Induction of tumoricidal macrophages from bone marrow cells of normal mice or mice bearing a colony-stimulating-factor-producing tumor

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Summary. Nonadherent cells of the bone marrow of C3H/ HeN mice were incubated for 3 days with the culture supernatant of an L-929 cell line containing macrophage-colony-stimulating factor. Approximately, 70% of the cells became phagocytic, adherent to plastic dishes and positive for nonspecific esterase staining. The adherent cells exhibited a weak tumoricidal activity against MM48 syngeneic mammary carcinoma cells, and the cytotoxicity was strongly augmented by the addition of bacterial lipopolysaccharide to the cytotoxicity assay. The cytotoxicity induced by lipopolysaccharide was also shown to be mediated by Thy1.2$^-$ and asialo-GM1$^+$ cells, and was abrogated by the addition of carrageenan. Macrophage-colony-stimulating-factor-producing (D66) and nonproducing (A23) variants were separated from the MM48 tumor line in in vitro culture following limiting dilution. There was no difference between these two variants in either the in vitro growth rate or the susceptibility to macrophage-mediated cytotoxicity. C3H/HeN mice inoculated i.p. with D66 survived longer than did those inoculated i.p. with A23. C3H/HeN mice bearing D66 or A23 as an ascitic form were given i.p. injections of *Nocardia rubra* cell wall skeleton (N-CWS). N-CWS significantly prolonged the survival period of mice bearing D66, whereas it exhibited no apparent antitumor effect on mice bearing A23. The increase in the cell number of D66 in the peritoneal cavity was significantly retarded, compared with that of A23. In contrast, the number of peritoneal macrophages increased more in D66-bearing mice than in A23-bearing mice. The increase in the peritoneal macrophage number was further augmented by an i.p. injection of N-CWS. Peritoneal macrophages of D66-bearing mice exhibited apparent tumoricidal activity against MM48 tumor cells in the presence of lipopolysaccharide, and the cytotoxicity was significantly augmented by i.p. injection of N-CWS. On the other hand, the responsiveness of peritoneal macrophages to lipopolysaccharide was found to be poor in A23-bearing mice and the tumoricidal activity was only weakly augmented by N-CWS. These results strongly suggest that M-CSF plays an important role not only in the maturation of macrophage progenitors but also in the induction and the accumulation of activated macrophages.

Introduction

Macrophages play an important role in the host's immune response to tumors. Some evidence indicates that macrophages, when properly activated, will kill neoplastic cells in vitro [21] and possibly in vivo. To achieve successful cancer immunotherapy using activated macrophages, it is necessary not only to activate macrophages but also to increase the number of macrophages and accumulate the cells at the tumor sites. However, few studies have demonstrated the mechanism(s) whereby macrophages are derived from the bone marrow [14, 27] and migrate to the tumor sites, while there have been many studies regarding the derivation of effector cells with natural killer cell activity [13, 15, 16, 29] or natural cytotoxic cell activity [7, 12] from bone marrow. Furthermore, it is still unclear how macrophage progenitors in bone marrow become tumoricidal as one of the final events of maturation and activation of cells of the macrophage lineage. This study was undertaken to investigate whether bone-marrow-derived macrophages, matured by macrophage-colony-stimulating factor (M-CSF), further require other stimuli to become tumoricidal macrophages, and to investigate a role for M-CSF in in vivo induction and accumulation of activated macrophages. The present study showed that both incubation with L-929 conditioned medium (CM), used as a source of M-CSF, and stimulation with lipopolysaccharide are essential for the induction of cytotoxic mature macrophages from bone marrow in vitro, and inoculation of a CSF-producing tumor resulted not only in the increase in the number of macrophage progenitors but also in the induction and the accumulation of macrophages possessing high potential to become tumoricidal macrophages at the tumor site.

Materials and methods

Animals and tumors. Male C3H/HeN mice 8–10 weeks old were obtained from Charles River Japan Inc (Kanagawa, Japan). MM48 mammary carcinoma cells and cells of two kinds of variants derived from the MM48 tumor line were passed twice a week in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS, General Scientific Laboratories, Los Angeles, Calif), 100 U/ml penicillin (Meiji Seika Kaisha, Ltd, Tokyo, Japan) and 100 µg/ml streptomycin (Meiji Seika) (10% FCS-RPMI medium).

Conditioned medium. L-929 cells (10^5 cells) were incubated in 10% FCS-RPMI medium in a 5% CO_2/95% humidified atmosphere at 37° C for 3 days. The supernatant was harvested, sterilized by filtration through a filter of 0.45 µm pore size (Sartorius, Göttingen, FRG) and kept at −20° C. The supernatant was designated as L-929 CM and was used as a source of standard M-CSF.

In the following experiment, each incubation was performed in a 5% CO_2/95% humidified atmosphere at 37° C unless otherwise stated.

Culture of nonadherent bone marrow cells. Mice were killed by cervical dislocation, and bone marrow cells were obtained by flushing the femoral shafts with ice-cold 5% FCS-RPMI medium using a 26-gauge needle. Bone marrow cells (15×10^6 cells) were placed in a 10-cm-diameter plastic dish (no. 25020, Corning) in 5 ml 5% FCS-RPMI medium. After a 2-h incubation, nonadherent bone marrow cells (NABMC) were separated from adherent cells, which were morphologically mature macrophages, by gentle pipetting. NABMC were washed once and resuspended in 10% FCS-RPMI medium at a cell density of 2×10^6 cells/ml. A 200-µl aliquot of the cell suspension containing 4×10^5 NABMC was added to each well of 96-well flat-bottomed microtiter plates (no. 25860, Corning) and incubated for 0–3 days with or without 10% L-929 CM. On day 3 of the culture, the number of NABMC cultured alone or with 10% L-929 CM was found to decline to 1.9×10^5 or 2.1×10^5 cells per well, respectively, when determined by counting the number of cells detached completely from a well by enzymatic digestion.

Cytotoxicity assay. As reported previously [22], 10^6 MM48 tumor cells were labeled with 5 µCi [^{125}I]iododeoxyuridine (Amersham, Buckinghamshire, England) for 4 h, washed three times, and resuspended in 10% FCS-RPMI medium at a cell density of 10^5 cells/ml. Medium of the well containing the cultured NABMC was removed by centrifugation and 100 µl 10% FCS-RPMI medium was added to the each well. In some experiments the well containing the cultured NABMC was washed with warmed 5% FCS-RPMI medium twice to remove nonadherent cells, and 100 µl of 10% FCS-RPMI medium was added. The nonadherent cells were removed, added to the other well, centrifuged, and then suspended in 100 µl 10% FCS-RPMI medium. Labeled MM48 (10^4 cells) suspended in 100 µl 10% FCS-RPMI medium were added to the well containing these effector cells and incubated for 24 h. Lipopolysaccharide from *Escherichia coli* (Difco Laboratories, Detroit, Mich) was added at a final concentration of 10 µg/ml. After incubation, 100 µl supernatant was collected and the radioactivity was counted in a gamma scintillation counter. The tumoricidal activity in triplicate cultures was calculated by the following formula.

$$\text{Tumoricidal activity (\%)} = \frac{\text{experimental }^{125}\text{I release (cpm)} - \text{spontaneous }^{125}\text{I release (cpm)}}{\text{total }^{125}\text{I release (cpm)}} \times 100$$

The spontaneous ^{125}I release, measured by incubation of target cells without effector cells, was less than 10% of the total radioactivity measured. In some experiments, the tumoricidal activity was determined in the presence of carrageenan (Sigma, St. Louis, Mo) at a final concentration of 100 µg/ml.

Assay of CSF and number of colony-forming units in culture. Fresh bone marrow (10^5 cells) from normal C3H/HeN mice was suspended in 1 ml McCoy 5A medium (Flow Laboratories, McLean, Va) containing 15% HI-FCS, 5% heat-inactivated horse serum (Flow Laboratories), 0.3% Bacto Agar (Difco Laboratories), penicillin and streptomycin. One milliliter of the cell suspension containing CSF samples at concentrations ranging from 10% to 40% was plated in a 35-mm-diameter plastic dish (Corning no. 25000). After a 7-day incubation, discrete cellular aggregates with more than 50 cells were scored as colonies [11]. To investigate in vivo production of CSF by a CSF-producing tumor variant, bone marrow cells were obtained from normal C3H/HeN mice or mice inoculated i.p. with 10^6 cells of the variant 7 days earlier, and cultured as described above for 7 days with 10% L-929 CM. The number of colonies derived from the bone marrow of tumor-bearing mice was compared with that formed by normal bone marrow cells.

Treatment with antibody and complement. NABMC were suspended in 0.5 ml 2% FCS-RPMI medium containing anti-Thy1.2 monoclonal antibody (Cedarlane, Ontario, Canada) or anti-(asialo-GM1) antibody (Wako Pure Chemicals, Osaka, Japan) at a dilution of 1:20 or 1:200, respectively, kept on ice for 60 min, washed once and then suspended in 0.5 ml 2% FCS-RPMI medium containing rabbit complement (Cedarlane) at a dilution of 1:10. The cell suspension was incubated at 37° C for 45 min. The treated NABMC were washed, incubated with 10% L-929 CM for 3 days and then assayed for tumoricidal activity against MM48 cells in the presence of 10 µg/ml lipopolysaccharide. NABMC cultured with L-929 CM for 3 days were also treated with antibodies and complement as described above and then applied for the cytotoxicity assay.

Separation of CSF-producing and non-CSF-producing variants from MM48 tumor line. One cell of the MM48 tumor line, suspended in 200 µl 10% FCS-RPMI medium, was seeded into a well of a microculture plate (no. 25860, Corning) and incubated at 37° C in a 5% CO_2 atmosphere for up to 14 days. The culture supernatants of the well containing growing clones were tested for CSF activity, and CSF-producing and non-CSF-producing clones were similarly applied to recloning using limiting dilution. Finally D66 and A23 subclones were established as CSF-producing and non-CSF-producing variants of MM48 tumor line.

Inoculation of A23 and D66 variants and treatment with Nocardia rubra cell wall skeleton. C3H/HeN mice were inoculated i.p. with 10^6 cells of the D66 or A23 variant, and injected i.p. with 100 µg *Nocardia rubra* cell wall skeleton (N-CWS) suspended in 0.5 ml 0.85% NaCl solution on days 4, 8 and 12 after tumor inoculation. Control mice were treated with 0.85% NaCl solution alone. Each group consisted of 16 mice. Mice were monitored daily for mortality.

Preparation of peritoneal macrophages. C3H/HeN mice were given an i.p. injection of 1 ml of 10% thioglycollate (Difco Laboratories). Four days after injection, peritoneal cells were obtained by lavage of the peritoneal cavity with Hanks'. medium (Nissui Pharmaceutical Co.) supplement-

ed with 5 U/ml heparin (Novo Industries A/S, Denmark), washed twice, and suspended in 10% FCS-RPMI medium. The cell population was analysed by Giemsa staining. To obtain peritoneal macrophages from tumor-bearing mice treated with N-CWS, C3H/HeN mice were given an i.p. injection of 0.5 ml 0.85% NaCl solution or 100 μg N-CWS 4 days after i.p. inoculation of 10^6 cells of A23 or D66. Three days after i.p. injection of 0.85% NaCl solution or N-CWS, peritoneal cells were obtained as described above. After counting the cell number and analyzing the cell population using Giemsa staining, the lavage fluid was repeatedly centrifuged at 50 g for 3 min until the tumor cell contamination in the fluid decreased to less than 10%. Peritoneal cells suspended in 10% FCS-RPMI medium were added to a well of a microculture plate (Corning no. 25850), so as to seed 4×10^5 macrophages in a well, and incubated for 2 h. After the incubation, nonadherent cells were removed by repeated washings of the wells and 100 μl 10% FCS-RPMI medium was added to each well. The resultant adherent cells were used as effector cells as described above.

Statistical evaluation. The mean difference between experimental and control groups was evaluated by Student's *t*-test. The difference in the survival rate of mice was examined by the log rank test. A *P* value less than 0.05 was regarded as statistically significant.
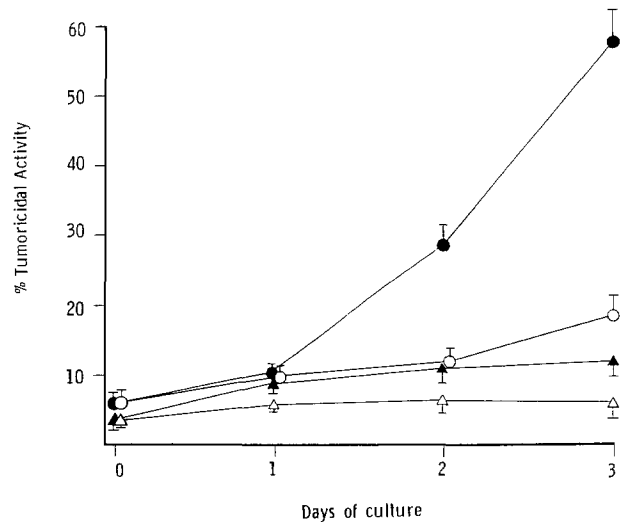
Results

Differentation and induction of tumoricidal activity of macrophage progenitors by M-CSF and lipopolysaccharide
When NABMC of C3H/HeN mice were incubated in the absence or presence of L-929 CM for 3 days, most of them became adherent, phagocytic and positive for nonspecific esterase staining (Table 1). The cultured cells were tested for tumoricidal activity against syngeneic MM48 tumor cells in the presence or absence of lipopolysaccharide. During the 3-day culture of NABMC, the number of NABMC declined to approximately 50% of the starting cell number whenever the cells had been cultured alone or with 10% L-929 CM, indicating that there was no significant difference in an effector-to-target-cell ratio between the culture of NABMC with medium alone and that with L-929 CM. The results are shown in Fig. 1. Freshly prepared NABMC exhibited very low cytotoxicity and the cytotoxicity was not augmented by the addition of lipopolysaccharide. NABMC cultured in medium alone also

Fig. 1. Induction of tumoricidal macrophages from NABMC by M-CSF and lipopolysaccharide. NABMC from C3H/HeN mice were cultured for up to 3 days with (*closed symbol*) or without (*open symbol*) 10% L-929 CM and assayed for tumoricidal activity against labeled MM48 tumor cells in the absence (*triangle*) or presence (*circle*) of lipopolysaccharide (10 μg/ml). *Bars*, SE ($n = 3$)

showed low cytotoxicity and it was only weakly augmented when tested in the presence of lipopolysaccharide. The induction of tumoricidal activity by lipopolysaccharide was more apparent when NABMC had been cultured in 10% L-929 CM. The coexistence of lipopolysaccharide in the cell culture during the stimulation with L-929 CM resulted rather in the suppression than in the augmentation of the induction of the cytotoxicity (Table 2). On the other hand, M-CSF was not necessary during the cytotoxicity test, because the removal of L-929 CM from the cell culture just before the cytotoxicity test did not affect the following induction by lipopolysaccharide of tumoricidal activity of the cells (Table 2). These results indicate that induction of tumoricidal macrophages from bone-marrow-derived macrophage progenitors requires both the matura-

Table 1. Differentiation of macrophage progenitors in NABMC

Macrophage function[a]	Before culture (%)	After culture with[b]	
		Medium alone (%)	L-929 CM (%)
Adherence	0	70	66
Phagocytosis	28	75	76
Esterase stain	39	71	71

[a] Freshly prepared or cultured NABMC of C3H/HeN mice were tested for adherence to plastic dishes, phagocytosis of zymosan particles and positiveness for nonspecific esterase staining
[b] NABMC of C3H/HeN mice were incubated with or without L-929 CM for 3 days

Table 2. Effect of L-929 CM and lipopolysaccharide (LPS) on the induction of tumoricidal activity of cultured NABMC

Expt.	Cultured with[a]		Cytotoxicity test with[b]		Tumoricidal[c] activity (%)
	L-929 CM	LPS	L-929 CM	LPS	
1	−	−	−	−	10.3 ± 0.5
	−	−	−	+	18.6 ± 2.4
	−	+	−	+	11.0 ± 0.4
	+	−	−	−	12.8 ± 0.8
	+	−	−	+	54.4 ± 1.1
	+	+	−	+	12.2 ± 0.7
2	−	−	−	+	18.7 ± 0.7
	+	−	−	+	62.2 ± 2.7
	+	−	+	+	57.8 ± 2.6

[a] NABMC were cultured for 3 days with or without 10% L-929 CM and/or LPS (10 μg/ml)
[b] After culture, the cells were assayed for tumoricidal activity against labeled MM48 cells with or without L-929 CM and LPS (10 μg/ml)
[c] Mean ± SE of triplicate cultures

tion with M-CSF and further stimulation with substances such as lipopolysaccharides. On the basis of these results, the following assays of tumoricidal activity of cultured NABMC were performed in medium containing lipopolysaccharide but not L-929 CM.

Characterization of the tumoricidal effector cells

After incubation of NABMC with 10% L-929 CM for 3 days, the cells were fractionated into adherent and non-adherent cells and tested for tumoricidal activity. As shown in Table 3, the majority of the cytotoxicity was mediated by adherent cells and was abolished by the addition of carrageenan. Removal of phagocytic cells contaminating in NABMC by carbonyl iron treatment followed by magnet stirring before culture with L-929 CM did not affect the induction of tumoricidal macrophage by M-CSF and lipopolysaccharide (data not shown), suggesting that the small number of mature macrophage contaminating NABMC did not play a major role in the induction of the cytotoxicity.

We next examined the surface marker of the precursors and the effector cells of the cytotoxicity induced by M-CSF and lipopolysaccharide. NABMC that had been treated with anti-Thy1.2 or with anti-(asialo-GM1) anti-

body and complement were incubated with 10% L-929 CM for 3 days and assayed for tumoricidal activity. The results are summarized in Table 4. The pretreatment of NABMC with these antibodies and complement before culture did not affect the induction of the tumoricidal activity. On the other hand, the cytotoxicity was strongly abolished when cells were treated with anti-(asialo-GM1) antibody and complement after the stimulation with L-929 CM. However, the effector cells of the cytotoxicity were still negative for Thy1.2 antigen, because anti-Thy1.2 treatment did not reduce the cytotoxicity.

Separation of CSF-producing and non-CSF-producing variants from the MM48 tumor line

In preliminary experiments, the number of colony-forming units in culture in bone marrow was found to increase significantly in C3H/HeN mice bearing the MM48 tumor, suggesting that MM48 is a CSF-producing line. CSF assay using culture supernatants revealed that MM48 tumor cells moderately produce M-CSF, compared with the production of M-CSF by L-929 cells (data not shown). Therefore, the MM48 tumor line was further cloned to obtain highly M-CSF-producing and M-CSF-nonproducing clones. When tested using normal C3H/HeN bone marrow cells as responder cells, it was found that the culture supernatant of the D66 subclone contained higher CSF activity than that of MM48 parent cells, whereas no apparent CSF activity was detected in the culture supernatant of the A23 subclone (Fig. 2). Giemsa staining revealed that the cells comprising these colonies were macrophages but not neutrophils (data not shown). In vivo production of M-CSF by D66 was confirmed by the fact that the number of colony-forming units in culture in the bone marrow significantly increased in mice bearing D66 as an ascitic form (Table 5). As also shown in Table 5, there was no significant difference in either the in vitro growth rate or the susceptibility to macrophage-mediated cytotoxicity.

Table 3. Fractionation of NABMC after culture with L-929 CM

Cell culture[a]	Cell fractionation[b]	Addition of[c] carrageenan	Tumoricidal[d] activity (%)
Medium	Unfractionated	–	8.9±0.3
L-929 CM	Unfractionated	–	21.4±1.6
L-929 CM	Nonadherent cells	–	–3.0±0.4
L-929 CM	Adherent cells	–	22.4±0.5
L-929 CM	Adherent cells·	+	2.2±0.8

[a] NABMC were cultured alone or with 10% L-929 CM for 3 days
[b] After culture, cells were fractionated by plastic dishes
[c] Carrageenan was added to the cell mixture at a final concentration of 100 μg/ml
[d] Cytotoxicity was assayed in the presence of lipopolysaccharide at a concentration of 10 μg/ml. Mean ± SE of triplicate cultures

Table 4. Phenotype of precursor and effector cells of bone-marrow-derived macrophage-mediated cytotoxicity

Before or after culture[a] with L-929 CM	Treatment of cells	Tumoricidal activity[b] (%)
Before culture	Medium	21.6±0.3
	C	26.0±2.1
	Anti-Thy1.2+C	27.2±3.1
	Anti-(asialo-GM1)+C	27.3±2.8
After culture	Medium	18.3±1.5
	C	17.6±2.3
	Anti-Thy1.2+C	17.6±0.2
	Anti-(asialo-GM1)+C	3.4±1.3

[a] NABMC from normal C3H/HeN mice were treated with antibodies and complement (C) before or after a 3-day incubation with 10% L-929 CM
[b] Cultured NABMC were tested for tumoricidal activity against labeled MM48 tumor cells in the presence of lipopolysaccharide (10 μg/ml). Mean ± SE of triplicate cultures
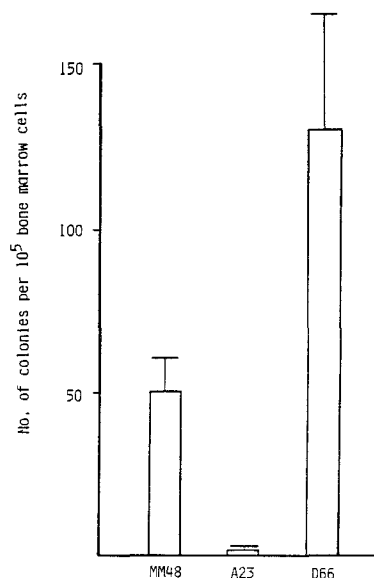
Fig. 2. CSF production by MM48 and its variant tumor cell lines. Culture supernatants of MM48 parent, A23 and D66 variant tumor cells were tested for CSF activity by a soft-agar colony assay system using 10^5 normal C3H/HeN bone marrow cells per dish. Bars, SE ($n = 6$)

Table 5. Characteristics of A23 and D66 variants of MM48 tumor line

Cell lines	In vitro growth rate[a] (no. of cells × 10⁻⁴/well) culture for			Susceptibility to macrophage--mediated cytotoxicity (% tumoricidal activity[b])	In vivo CSF production[c] (no. of CFU-c in the bone marrow) (% control)
	1 day	2 days	3 days		
A23	9.0	13.4	94.5	39.9	110.3
D66	8.5	18.0	97.0	42.0	239.6

[a] A23 or D66 variant (10^4 cells) was incubated in vitro and the numbers of viable cells were counted by the trypan-blue-dye-exclusion test on days 1, 2 and 3 of incubation

[b] Peritoneal exudate cells were obtained from C3H/HeN mice injected ip with 1 ml 10% thioglycollate 4 days earlier. After 2h incubation of peritoneal exudate cells, nonadherent cells were removed by gentle pipetting and the resultant adherent cells were tested for tumoricidal activity against ^{125}I-dU labeled A23 or D66 cells in the presence of lipopolysaccharide (10 µg/ml)
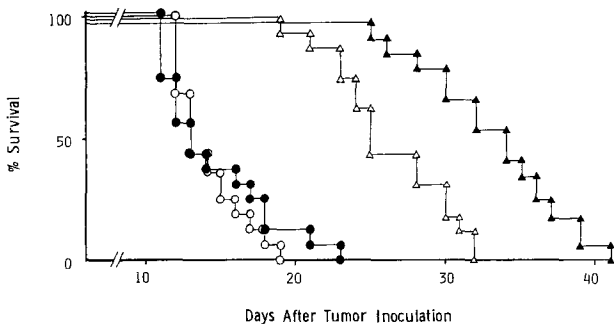
[c] A23 or D66 (10^6 cells) was inoculated i.p. into C3H/HeN mice. Seven days later, bone marrow cells were obtained from these mice and tested for the number of colony-forming units in culture (CFU-c) by soft-agar colony assay. Data represent percentage of those shown by normal syngeneic mice

Survival of mice bearing the CSF-producing or non-CSF-producing tumor variant and antitumor effect of N-CWS

C3H/HeN mice were inoculated i.p. with 10^6 cells of the D66 or A23 variants of the MM48 tumor line. As shown in Fig. 3, mice bearing D66 survived significantly longer than those bearing A23. The survival period of D66-bearing mice was apparently prolonged by i.p. injection of N-CWS, while no apparent prolongation of the survival period was obtained by N-CWS in A23-bearing mice.

Effect of N-CWS on the number of peritoneal macrophages and on their ability to become tumoricidal macrophages in mice bearing the CSF-producing or non-CSF-producing tumor variant

Seven days after i.p. injection with cells of the D66 or A23 variants, the peritoneal cavities were lavaged and the cell population including tumor cells was examined by Giemsa staining. As shown in Table 6, the cell number of D66 was significantly less than that of A23, and was further reduced when mice bearing D66 were given an i.p. injection of N-CWS 4 days after tumor inoculation. N-CWS-induced tumor-growth suppression in the peritoneal cavity was also detected in mice bearing A23. However, the effect was less than that shown in D66-bearing mice. On the other hand, the number of peritoneal macrophages of mice bearing D66 was found to be more than that of mice bearing A23.

An i.p. injection of N-CWS resulted in the apparent increase in the peritoneal macrophage number in both D66- and A23-bearing mice. Peritoneal macrophages of these mice were tested for tumoricidal activity against MM48 tumor cells in the absence or presence of lipopolysaccharide. The results are summarized in Table 7. When tested in the absence of lipopolysaccharide, peritoneal macrophages obtained from D66-bearing mice exhibited weak though

Table 6. Correlation between tumor burden and macrophage number in the peritoneal cavity of tumor-bearing mice treated with N-CWS

Inoculated with[a]	N-CWS injection[b]	No. of cells in the peritoneal cavity ($\times 10^{-6}$)[c]	
		Tumor cells	Macrophages
A23	−	265.0	12.5
	+	185.0	37.5
D66	−	76.3	38.8
	+	33.7	56.3

[a] C3H/HeN mice were inoculated i.p. with A23 or D66 tumor cells

[b] Four days after tumor inoculation, C3H/HeN mice were given i.p. injection of 100 µg N-CWS

[c] Seven days after tumor inoculation, mice were killed and cells in the peritoneal cavities were analysed. Data represent the mean of three mice

Table 7. Tumoricidal activity of peritoneal macrophages from tumor-bearing mice treated with N-CWS

Treatment of mice[a]	Tumoricidal activity[b] (%)	
	− LPS	+ LPS
None	10.6	29.4
A23	10.8	11.2
A23 + N-CWS	14.7	27.8
D66	18.6	39.3
D66 + N-CWS	20.8	61.5

[a] C3H/HeN mice were inoculated i.p. with 10^6 tumor cells and injected i.p. with 100 µg N-CWS 3 days later

[b] Peritoneal macrophages were obtained 7 days after tumor inoculation and tested for tumoricidal activity against MM48 tumor cells in the absence or presence of lipopolysaccharide (LPS) (10 µg/ml)

Fig. 3. Treatment of C3H/HeN mice bearing an A23 or D66 tumor with N-CWS. C3H/HeN mice were given an i.p. inoculation of 10^6 A23 (O, ●) or D66 (△, ▲) variant cells on day 0 and i.p. injections of 100 µg N-CWS on days 4, 8 and 12 (*closed symbols*). Statistical significance in the survival rate: O, △, $P < 0.01$; O, ●, not significant; △, ▲, $P < 0.05$ Each group consists of 16 mice

higher cytotoxicity than that shown by cells of A23-bearing mice. The addition of lipopolysaccharide to the cytotoxicity assay resulted in the apparent augmentation of the cytotoxicity when peritoneal macrophages of D66-bearing mice were used as effector cells, while cells of A23-bearing mice did not respond to lipopolysaccharide. An i.p. injection of N-CWS caused only a weak augmentation of the cytotoxicity in cases of both A23- and D66-bearing mice when tested in the absence of lipopolysaccharide. However, N-CWS-stimulated peritoneal macrophages of D66-bearing mice exhibited high responsiveness to lipopolysaccharide, resulting in the marked elevation of cytotoxicity tested in its presence. The N-CWS-induced augmentation of the ability to become tumoricidal macrophages in response to lipopolysaccharide was more apparent in D66- than in A23-bearing mice.

Discussion

To analyze the ontogeny and the dynamics of effector macrophages with tumoricidal activity, bone marrow cells have been intensively investigated focusing on macrophage precursors [26, 28]. In most of the studies, bone marrow cells have been cultured in medium containing L-929 CM as the source of M-CSF to mature cells of the macrophage lineage. In their reports, however, direct cytotoxicity of bone-marrow-derived macrophages against natural killer (NK)-resistant tumor cells cannot be induced when normal bone marrow cells are incubated with L-929 CM alone. In the report of Lohmann-Matthes et al. [17], normal murine bone marrow cells cultured with L-929 CM for 5 days were shown to be able to exhibit antibody-dependent cell-mediated cytotoxicity but not direct cytotoxicity against a variety of tumor cells. Fisher et al. [8] succeeded in inducing direct cytotoxicity of bone-marrow-derived macrophages against spontaneous mammary carcinoma cells by incubation of bone marrow cells with L-929 CM only when bone marrow cells were obtained from mice bearing the mammary tumor but not from normal mice.

The results of this study clearly indicate that bone-marrow derived macrophages matured by M-CSF can be rendered tumoricidal by the additional stimulation with lipopolysaccharide. When cultured alone, 70% NABMC became adherent, phagocytic and nonspecific-esterase-positive mononuclear cells, comparable with macrophages. The cells cultured alone were also considered to be positive for Fc receptors, because they exhibited apparent antibody-dependent cellular cytotoxicity (data not shown). However, the direct cytotoxicity of these cells was very low, and was only slightly augmented by the addition of lipopolysaccharide. When NABMC were cultured with L-929 CM, macrophage progenitors differentiated to mature macrophages, as did the cells cultured alone, but they still did not exhibit tumoricidal activity. However, the cells cultured with L-929 CM exhibited apparent cytotoxicity when tested in the presence of lipopolysaccharide, indicating that the incubation with M-CSF followed by the additional stimulation with lipopolysaccharide is essential for the generation of tumoricidal macrophages from bone marrow in vitro. These results indicate that among various kinds of macrophage function, adherence, phagocytosis and expression of Fc receptors are easily induced in vitro during culture of macrophage progenitors, but they require more potent stimuli, including M-CSF, to differentiate to cells capable of becoming tumoricidal macrophages in response

to lipopolysaccharide or other stimulators. However, the long-therm stimulation of macrophage progenitors with lipopolysaccharide may cause down-regulation of the formation of CSF receptors, because lipopolysaccharide has been shown not only to stimulate phagocytosis and related functions but also to inhibit CSF-induced proliferation of bone-marrow derived macrophages [6].

Several investigators have reported that cells with NK cell activity can be generated from bone marrow by in vitro culture of bone marrow cells with M-CSF, interferon or interleukin 2. Lohmann-Matthes et al. described how cells differentiating to promonocytes in response to M-CSF have functional characteristics comparable to NK cells capable of mediating antibody-dependent cellular cytotoxicity [17, 18, 20, 25]. Clésson et al. have also described that bone-marrow-derived diffuse colonies induced by M-CSF, the majority of which are morphologically defined as myelomonocytic cells, have NK cell activity [4, 5] and that the activity is enhanced by interferon [3]. Other investigators have shown that NK cells [13, 15, 16, 29] or natural cytotoxic cells [7, 12] can be generated from bone marrow by interferon or interleukin 2 in vitro and in vivo [23, 24]. In this study, phenotypic analysis revealed that effector cells of the cytotoxicity induced by M-CSF and lipopolysaccharide was Thy1.2$^-$ and asialo-GM1$^+$, resembling NK cells. However, these cells were found to be adherent, phagocytic and sensitive to carrageenan, indicating that they are macrophages. Furthermore, no apparent NK cell activity was detected in NABMC cultured with L-929 CM when tested by 4-h ^{51}Cr-release assay using YAC-1 tumor cells as the target (data not shown). Therefore, it seems likely that lipopolysaccharide-induced cytotoxicity of NABMC cultured with L-929 CM is mainly mediated by asialo-GM1$^+$ macrophages.

To investigate the influence of M-CSF on the induction and the accumulation of macrophages possessing a high potential to become tumoricidal macrophages at the tumor site, highly CSF-producing and non-CSF-producing variants, separated from the MM48 moderately CSF-producing tumor line, were inoculated into syngeneic mice. When inoculated i.p. into C3H/HeN mice, the highly CSF-producing variant D66 grew more slowly than A23, the non-CSF-producing variant, whereas there was no difference in either the in vitro growth rate or the susceptibility to macrophage-mediated cytotoxicity between these two variants. On the other hand, peritoneal macrophages increased in mice bearing D66 more than in those bearing A23. An i.p. of N-CWS, known to be able to stimulate macrophages [19], resulted not only in the further accumulation of peritoneal macrophages but also in the retardation of the tumor growth more potently in mice bearing D66 than in mice bearing the A23 variant. The ability of peritoneal macrophages to become tumoricidal in response to lipopolysaccharide was augmented when mice were inoculated i.p. with D66 and was further potentiated by an i.p. injection of N-CWS. These results clearly reflected the survival period of tumor-bearing mice. Mice bearing D66 survived longer than did those bearing A23, and the antitumor effect of N-CWS clearly developed in D66- but not A23-bearing mice. These findings suggest that M-CSF not only induces the proliferation and the maturation of macrophage progenitors in bone marrow but also stimulates and accumulates tissue macrophages, and that M-CSF cooperates with macrophage activators, resulting in the further stimulation of macrophages. A pre-

liminary experiment revealed that N-CWS did not alter either the in vitro proliferation rate or the in vitro CSF-producing capacity of the D66 variant, indicating that the N-CWS-induced prolongation of the survival period of mice bearing D66 is not due to either the growth suppression or the enhancement of CSF production of D66 by N-CWS.

There have been several studies of hematopoietic events during tumor growth. Fisher et al. [8, 9] have reported that cytotoxic macrophages can be induced from bone marrow of C3H/HeN mice bearing a syngeneic mammary tumor more efficiently than from that of normal mice. Other investigators have shown that the number of colony-forming units in culture in bone marrow increases in mice bearing Lewis lung carcinoma (3LL) or a mammary tumor [1, 2, 10]. In preliminary experiments, we found that 3LL is a moderately CSF-producing tumor line and confirmed that s.c. inoculation of a CSF-producing tumor, such as MM48 and 3LL, resulted in the increase in the number of macrophage progenitors in bone marrow possessing high potential to become tumoricidal macrophages, while P815 mastocytoma, a non-CSF-producing tumor line, did not provoke this response when inoculated s.c. in DBA/2 mice (data not shown). In this study, in vivo production of M-CSF by D66, the highly CSF-producing variant of MM48, was also confirmed by the fact that i.p. inoculation of D66 resulted in the increase in the number of macrophage progenitors in bone marrow.

Finally this study investigated the influence of the M-CSF-producing tumor inoculation on the functions of immature and mature cells of the macrophage lineage, and the results strongly suggest that exogenous M-CSF administration may result in benefical effects on cancer immunotherapy using macrophage activators. Further studies are now underway.

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