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Isolation of a Factor from Cancer Ascitic Fluid Increasing Susceptibility of Mice to *Listeria* Infection (41705)

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Abstract. A low-molecular-weight factor (<1000) which impairs the capacity of normal mice to resist *Listeria* infection has been isolated from the ascitic fluid of tumor-bearing mice. The factor showed potent suppressive activity when as little as 0.2 ml of $1:10^5$ dilution of the factor was given iv to mice. The factor impaired stage 2 (72 hr after infection) of *Listeria* elimination in the liver when circulating monocytes migrate from the blood to the liver to begin eliminating the bacteria, but not stage 1 (within 24 hr after infection) when *Listeria* elimination depends on resident liver macrophages. The factor also inhibited macrophage accumulation in the peritoneum of mice after ip injection of phytohemagglutinin and macrophage chemotaxis *in vitro*. The factor was heat labile and lost its suppressive activity after being heated at 56°C for 30 min. The factor was detectable in the ascitic fluid rather late after tumor implantation and its production was closely related to tumor growth.

It has become increasingly evident that macrophages play important roles in tumor surveillance, as shown in their tumoricidal activity both *in vivo* and *in vitro* (1–4). However, additional evidence shows that immunosurveillance systems fail to detect and destroy malignant neoplasms (5, 6) and that the failure of tumor-bearing hosts to eliminate their tumors can be attributed to immunosuppression (7). The presence of humoral factors with immunosuppressive properties in many tumor-bearers has been suggested (7–11). However, the principles impairing immunosurveillance against malignant neoplasms have not yet been fully characterized.

The major purpose of this experiment was to investigate the way in which malignant neoplasms avoid the immunosurveillance systems through the isolation and characterization of factors from tumor-bearers which suppress function of macrophages and lymphoid system. We first attempted to isolate factors from murine cancer ascitic fluids which suppress resistance of mice to *Listeria* infection, since the macrophage is the only cell known to destroy *Listeria* in mice (12) and also because the macrophage system serves a dual role in the defense against microbial and neoplastic growth (3, 13). In a previous experiment, we isolated a low-molecular-weight factor (4000–10,000) from murine cancer ascitic fluid which significantly suppresses the ability of mice to resist *Pseudomonas* infection (14). This paper is the second of a series and describes the isolation of another low-molecular-weight factor (<1000) from murine cancer ascitic fluid, which suppresses mice from resisting *Listeria* infection and inhibits the mobility of macrophages both *in vivo* and *in vitro*.

Materials and Methods. *Mice.* Six- to eight-week-old outbred ddY mice purchased from Funabashi Animal Laboratory and 6- to 8-week-old inbred C3H/He mice obtained from the Experimental Animal Center of Tohoku University were used.

Cancer ascitic fluids. Ehrlich ascitic fluid obtained from ddY mice through biweekly transfers of tumor cells free of LDH virus were used throughout the experiment. A parallel experiment was conducted with methylcholanthrene-induced ascitic tumor (MC-A) fluid from C3H/He mice and ascitic fluid of FM-3A, an ascitic form tumor cell line derived from spontaneous mammary carcinoma of C3H/He mice, to confirm the results obtained from Ehrlich cancer ascitic fluids. Cancer ascitic fluids were collected 13 days after the ip implantation of 1×10^5 tumor cells and immediately centrifuged at 17,000*g* for 20 min. The obtained supernatants were centrifuged at 150,000*g* for 3 hr and then stored at −20°C. To know the approximate molecular size of the suppressive factor, an ultrafiltration ex-

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periment was conducted through a Diaflow membrane (Amicon, UM-10 and UM-2). Unless otherwise stated, 100-ml aliquots of ascitic fluid supernatant was used and the obtained residues on the membrane were washed three times with 200 ml of M/100 phosphate-buffered saline (PBS) pH 7.2.

Control peritoneal washing fluids. Noncancer peritoneal washing fluids were obtained by first injecting 5 ml of PBS into the peritoneum of the mice. The fluid was extracted 5 hr later, centrifuged in the same manner as the cancer ascitic fluids, stored at −20°C, and used as the working control. Noncancer ascitic fluids obtained by other methods (e.g., repeated ip injection of Freund's complete adjuvant) were also examined.

Bacteria. Listeria monocytogenes, isolated from the spinal fluid of a human patient with *Listeria meningitis* and passed several times through mice via the ip route, was grown in HI broth and stored on HI agar slants at 4°C. Before infection, stocked bacteria were grown again on the HI agar, collected and diluted in a standard fashion with PBS for iv injection. The organism had a median lethal dose (LD_{50}) of 4–6 × 10^5 when given iv to the ddY mice.

Evaluation of activities suppressing antibacteria resistance. Two-tenths milliliter of cancer ascites or crude preparations from cancer ascites dissolved in PBS, was injected iv 24 hr before infection. The control mice received 0.2 ml of control peritoneal washing fluids. After infection, the number of surviving mice was checked for 20 consecutive days. The number of survivors in the treated groups was compared with that of the control groups by means of chi-square analysis. If the P value obtained was below 0.05, the assayed materials were considered to have suppressive activity. The number of bacteria in the livers of treated and control mice was also counted to determine the effect of cancer ascites or crude preparations from cancer ascites on bacteria growth *in vivo.* This was done by plating 10-fold serial dilutions of liver homogenates on HI agar.

The direct effect of the crude preparations on bacteria growth was also examined *in vitro.* The number of bacteria in HI broth containing either the crude preparations from Ehrlich ascitic fluid or control peritoneal washing fluid was determined by plating 10-fold serial dilutions of broth on agar.

Kinetics of inflammatory response to phytohemagglutinin in the peritoneum of mice. Twentyfour hours after iv injection of the crude preparations, mice in groups of five were injected ip with 1.5 ml of 35 μg, purified phytohemagglutinin (PHA, Gibco, Grand Island, N.Y.) diluted in sterile PBS. After various time intervals, the mice were killed by cervical dislocation and their peritoneal cavities were lavaged vigorously with 5.0 ml of PBS supplemented with 5 u/ml heparin (Upjohn, Kalamazoo, Mich.). The number of peritoneal exudate cells was counted by a hemocytometer and the remaining cell suspensions were centrifuged at 300g for 10 min. The pellets were then streaked over glass slides coated with a mixture of ovalbumin and glycerol and were used for morphological characterization of the cells.

Quantification of macrophage chemotaxis in vitro. Macrophage chemotaxis was quantified in a Boyden chamber according to the method described by Pike and Snyderman (15). Peritoneal macrophages were obtained from the mice 2 days after ip injections of 35 μg of PHA. The washed cells from several mice were pooled and prepared to contain 3–4 × 10^6 macrophages/ml in RPMI 1640 at pH 7.0 (Gibco, Grand Island, N.Y.). One milliliter of the cell suspension was placed in the upper compartment of a Boyden chamber. Either endotoxin-activated mouse serum diluted in RPMI 1640 (3% v/v) or medium alone was placed in the lower chamber. A polycarbonate filter with 5-μm pores (Nucleopore, Wallabs, San Rafael, Calif.) separated the two chambers. After incubation at 37°C for 4 hr in humidified air under 5% CO_2, the chambers were emptied and the filters removed. The filters were then fixed in ethanol and stained with hematoxylin. Chemotaxis was quantified by averaging the number of macrophages which had migrated completely through the filter in 20 oil immersion (×1000) fields. The effect of the crude preparations from cancer ascitic fluid on macrophage chemotaxis was determined by conducting the chemotaxis with the presence of the preparation in the upper compartment of the chamber.

Determination of phagocytic and bactericidal activities of macrophages. Peritoneal cells, obtained by rinsing peritoneal cavities with 5 ml of RPMI 1640 containing 10% fetal

calf serum and 5 u/ml of heparin, were cultured in plastic dishes (Falcon 3001, Calif.) at 37°C for 30 min and cells adhered to the bottom of the dishes were used as peritoneal macrophages for the test. For the preparation of spleen macrophages, a spleen was minced by razors and the cells were passed through a stainless-steel mesh. Then, the cells were cultured in plastic dishes and cells which adhered to the bottom were used as spleen macrophages. The number of macrophages cultured in a dish was 2–4 $\times$ 10^6 cells.

After receiving *Listeria* infection at 37°C for 30 min, macrophage cultures were washed five times with PBS and reincubated at 37°C in the presence of penicillin (50 u/ml) and streptomycin (50 mg/ml) to kill extracellularly residing bacteria. After 4 hr of the culture in the presence of antibiotics, the macrophage cultures were washed five times with PBS to decrease the effects of the antibiotics and were disrupted osmotically in cold water. Then, the number of bacteria in the supernatant of the disrupted cell homogenates was determined by plating serially 10-fold diluted solution on HI agar. The phagocytic and bactericidal activities of macrophages were evaluated by comparing the number of intracellular bacteria in the macrophages cultured for 30 min to the number of inoculated bacteria and by comparing the number of intracellular bacteria in the macrophages cultured for 4 hr to that of macrophages cultured for 30 min, respectively.

Results. *Suppression of anti-bacterial resistance in mice by iv injection of cancer ascitic fluids.* The ability of cancer ascitic fluids obtained from three different tumors to suppress resistance to bacteria infection was examined by injecting 0.2 ml of ascitic fluids iv or their dilutions into normal mice one day ahead of the iv injection of 2.6 $\times$ 10^6 *Listeria monocytogenes* (Table I). The iv injection of $1:10^1$ dilution of any of three different cancer ascitic fluids resulted in the death of all mice within 6 days after infection, whereas 80% of the control mice survived more than 20 days. Thus a striking difference was found in the resistance to *Listeria* infection between control mice and cancer ascitic fluids-treated mice. The potency of the suppressive properties of the cancer ascitic fluids on bacteria resistance was clearly demonstrated and an injection of as little as 0.2 ml of $1:10^5$ dilution of Ehrlich ascitic fluids resulted in a significant reduction in the survival rate (Table I). All of the mice which received cancer ascitic fluids, but no *Listeria* infection, retained quite healthy appearances during the 20-day observation period. Only iv injections of both cancer ascitic fluids and bacteria produced definite and reproducible effects.

Characterization of the suppressive factor. In order to calculate the approximate molecular size of the suppressive factor, an ultrafiltration experiment was conducted through a Diaflow ultrafiltration membrane ("Amicon" UM-10, 10,000 Da exclusion). Suppressive activity was found both in the dialyzate and nondialyzate (Table II). However, the characterization of only the dialyzable factor was carried out in this experiment. This

TABLE I. SUPPRESSIVE EFFECT OF CANCER ASCITIC FLUIDS ON THE CAPACITY OF ddY MICE TO RESIST *Listeria* INFECTION[a]

Ascitic fluids	Dilution of ascites	Survival days	No. of Survivors / No. infected	Mean days of survival
Ehrlich	$1:10^1$	2, 2, 2, 3, 3, 3, 4, 4, 5, 6	0/10*[,b]	3.4
	$1:10^3$	3, 3, 3, 3, 3, 4, 4, 4, 5, 7	0/10*	3.9
	$1:10^5$	3, 3, 3, 4, 4, 5, 5, 6, 6, 8	0/10*	4.7
	$1:10^7$	4, 4, 5, 6, 8, s, s, s, s, s[c]	5/10	>12.7
MCA	$1:10^1$	3, 3, 3, 3, 4, 4, 4, 5, 5, 6	0/10*	4.0
FM-3A	$1:10^1$	2, 2, 2, 3, 3, 3, 3, 3, 3, 3	0/10*	2.7
Control[d]	$1:10^1$	5, 6, s, s, s, s, s, s, s, s	8/10	>17.1

[a] 2.6 $\times$ 10^6 *Listeria* was injected iv 24 hr after iv injection of ascitic fluids.
[b] Statistically significant ($P < 0.05$) by chi-square analysis.
[c] Survivors at Day 20 postinfection.
[d] Mice were treated iv with the peritoneal washing fluid from normal mice.

TABLE II. EFFECT OF EHRLICH ASCITIC FLUID FRACTIONS ON THE CAPACITY OF ddY MICE TO RESIST *Listeria* INFECTION[a]

Crude preparations from ascitic fluid			No. of survivors	Mean days of
Fractions	Dilution	Survival days	No. inoculated	survival
Dialyzate (<10,000)	$1:10^1$	3, 3, 3, 4, 4, 5, 5, 6, 6, 8	0/10*[b]	4.7
Fraction[c] (10,000–1000)	$1:10^1$	3, 4, 4, 7, 7, 8, 10, s, s, s[d]	3/10	>10.3
	$1:10^3$	4, 4, 5, 6, 7, 8, s, s, s, s	4/10	>11.4
Fraction (<1000)	$1:10^3$	3, 3, 3, 4, 4, 4, 5, 5, 6, 6	0/10*	4.3
	$1:10^5$	3, 3, 4, 4, 5, 5, 5, 6, 6, 7	0/10*	4.8
Non-dialyzate (>10,000)	$1:10^1$	3, 3, 3, 4, 4, 4, 5, 5, 6, 8	0/10*	4.5
Control[e]	$1:10^1$	4, 4, 5, 5, 6, s, s, s, s, s	5/10	>12.4

[a] 3.0×10^6 *Listeria* was iv injected 24 hr after iv injection of Ehrlich ascitic fluid fractions.
[b] Statistically significant ($P < 0.05$) by chi-square analysis.
[c] Adjusted to the volume of the original ascitic fluid and used for treatment after receiving several 10-fold dilutions.
[d] Survivors at Day 20 postinfection.
[e] Mice were treated iv with the peritoneal washing fluid from normal mice.

was accomplished by further separating the dialyzable factor into two fractions, one with molecular weight 10,000–1000 and the other less than 1000 by ultrafiltration using "Amicon" UM-2 (1000 Da exclusion). The suppressive factor against *Listeria* infection in mice could be detected in the fraction with molecular weight of less than 1000 but not in the fraction with molecular weight of 10,000–1000. This higher-molecular-weight fraction (10,000–1000) is known to contain a factor which suppressed resistance of mice to *Pseudomonas* infection (14).

The heat resistance of the lower-molecular-weight fraction (<1000) was also examined. The factor was found to be heat labile, losing its suppressive activity after being heated at 56°C for 30 min. The original ascitic fluid, on the other hand, was heat resistant and preserved its suppressive activity after being heated at 70°C for 10 min (Table III). Furthermore, when the effect of the factor (<1000)

TABLE III. EFFECT OF HEATING ON THE SUPPRESSIVE CAPACITY OF THE DIALYZABLE FACTOR TO RESIST *Listeria* INFECTION[a] IN ddY MICE

Factors	Dilution	Heating	Survival days	No. of survivors / No. inoculated	Mean days of survival
Ehrlich ascitic fluids	$1:10^1$	Nonheating	2, 3, 3, 3, 3, 3, 3, 3, 3, 3	0/10*[b]	2.9
		56°C for 30 min	2, 2, 2, 3, 3, 3, 3, 3, 3, 3	0/10*	2.7
		70°C for 10 min	2, 2, 2, 2, 3, 3, 4, 4, 4, 6	0/10*	3.2
Dialyzable factor (<1000)	$1:10^3$	Nonheating	4, 4, 4, 4, 4, 5, 5, 5, 5, 5	0/10*	4.5
		56°C for 30 min	4, 4, 5, 5, 5, s, s, s, s, s[c]	5/10	>12.3
		70°C for 10 min	4, 4, s, s, s, s, s, s, s, s	8/10	>16.8
Control[d]	$1:10^1$		4, 4, 4, 4, 6, s, s, s, s, s	5/10	>12.2

[a] 4.3×10^6 *Listeria* was infected iv 24 hr after iv injection of factors.
[b] Statistically significant ($P < 0.05$) by chi-square analysis.
[c] Survivors at Day 20 postinfection.
[d] Mice were treated iv with the peritoneal washing fluid from normal mice.

on the growth of *Listeria monocytogenes* was examined *in vitro* by cultivating 1×10^6 cells in HI broth at 37°C both in the presence and absence of the factor, the factor had no significant effect on the growth of the bacteria. The same experiment was done with Ehrlich ascitic fluid and this fluid also had no significant effect on the growth of the organism *in vitro* (data not shown).

Production and potency of the factor from cancer ascitic fluids. The production of the factor in ascitic fluid was examined during the course of tumor growth in mice implanted with Ehrlich ascitic tumors. When mice received 0.2 ml of $1:10^3$ dilution of the factor derived from Ehrlich ascitic fluids collected on the 13th or 15th day after tumor implantation, they all died within 5 days after 2×10^6 *Listeria* infection. However, when mice received the same amount of an even higher concentration ($1:10^1$) of the factor prepared from ascitic fluid collected on the 5th day after tumor implantation, 60% of the mice survived after infection which is comparable to the results of the control group (Table IV). These results suggest that the suppressive factor is liberated into or accumulated in the ascitic fluid during the later stages of tumor growth. A quantitative titration of the crude preparations from the ascitic fluid was also performed to determine their suppressive potency and their mode of production or accumulation following the same procedure. While the preparations from ascitic fluid obtained on Day 9 caused all the infected mice to die at a $1:10^2$ dilution, only a $1:10^5$ or more dilution of preparation obtained on Days 13 and 15 produced the same results (Table IV), thus suggesting a peak in production or accumulation of the factor in the ascitic fluid on Days 13 or 15, almost 3 days before the tumor death of the mice.

The factor prevents the migrated macrophages in mice liver from eliminating Listeria. It is known that in normal mice more than 90% of the *Listeria* injected iv are trapped in the liver within a few minutes after infection and are killed there, first by resident liver macrophages within 24 hr after infection (stage 1), and then by macrophages migrated from the blood to inflammatory sites of liver (stage 2) (12, 16–18). To find out whether the factor has any effect on stage 1, stage 2, or both stages, changes in *Listeria* elimination patterns in the liver were examined in the factor-treated mice by counting the number of viable bacteria in the liver at various times after infection. An iv injection of $1:10^5$ dilution of the factor given 24 hr before infection vigorously suppressed *Listeria* elimination at stage 2 but did not effect stage 1 (Fig. 1).

Inhibition of macrophage accumulation in vivo and chemotaxis in vitro by the factor. To discover whether or not the impairment of

TABLE IV. POTENCY OF THE DIALYZABLE FACTOR OBTAINED FROM EHRLICH ASCITIC FLUIDS COLLECTED AT VARIOUS TIMES AFTER TUMOR IMPLANTATION TO SUPPRESS HOST CAPACITY TO RESIST *Listeria* INFECTION IN ddY MICE[a]

Days of sampling	Dilution of factor	Survival days	No. of survivors / No. inoculated	Mean days of survival
5	$1:10^1$	4, 5, 5, 6, s, s, s, s, s, s[b]	4/10	>14.0
9	$1:10^1$	3, 3, 3, 3, 3, 4, 4, 4, 5, 5	0/10*,[c]	3.7
	$1:10^2$	3, 3, 3, 4, 4, 4, 5, 5, 6, 6	0/10*	4.3
	$1:10^3$	4, 4, 5, 5, 5, 6, s, s, s, s	4/10	>10.9
13	$1:10^3$	3, 3, 3, 3, 3, 3, 4, 4, 5, 5	0/10*	3.6
	$1:10^6$	3, 3, 4, 4, 4, 4, 5, 5, 5, 6	0/10*	4.3
	$1:10^8$	4, 4, 5, 5, 6, s, s, s, s, s	5/10	>12.4
15	$1:10^3$	3, 3, 3, 3, 3, 4, 4, 4, 5, 5	0/10*	3.7
	$1:10^5$	3, 3, 4, 4, 4, 5, 5, 5, 6, 6	0/10*	4.5
	$1:10^6$	3, 4, 4, 4, 5, 5, 6, s, s, s	3/10	>9.1
Control[d]	$1:10^1$	4, 5, s, s, s, s, s, s, s, s	8/10	16.9

[a] 2.0×10^6 *Listeria* was given iv 24 hr after injection of membrane filtrate from Ehrlich ascitic fluids.
[b] Survivors at Day 20 postinfection.
[c] Statistically significant ($P < 0.05$) by chi-square analysis.
[d] Mice were treated with the peritoneal washing fluid from normal mice.

Listeria elimination at stage 2 by the factor is associated with macrophage accumulation at inflammatory sites of the liver, effects of the factor on macrophage mobility was first examined *in vivo*. When the number of macrophages accumulated in the peritoneum after PHA stimulation was examined in the factor-treated mice (iv, 24 hr ahead) and control mice, significant inhibition of macrophage accumulation was found 24 and 48 hr after PHA stimulation in the factor-treated mice (Table V). Further, we examined whether the factor has any direct suppressive effects on macrophage mobility by an *in vitro* test using the Boyden chamber (15). When macrophage chemotaxis was examined in the presence of $1:10^1$, $1:10^3$, and $1:10^5$ dilutions of the factor in the upper compartment of the chamber, significant inhibition in chemotaxis was observed at $1:10^1$ and $1:10^3$ dilution of the factor (Table VI). In repeating the same experiment with the same lot, $1:10^3$ dilution always gave higher inhibition than $1:10^1$ dilution, as will be later discussed. These *in vivo* and *in vitro* experiments were repeated using two other lots of the factor obtained in a similar manner from Ehrlich cancer ascitic fluids and yielded identical results (data not shown).

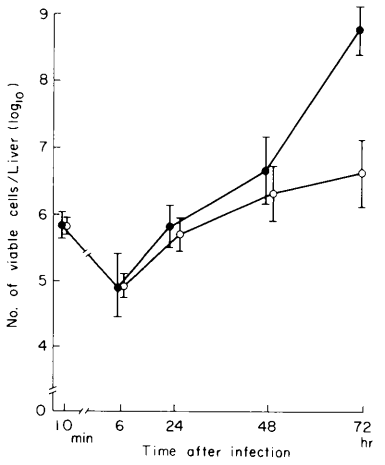

FIG. 1. The suppressive effect of dialyzable factor (<1000) on *Listeria* elimination in the liver of mice. *Listeria* (1.8×10^6) was injected iv 24 hr after an iv injection of 0.2 ml of $1:10^5$ dilution of the factor or $1:10^1$ dilution of control peritoneal washing fluid. Livers were separated aseptically 10 min, 6, 24, 48, and 72 hr after infection. Then, they were homogenized and the number of viable bacteria in the liver was counted on an agar plate. (○) Control peritoneal washing fluid-treated mice; (●) dialyzable factor-treated mice.

TABLE V. EFFECT OF THE FACTOR ON MACROPHAGE ACCUMULATION IN THE PERITONEAL CAVITY OF MICE

Treatment	No. of accumulated macrophage $\times 10^6 \pm$ SD[a]	
	24 hr[b]	48 hr
Dialyzable factor[c]		
$1:10^3$	0.4 ± 0.1*[d]	0.8 ± 0.2**[e]
Control[f]		
$1:10^1$	1.6 ± 0.2	1.8 ± 0.3

[a] Number of accumulated macrophages was determined by subtracting the average number of macrophages found in the peritoneum of unstimulated mice ($1.0–1.3 \times 10^6$ cells) from the number of macrophages found in the peritoneum of stimulated mice.

[b] Hours after ip injection of 35 μg of PHA.

[c] Dialyzable factor (0.2 ml/mouse) was given iv 24 hr before ip injection of PHA.

[d] Statistically significant by Student's *t* test ($P < 0.05$).

[e] Statistically significant by Student's *t* test ($P < 0.01$).

[f] Peritoneal washing fluid from normal mice (0.2 ml/mouse) was given iv instead of the dialyzable factor.

The factor does not show any effect on phagocytic and bactericidal activities of macrophages. In the first experiment, phagocytic and bactericidal activities of peritoneal and spleen macrophages from mice treated iv 1 day before the test with 0.2 ml of $1:10^3$ dilution

TABLE VI. SUPPRESSIVE EFFECT OF THE FACTOR ON MACROPHAGE CHEMOTAXIS *in Vitro*[a]

Dilution of the factor	No. of migrated macrophages	Percentage inhibition[b]
$1:10^1$	60.5 ± 9.2*[c]	30
$1:10^3$	37.8 ± 7.2**[d]	71
$1:10^5$	67.1 ± 7.0	18
Positive control[e]	76.9 ± 4.9	
Control[f]	21.5 ± 2.0	

[a] 3×10^6 peritoneal macrophages were seeded on the filter and incubated at 37°C for 4 hr.

[b] The percentage of macrophage chemotaxis inhibition was determined according to the following formula: {1 − [(test − control)/(positive control − control)]} × 100.

[c] Statistically significant ($P < 0.05$) by Student's *t* test.

[d] Statistically significant ($P < 0.01$) by Student's *t* test.

[e] Chemotaxis was made in the presence of only activated serum in the lower compartment.

[f] Macrophage migration was performed in the absence of both the factor and activated serum.

of the factor or $1:10^1$ dilution of the control peritoneal washing fluid were examined. After infection with 1.6×10^6 or 4.4×10^6 *Listeria,* when the number of viable intracellular bacteria of infected peritoneal and spleen macrophages ($3–4 \times 10^6$ cells, see Table VII) was examined 30 min and 4 hr after incubation, no significant difference was found in the number of intracellular bacteria at either time between macrophages from the factor-pretreated mice and those from the control mice (Table VII, 1).

In the next experiment, the direct effect of the factor on phagocytic and bactericidal activities of peritoneal macrophages was examined. After culturing the peritoneal macrophages from normal mice for 2 hr in the presence of the factor ($1:10^3$/ml) or the control peritoneal washing fluid ($1:10^1$/ml), the macrophage cultures were infected with 2.0×10^6 *Listeria.* Then, the number of intracellular bacteria of infected macrophages was examined 30 min and 4 hr after culture. There was no significant difference in the phagocytic and bactericidal activities between macrophages cultured in the presence of the factor and those cultured in the presence of the control peritoneal washing fluid (Table VII, 2). Since the macrophages precultured 24 hr showed depressed bactericidal activity compared to that of freshly isolated normal peritoneal macrophages when the test was performed in the presence of control peritoneal washing fluid (Table VII), it is evident that this test system is able to detect differences of phagocytic and bactericidal activities of various states of macrophages.

Discussion. The present study demonstrated that murine cancer ascitic fluids contain a low-molecular-weight product (<1000) capable of suppressing the capacity of mice to resist *Listeria* infection as well as inhibiting macrophage mobility. The fraction (10,000–1000) from the same cancer ascitic fluid, which was shown earlier to suppress the capacity of mice to resist *Pseudomonas* infection (14), did not inhibit mice from resisting *Listeria* infection, suggesting that these two different fractions from the same cancer ascitic fluid affect the anti-*Pseudomonas* and anti-*Listeria* defense systems differently.

The potency of the factor (<1000) produced in cancer ascitic fluid was indicated by the significantly suppressed defense system of mice against *Listeria* infection when as little as 0.2 ml of $1:10^5$ dilution of the factor was administered iv (Table IV). Production or accumulation of the factor in cancer ascitic fluid was rather slow. It became detectable on the ninth day and reached its peak potency 13 days after tumor implantation (Table IV) suggesting that the inhibitory factor was produced

TABLE VII. EFFECTS OF THE FACTOR ON PHAGOCYTIC AND BACTERICIDAL ACTIVITIES OF MACROPHAGES

Experiment number	Treatment with the factor	Source of macrophages	Doses of *Listeria*	No. of intracellular bacteria ($\log_{10}$)/culture	
				30 min	4 hr
1[a]	+	Peritoneum	1.6×10^6	3.87 ± 0.09[b]	2.45 ± 0.07
	−	Peritoneum	1.6×10^6	3.82 ± 0.08	2.42 ± 0.09
	+	Spleen	4.4×10^6	3.54 ± 0.09	2.56 ± 0.08
	−	Spleen	4.4×10^6	3.48 ± 0.08	2.51 ± 0.04
2[c]	+	Peritoneum	2.0×10^6	4.56 ± 0.10	3.61 ± 0.12
	−	Peritoneum	2.0×10^6	4.63 ± 0.09	3.59 ± 0.10*[,d]
Control[e]	−	Peritoneum	2.0×10^6	4.65 ± 0.11	4.32 ± 0.11

[a] The test was made with macrophages obtained from mice receiving iv infection of the factor (+) or the control peritoneal washing fluid (−) 1 day before the test.

[b] Figures represent mean ± SD of five identically prepared cultures.

[c] The test was made with macrophages from untreated normal control mice in the presence of either the factor (+) or the control peritoneal washing fluid (−) in the culture.

[d] Statistically significant ($P < 0.01$) by Student's t test when compared to the number of intracellular bacteria of control peritoneal macrophages 4 hr after culture.

[e] The test was made in the presence of the control peritoneal washing fluid with normal mice peritoneal macrophages which were precultured for 24 hr before the test.

or accumulated in the ascitic fluid progressively with time as the tumor grew.

Prominent suppression of *Listeria* elimination in the liver of mice pretreated with the factor at stage 2 rather than at stage 1 (Fig. 1) indicates that the factor affects macrophages, migrated to the liver from blood but not resident liver macrophages, since *Listeria* elimination in the liver of mice at stage 1 and 2 is known to depend on resident liver macrophages and macrophages migrated to the liver from blood, respectively (12, 16–18). Two ways in which the factor suppresses the functions of migrated macrophages were postulated, (i) suppression of macrophage accumulation at inflammatory sites, (ii) suppression of phagocytic and bactericidal activities of migrated macrophages. Significant inhibition of macrophage accumulation in the peritoneal cavity of mice (Table V) and also in macrophage chemotaxis by the factor (Table VI) indicates that the factor is able to suppress macrophage mobility, thus supporting the first proposal. The second proposal is unlikely, since the factor did not cause any direct or indirect suppression of the phagocytic and bactericidal activities of both peritoneal and spleen macrophages (Table VII).

Optimal inhibition of macrophage chemotaxis constantly occurred when a highly diluted ($1:10^3$) rather than a less diluted ($1:10^1$) form of the factor was used (Table VI). We do not yet have an explanation for this, but it may be ascribed to another antagonistic factor contained in the fluid.

There are many studies on products from tumor cells or tumor-bearers which subvert host defense mechanisms (7, 11, 19, 20), including factors which suppress macrophage functions (9, 10, 15, 21–23). However, characterization of many of these tumor factors still remains inadequate. Although North reported that a factor, having a suppressive activity on the capacity of mice to resist *Listeria* infection in the sera of tumor-bearing mice (13), several features distinguish it from our factor. In addition to its larger molecular size (12,000 or less), the North's factor appears in the sera of mice as early as 8 hr and reaches peak potency at 24 hr after tumor implantation, whereas our factor became detectable in ascitic fluid after 9 days of tumor implantation (Table IV). Nelson and his colleagues reported that a low-molecular-weight factor (mol wt < 1000) from *in vitro* tumor cell culture supernatants suppressed various macrophage functions (24) such as phagocytosis and the migration. However, the physicochemical similarity between Nelson's factor and ours still remains unresolved. The other important molecules to be discriminated from ours are prostaglandins which have been shown to affect various immunological functions (25, 26). Since our factor suppresses macrophage mobility (Table VI), it is likely that our factor is different from prostaglandins which are known to stimulate rather than suppress macrophage mobility (27).

The striking potency of our factor to inhibit macrophage functions raised the possibility that its effects could be attributed to a replicating agent, especially a virus, because many viruses are known to affect immune functions (28). LDH virus in particular is commonly known to be associated with many tumors and to alter macrophage functions (29, 30). Our laboratory has been very careful in eliminating the LDH virus from all our tumor cell inoculum (14), and by periodical examinations (30), tumors used in this study appear to be free of LDH virus. Furthermore, possible contamination by LDH virus was ruled out by comparing the serum LDH levels in the mice before and after iv injections of the factor, which was a UM-2 filtrate (<1000).

Further characterization of the factor is now in progress and will be reported in the near future.

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