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Isolation of a Factor from Cancer Ascitic Fluid Increasing Susceptibility of Mice to *Pseudomonas* Infection (40994)

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Abstract. A factor which impairs the capacity of normal recipient mice to resist *Pseudomonas* infection has been isolated from the ascitic fluids of tumor-bearing mice. This factor was dialyzable (molecular weight between 4000 and 10,000), showed potent suppressive activity when as little as 10 μg was given ip and was resistant to heating at 56° for 30 min. The factor in the ascitic fluids was detectable rather late after tumor implantation and the production of the factor in the ascitic fluids was closely related to the tumor growth.

Many clinical observations on the frequent occurrence of severe infections in cancer patients, caused by opportunistic microorganisms such as *Pseudomonas aeruginosa* (1, 2) and *Candida albicans* (3), which do not usually cause infection in a normal person, may suggest that these microorganisms easily invade cancer patients. The mechanism of easy invasion, however, still remains unclear.

Immunosuppression and the role of suppressive factors in cancer are already known and they were well reviewed by Kamo and Friedman (4). However, the details of the relationship between tumor-bearing and antibacterial resistance still remain unresolved. In 1976, North *et al.* (5) reported that mice implanted with tumors were easily weakened in their resistance to the infection of *Listeria* and *Yersinia* and that the impairment of bacterial resistance was transferable to normal recipients by the serum of tumor-bearing donor mice. On this experimental basis, they also suggested a possibility that the suppression of the capacity to resist a spectrum of microorganisms by the suppressive factors produced in a tumor-bearing host is responsible for the higher incidence of death from natural infection in terminal cancer patients. However, the principle of impairment of the defense mechanism has not yet been fully characterized.

This experiment was designed to investigate the mechanism by which opportunistic microbes establish their easy invasion and cause severe infection in tumor bearers through the isolation of such a suppressive factor(s). In cancer ascitic fluids several biologically active factors, such as inhibitors of interferon induction (6) and inhibitors of antibody formation (7), have already been isolated and described. Through isolation and characterization of these suppressive factors, the final purpose of this series of works is to establish the *in vivo* effect of these humoral factors, in particular, their effects on lymphoid cells. The present paper is the first of a series, and is concerned only with the fact that cancer ascitic fluids obtained from mice possess potent activity in suppressing the capacity of mice to resist *Pseudomonas aeruginosa* infection and the isolation of such a specific suppressive factor from cancer ascitic fluids.

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

Cancer ascitic fluids. Ehrlich ascites tumor maintained by biweekly transfers in ddY mice were used throughout the experiment. A further comfirmative experiment was conducted with methylcholanthrene-induced ascitic tumor (MCA) and FM-3A ascitic tumor, a spontaneous mammary tumor of C3H/He mice (8). Cancer ascitic fluids were collected on the 14th day (if undescribed) after ip implantation of 1×10^5 cells. Obtained fluids were immediately centrifuged at 17,000*g* for 20 min to remove

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the cells and then the supernatants were ultracentrifuged at 150,000*g* for 3 hr. The final supernatants were stored at −20° until use.

Control ascitic fluids. Noncancer ascitic fluids were obtained by the repeated ip injection of 0.1 ml of Freund's complete adjuvant (Difco Lab., Detroit, Mich.) according to the method of Elleman *et al.* (9). The fluids obtained 3 days after the last injection were centrifuged as for the cancer ascitic fluids and stored at −20°.

Bacteria. Pseudomonas aeruginosa isolated from the urine of a leukemic patient and given several passaged through mice, was grown in HI broth and stored on HI agar slant at 4°. Before infection, stock bacteria were grown again on the BHI agar, collected, and diluted in a standard fashion 0.01 *M* phosphate-buffered saline, pH 7.2 (PBS), for iv infection. The organism had a median lethal dose (LD_{50}) of $6-8 \times 10^6$ by the iv route in ddY mice.

Evaluation of suppressive activity on antibacterial resistance. Two-tenths milliliter of cancer ascites or crude preparations, which underwent semipurification, was dissolved in PBS and injected iv 24 hr before infection and the control mice received 0.2 ml of ascitic fluids induced by adjuvant in normal mice. After infection, the number of mice that survived was checked for 20 days. When the number of survivors in the treated groups was compared with that of the control group by means of χ^2 analysis and the *P* value obtained was below 0.05, the assayed materials were considered to have suppressive activity. The number of bacteria in the kidneys of treated and control mice was enumerated to examine the effect of cancer ascites on the bacterial growth *in vivo*. This was done by plating 10-fold serial dilutions of kidney homogenates on the nalidixic acid−cetrimide agar (10).

The direct inhibitory effect of cancer ascites on bacterial growth was examined *in vitro* by determining the number of bacteria in HI broth with the presence of Ehrlich ascites, control ascites, or PBS by plating 10-fold serial dilution of broth on agar.

Results. (1). Suppression of antibacterial resistance in mice by iv injection of cancer ascitic fluids. The ability of cancer ascites to suppress the capacity to resist bacterial infection was examined by an iv injection of 0.2 ml of ascitic fluids obtained from tumor-implanted donor mice to normal mice, 1 day ahead of the iv injection of 6.0×10^6 *Ps. aeruginosa* (Table I). Only iv injection of cancer ascites and bacteria gave definite and reproducible results. The iv injection of any of three different cancer ascites resulted in the death of all mice within 7 days after infection, 80% within 5 days, whereas 60% of the control mice survived more than 20 days. Thus a striking difference was found in the resistance to *Ps. aeruginosa* infection between control and cancer ascites-treated mice. All of the mice which received cancer ascites but not *Ps. aeruginosa* infection retained a quite healthy appearance during the observation period of 20 days.

(2). Cancer ascitic fluids interfere with the bacterial elimination system. To investigate whether the suppression of antibacterial resistance by cancer ascites was expressed through its direct effect on the

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

Ascitic fluids	Survival days	No. of survivors/ No. inoculated	Mean days of survival
Ehrlich	2,2,2,2,3,3,3,5,5,7,	0/10[c]	3.4
MCA	2,2,3,3,3,3,5,5,7,7,	0/10[c]	4.0
FM-3A	2,2,2,3,3,3,5,7,7,8,	0/10[c]	4.2
Control	4,4,5,7,s,s,s,s,s,s,[b]	6/10	>14.0

[a] 6.0×10^6 *Pseudomonas* was given iv 24 hr after iv injection of ascitic fluids.
[b] Survivors at Day 20 postinfection.
[c] Statistically significant ($P < 0.05$) by chi-square analysis.

bacteria or through a host-mediated mechanism, the effect of Ehrlich ascites on the growth of *Ps. aeruginosa* was first examined *in vitro*. When 1×10^6 *Ps. aeruginosa* in HI broth was cultured at 37° in the presence of Ehrlich ascites or control ascites, no significant difference of bacterial growth was found (data not shown). Next, the *in vivo* effect of cancer ascites on the bacteria growth was examined. When growth of *Ps. aeruginosa* in the kidneys of mice (11) treated with Ehrlich ascites or control ascites was examined after infection, the number of *Ps. aeruginosa* in the kidneys of mice treated with Ehrlich ascites was significantly higher ($P < 0.05$) than that of control mice (Fig. 1). These results suggest that the suppression of host resistance to *Ps. aeruginosa* by cancer ascites was expressed through the suppression of the bacterial elimination system of the host rather than its direct effect on the growth of bacteria.

(3). Production and potency of such a suppressive factor in cancer ascites. The production of such a suppressive factor in ascitic fluids was examined along the course of tumor growth in mice implanted with Ehrlich ascitic tumor. When mice received 0.2 ml of Ehrlich ascites fluid collected on the 12th or the 16th day of tumor implantation, they died within 6 to 8 days after 8×10^6 *Ps. aeruginosa* infection, whereas when mice received 0.2 ml of the fluids collected on the 5th or the 7th day of tumor implantation, 50 and 60% of mice survived after infection, comparable to that of the control (Table II). These results suggest that the suppressive factor is liberated into or accumulates in the ascitic fluids during the late stage of tumor growth. When a quantitative titration of the suppressive potency of the fluids was made following the same procedure, the ascites obtained on Day 12 caused the infected mice to die at a 10^{-3} dilution and those of Days 14 and 16 at a 10^{-5} dilution, suggesting a peak of production or accumulation of the suppressive factor on Days 14 or 16, almost 2 days before the tumor death of the mice.

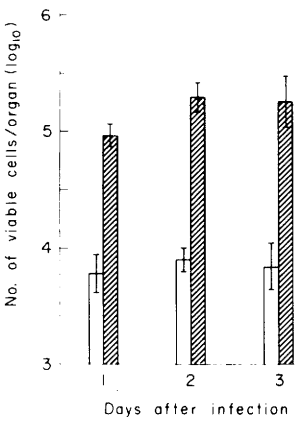

FIG. 1. Effect of Ehrlich ascites on the growth of *Pseudomonas* in kidney of mice. 6.0×10^6 *Pseudomonas* was infected iv 24 hr after iv injection of Ehrlich ascites or control ascites. Kidneys were separated aseptically on 1, 2, and 3 days after infection, homogenized, and the number of viable bacteria in the kidney was enumerated on the agar. ▨, Ehrlich ascites-treated mice; □, control ascites-treated mice.

(4) Characterization of the suppressive factor. To know the approximate molecular size of the suppressive factor, the ultrafiltration experiment was first conducted through a Diaflow ultrafiltration membrane ("Amicon" UM-10, 10,000 dalton exclusion). The suppressive activity was found in the dialyzate but not in the nondialyzate (Table III). Further purification of the suppressive factor in the dialyzate was conducted by gel filtration through Sephadex G-25 (Fig. 2). The result clearly shows that the suppressive factor in mice against *Ps. aeruginosa* infection can be detected in Peak 1 but not in Peak 2 (Table III). When the suppressive effect of Peak 1 was tested in the same mice against *Listeria* infection, even a 200-μg administration did not show such an effect. Peak 2, in contrast, contained a suppressive factor directed to *Listeria* infection as will be reported in a forthcoming paper. Moreover, the fact that as little as 10 μg of this preparation could render the mice sensitive to infection should be noticed. From the result of the gel filtration (Fig. 2), the molecular size of the suppressive factor seems to be between 4000 and 10,000 daltons.

The heat resistance of the suppressive factor was examined by determining its ef-

TABLE II. POTENCY OF EHRLICH ASCITIC FLUIDS COLLECTED AT VARIOUS TIMES AFTER TUMOR IMPLANTATION TO SUPPRESS HOST CAPACITY TO RESIST *Pseudomonas* INFECTION IN ddY MICE[a]

Days of sampling	Dilution of ascites	Survival days	No. of survivors/ No. inoculated	Mean days of survival
5	1:10	3,4,7,9,12,s,s,s,s,s,[b]	5/10	>13.5
7	1:10	3,5,6,7,s,s,s,s,s,s,	6/10	>14.1
12	$1:10^3$	2,2,3,3,3,4,4,5,6,6,	0/10[c]	3.8
	$1:10^5$	3,3,4,5,6,6,s,s,s,s,	4/10	>10.7
14	$1:10^5$	2,3,3,3,3,4,5,5,6,6,	0/10[c]	4.0
	$1:10^7$	4,4,5,6,8,8,10,s,s,s,	3/10	>10.5
16	$1:10^3$	1,2,2,3,3,3,3,4,4,6,	0/10[c]	3.1
	$1:10^5$	2,2,2,3,3,4,4,5,5,8,	0/10[c]	3.8
	$1:10^7$	3,4,4,5,6,8,8,s,s,s,	3/10	>9.8
Control	1:10	4,5,7,9,11,s,s,s,s,s,	5/10	>13.6

[a] 8.0×10^6 *Pseudomonas* was given iv 24 hr after injection of Ehrlich ascites.
[b] Survivors at Day 20 postinfection.
[c] Statistically significant ($P < 0.05$) by χ^2 analysis.

fect on the survival rate after infection. Table IV shows that the suppressive factor was resistant to heating at 56° for 30 min whereas it lost activity by heating at 70° for 10 min. When the same experiment was conducted with original cancer ascites, the same results were obtained.

Discussion. Extensive information on the frequent occurrence of severe infection in cancer patients (12–14) suggested the presence of suppressed host defense activity such as the generalized defective immune response observed both in men and experimental animals bearing a variety of tumors (4, 15). It also seems clear that humoral factors that exert immunosuppressive effects in a variety of test systems are present in many tumor bearers (4, 16, 17). However, the principle of impairment of the defense mechanism against microbial infection in men and animals bearing tumors has not yet been fully characterized. The main purpose of this experiment was to discover the mechanism by which *Ps. aeruginosa*, an opportunistic microorganism, established its invasion and growth in tumor-bearing mice by the isolation and characterization of a factor(s) from cancer ascites which suppressed host resistance to bacterial infection in mice.

The present study shows that iv injection of cancer ascites induced a marked sup-

TABLE III. EFFECT OF EHRLICH ASCITIC FLUID FRACTION ON HOST CAPACITY TO RESIST *Pseudomonas* IN ddY MICE[a]

Crude preparations from ascitic fluids	Survival days	No. of survivors/ No. inoculated	Mean days of survival
Nondialysate[b]	5,5,6,8,9,s,s,s,s,s,[c]	5/10	>13.3
Dialysate[b]	2,3,3,4,4,4,4,5,5,6,	0/10[d]	4.0
Peak 1			
(100 μg/mouse)	3,3,3,3,4,4,4,5,6,8,	0/10[d]	4.3
(10 μg/mouse)	3,3,3,3,4,4,5,5,6,7,	0/10[d]	4.3
Peak 2			
(100 μg/mouse)	4,5,7,8,10,12,s,s,s,s,	4/10	>12.6
(10 μg/mouse)	5,6,7,10,11,s,s,s,s,s,	5/10	>13.9
Control	5,6,7,8,12,s,s,s,s,s,	5/10	>13.8

[a] 6.0×10^6 *Pseudomonas* was given iv 24 hr after iv injection of Ehrlich ascitic fluid fractions.
[b] Adjusted to the volume of original ascitic fluids and used for treatment.
[c] Survivors at Day 20 postinfection.
[d] Statistically significant ($P < 0.05$) by χ^2 analysis.

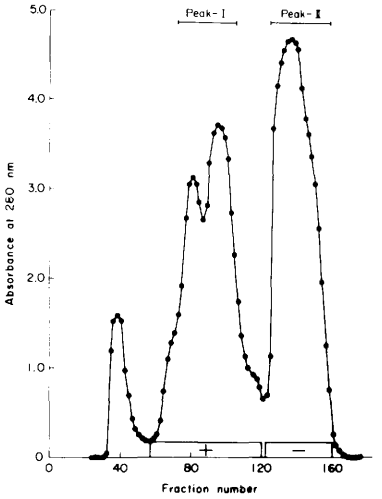

FIG. 2. Separation of a suppressive factor to anti-*Pseudomonas* resistance from Ehrlich ascites on Sephadex G-25. The low-molecular-weight fraction of Ehrlich ascites, lyophilized after dialyzation with hollow fiber (molecular weight cut-off 10,000, HIP-10, Amicon), was applied to the Sephadex G-25 column (3.2 × 84) previously equilibrated with 10 m*M* Tris–HCl buffer (pH 7.3). The column was eluted with the same buffer and 7 ml of the elutes was collected with each fraction. A pool of the fractions 60–120 showed positive suppressive activity but a pool of fractions 122–160 did not show such an effect.

pression of resistance to *Ps. aeruginosa* infection in normal syngeneic and allogeneic recipient mice. The suppressive activity was very potent; as little as 0.2 ml of $1:10^5$ dilution of cancer ascites caused a striking suppression. The speed of the appearance of suppressive activity in cancer ascites seemed to be rather slow (it became detectable on the ninth day after tumor implantation, dat not shown). However, the potency of the suppressive activity in the cancer ascites seemed to increase progressively as the tumor grew (100 times higher in 16-day ascites compared to 12-day ascites in the suppressive activity, Table II). This was in a big contrast to the factor reported by North (5), found in the serum of tumor transplanted mice and shown to suppress the capacity to resist *Listeria* infection. North's factor appears in the serum of mice as early as 8 hr after tumor implantation, reaches a peak potency at 24 hr, and begins to decrease at 9 days of tumor implantation. In this experiment, a possible contamination of LDH virus, which can induce immunosuppression in mice (18), was ruled out by testing serum LDH level in mice receiving ip injection of the principle, which is a UM-10 filtrate. The factor we isolated from Ehrlich ascites (MW, 4000–10,000) showed a suppressive effect on the resistance only to *Ps. aeruginosa* but not to *Listeria*. The suppressive effect on the resistance to *Listeria* was found in another fraction with lower-weight molecules (MW, 800, will be reported in a forthcoming paper). These results suggest that the suppressive factors we isolated from Ehrlich ascites are different from the serum factor reported by North *et al.* (5). Moreover, our factor, with the molecular weight of between 4000 and 10,000, seems to be differ-

TABLE IV. HEATING EFFECT ON THE FACTOR TO SUPPRESS THE CAPACITY TO RESIST *Pseudomonas* INFECTION IN ddY NORMAL RECIPIENT MICE[a]

Crude preparations from cancer ascitic fluids	Heating	Survival days	No. of survivors/ No. inoculated	Mean days of survival
Cancer ascitic	Nonheating	2,2,2,2,3,3,3,4,4,5,	0/10[c]	3.0
fluids	56° for 30 min	2,2,2,3,3,3,4,4,5,6,	0/10[c]	3.4
($1:10^2$)	70° for 10 min	3,4,5,7,8,s,s,s,s,s,[b]	5/10	>12.8
Peak 1	Nonheating	2,2,3,3,3,4,4,4,5,6,	0/10[c]	3.6
(10 μg/mouse)	56° for 30 min	2,2,3,3,3,3,4,5,5,6,	0/10[c]	3.6
	70° for 10 min	3,4,5,6,s,s,s,s,s,s,	6/10	>13.8
Control		3,4,4,5,6,s,s,s,s,s,	5/10	>12.2

[a] 8.3×10^6 *Pseudomonas* was given iv 24 hr after iv injection of Peak 1.
[b] Survivors at Day 20 postinfection.
[c] Statistically significant ($P < 0.05$) by χ^2 analysis.

ent in molecular size from corticosteroids which are known to show potent activity in suppressing host antibacterial resistance (19). Details of the way of suppression by the factor are still unclear, although it is evident that the suppressive activity is expressed through a host-mediated mechanism but not through its direct effect on bacterial growth.

Further characterization of the factor and elucidation of the mechanism of its action are now in progress.

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