fujii *et al.* for RL-1 lymphoma cells *in vitro* treated with lymphokine-activated polymorphonuclear leukocytes [24]. Indeed, in the present paper, peritoneal cells from lysozyme-treated mice also inhibited the growth of the pulmonary metastatic tumor (Table 3). The i.v. injection of peritoneal cells obtained from lysozyme-treated mice causes a statistically significant and pronounced reduction of the weight of lung metastases by 56% as compared with controls treated with peritoneal cells collected from intact CBA mice,

Table 3. Effects of peritoneal resident cells of lysozyme-treated mice on lung metastasis formation in mice whose primary tumor was surgically removed 12 days after implantation

Treatment of peritoneal cell donors	Lung metastases			
	Total number	%Var	Weight (mg)	% Var
None	24.4 ± 3.9	—	24.9 ± 4.8	—
Lysozyme	20.7 ± 3.4	−15	10.9 ± 2.9*	−56

*Means significantly different from control value, Mann–Whitney test [26] and *t*-test for grouped data [27], $P = 0.05$.
%Var: percentage variation as compared to controls.
Each value is the mean ± S.E. obtained in groups of 10 CBA mice, implanted with 10^6 MCa mammary carcinoma cells on day 0, and undergoing surgical amputation on the whole of the tumored leg on day 12. Each animal received i.v., 24 h after surgery, 3×10^5 peritoneal resident cells obtained from the peritoneal cavity of CBA mice treated daily for 7 consecutive days with 100 mg/kg/day lysozyme.

when treatment is performed 24 h after surgical removal of the primary tumor.

These data indicate that lysozyme antitumor activity can be mediated by eliciting the cytotoxic action of the macrophages and lymphocytes for tumor cells lodged either s.c. or in the lungs. Indeed, the presently reported data do not indicate whether macrophages or lymphocytes from lysozyme-treated mice directly kill tumor cells or do it by the release of factors (monokines and/or lymphokines) responsible for a more general (specific) elicitation of host responses. The examination of the antitumor effects of plasma samples (containing soluble factors?) helped to overcome this question, and to support the hypothesis put forward. The effect of the concomitant inoculation s.c. or i.v. of tumor cells previously kept at 37°C for 30 min with samples of whole plasma obtained from mice orally treated with lysozyme on the growth of the subcutaneous tumor and on the development of artificially induced lung metastases are reported in Table 4. Either the weight of primary tumors or lung tumor weight are significantly reduced in the groups of mice receiving tumor cells and whole plasma of lysozyme-treated mice as compared to the group of controls for which a sample of plasma of normal CBA mice was employed. Besides the high variability of individual tumor metastatic weights in the animals, the tendency to a marked reduction in both treated groups as compared to untreated controls is clear. The reduction in the primary tumor growth is of the same magnitude as that observed using the concomitant inoculation of tumor cells and peritoneal resident cells (see Fig. 1). Data reported in Table 5 further show that an effect on MCa lung metastases can be achieved also by *in vivo* treatment of tumor-bearing hosts. The development of spontaneous lung metastases in mice carrying an i.m. implant of MCa mammary carcinoma is significantly reduced by i.p. injections, in early stages of tumor growth, of whole plasma obtained from lysozyme-treated hosts. The absence of direct cytotoxicity of plasma components to tumor cells resulted from the lack of loss of tumor cell viability or of their number, measured by trypan blue exclusion, at the end of the incubation time.

Taken together, these data give evidence for the elicitation of host responses in lysozyme-treated

Table 4. Effects of whole plasma samples from lysozyme-treated mice on primary tumor growth and on the formation of artificial lung metastases of MCa mammary carcinoma

Treatment of plasma donors	Primary tumor weight (mg)		Metastasis weight (mg)	
	Day 12	Day 17	mean ± S.E.	(min–max)
Controls	628 ± 44	1461 ± 81	55 ± 26	(7–199)
Lysozyme 25	438 ± 133*	1081 ± 76*	6 ± 1*	(3–10)
Lysozyme 100	437 ± 50*	1073 ± 73*	11 ± 3	(4–33)

*Means statistically different from the group of controls, Mann–Whitney test [26] and *t*-test for grouped data [27], $P = 0.01$.
2×10^7 MCa mammary carcinoma cells were kept at 37°C for 30 min in the presence of 1 ml of samples of whole plasma obtained from mice untreated (controls) or treated with lysozyme at 25 mg/kg/day (lysozyme 25) or 100 mg/kg/day (lysozyme 100). At the end of incubation, aliquots of 0.1 ml were implanted s.c. in groups of 10 CBA mice for the evaluation of primary tumor growth. The effects on lung metastases were determined on day 14 in groups of 10 CBA mice inoculated i.v. with aliquots of 0.025 ml of the incubated mixture.

Table 5. Effects of whole plasma samples from lysozyme-treated mice on spontaneous lung metastasis formation in mice bearing MCa mammary carcinoma

Treatment group	Metastasis weight (mg) Mean ± S.E.	(min–max)	Statistics vs. controls
Controls	22 ± 7	(6–49)	
Lysozyme 25	8 ± 3	(0.5 ± 22)	$P = 0.002$
Lysozyme 100	9 ± 2	(3–23)	$P = 0.047$

Statistical analysis: Mann–Whitney test [26].
Groups of eight CBA mice, inoculated i.m. with 10^6 MCa mammary carcinoma cells on day 0, received on day −1 and on day 1 an i.p. injection of 0.275 ml/animal of a sample of whole plasma obtained from syngeneic animals untreated (controls) or treated with lysozyme at 25 mg/kg/day (lysozyme 25) or 100 mg/kg/day (lysozyme 100) on day −1 and on day 1. The examination of the weight of the lung metastatic tumor was performed on day 18.

mice, which can account for the antineoplastic effects observed. Furthermore, the examination of the effects of lysozyme on the growth of solid malignant neoplasms reported in the present study and those reported in the literature evidence for this substance interesting opportunities of application. It appears that a simple harmless treatment with lysozyme can be used in those situations in which a positive modulation of host responses is needed; this hypothesis is supported by the increased therapeutic efficacy of cisplatin treatment in mice concomitantly fed a diet enriched with lysozyme [2, 3]. Indeed, the characterization of the nature of the immunoactivation of hosts fed lysozyme can help to better modulate its action and to ascertain whether other substances, such as lysozymes of different source or endowed with different enzymatic activity, do possess antitumor activity better than that reported up today for the hen egg-white lysozyme. The determination of the nature of the cell(s) and/or factor(s) principally involved in the antitumor action of lysozyme would also help to improve its therapeutic activity, and work in this direction is in progress.

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