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Role of Mononuclear Phagocyte Function in Endotoxin-induced Tumor Necrosis*

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Abstract—*The temporal susceptibility of tumors to induction of necrosis and regression by endotoxin was investigated further with a focus on the role of the putative mediator, tumor necrosis factor (TNF). Production of this factor was shown earlier to require prior activation of the mononuclear phagocytic system (MPS). Transplants of Meth A sarcoma or MOPC315 plasmacytoma had no consistent effect on parameters of MPS function such as hepatosplenomegaly, carbon clearance and non-specific antibacterial resistance at times that they were sensitive to induction of necrosis. Moreover, TNF, quantified by its necrotizing and regressing activity* in vivo, *could not be detected in the serum of tumor hosts after a necrotizing dose of endotoxin, while much smaller volumes of serum with TNF (TNS) of appropriately treated donor mice showed activity. As repeated incubation of TNS with Meth A cells at 37°C hardly removed its* in vivo *activity against Meth A, immediate absorption of produced TNF to the tumor cell mass seems a less likely cause. Cytostatic activity, another property attributed to TNF, was hardly increased in post-endotoxin tumor host serum, while TNS is highly cytostatic. It is concluded that induction of tumor necrosis is not dependent on MPS activation. A role of TNF as mediator of the effects of endotoxin still remains uncertain. Furthermore, the present and other data suggest that TNF, like endotoxin, probably acts by an indirect mechanism against tumors* in vivo.

INTRODUCTION

THE CAPACITY of endotoxin to induce hemorrhagic necrosis and regression of different tumors in guinea pigs and mice has been well known for a long time [1–3]. The susceptibility to endotoxin was shown to be restricted to fully established tumors [4, 5]. Small tumors were rather resistant. As endotoxin itself is not toxic for tumor cells *in vitro*, an indirect mode of action was suggested [6] and confirmed by subsequent studies [7–9]. A putative mediator of the effects of endotoxin is tumor necrosis factor (TNF; [7]). High levels of TNF could be elicited by endotoxin in the circulation of mice previously treated with macrophage-activating agents like Bacille Calmette-Guérin, *Corynebacterium parvum* or zymosan. This pretreatment appeared to be indispensible, indicating that TNF could be a product of activated macrophages [7, 10]. This has been confirmed by data showing that only coryneform strains which activated the MPS, as measured by hepatosplenomegaly, carbon clearance and bactericidal capacity, supported the elicitation of TNF by endotoxin [10, 11]. On the other hand, tumor necrosis following injection of endotoxin may occur without a need for pretreatment with macrophage-activating agents. Various tumors, however, have been found to stimulate or depress MPS activity, dependent on the stage of tumor development [12–15]. Therefore the temporal sensitivity of the tumors may be related to their influence on the MPS at the time of endotoxin administration. This was the subject of the present study.

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Abbreviations: CFU: colony-forming units; i.v.: intravenous(ly); MPS: mononuclear phagocytic system; NMS: normal mouse serum; s.c.: subcutaneous(ly); TNF: tumor necrosis factor; TNS: tumor necrosis serum.

MATERIALS AND METHODS

Animals and tumors

Inbred female BALB/c mice, 10–12 weeks old, were used for tumor experiments. They were bred and maintained in our own facilities. Female

Swiss random mice were used for the production of serum with TNF (TNS) unless otherwise stated. They were obtained from the Central Institute for the Breeding of Laboratory Animals, CPB, Zeist, The Netherlands. The Meth A fibrosarcoma, syngeneic to BALB/c, was obtained from the Clinical Research Centre (Harrow, Middlesex, U.K.); the syngeneic IgA plasmacytoma MOPC315 was kindly provided by Dr. J. Ràdl (Institute for Experimental Gerontology, TNO, Rijswijk, The Netherlands). The tumors were maintained in ascitic form by serial intraperitoneal passage in BALB/c mice. For tumor experiments tumor cells were harvested in Eagle's minimal essential medium and washed by low-gravity centrifugation to remove erythrocytes. Cell viability was determined by trypan blue exclusion. Desired numbers of viable tumor cells were injected subcutaneously (s.c.) in a volume of 0.05 ml on the ventral side of the abdomen.

Preparation of TNS

TNS was prepared according to Green *et al.* [10] by intravenous (i.v.) injection of 1 mg *C. parvum* (Coparvax, strain 6134, Lot CA 749, Burroughs Wellcome, Beckenham, Kent, U.K.) followed by an i.v. injection of 25 μg endotoxin 14 days later. Serum was prepared from blood obtained 90 min after injection of endotoxin. Sera were heated at 56°C for 10 min, as TNF is stable at this temperature [7], and stored at -20°C.

Determination of necrosis and tumor growth

Mice with s.c. growing tumors were injected i.v. with 25 μg of endotoxin (LPS w from *Escherichia coli* 0111:B4; Difco Laboratories, Detroit, MI, U.S.A.) or 0.05–0.5 ml TNS. Tumor size and extent of necrosis were measured with a caliper, starting at the day of injection of endotoxin or serum. Tumor size was given as mean diameter (mm). Extent of necrosis was expressed as 100 times the ratio of the mean diameters of necrotic area and tumor 2 days after injection of endotoxin or serum.

Assays for MPS activity

Carbon clearance was determined to measure the pinocytic activity of the MPS [16]. Mice were injected i.v. with 0.2 ml of a carbon suspension (160 mg/kg body weight; Pelikan ink C11/1431a, G. Wagner, Hannover, F.R.G.) in phosphate-buffered saline (PBS; pH 7.2) with 1% (w/v) gelatin. Blood samples were collected immediately after carbon gift and 10 min later. The carbon content of lysed blood was measured in a spectrophotometer at 620 nm. The phagocytic index was expressed as

$$K = (\log C_0 - \log C_t)/t,$$

where C_0 and C_t are the carbon concentrations at zero time and time t. The corrected phagocytic index was calculated by multiplication of $\sqrt[3]{K}$ with the ratio of body weight and weight of liver and spleen.

The bactericidal capacity of the MPS was determined by a modified listeria clearance test [17]. Mice were injected i.v. with about 3×10^4 viable *L. monocytogenes* (strain L 347, type 4b). Spleens were removed 1 or 2 days later, weighed and homogenized in PBS in a Potter–Elvehjem apparatus with a narrow fitting pestle. Homogenates were adjusted to a fixed volume and the number of viable listeria were determined by the method of Miles and Misra [18] on trypticase soy agar plates (BBL, Division of BioQuest, Cockeyville, MD, U.S.A.) and expressed as $\log_{10}$ colony-forming units (CFU) per spleen.

Assay for cytostatic activity

The *in vitro* cytostatic activity of TNS and sera of tumor-bearing mice was determined as previously described by measurement of the incorporation of [^{3}H]-thymidine into Meth A cells cultured with the sera for 40 hr [19]. Sera were heated prior to assay (56°C for 10 min). This treatment was shown to reduce the non-specific cytostatic activity of normal mouse serum (NMS; [20]) and to eliminate interferon induced by endotoxin [21].

Statistical analysis

Data were expressed as their arithmetic means ± S.E.M. and analysed for significance by Student's t test.

RESULTS

Time-dependence of antitumor activities

Injection of 25 μg of endotoxin into mice with tumor transplants of different age showed that susceptibility of MOPC315 and Meth A tumors to the induction of necrosis and regression increased initially with age of the tumors (Table 1). Eight-day-old MOPC315 tumors and especially 9-day-old Meth A tumors were very sensitive. Fifteen-day-old tumors were already necrotic before injection. Nevertheless, the black-stained necrosis induced by endotoxin could be clearly distinguished from necrosis in control tumors and was very extensive. Little or no effects on subsequent tumor growth was observed. Susceptibility of the tumors to the antitumor effects of TNS appeared also to be time-dependent (Table 2).

Compared to Meth A sarcoma a dose of 10^6 viable MOPC315 cells appeared to result in much larger tumors at day 9. Therefore in further experiments we used an inoculum of 3×10^5 MOPC315 cells. This resulted in tumors which at

Table 1. Antitumor effects of endotoxin against MOPC315 and Meth A tumor transplants of different age

Tumor	Day of treatment*	Treatment	Tumor size at day of treatment	Necrosis Incidence	Necrosis Extent	Incidence of: growth stop†	Incidence of: regression‡
MOPC315	5	endotoxin	0.65 ± 0.01	0/5		0/5	0/5
	7	endotoxin	0.89 ± 0.01	2/5	28.3 ± 2.4	n.d.§	0/5
	8	endotoxin	1.06 ± 0.01	5/5	63.6 ± 6.7	1/5	2/5
	15	endotoxin	2.04 ± 0.03	5/5	78.4 ± 4.9	0/5	0/5
	5, 7 or 8	saline		0/15		0/10	0/15
	15‖	saline		5/5	42.5 ± 3.7	0/5	0/5
Meth A	5	endotoxin	0.61 ± 0.02	2/10	33.7 ± 6.4	5/10	1/10
	7	endotoxin	0.69 ± 0.02	1/5	56.3	2/5	0/5
	9	endotoxin	0.73 ± 0.02	4/5	57.8 ± 2.6	4/5	4/5
	15	endotoxin	1.12 ± 0.03	5/5	73.7 ± 2.2	2/5	0/5
	5, 7 or 9	saline		0/20		1/20	0/20
	15	saline		5/5	53.6 ± 3.5	0/5	0/5

*BALB/c mice received 10^6 viable tumor cells s.c. at day 0.
†No increase of tumor size for 2 days following injection of endotoxin.
‡Complete disappearance of the tumor within 2 weeks after injection of endotoxin.
§Not determined.
‖At day 0 3×10^5 viable MOPC315 cells were injected s.c.

Table 2. Susceptibility of MOPC315 and Meth A tumor transplants of different age to induction of tumor necrosis by TNS

Tumor	Day of treatment*	Treatment	Necrosis Incidence	Necrosis Extent	Incidence of: growth stop†	Incidence of: regression‡
MOPC315	5	TNS	0/5		0/5	0/5
	5	NMS	0/5		0/5	0/5
	7	TNS	3/5	53.0 ± 6.2	0/5	0/5
	7	NMS	0/5		0/5	0/5
Meth A	5	TNS	3/10	39.7 ± 4.8	2/10	2/10
	5	NMS	0/10		0/10	0/10
	7	TNS	10/10	56.7 ± 3.0	6/10	5/10
	7	NMS	0/10		0/10	0/10

*BALB/c mice received 10^6 viable tumor cells s.c. at day 0.
†No increase of tumor size for 2 days following injection of serum.
‡Complete disappearance of the tumors within 2 weeks after injection of serum.

day 9 were susceptible to the induction of necrosis and regression by endotoxin (data not shown).

Effect of the tumors on the MPS

Mice bearing 9-day-old Meth A or MOPC315 tumors had significantly increased spleen weights compared to tumor-free controls. Liver weights were only enhanced in Meth A-bearing mice (Fig. 1). The capacity of these mice to clear an i.v. gift of colloidal carbon from the circulation was again only significantly enhanced in the Meth A sarcoma-bearing mice, while the corrected phagocytic index α was not changed in either case (Fig. 2). The influence of the tumors on the listericidal capacity of the mice is indicated in Fig. 3. Both 5 and 15 days after inoculation of Meth A or MOPC315 cells, mice showed an enhanced capacity to reduce the growth of listeria in their spleens, 1 as well as 2 days after i.v. challenge infection. In mice with 9-day-old tumors, however, this only became apparent 2 days after challenge with listeria. On days 5 and 15 enhanced clearance seemed to parallel the

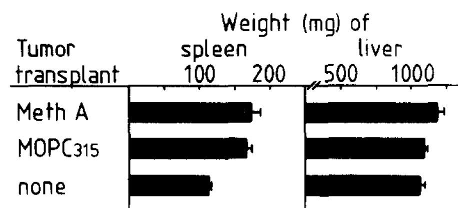

*Fig. 1. Weight of spleen and liver of mice 9 days after s.c. transplantation of 10^6 Meth A cells or 3×10^5 MOPC315 cells (n = 10). Bars indicate mean ± S.E.M. *P < 0.001 compared to tumor-free controls; **P < 0.025 compared to tumor-free controls.*

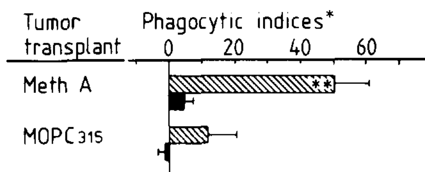

Fig. 2. *Carbon clearance of BALB/c mice* 9 days *after s.c. inoculation of* 10^6 *viable Meth A or* 3×10^5 *viable MOPC315 cells.* * *Phagocytic indices* K (▧) *and* α (■) *of* 10 *mice have been expressed as percentage over or below values measured in saline-treated control mice.* **, P < 0.001.

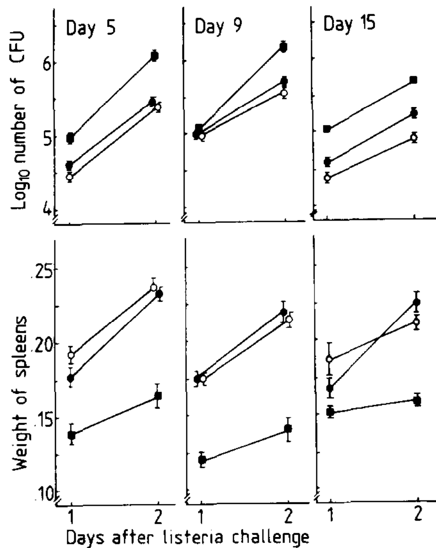

Fig. 3. *Spleen weights and splenic bactericidal capacity of tumor-bearing mice. Groups of* 10 *mice bearing* 5- *or* 9-*day-old transplants of Meth A sarcoma* (●) *or MOPC315 plasmacytoma* (○) *and tumor-free controls* (■) *were injected i.v. with listeria and spleen weights and their content of viable listeria were determined* 1 *and* 2 days *later. Means* ± *S.E.M. are given. In tumor-bearing mice the numbers of CFU per spleen were significantly lower in all instances but* 1 day *after listeria challenge at day* 9 (P < 0.005). *The spleen weights were enhanced in all instances* (P < 0.01), *with the exception of spleen weights of Meth A-bearing mice* 24 hr *after challenge with listeria on day* 15.

increase of spleen weights. On day 9, however, an increase of spleen weights without an increase of listeria clearance was observed.

In vitro *and* in vivo *antitumor activity of serum of tumor-bearing mice after injection of endotoxin*

Mice with 9-day-old Meth A or MOPC315 tumors were injected i.v. with endotoxin or saline and blood was collected 90 min later and serum was prepared. The cytostatic activity of these sera for Meth A cells *in vitro* was compared with that of TNS from Swiss random and BALB/c mice. While the cytostatic activity of serum from saline-treated normal BALB/c mice was equal to that of Swiss random mice, TNS of the latter was significantly more inhibitory than TNS of BALB/c mice (Fig. 4). Compared to serum of

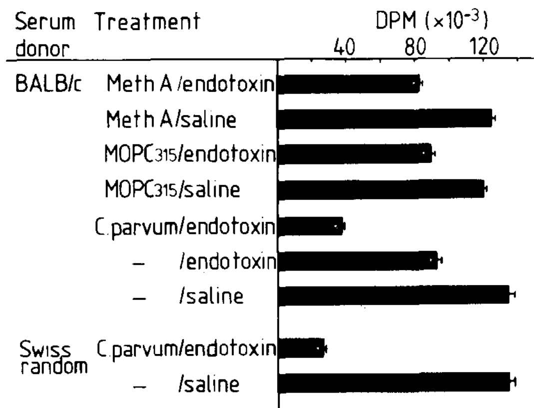

Fig. 4. In vitro *cytostatic activity for Meth A sarcoma cells of sera from mice bearing* 9-*day-old s.c. tumor transplants. Bars indicate mean dpm (multiplied by* 10^3) ± *S.E.M. of individual sera of* 5 *mice.* *P < 0.001 *compared to tumor/saline- and –/saline-treated mice. TNS of BALB/c mice was significantly less inhibitory than TNS of Swiss random mice* (P < 0.001).

normal mice, the presence of a tumor only slightly enhanced the cytostatic activity. A single injection of endotoxin into both normal and tumor-bearing BALB/c mice augmented significantly the cytostatic activity of their serum to about the same extent, but compared to TNS the cytostatic activity was small. All sera were tested for their antitumor effect *in vivo* in an amount of 0.5 ml. None of the sera from the tumor-bearing mice had significant necrotizing or growth-reducing effects on 9-day-old Meth A sarcoma transplants (data not shown).

To get an impression of the sensitivity of the *in vivo* assay for TNF, graded amounts of TNS were injected into Meth A-bearing mice (Table 3). Necrosis and regression were still observed after injection of 0.1 ml TNS, but 0.05 ml had only a minor effect.

Since TNF might be absent from the blood of tumor-bearing mice as a consequence of absorption to the tumor, the effect of incubation of TNS with the tumor target cells on its *in vivo* antitumor activity was evaluated (Table 4). The extent of necrosis and the incidence of regression induced by TNS were moderately reduced by this procedure. NMS tended to gain slight antitumor effects upon incubation with Meth A cells.

DISCUSSION

In this study the time-restricted susceptibility of tumors to the damaging effects of endotoxin described in the literature [4, 5] could be confirmed with both tumors used (Table 1). In addition, a temporal susceptibility to the effects of TNS was observed (Table 2). The latter points at an indirect mode of action of TNF not related to the toxic activity of TNF against tumor cells as observed *in vitro* [7, 22]. The observation that

Table 3. Effect of graded doses of TNS on 9-day-old Meth A transplants

Amount of TNS (ml)*	Necrosis Incidence† dark	light	Extent	Incidence of: growth stop‡	regression§
0.5	5/5	0/5	68.0 ± 3.1	5/5	3/5
0.3	5/5	0/5	61.4 ± 3.6	5/5	3/5
0.1	1/5	3/5	48.6 ± 5.4	2/5	1/5
0.05	0/5	0/5		2/5	0/5
0.5 ml NMS	0/5	0/5		0/5	0/5

*Injected i.v. 9 days after s.c. injection of 10^6 Meth A cells.
†For better discrimination of quantitative effects incidence of black-brown necrosis was scored separately from lighter coloured necrosis.
‡No increase of tumor size within 2 days of injection.
§Complete disappearance of the tumor before day 28.

Table 4. Effect of incubation of TNS with Meth A cells in vitro *on its antitumor effects* in vivo

Serum	Incubation with Meth A*	Necrosis Incidence† dark	light	Extent	Incidence of: growth stop‡	regression§
TNS	0×	5/5	0/5	77.1 ± 1.7	5/5	5/5
	1×	4/4	0/4	63.0 ± 3.8‖	4/4	3/4
	2×	4/5	1/5	64.1 ± 5.4	5/5	3/5
NMS	0×	0/4	0/4		1/4	0/4
	1×	0/5	1/5	49.3	3/5	1/5
	2×	0/5	1/5	57.5	1/5	0/5

*One volume of packed Meth A cells was incubated with 2 vol. of heated TNS or NMS for 1 hr at 37°C with regular stirring. After centrifugation part of the supernatant was incubated similarly with fresh Meth A cells once again. Antitumor effects were determined after i.v. injection of 0.5 ml supernatant into mice with 9-day-old Meth A sarcoma transplants.
†For better discrimination of quantitative effects incidence of black-brown necrosis was scored separately from lighter coloured necrosis.
‡No increase of tumor size within 2 days of injection.
§Complete disappearance of the tumor before day 28.
‖$P < 0.02$ compared to non-incubated TNS.

repeated incubation of TNS with the tumor target cells at 37°C hardly affected its *in vivo* activity (Table 4) also supports this idea. The slight loss of activity may be attributed to unintentional dilution. Other data that suggest an indirect antitumor action of TNF *in vivo* are the inhibition of TNS-induced necrosis by adrenergic receptor antagonists [7] and the presence of extensive vascular changes within the tumor after injection of TNS before the onset of tumor cell degeneration [23].

The putative mediation of the antitumor effects of endotoxin by TNF, and the necessity of an activated MPS for its production, prompted us to study the effects of both tumors on various MPS functions. Mice with 9-day-old Meth A transplants showed significant hepatosplenomegaly and increased carbon clearance capacity, whereas comparable MOPC315-bearing mice had enhanced spleen weights only (Figs 1 and 2). This suggests that hepatomegaly and increased phagocytosis are probably not prerequisite to induction of necrosis by endotoxin. Only in mice with 9-day-old tumors was listeria clearance not enhanced 1 day after listeria challenge (Fig. 3). Induction of necrosis by endotoxin, however, is clearly apparent within 1 day, indicating that enhanced bactericidal activity is also not relevant for induction of necrosis. According to Berendt *et al.* [24], it may be involved in the subsequent tumor regression. The severe necrosis induced in 15-day-old tumors, however, was not followed by regression, despite the presence of a highly activated MPS. This suggests involvement of other systems such as tumor-specific T suppressor cells [24].

The obvious absence of a relation between MPS activity and inducibility of necrosis questions mediation of the endotoxin-induced necrosis in tumor-bearing mice by systemically released TNF. Moreover, the degree of MPS activation induced by both tumors does not surpass that of the *C. parvum* strain NCTC 10387, which was shown to support hardly any TNF production by endotoxin [10, 11]. Much better stimulating coryneforms such as *C. parvum* Wellcome strain

6134 appeared to be needed. Furthermore, TNF could not be demonstrated in the circulation of tumor-bearing mice using 0.5 ml of their sera, while 0.1 ml of TNS elicited unequivocal effects in the same assay (Table 3). Nevertheless, low levels of TNF sufficient for induction of necrosis but not for detection in a transfer assay may be formed in tumor-bearing mice. Moreover, low levels of TNF may be the result of preferentially local production of TNF within the tumor and/or immediate involvement and consumption of the factor in effector mechanisms.

Besides necrotizing activity *in vivo*, TNS was shown to have cytolytic or cytostatic activity against several tumor cell lines *in vitro* [7, 22]. All activities have been attributed to TNF [22]. Our experiments confirmed both activities of TNS (Table 3, Fig. 4), but necrotizing as well as substantial cytostatic activity were absent from serum of endotoxin-treated tumor-bearing mice. This might imply that the latter serum is devoid of TNF. The observation, however, that about 40% of the cytostatic activity of TNS can be removed by a single absorption with Meth A cells at 4°C [20] while the necrotizing activity is rather refractory to absorption suggests that different factors or several cytostatic factors are involved. The latter is supported by data showing that only one out of three distinct cytotoxic factors isolated from TNS had necrotizing activity [25]. The observed prevention of endotoxin-induced hemorrhagic necrosis, without affecting endotoxin-induced mitotic arrest within Meth A tumors by the α-adrenergic receptor antagonist phenoxybenzamine, is also compatible with this idea. Absorption of cytostatic factors other than TNF to the tumor may thus explain the low cytostatic activity of post-endotoxin sera of tumor-bearing mice and the mitotic arrest of Meth A tumors upon injection of endotoxin or TNS [23, 26].

Regarding the effect of Meth A tumors on MPS function, we have observed normal bactericidal and increased pinocytic activity simultaneously (Figs 2 and 3). This is at variance with recent data showing a linear correlation between resistance against infective agents and carbon clearance in mice after administration of various immunostimulants and/or an immunosuppressive agent [27]. Our results suggest at least that different mechanisms are underlying both MPS functions.

It can be concluded that TNF, like endotoxin, probably acts by an indirect mechanism against tumors *in vivo*. Furthermore, mediation of the antitumor effects of endotoxin by TNF still remains uncertain, though additional factors seem to be involved.

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