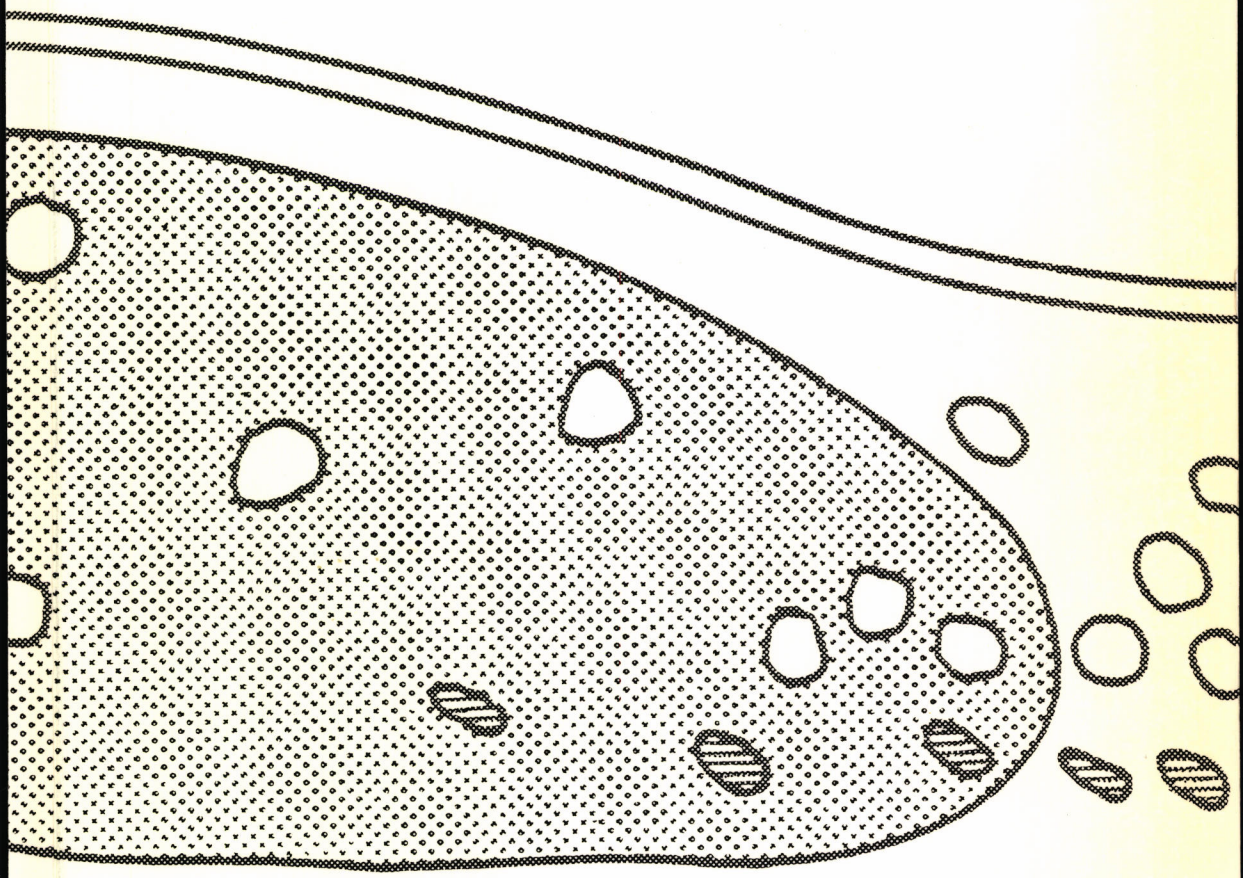
Endotoxin-induced necrosis in murine Meth A sarcoma

A histopathological study



C. Frieke Kuper

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Door endotoxine geïnduceerde necrose van het Meth A sarcoom in de muis.
Een histopathologisch onderzoek.

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor in de geneeskunde aan de Rijksuniversiteit te Utrecht, op gezag van de Rector Magnificus Prof. Dr. J.A. van Ginkel, volgens besluit van het College van Decanen in het openbaar te verdedigen op dinsdag 25 november 1986 des namiddags te 4.15 uur

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CONTENTS

* Data presented in these chapters have been previously published in the PhD thesis of N. Bloksma, Utrecht, The Netherlands (1982). They are included in this thesis because of consistency with and better understanding of the remaining data.

ABBREVIATIONS

AP	acid phosphatase
B cell (lymphocyte)	bursa equivalent-derived lymphocyte
CN	coagulation necrosis
Cp	Corynebacterium parvum
GT	granulation tissue
HN	hemorrhagic necrosis
i.p.	intraperitoneal(ly)
i.t.	intratumorally
i.v.	intravenous(ly)
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
LPSw	lipopolysaccharide, prepared according to Westphal (phenol/water extraction)
MEM	minimal essential medium
MDP	muramyl dipeptide
nm	not measured
NMS	normal mouse serum
NSE	non-specific esterase
p	probability
PALS	periarteriolar lymphocyte sheath
PBS	phosphate buffered saline
rTNF	recombinant tumor necrosis factor
s.c.	subcutaneous(ly)
SEM	standard error of the mean
T cell (lymphocyte)	thymus-derived lymphocyte
TNF	tumor necrosis factor
TNS	tumor necrosis serum

CHAPTER 1

GENERAL INTRODUCTION

Endotoxins.

Bacterial endotoxins were first defined by their ability to produce fever. Since then, interactions of these agents with almost all host humoral and cellular mediation systems have been defined and characterized in parallel with aspects of the biochemical nature of endotoxins (reviewed e.g. by Thomas, 1954; Morrison and Ryan, 1979; Rietschel et al., 1982; Nowotny, 1985). The interactions lead to multiple biologic effects among which is antitumor activity. Endotoxins are components of the outer membrane of gram-negative bacteria. They are unique to these organisms. Although endotoxins from different groups of gram-negative bacteria vary widely in their chemical fine structure, they are built up according to a common principle. In contrast to bacterial exotoxins, different endotoxins induce similar biological reactions. It must, however, be stressed that the actions of endotoxins on biological systems depend on various factors. Host factors that influence the effects of endotoxin are species, genetic make up, nutritional, hormonal and disease states and past contact with endotoxin (Westphal, 1975; Greer and Rietschel, 1978). In addition, the bacterial source, purity and composition of the endotoxin preparation determine its biological effect (Rietschel et al., 1982).

Endotoxins contain lipopolysaccharide and protein. Almost all effects can be attributed to the lipopolysaccharide portion. Purified protein-free lipopolysaccharide (LPS) can be prepared by phenol-water extraction according to Westphal et al. (1952). This LPS is considered artefactual since it does not occur in the environment as such. It consists of a polysaccharide part with a covalently bound lipid moiety. The lipid moiety, called lipid A, has a relatively constant composition and structure among the various bacterial sources. In a large group of gram-negative bacteria, lipid A consists of an ester-linked glucosamine disaccharide with two phosphate residues, and four ester- and amide-linked fatty acids (Rietschel et al., 1977). Lipid A

was shown to be the toxic moiety of complete LPS. It is attached to the polysaccharide component through 2-keto-3-deoxyoctonate or 2-deoxy-3-manno-octulosonic acid (KDO). Rough mutant strains from several gram-negative bacteria were found to synthesize LPS with incomplete polysaccharide. The most defective mutants (Re) synthesize LPS consisting of KDO residues and lipid A only (Galanos, 1975; Westphal, 1975). This LPS in aqueous solutions still had strong antitumor effects (Bloksma et al., 1984b). Hydrolysis of KDO-lipid A of *Salmonella typhimurium* Re, according to the procedures described by Ribi et al. (1982), yielded a preparation virtually free of KDO and with about one-half of the phosphate content of the KDO-lipid A. In vivo studies demonstrated that the hydrolyzed endotoxin was almost non-toxic and that in aqueous solutions it had no significant antitumor effects (Ribi et al., 1982; Bloksma et al., 1984a). The polysaccharide portion of LPS consists of a 'core oligosaccharide', directly linked to KDO and a chain of a polymer of oligosaccharide molecules, the 'O-polysaccharide'. The latter has been found to be largely responsible for the antigenic heterogeneity of LPS.

The phenomenon of endotoxin-induced tumor necrosis.

History

In the last century, attention was attracted to the beneficial effects of bacterial infections on tumor-bearing patients (Busch, 1866; Coley, 1898 in: Morrison and Ulevitch, 1978). Coley systematically explored the potential use of a mixture of *Streptococcus erysipelas* (*Streptococcus pyogenes*) and *Serratia marcescens* culture filtrates on the management of inoperable tumors. These so-called 'Coley toxin' preparations were applied by numerous physicians, but the marked variability of the therapeutic outcome diminished the interest in the preparations (reviewed by Shear and Perrault, 1943).

The experimental work of Gratia and Lintz (1931) renewed the interest for the antitumor effects of bacterial preparations. They showed that culture filtrates of *Escherichia coli* induced hemorrhage, necrosis and complete regression of transplanted liposarcomas in the guinea pig. Since then, similar experiments were done with several types of tumors, animal species

and hemorrhage- and necrosis-inducing agents by numerous workers (reviewed by Shear and Perrault, 1943; Morrison and Ryan, 1979). An overview of the results will be given here, arranged by subject rather than in chronological order. Various factors were found to influence the reaction of the tumor to the tested agents.

Firstly, the tumor-animal model used was of importance. In mice, transplanted sarcomas appeared more susceptible than carcinomas. The susceptibility of tumor transplants was found superior to that of induced primary or spontaneous tumors (Duran-Reynals, 1935; Andervont, 1936; Shear and Perrault, 1943; Bober et al., 1976). The tumor transplants used in these studies were inoculated in the subcutis or dermis. Intraperitoneally transplanted ascitic tumors showed no growth inhibition (Parr et al., 1973). The effectiveness of endotoxins depended also on the size or age of the tumor. Very young, small tumors were hardly susceptible. Generally, the larger the tumor, the more severe and extensive was the hemorrhage (Andervont, 1936; Parr et al., 1973; North, 1981). However, large ulcerated tumors had a decreased reactivity, attributed to induction of resistance against the hemorrhage-inducing agent by bacterial contamination of the ulcerated tumor (Gardner et al., 1939 in: Shear and Perrault, 1943). In addition, marked differences in susceptibility were found between various tumor-bearing mouse strains (Andervont, 1936). As differences in antigenicity and in sensitivity to the toxicity of endotoxins were found between several healthy animal species (Westphal; 1975), it can be expected that the antitumor effects also vary between species.

Secondly, the source and methods of preparation of the hemorrhage- and necrosis-inducing bacterial compounds were of importance. Most of the early work was done with crude filtrates of bacterial cultures, often obtained from mixed cultures of several gram-positive and -negative strains. Already in the 1940's it was suggested that at least one of the components, active in tumor necrosis and present in 'Coley toxin' preparations was endotoxin (Zahl et al., 1942). This was confirmed by work of Shear and coworkers (1943). As is outlined in the previous pages, endotoxins from various bacterial sources differ in their interactions with the host mediation systems. The presence of components which affect particle size and solubility of the preparations might play a crucial role in this (Rietschel et al., 1977). It is therefore not surprising that the crude bacterial filtrates had markedly

divergent antitumor activity. The availability of purified endotoxins and its components permitted investigations of the relation between the beneficial antitumor effects of endotoxin and its much emphasized, serious side effects. Up till now, only a few studies demonstrated a separation between toxicity and antitumor activity (Ribi et al., 1982; Bloksma et al., 1984b).

Thirdly, the route of application was shown to determine the antitumor activity of preparations of gram-negative bacteria. Culture filtrates or purified endotoxin were effective when injected intravenously, intratumorally or intraperitoneally, but less effective when administered orally or subcutaneously (Shear and Perrault, 1943; O'Malley et al., 1962; Parr et al., 1973; Yarkoni et al., 1979). Multiple injections were no more effective than a single treatment (reviewed by Shear and Perrault, 1943; O'Malley et al., 1962).

Not only preparations of gram-negative bacteria were effective, but also intravenous injection of rattle snake venom (Apitz, 1933) and of glycogen (Stetson, 1951), and intraperitoneal injection of several vasoactive agents among which were histamine and serotonin (Pradhan et al., 1957), and double stranded RNA's (Parr et al., 1973), all induced tumor hemorrhage and necrosis. These data have to be evaluated critically because contamination of these agents with endotoxins was not excluded. The macrophage product, tumor necrosis factor (TNF), induced by endotoxins, also had the same antitumor properties (Carswell et al., 1975). Preparations of TNF appeared not to be free of contaminating endotoxin (specified as less than 0.022 μg/ml as measured by the rabbit pyrogenicity test; Carswell et al., 1975). Even a temporary mechanically induced vascular occlusion induced necrosis in tumors (Youngner and Algire, 1949). Muramyl dipeptide was not effective when injected alone but highly potentiated the antitumor effects of toxic endotoxin. In addition, combination of muramyl dipeptide with detoxified endotoxin, both ineffective alone, had strong antitumor activity (Bloksma et al., 1984b).

Besides therapeutic activity, endotoxins were shown to have, under certain circumstances, prophylactic properties (Parr et al., 1973). On the other hand, a few authors reported potentiation of the tumor growth when endotoxin was given concomitantly with tumor transplantation (Kearney and Harrop, 1980).

Morphology

Most morphologic descriptions of endotoxin-induced tumor damage are found in the older studies using filtrates of bacterial cultures, containing undefined amounts of endotoxin besides many possible contaminants. So the phenomena observed cannot be reliably attributed to the effects of endotoxin alone. Moreover, size and age of the tumors were not always explicitly mentioned. Although recent studies have shown that the dose of endotoxins (Bloksma et al., 1984c) as well as the size (age) of the tumors (North, 1984; Bloksma et al., 1984a) determined the effectivity of the treatment, morphologic effects observed in older studies were remarkably consistent. Only periods between treatment and appearance of effects varied somewhat.

A discolouration of the tumor, bluish (Andervont, 1936) or red (Parr et al., 1973), was the first macroscopic effect, appearing within hours after treatment. Within 24 h, the discolouration darkened and the underlying tumor tissue became soft (Parr et al., 1973). On section, the tissue was hemorrhagic (reviewed by Shear and Perrault, 1943). Tumors that eventually regressed completely, hardened in the next days and collapsed, leaving a dry scab that eventually fell off (Parr et al., 1973; Bloksma, 1982). When regression was incomplete, the remaining vital tissue was seen in the periphery of the tumor (Shear and Perrault, 1943). Microscopically, the most prominent phenomenon within and sometimes immediately around the tumor was a circulatory disturbance, described as hyperemia, congestion or vasodilatation and observed within hours, which was consistent with the macroscopically observed discolouration (Apitz, 1933; Andervont, 1936; Algire et al., 1947; Algire and Legallais, 1951; Stetson, 1951; Parr et al., 1973). This was followed by extravasation of erythrocytes within 24 h. A detailed description of both untreated and treated tumors (a carcinoma), has been given by Apitz (1933). He observed hemorrhage especially at the border between spontaneously necrotic and vital tumor tissue and edematous swelling of tumor cells in both the hemorrhagic and non-hemorrhagic areas. In sarcomas, hemorrhage was usually found throughout the tumor (reviewed by Shear and Perrault, 1943; Andervont, 1936). Only Andervont (1936) described vascular rupture with extensive hemorrhage. Capillary thrombosis, varying from very slight to marked, was observed in almost all the above-mentioned studies,

early after treatment. There was little discussion on the role of inflammatory cell infiltrates. Leukocytes were seen within the tumor capillaries, 2 h after intravenous injection of bacterial culture filtrates (Andervont, 1936). North (1981) stated that host cells were far outnumbered by tumor cells, after treatment with endotoxin, leaving a direct cytotoxic action of the inflammatory cells doubtful. In a study on intralesional administration of endotoxin in saline, coagulative and colliquative necrosis of the tumor with marked infiltration of polymorphonuclear inflammatory cells has been described 3 days after treatment, leaving nothing but granulation tissue at day 7 (Yarkoni et al., 1979).

Theories on endotoxin-induced tumor necrosis

The mechanisms underlying the antitumor activity of endotoxin are still a matter of debate, although its effects have been studied systematically since the 1930's. It is, however, generally agreed that endotoxin acts indirectly via host mediation systems. The many theories that have been proposed have been reviewed e.g. by Bloksma (1982). They are briefly outlined here.

Vascular effects. In morphologic studies, antitumor effects of endotoxin have been attributed to a hemorrhagic reaction. The changes observed in the tumor were often related to or identified with a local Shwartzman reaction. The latter has been described as a severe hemorrhagic reaction with necrosis observed in rabbits, which were primed with an endotoxin preparation into the skin and about 24 h later were injected intravenously with the same or an unrelated endotoxin preparation (Shwartzman, 1928). The tissue injury has been attributed to occlusion of the local vessels by thrombi, generated by the endotoxin-activated coagulation system. Tumor necrosis could be provoked by a single (systemic or local) injection. Although there are similarities between the two reactions, there are also some important differences. A local Shwartzman reaction could only be induced in T-cell deficient nude mice (De Clerq and De Somer, 1980). Moreover, agents or conditions which could modulate incidence and severity of the reactions differed. For instance, anticoagulants strongly inhibited the Shwartzman reaction (Thomas et al., 1955), while these agents did not or only occasionally inhibited

induction of tumor necrosis by endotoxins (Parr et al., 1973; Bloksma et al., 1984c). The varying effects of anticoagulants on both reactions can be associated with differences in histopathology. Marked thrombosis was always observed in local Shwartzman reactions, but was not consistent in tumor necrosis. While in the Shwartzman reaction, decreased blood supply by intravascular thrombi was held responsible for hypoxia and necrosis of the affected tissue, tumor necrosis might be due to stasis of blood flow as a consequence of an abnormal reaction of the tumor vasculature to endotoxin. This theory is based on the observation that the tumor was especially sensitive to changes in blood flow, either induced mechanically or by hypo- and hypertensive agents (Algire et al., 1947; Youngner and Algire, 1949; Pradhan et al., 1957). Endotoxin was shown to cause severe hypotension (Algire and Legallais, 1951). Moreover, endotoxin could release vasoactive agents such as adrenaline, serotonin and histamine (Rosenberg et al., 1959; Emerson, 1985) all found to induce tumor necrosis (Pradhan et al., 1957). It is still not clear why the tumor vasculature is more sensitive than normal tissue vasculature to systemic vascular changes induced by endotoxin.

Metabolic effects. Although in most morphologic studies the hemorrhagic reaction within the tumor was considered as the prominent factor in the induction of tumor necrosis, some doubt was raised by Apitz (1933), as necrosis also occurred in the non-hemorrhagic area. In his opinion, at least part of the necrosis was caused by some unknown, but non-vascular effect on the tumor cells. Later, Jones (1979) postulated that endotoxin-induced tumor cell damage was a result of selective mitochondrial injury within tumor cells. This theory was based on the observation that endotoxin caused an uncoupling of oxidative phosphorylation in a murine tumor transplant. Metabolic products from adipose tissue and also catecholamines were thought to be potential uncoupling agents. Although morphological aspects of mitochondrial damage were not reported, a role for metabolic changes in tumor necrosis cannot be excluded.

TNF-mediated effects. Another, rather commonly adopted theory on the mechanism of endotoxin-induced tumor necrosis and regression is that the effects are mediated by a single factor, produced by macrophages upon contact with endotoxin. This factor has been called tumor necrosis factor (TNF). Serum with high levels of TNF could be obtained from blood withdrawn about 2 h after injection of toxic endotoxins in animals on the condition

that they had been pretreated with a strong activator of the mononuclear phagocyte system (Carswell et al., 1975; Green et al., 1977; Bloksma et al., 1982a). Such sera or partially purified fractions of TNF could mimic the antitumor activity of toxic endotoxins completely after iv administration. In vitro these sera or their fractions appeared cytostatic or cytolytic to a great variety of tumor cell lines, but not to normal cells (Carswell et al., 1975; Old, 1976; Green et al., 1977). Therefore it was stated that endotoxin would release TNF upon injection in tumor-bearing animals in such amounts, that it would cause necrosis and complete regression by its direct cytotoxic antitumor action. Considerable circumstantial data, however, have cast doubt on the role of TNF as a unique as well as directly acting mediator (Bloksma et al., 1984c).

Recently, TNF has been produced by means of recombinant DNA techniques (Pennica et al., 1984). Because in vivo activity appeared much more pronounced than in vitro activity on a per tumor cell basis, it was suggested that TNF would act by indirect mechanisms in vivo, as does endotoxin (Sugarman et al., 1985). Recent data indicated that TNF is identical to cachectin (Beutler et al., 1985a). This macrophage factor is thought to be responsible for the cachexia observed in organisms with gram-negative sepsis, parasite infections and large tumor burdens (Torti et al., 1985; Beutler et al., 1985b). Its role as mediator of the toxic effects of endotoxins was confirmed by experiments showing that antibodies to TNF/cachectin protected against the lethal effects of endotoxin (Beutler et al., 1985b).

Complex, partly immune-dependent effects. Endotoxin was shown to have effects on almost all elements of the immune system (reviewed e.g. Morrison and Ryan, 1979; Nowotny, 1985). Nevertheless, endotoxin-induced tumor necrosis seemed not to be immunologically mediated because it could be induced in both immunogenic and non-immunogenic tumors, in the absence of immunocompetent T-lymphocytes and in the presence of established tumor-specific T-suppressor cells (Parr et al., 1973; North, 1981; Kodama et al., 1982).

The immune system, however, probably plays a key role in the induction of complete regression following tumor necrosis. Non-immunogenic tumors generally did not regress and resumed growth after a slight delay. Also immunogenic tumors usually did not show complete regression, unless endotoxin was injected during a state of concomitant immunity (Berendt et al., 1978b;

North, 1981 and 1984). Based on these data North (1984) has stated that induction of tumor necrosis and complete regression by endotoxin are separate, although related events.

The Meth A fibrosarcoma.

The tumor model used in the experiments described in this thesis was comparable with that used in the TNF experiments of Carswell et al. (1975) and similar to the majority of the experiments of Bloksma et al. (1982; 1984c). The tumor has been induced by methylcholanthrene in female BALB/c mice and has been classified as strongly immunogenic (Old et al., 1962; Livingston et al., 1983; 1985). Despite its immunogenicity, Meth A showed an almost undisturbed progressive growth in syngeneic and semisyngeneic mice. Growth rates appeared essentially the same as those observed in T-cell-depleted and thymus-less nude mice (North, 1984). Upon intradermal transplantation of 10^6 Meth A cells in semisyngeneic mice an evanescent delayed hypersensitivity reaction (optimal around day 5; Giacomo and North, personal communication) followed by a transient period of T cell specific concomitant immunity between day 6 and 9 was demonstrated (Berendt et al., 1978b; North, 1981; 1984). The observed rapid decay of concomitant immunity after day 9 has been attributed to the establishment of specific suppressor T lymphocytes (North and Bursuker, 1984; Bursuker and North, 1984).

Meth A tumors appeared not to induce specific antibodies in syngeneic, allogeneic as well as xenogeneic animals under normal conditions. Specific antibodies could be induced by pretreatment with cyclophosphamide combined with defined adjuvants (Livingston et al., 1983; 1985).

An enhanced nonspecific bactericidal capacity was observed in the spleen of BALB/c mice with 5-, 9- and 15-day old Meth A tumors (Berendt et al., 1978a; Bloksma et al., 1984e).

Meth A sarcoma is considered to be a virtually non-metastasizing tumor (North, 1984; Reichert et al., 1985).

Other properties of Meth A sarcoma are its relatively high resistance to gamma-irradiation and cytostatic agents like cyclophosphamide (North, 1984).

Purpose of the study.

The experiments described in this thesis were aimed at further elucidation of the mechanisms leading to the induction of necrosis in solid Meth A tumors after i.v. injection of endotoxin.

The macroscopic observations that α- and β-adrenoceptor antagonists modulated endotoxin-induced antitumor effects in vivo (Bloksma et al., 1982b), were substantiated by studying the histopathological events (Chapter 2). Furthermore, we compared the histopathology of tumor necrosis induced by endotoxin with that induced by agents acting at α- or β-adrenergic or serotonin receptors (Chapter 3).

As the role of inflammatory cells in the induction of tumor necrosis by endotoxin is still far from clear, the effect of endotoxin on inflammatory cells within the tumor was studied by means of cytocentrifuge preparations of enzymatically dispersed tumors (Chapter 4). Centres and margins of the tumors were processed separately, because previous studies (Bloksma et al., 1983b; Chapters 2 and 3) had shown that tumor cell damage inflicted upon these areas by endotoxin, differed markedly.

The frequent observation that induction of tumor necrosis by endotoxin depended on the age or size of the tumor, prompted a microscopical study in order to verify the macroscopical data and to acquire more insight in the conditions within the tumor defining susceptibility to induction of necrosis by endotoxin. The histological examination consisted of a morphometric study of histologic changes in the tumors and regional lymph nodes (Chapter 5), and an ultrastructural study with special attention to the effects on tumor vascular structures and inflammatory cells (Chapter 6).

In the last two experimental chapters, effects of combinations of muramyl dipeptide (MDP), a potentiator of the endotoxin effects, with toxic and detoxified endotoxin of *Salmonella typhimurium* Re were studied. The histopathological effects of these combinations were compared with those of the separate constituents to unravel the mechanism underlying the potentiation by MDP (Chapter 7). Because the spleen is known to be involved in the toxic and immunological effects of toxic endotoxin and to reflect changes in the immune status of the host during tumor growth, splenic changes of tumor-bearing mice upon treatment with the combinations were investigated to find out whether these could be related to antitumor action (Chapter 8).

CHAPTER 2

INFLUENCE OF ADRENOCEPTOR BLOCKADE ON ENDOTOXIN-INDUCED HISTOPATHOLOGICAL CHANGES IN MURINE METH A SARCOMA

C. Frieke Kuper, Nanne Bloksma, Frans M.A. Hofhuis, Joost P. Bruyntjes and Jan M.N. Willers

SUMMARY. Histological examination of subcutaneous murine Meth A sarcomata 4 h after administration of endotoxin revealed an overt but superficial hyperemia, which had largely disappeared after 24 h. At this time hemorrhagic necrosis could be observed in that region mainly featured by degenerating tumor cells, diffuse hemorrhage and blood vessels filled with a yellow substance probably derived from disintegrated erythrocytes. Administration of the α-adrenoceptor antagonist phenoxybenzamine or the β-adrenoceptor antagonist propranolol caused decrease of hyperemia at 4 h but an increase at 24 h especially after phenoxybenzamine. Only the latter agent prevented the induction of hemorrhagic necrosis by endotoxin. Endotoxin reduced tumor size within 24 h, which was prevented by both adrenoceptor blocking agents. Administration of these agents alone even enhanced tumor size. Mitotic activity of Meth A sarcoma was greatly reduced already 4 h after endotoxin injection. This was not significantly changed by both adrenoceptor antagonists, while these agents alone probably stimulate tumor cell division between 4 and 24 h after administration. The histological data are discussed in relation to earlier data on the influence of adrenoceptor blockade on endotoxin-induced hemorrhagic necrosis and tumor regression.

INTRODUCTION

Intravenous injection of endotoxin in BALB/c mice bearing Meth A sarcoma causes hemorrhagic necrosis and growth retardation of the tumor, followed by

complete regression in part of the mice. Both hemorrhagic necrosis and regression have been attributed to an induced factor, called tumor necrosis factor (TNF; Carswell et al., 1975; Hoffmann et al., 1978). On the other hand, Berendt et al. (1978a, 1978b) consider hemorrhagic necrosis and tumor regression to be separate events.

In a previous paper the effect of α- and β-adrenoceptor blockade on anti-tumor effects of endotoxin was described (Bloksma et al., 1982b). Pretreatment with the α-adrenoceptor blocking agent phenoxybenzamine obliterated the growth retardation of the tumor induced by endotoxin and diminished the frequency of regression. Furthermore both incidence and extent of hemorrhagic necrosis were reduced. Although in general a large hemorrhagic necrotic area was attended with tumor regression, no such relation was found following pretreatment with phenoxybenzamine. Pretreatment with a high dose of the β-adrenoceptor blocking agent propranolol potentiated the effect of endotoxin on tumor growth but did not influence the incidence and extent of hemorrhagic necrosis induced by endotoxin.

In the present report we describe the histopathological events in Meth A sarcoma after administration of endotoxin and the effect of α- or β-adrenoceptor blockade prior to endotoxin administration.

MATERIALS AND METHODS

Materials. Endotoxin or LPS w from *E. coli* 0111:B4 was obtained from Difco Laboratories (Detroit, MI, U.S.A.) and DL-propranolol. HCl from Sigma Chemical Company (St. Louis, MO, U.S.A.). Phenoxybenzamine (Dibenyline) was kindly provided by Smith, Kline & French Laboratories Ltd. (The Hague, The Netherlands).

Tumor. The Meth A fibrosarcoma was obtained from the Clinical Research Centre (Harrow, Middlesex, U.K.) and maintained in ascitic form by serial intraperitoneal (i.p.) passage in syngeneic BALB/c mice. Cells from 5 to 6 days old tumor transplants were collected in Eagle's minimal essential medium and washed at least three times by low gravity centrifugation till

free from erythrocytes. Viable cell numbers were determined by trypan blue exclusion.

Animals and injections. Female BALB/c mice were bred and maintained at the Laboratory of Microbiology (Utrecht, The Netherlands). They were used at an age of about 12 weeks (weight approximately 20 g).

Groups of 4 mice were injected subcutaneously (s.c.) with 10^6 viable Meth A cells on the abdomen. At day 9 they received an intravenous (i.v.) injection of 25 μg endotoxin. Phenoxybenzamine (300 μg) and propranolol (1 mg) in 0.5 ml saline were administered i.p. 15 min before endotoxin. Controls received saline.

Histological procedures. Tumors were carefully removed 4 or 24 h after endotoxin injection and fixed in phosphate-buffered 4% formaldehyde solution. Paraffin-embedded sections (5 μm) made in dorso-ventral direction from the central part of the tumor, were stained with haemotoxylin and eosin and examined microscopically. Combined hemoglobin-hemosiderin staining was performed according to Puchtler and Sweat (1963) with potassium, ferrocyanide, tannin, phosphomolybdenic acid and phloxin.

Quantitation of morphology of the tumors. Areas of the tumor sections or parts of them were determined with a planimeter on photomicrographs taken at known magnification. Hemorrhagic necrosis was scored as moderate if less than 5% of the surface of the tumor section consisted of tissue with features of hemorrhagic necrosis and as marked if the necrotic area was more extended. Hyperemia was scored normal if less than 25 transections of blood-vessels could be observed in the whole tumor at 100x magnification. It was classified as moderate if the number of transections was between 25 and 60 and as marked if this number was over 60.

Mitotic indices were determined by means of an ocular square grid at 1000x magnification. In this way about 45-55 cells per square were observed, representing 0.01 mm^2 of the tumor section. The numbers of dividing cells at 10 different sites of each tumor section (Fig. 1) were counted. The mitotic

index was calculated as number of dividing cells per square mm. From this number and the area of the whole section the number of dividing cells per section was calculated.

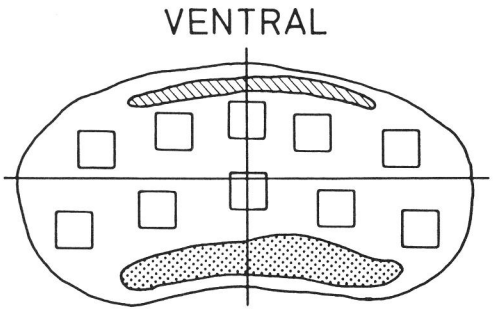


Fig. 1. Schematic survey of a Meth A sarcoma section with hemorrhagic necrosis (≋). The dotted area indicates coagulation necrosis. Squares indicate the sites of the tumor at which dividing cells were counted.

Statistics. Means and standard error of the mean of mitotic indices and tumor section surfaces were determined. Statistical significance was determined by the Mann-Whitney U-test (Siegel, 1959).

RESULTS

Histological features of tumors with hemorrhagic necrosis induced by endotoxin.

Twenty-four hours after i.v. injection of endotoxin in tumor-bearing mice the centre of the tumors showed a clearly visible blackening. Histological examination revealed scattered areas of coagulation necrosis and an extensive region of coagulation necrosis on the base in all tumors, irrespective of the treatment (Fig. 2a). Four hours after injection of endotoxin overt

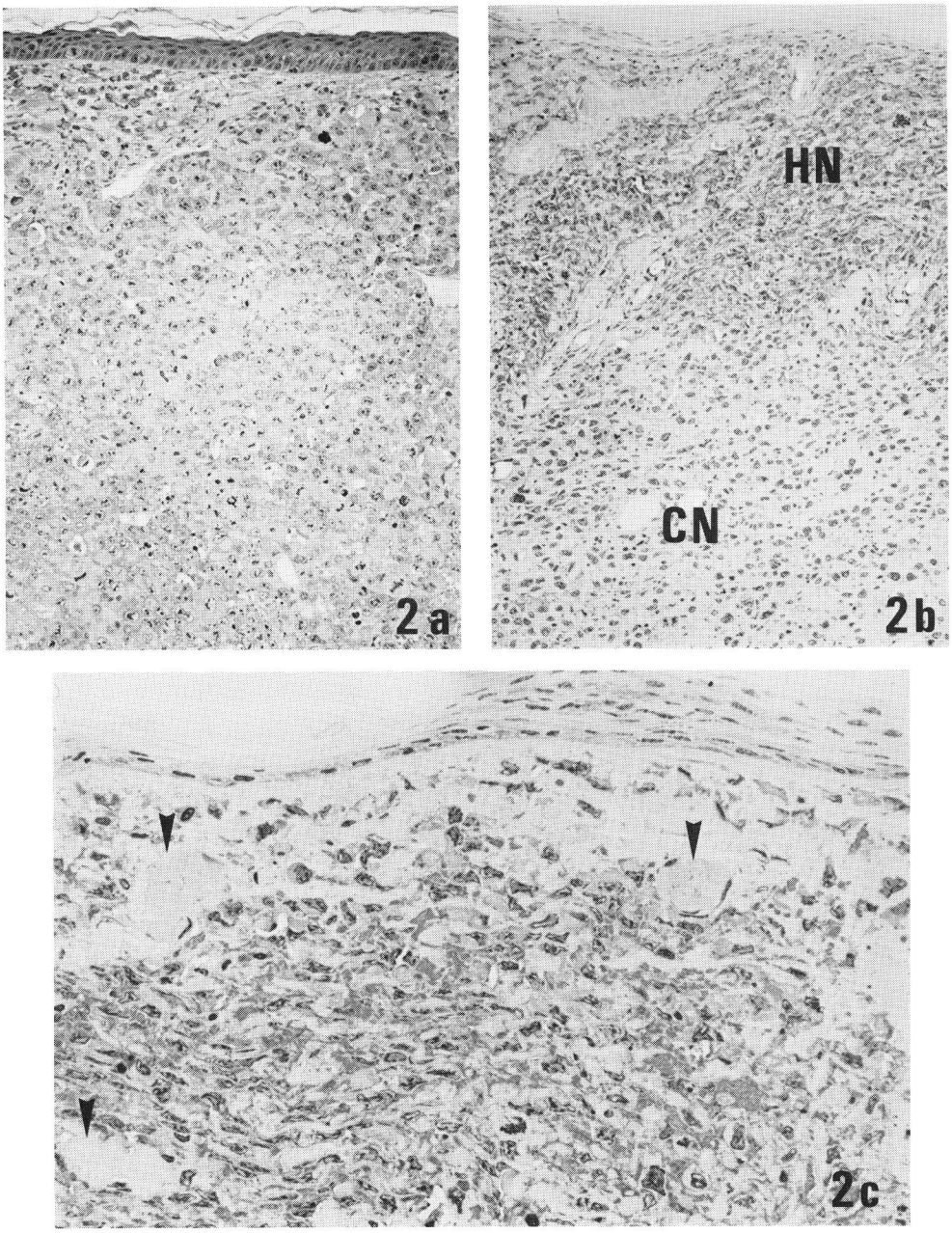


Fig. 2. Histology of Meth A sarcoma 24 h after injection of saline (a, 55x), endotoxin (b, 55x; c, 160x). HN: area with hemorrhagic necrosis; CN: area with coagulation necrosis; arrow head: parallel alignment of tissue; arrow: dilated vessels filled with yellow granular material.

dilation of bloodvessels was observed mainly at the skin side of the tumor. At this site hemorrhagic necrosis is apparent at 24 h, characterized by parallel layers of degenerating tumor cells with elongated hyperbasophilic nuclei, diffuse hemorrhage and blood vessels filled with a yellow granular substance which was also visible in unstained sections (Fig. 2b and c). This material stained weakly for hemosiderin. Besides hemorrhagic necrosis in the remaining part of the tumor marked degenerative changes like pyknosis, karyorrhexis and cytoplasmic eosinophilia were observed. In the areas with hemorrhagic necrosis and degeneration little or no inflammatory infiltrate could be observed.

Influence of adrenoceptor blockade on endotoxin-induced hemorrhagic necrosis and hyperemia.

Already 4 h after a single injection of endotoxin one mouse showed some hemorrhagic necrosis, while no hemorrhagic necrosis was observed in mice of the other groups (Table 1).
One day after administration of endotoxin all mice showed moderate to marked hemorrhagic necrosis. Pretreatment with the α-adenoceptor antagonist phenoxybenzamine prevented the induction of hemorrhagic necrosis. The β-adrenoceptor antagonist propranolol did not considerably alter the effects of endotoxin. In the groups of animals only treated with phenoxybenzamine or propranolol one mouse showed hemorrhagic necrosis.

Endotoxin induced hyperemia 4 h after administration, which had almost completely disappeared after one day. Pretreatment with phenoxybenzamine tended to impair the induction of hyperemia by endotoxin after 4 h, but after 24 h hyperemia was very pronounced as expressed both in numbers of transected vessels and in extent of dilation. Incidentally fibrin deposition was observed. β-Adrenoceptor blockade with propranolol prior to endotoxin caused about the same effect on hyperemia as pretreatment with phenoxybenzamine 4 and 24 h after endotoxin. At 24 h however vessels were not extremely dilated. Phenoxybenzamine and propranol alone did not cause hyperemia.

Table 1 - Effects of adrenoceptor antagonist injection prior to endotoxin on hemorrhagic necrosis and hyperemia of Meth A sarcoma

Treatment combinations	Hours after last injection	Hemorrhagic necrosis			Increase of hyperemia		
		none	moderate	marked	none	moderate	marked
Saline/ endotoxin	4	3[a]	1	0	0	1	3
	24	0	1	3	3	1	0
Phenoxybenzamine/ endotoxin	4	4	0	0	0	3	1[b]
	24	4	0	0	0	1	3[b]
Propranolol/ endotoxin	4	4	0	0	1	2	1[b]
	24	1	0	3	0	2	2
Phenoxybenzamine/ saline	4	4	0	0	3	1	0
	24[c]	2	1	0	2	1	0
Propranolol/ saline	4	4	0	0	4	0	0
	24	3	1	0	4	0	0
Saline/ saline	4	4	0	0	4	0	0
	24	4	0	0	4	0	0

a. Figures indicate incidence based on 4 mice in each experiment

b. In these instances vessels were extremely dilated

c. n = 3

Influence of endotoxin and/or adrenoceptor blockading agents on mitotic activity and size of the tumor.

Tumor size was measured from sections of tumors excised 4 and 24 h after endotoxin or saline injections. The tumor size was not changed at 4 h but 24 h after endotoxin tumor size was reduced (Table 2).
This decrease was nullified by pretreatment with phenoxybenzamine. Injection of the latter agent without endotoxin increased tumor size significantly above the size of control tumors. Propranolol treatment enhanced tumor size even in endotoxin treated mice.

The mitotic index was the same in control tumors at 4 and 24 h and this was not changed by a single i.p. injection with phenoxybenzamine or propranolol (Table 2). Endotoxin reduced the mitotic index 5 - 10 fold. This effect was not inhibited by prior α- or β-adrenoceptor blockade. The total number of dividing cells per tumor section was about the same 4 and 24 h after injection of saline but was decimated after injection of endotoxin.

Table 2 - Tumor size and mitotic activity after administration of endotoxin and/or adrenoceptor blocking agents

Treatment combinations	Hours after last injection	Surface of tumor section (mm^2)[a]	Mitotic index[a]	Number of mitoses per tumor section[a]
Saline/ endotoxin	4	13.7±1.3	0.50±0.28[b]	6.3± 3.8[b]
	24	9.6±0.7[b]	0.75±0.25[b]	6.9± 1.3[b]
Phenoxybenzamine/ endotoxin	4	18.5±4.1	0.75±0.48[b]	12.7± 7.8[b]
	24	13.9±0.8[c]	0.00±0.00[b]	0.0± 0.0[b]
Propranolol/ endotoxin	4	18.7±3.8	1.00±0.41[b]	15.8± 6.1[b]
	24	17.3±2.1[c]	0.80±0.50[b]	17.9± 7.8[b]
Phenoxybenzamine/ saline	4	16.3±1.9	4.00±0.70	69.1±20.5
	24[e]	29.8±2.8[d]	4.67±1.20	135.6±27.1[c]
Propranolol/ saline	4	14.5±1.6	5.00±0.65	69.9± 6.8
	24	24.5±3.2[d]	5.30±0.75	124.7±20.1
Saline/ saline	4	13.9±0.9	5.00±0.71	59.2± 5.7
	24	13.2±1.8	5.50±1.19	68.5±13.0

a. Mean ± SEM

b. $P < 0.015$ with respect to saline/saline treated controls

c. $P < 0.015$ with respect to saline/endotoxin treated controls

d. $P < 0.030$ with respect to saline/saline treated controls

e. n = 3

Both α- and β-adrenoceptor blockade considerably enhanced the number of mitotic cells. These effects were completely eliminated if the injections of the blocking agents were followed by endotoxin.

DISCUSSION

The histopathological effects of endotoxin on Meth A sarcoma in mice and the influence of prior adrenoceptor blockade were studied. Tumors grown during 9 days after a s.c. injection of 10^6 viable Meth A cells consisted of very vital and well vascularized tissue with some scattered areas of necrosis. Within 4 h after administration of endotoxin a marked vasodilation in the tumor was observed. This was decreased by prior injection of either

phenoxybenzamine or propranolol (Table 1). These agents, when given alone, decrease or enhance peripheral resistance respectively (Weiner, 1980b). This indicates that the vasodilating activities of endotoxin and phenoxybenzamine do not potentiate each other. It seems that phenoxybenzamine acts to delay the endotoxin-induced hyperemia. Hyperemia had turned to normal 24 h after endotoxin. This was antagonized by prior treatment with propranolol or phenoxybenzamine. At that moment the bloodvessels of tumors of mice treated with phenoxybenzamine and endotoxin were even extremely dilated. This effect has to be attributed to a combined action of endotoxin and the α-antagonist as both agents have no such effects of their own. Hyperemia 24 h after administration of phenoxybenzamine and endotoxin is accompanied by fibrin deposition in some of the vessels, but no further features of hemostasis could be observed. On the other hand tumors of the animals merely treated with endotoxin showed many characteristics of severe hemostasis. Consequences of this checked blood flow are frequently offered as explanation to account for hemorrhagic necrosis (Shear and Perrault, 1944; Barrett, 1942; Algire and Legallais, 1951). It is quite possible that phenoxybenzamine prevents the induction of hemorrhagic necrosis by counteracting hemostasis. Other factors, however, are likely to be involved as both vasodilating and vasoconstricting compounds are among the substances that induce hemorrhagic necrosis (Pradhan et al., 1957). Although phenoxybenzamine and propranolol are agents with opposite vasoactivity, both induced hemorrhagic necrosis in one out of four tumors (Table 1). This result is rather paradoxical as α-adrenoceptor blockade with phenoxybenzamine prevented endotoxin-induced hemorrhagic necrosis while β-adrenoceptor blockade with propranolol did not. Moreover the specific β-adrenoceptor agonist isoproterenol induced hemorrhagic necrosis of murine sarcoma 37 (Pradhan et al., 1957) and of our Meth A sarcoma (Chapter 3). Further experiments will be necessary to unravel these controversial data.

Hemorrhagic necrosis of the tumors was attended with a typical yellow colour of the necrotic area which weakly stained for hemosiderin, indicating disintegration of erythrocyte-derived hemoglobin. Induction of hemorrhagic necrosis by endotoxin is known to be followed by tumor regression in a certain number of instances (Carswell et al., 1975; Berendt et al., 1978a, 1978b). We could easily observe hemorrhagic necrosis within 24 h after endotoxin administration (Table 1), but only a tendency of tumor regression

when sizes of tumor sections of saline/endotoxin- (9.6 ± 0.7 mm^2) and saline/saline treated mice (13.2 ± 1.8 mm^2) were compared (Table 2). Measuring tumor sizes later after endotoxin administration will probably give more clear results.

Tumor regression cannot be directly related to the mitotic activity of tumors as tumors of phenoxybenzamine/endotoxin-treated mice had about the same mitotic index as tumors of saline/endotoxin-treated mice (Table 2), although phenoxybenzamine antagonizes endotoxin-induced tumor regression (Bloksma et al., 1982). Moreover endotoxin induced a reduction of mitotic activity in all tumors observed, while it induces regression in part of the mice in our system and those of others (Carswell et al., 1975; Berendt et al., 1978a, b). The considerable cytostatic activity of endotoxin in vivo is probably an indirect effect, as endotoxin itself is not cyto-static for tumor cells in vitro (Shapiro, 1940; Carswell et al., 1975). Strong in vitro cytostatic activity was observed with endotoxin-induced tumor necrosis serum (TNS) obtained from mice pretreated with *Corynebacterium parvum* (Bloksma et al., 1980a). This cytostasis was attributed to induced heat-stable factor(s) (Bloksma et al., 1980b). It is likely that endotoxin induces such cytostatic principles in tumor-bearing mice too.

The decreased mitotic activity caused by endotoxin was not influenced by previous adrenoceptor blockade as can be concluded from both mitotic index and numbers of mitoses per section. The data from Table 2, however, indicate that both adrenoceptor antagonists alone must have stimulated cell division to a considerable degree between 4 and 24 h after their administration. Stimulation of the tumor growth by a single injection of phenoxybenzamine is in line with previous in vivo results (Bloksma et al., 1982b). On the other hand a single injection of propranolol did not affect in vivo tumor growth in these experiments, suggesting an only temporary and reversing growth stimulating effect.

In conclusion our results show that α-adrenoceptor blockade with phenoxybenzamine inhibits the induction of hemorrhagic necrosis and reduction of tumor size by endotoxin, while endotoxin-induced diminution of mitotic activity is not changed within the observation period. The reduction of tumor size was opposed by both the α- and β-antagonist. Both adrenoceptor blocking agents alone strongly enhanced mitosis and tumor size shortly after administration.

CHAPTER 3

ROLE OF VASOACTIVE AMINES IN THE ANTITUMOR ACTIVITY OF ENDOTOXIN

Nanne Bloksma, C. Frieke Kuper, Frans M.A. Hofhuis and Jan M.N. Willers

SUMMARY. To estimate a possible role of vasoamines in the antitumor action of endotoxin, effects of isoproterenol, serotonin and adrenaline on subcutaneously transplanted murine Meth A sarcoma and the capacity of these agents to elicit antitumor factors were studied. Macroscopically all agents induced tumor necrosis and a temporal tumor growth inhibition, but only endotoxin was capable of induction of complete tumor regression. Histology showed that all agents induced hyperemia by 4 h and hemorrhagic necrosis by 24 h. The latter was located superficially at the outside of the tumors. Only serotonin and especially endotoxin induced substantial non-hemorrhagic necrosis in the remaining part of the tumors. Endotoxin induced a profound inhibition of the mitotic activity within the tumor, the effect of other agents was considerably less. Only endotoxin induced high levels of tumor necrosis factor, heat stable cytostatic factors and interferon in the circulation of mice treated with *Corynebacterium parvum* 14 days earlier.

It is concluded that these and other data provide indirect but circumstantial evidence for a role of vasoamines in the induction of hyperemia and hemorrhagic necrosis by endotoxin. The latter two effects are probably causally related. It is suggested that non-toxic vasoamines may be useful adjuvants to other treatments of cancer.

INTRODUCTION

The potency of endotoxin to cause necrosis and regression of tumors is well known for many years (Gratia and Linz, 1931; Shear et al., 1943; Nowotny et al., 1971; Parr et al., 1973). Effects are indirect and mediated by the host

(Shapiro, 1940; Männel et al., 1979). According to Carswell et al., (1975) induction of tumor damage may be mediated by tumor necrosis factor (TNF). TNF is present in sera from mice sequentially treated with *C. parvum* and endotoxin and mimics the antitumor effects of endotoxin. Recently we found that induction of necrosis and regression by endotoxin was inhibited by prior administration of the α-adrenergic receptor blocking agents phentolamine and phenoxybenzamine but not the β-receptor antagonist propranolol (Bloksma et al., 1982a). On histological examination it was found that phenoxybenzamine completely prevented induction of hemorrhagic necrosis, but substantial non-hemorrhagic necrosis remained apparent (Chapter 2). This suggests that endotoxin induced two kinds of tumor necrosis and that hemorrhagic necrosis may be mediated by an adrenergic mechanism. As phenoxybenzamine and phentolamine not only antagonize reactions at α-receptors but also reactions at serotonin receptors, a serotonergic mechanism might also be involved. This approach is supported by earlier findings that adrenaline and serotonin are released upon injection of endotoxin (Rosenberg et al., 1959) and that both agents induce tumor damage in a mouse sarcoma (Pradhan et al., 1957).

The aim of this study is to compare the histopathology of necrosis of Meth A sarcoma induced by endotoxin with those induced by agents acting at α, β or serotonin receptors, namely adrenaline, isoproterenol and serotonin. As previous experiments have shown that endotoxin-induced release of TNF and especially interferon was impaired by α-receptor antagonists (Bloksma et al., 1982b) the capacity of the agonists to elicit these factors was studied too.

MATERIALS AND METHODS

Materials. The following drugs were used: L-adrenaline (L-epinephrine), L-isoproterenol hydrochloride and serotonin (5-hydroxytryptamine.HCl) all from Sigma Chemical Company (St. Louis, MO, U.S.A.); endotoxin (LPSw from *E. coli* 0111:B4) from Difco Laboratories (Detroit, MI, U.S.A) and *C. parvum* (*Propionibacterium acnes*; Coparvax Lot CA 761) from The Wellcome Research Laboratories (Beckenham, Kent, U.K.). All agents but adrenaline were administered intravenously (i.v.) in 0.5 ml saline.

Adrenaline was dissolved in phosphate-buffered saline (PBS) and a few drops of 0.1 N HCl. The pH was adjusted to 7.0 with 0.1 N NaOH.

Animals and tumors. Sera were prepared in female Swiss random mice of about 12 weeks of age (mean weight 30 g) obtained from the Central Institute for the Breeding of Laboratory Animals (CPB, Zeist, The Netherlands). Tumor experiments were performed on female BALB/c inbred mice of the same age (mean weight 20 g). They were bred and maintained in our own facilities. The Meth A fibrosarcoma, syngeneic to BALB/c mice, was obtained from the Clinical Research Centre (Harrow, Middlesex, U.K.) and was maintained by serial intraperitoneal passage. Cells from 5-to 6-day-old transplants were harvested in Eagle's minimal essential medium and washed three or more times by centrifugation to remove erythrocytes. Cell viability was determined by trypan blue exclusion.

Preparation of the sera. Tumor necrosis serum (TNS) was prepared according to Green et al. (1977). Mice received *C. parvum* (33 mg/kg body weight) i.v. followed by endotoxin (1.25 mg/kg body weight) i.v. two weeks later. Serum was prepared from blood collected 90 min after endotoxin, pooled and stored at -20°C.

Other sera were prepared by omission of *C. parvum* and/or substitution of endotoxin for adrenaline (0.5 mg/kg), isoproterenol (10 mg/kg), serotonin (10 mg/kg) or saline. Sera were heated (56°C, 10 min) prior to all assays excepting determination of interferon. TNF is stable at this temperature (Carswell et al., 1975).

Tumor assays. Mice received a subcutaneous injection with 10^6 viable Meth A cells in the abdomen. After 9 days they were injected i.v. with a test agent. Tumor size was measured with a caliper and expressed as mean diameter [(length + breadth)2]. Necrosis was measured 2 days after injection and expressed as 100 times the quotient of the mean diameters of necrotic area and tumor.

The in vitro cytostatic activity of the sera against Meth A cells was determined by measuring the incorporation of [^{3}H] thymidine into the tumor cells after culture with serum for 40 h as described earlier (Bloksma et al., 1980a).

Histology. Tumors were fixed in 4% phosphate-buffered formalin. Paraffin-embedded sections (5 μm) made in dorso-ventral direction from the central part of the tumor were stained with hematoxylin-eosin and examined microscopically without previous knowledge of the treatment. Hyperemia was expressed as the number of vessel transections with a diameter of $\geq$ 20 μm/section. Counts surmounting 60 were indicated with > 60. Dividing tumor cells were counted with the aid of an ocular grid at 1000x magnification on 10 predetermined sites, representing all parts of the section.

Quantitation of interferon. Interferon was assayed by the plaque reduction method (Epstein and McManus, 1980) using mouse L cells and vesicular stomatitis virus.

Interferon titres were adjusted to the units of the reference mouse interferon standard 6002-902-026 (National Institutes of Health, Bethesda, MD, U.S.A).

Detection of endotoxin. To exclude a possible role of contaminating endotoxin in the necrosis induced by serotonin, adrenaline and isoproterenol these preparation were investigated photometrically for the presence of endotoxin. Limulus amebocyte lysate (Associates of Cape Cod Inc., Woods Hole, MA, U.S.A.) was reconstitued with 2.5 ml pyrogen-free distilled water. Standard endotoxin of the same company and chromogenic substrate S 2423 (KabiVitrum, Stockholm, Sweden) were reconstituted according to the manufacturers' directions. Equal volumes (30 μl) of appropriately diluted samples in saline and Limulus amebocyte lysate reagent were incubated in flat-bottomed wells of microtiter plates (96 wells; Greiner, Nürtingen, F.R.G.) at 37°C for 45 min. After addition of 60 μl of S 2423, plates were incubated for 4 min at 37°C followed by addition of 60 μl glacial acetic acid. Absorbance was measured in a Titertek Multiskan photometer at 405 nm. Endotoxin concentrations in the samples were read from the standard curve.

Statistics. When appropriate, data have been expressed as mean $\pm$ SEM. Significance analysis was done by Student's t-test or the Mann-Whitney U test (Siegel, 1959), indicated in the legend. p-values over 0.05 were considered not significant.

RESULTS

Macroscopical antitumor effects of endotoxin and vasoactive amines.

All agents but saline and PBS induced a marked reddening of the tumors 4 h after injection. Upon bisecting these tumors the cut surface was very bloody, which is indicative of hyperemia (Robbins and Cotran, 1979). Upon i.v. injection of endotoxin, a high incidence of extensive tumor necrosis could be observed by 24 h. This was mostly followed by stagnation of tumor growth and sometimes by complete regression (Table 1). Necrosis induced by isoproterenol and serotonin was less frequent and less extensive. Tumor growth was moderately inhibited and no regressions occurred. Non-lethal doses of adrenaline injected i.v. caused no tumor damage (data not shown), but administration of adrenaline into the tumor in a volume of 0.05 ml caused necrosis with little consequences for tumor growth (Table 1). To exclude a role of contaminating endotoxin in the necrosis induced by the vasoactive agents, the endotoxin content of these preparations was determined. The injected amount of these preparations contained less than 0.1 ng of endotoxin corresponding with a potency of < 0.3 EU of the control standard endotoxin. The vasoactive agents did not interfere with endotoxin determination as was assessed by use of endotoxin-spiked preparations.

Microscopical antitumor effects of endotoxin and vasoactive agents.

Tumor-bearing mice were injected with a necrotizing dose of endotoxin or vasoactive amines. Endotoxin and adrenaline caused a marked hyperemia by 4 h, while isoproterenol and serotonin caused less effect (Table 2). The hyperemia was mainly located superficially on the outside of the tumor and had disappeared by 24 h. At that time hemorrhagic necrosis was observed at the same site. The extent of hyperemia and hemorrhagic necrosis corresponded very well. After injection of endotoxin severe non-hemorrhagic necrosis as judged by pyknosis, karyorrhexis and cytoplasmic eosinophilia was observed in the remaining part of the tumor sections. Tumor sections of mice treated with adrenaline or isoproterenol had no more non-hemorrhagic necrosis than control tumors, having over 50% vital tissue. Serotonin had an intermediate effect.

Table 1 - Necrosis growth inhibition and regression of Meth A sarcoma induced by several agents

Agent	Dose[a] (mg/kg)	Necrosis		Incidence[b] of	
		Incidence[b]	Extent[c]	Growth inhibition[d]	Regression[e]
Endotoxin	1.25	9/10	57 ± 4	8/10	3/10
Isoproterenol	5	1/5	38	0/5	0/5
	10	4/10	42 ± 10	3/10	0/10
Serotonin	10	7/10	43 ± 5	1/10	0/10
	30	6/10	47 ± 4	4/10	0/10
Saline	-	1/20	37	0/20	0/20
Adrenaline	0.5	10/10	46 ± 4	3/10	0/10
	0.15	5/5	47 ± 3	1/5	0/5
	0.05	3/5	54 ± 6	1/5	0/5
PBS	-	0/10		0/10	0/10

a. I.v. except for adrenaline and PBS which were administered intratumorally

b. Number of affirmative cases/total

c. (Mean diameter necrotic area/mean diameter tumor) x 100

d. No increase of tumor size for at least 2 days after injection (observed exclusively in necrotic tumors)

e. Complete disappearance of the tumor within 12 days after injection

Table 2 - Histology of Meth A sarcoma 4 and 24 h after i.v. administration of tumor necrotizing agents

Agent	Dose (mg/kg)	Time	Hyperemia[a] (mean ± SEM)	Mitotic index[b]	Hemorrhagic necrosis	
					Incidence	Extent[g]
			EXPERIMENT 1			
Endotoxin	1.25	4[c]	>60 ± 0[d]	0.33 ± 0.33		
		24	3 ± 1	0.00 ± 0.00[e]	4/4	9 ± 3
Isoproterenol	10	4	31 ± 6[d]	1.50 ± 0.50		
		24	12 ± 4	1.45 ± 0.48[e]	3/4	4 ± 0
Serotonin	10	4	>43 ± 11	0.25 ± 0.25[d]		
		24	19 ± 6	1.75 ± 0.48	3/4	6 ± 1
Saline	-	24	6 ± 3	3.25 ± 0.48	0/4	
			EXPERIMENT 2			
Endotoxin	1.25	4	>60 ± 0[d,e]	0.50 ± 0.29[e]		
		24	11 ± 8	0.00 ± 0.00[e]	3/4	12 ± 4
Saline	-	4	9 ± 5	2.50 ± 0.29		
		24	6 ± 1	3.00 ± 0.41	0/4	
Adrenaline[f]	0.25	4[c]	>60 ± 0[d,e]	0.67 ± 0.33[e]		
		24	13 ± 8	1.75 ± 0.28	3/4	15 ± 11
Saline	-	4	3 ± 1	1.00 ± 0.00[e]		
		24	17 ± 8	1.25 ± 0.48[e]	0/4	

a. Number of dilated vessel transections/section
b. Number of dividing cells/mm^2 tumor section. (mean ± SEM)
c. n = 3
d. $p < 0.03$ compared to 24 h (U-test)
e. $p < 0.03$ compared to saline-elicited controls (U-test)
f. Administered into the tumor
g. (Mean diameter necrotic area/mean diameter tumor) x 100

The mitotic activity within the tumor was considerably reduced by endotoxin at both times. Serotonin inhibited to the same degree only by 4 h. In all other instances less, but still considerable, inhibition of mitotic activity was induced when compared to saline-treated controls. In none of the sections could substantial inflammatory infiltrates be detected within the observation period.

In vivo and in vitro antitumor activity of sera from mice treated with tumor necrotizing agents.

Injection of 0.5 ml TNS into Meth A-bearing mice caused extensive tumor necrosis in all animals and complete regression in 4 out of 5 mice (Fig.1).

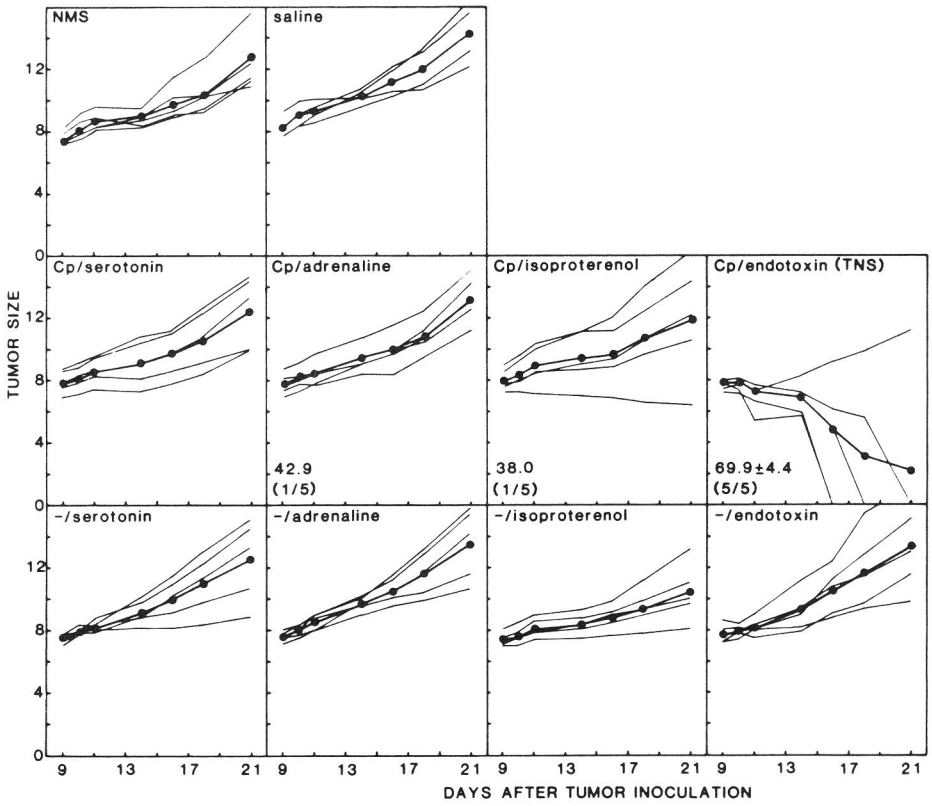


Fig. 1. Growth of Meth A sarcoma after i.v. injection of saline or serum of normal or *C. parvum* (Cp)-treated mice elicited with a tumor necrotizing agent or saline (NMS). Thin lines indicate tumor size of individual mice, heavy lines with circles the mean of each group. Figures give the mean % of hemorrhagic necrosis ± SEM and the incidence (in brackets)

None of the sera of mice treated with *C. parvum* and an vasoactive agent caused serious tumor damage. Only a moderate necrosis in 20% of mice was observed on injection of sera of mice treated with *C. parvum* and adrenaline or isoproterenol. Normal mouse serum (NMS) and sera of mice merely treated with endotoxin, serotonin or adrenaline had no significant antitumor effect, but serum of isoproterenol-treated mice retarded tumor growth sligthly.

The cytostatic activity of NMS against Meth A cells in vitro appeared to be high when compared to growth in medium alone (Fig. 2). Serum of mice treated with *C. parvum* was significantly more cytostatic than NMS, as were sera from mice merely treated with endotoxin, isoproterenol or serotonin. Compared to sera of mice treated with *C. parvum* alone, an additional injection with serotonin, isoproterenol or especially endotoxin enhanced cytostatic activity of the sera considerably.

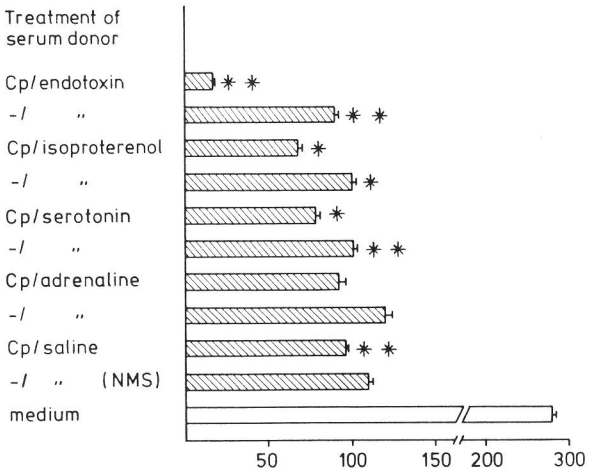


Fig. 2. Cytostatic activity on Meth A cells in vitro of sera of mice treated with *C. parvum* (Cp) and/or a tumor-necrotizing agent (shown as reduced [^{3}H]thymidine incorporation). Sera of 6 mice were tested in quadruplicate for each group. *, $P < 0.001$ (t-test) compared to Cp/saline. **, $P < 0.02$ compared to -/saline (NMS). All necrotizing agents elicited stronger cytostatic activity in Cp-treated mice than in normal mice ($P < 0.001$).

Induction of interferon by tumor necrotizing agents

Substantial levels of interferon could be detected in sera from mice treated with endotoxin 90 min earlier (Table 3). Pretreatment with *C. parvum*

Table 3 - Determination of interferon in TNS and other sera

Treatment of serum donors	Interferon (U/0.1 ml) Individual mice				Mean
Cp[a]/endotoxin (TNS)	>5952	>5952	>5952	5939	>5949[c,d,e]
-/endotoxin	4387	2785	2698	2079	2987[c,d]
Cp/isoproterenol	158	135	118	<62	<118
-/isoproterenol	629	322	301	<62	<329
Cp/serotonin	249	135	125	100	152[d]
-/serotonin	441	<62	60	3	<142
Cp/adrenaline	93	<62	<62	-[b]	<72
-/adrenaline	<62	<62	<62	-[b]	<62
Cp/saline	316	64	<62	<62	<126
-/saline	<62	<62	<62	<62	<62

a. *C. parvum*

b. I.v. injection of adrenaline was lethal to the majority of the mice. Therefore 10 normal and 10 Cp-treated mice were injected with adrenaline. Only 3 mice of each group survived

c. Significantly different from Cp/saline; $p = 0.014$ (U-test). For determination of significance values <62 and >5952 were equalized to 62 and 5952 respectively

d. Significantly different from -/saline; $p = 0.014$

e. Significantly different from sera of mice not pretreated with Cp; $p = 0.014$

augmented the interferon yield significantly. Elicitation of *C. parvum*-treated mice with isoproterenol or serotonin induced hardly more interferon than injection of saline. Isoproterenol tended to induce more interferon in normal than in *C. parvum*-treated mice. Adrenaline appeared incapable of inducing interferon.

DISCUSSION

The antitumor action of vasoactive amines was investigated as to their possible involvement in the antitumor action of endotoxin. Endotoxin induced necrosis of Meth A sarcoma mostly followed by growth inhibition. A number of tumors regressed completely. Isoproterenol, serotonin and adrenaline induced much less necrosis with only minor consequences as to tumor growth. The negligible quantities of endotoxin in these preparations, however, suggest that the effects observed are due to the vasoactive agents themselves.

Histology was in line with macroscopic effects. Endotoxin caused stronger hyperemia and hemorrhagic necrosis and a more profound inhibition of mitotic activity, while extensive non-hemorrhagic necrosis was only induced by endotoxin. So at most part of the antitumor action of endotoxin might be mediated by vasoamines. The moderate tumor damage resulting from injection of high, unphysiological doses of vasoamines does not rule out a role of these agents in the antitumor action of endotoxin. Endotoxin is known to cause the release of various vasoamines (Rosenberg et al., 1959; Endo, 1982) and also to increase sensitivity to their effects (Kuratsuka et al., 1975; 1978). These properties and the well known synergic activity of different vasoamines (Munoz and Bergman, 1968; Foon et al., 1976) may endow endotoxin with the property to induce hemorrhagic necrosis. Besides this action, other properties of endotoxin, not innate to vasoamines, such as its strong immunoadjuvanticity and macrophage activating capacity, probably contribute to its activity. Interferon, TNF and cytostatic factors, when induced in the tumor-bearing mouse, might also be involved. The amount of interferon found to be induced in tumor-bearing mice was shown to exert some activity in our Meth A system (Bloksma, 1982; Bloksma et al., 1983). However, we, and as far as we known others, have not been able to detect TNF in the circulation of

endotoxin-injected Meth A-bearing mice, while the factor could not be absorbed from TNS by Meth A cells (Bloksma et al., 1984).
The local vasodilating effect we observed after injection of adrenaline into Meth A is quite opposite to the usual effect of adrenaline in peripheral healthy tissue (Weiner, 1980a). On the other hand, Wickersham et al., (1977) observed no response of tumor vessels to topically administered adrenaline and noradrenaline, and Hafström et al., (1980a) reported enhanced blood perfusion of liver tumors in rats after injection of noradrenaline. This was attributed to the lack of smooth muscles and adrenergic nerves in the vessel walls of tumors (Krylova, 1969; Mattsson et al., 1977).

The endotoxin-induced vasodilation which preceded hemorrhagic necrosis in murine sarcomas and in adrenaline-prepared rabbit skin was shown to result in an almost complete to complete standstill of blood flow (Algire et al., 1947; Zweifach et al., 1956), suggesting that tissue damage is more or less due to ischemic hypoxia. A same mechanism may underlie hemorrhagic necrosis of tumors induced by adrenaline, isoproterenol and serotonin.

The absence of clear inflammatory infiltrates within the tumor 4 h as well as 24 h after injection of all agents, while tumor damage is overt at the later time, suggests that these effects are probably due to humoral mechanisms. Our data show that vasoamines may also exert antitumor activity by other means than recruitment of inflammatory cells into tumor tissue as suggested by others (Gershon et al., 1975; Lynch and Salomon, 1977; Askenase, 1977). The induction of tumor necrosis by the selective β-receptor agonist isoproterenol (Weiner, 1980b) is seemingly in contrast with the observation that α- but not β-adrenoceptor antagonists inhibited the induction of tumor necrosis by endotoxin (Bloksma et al., 1982a; Chapter 2). Different mechanisms are probably involved. Moreover, agonists at α- and β-receptors do not always elicit opposite reactions. Among other Beets and Paul (1980) showed that selective α- as well as β-adrenoceptor agonists induced identical vascular reactions in the guinea pig skin by entirely different mechanisms.

Several data point at a need for adrenaline and functional adrenergic receptors in the elicitation of TNF and interferon by endotoxin. TNF could not be elicited in adrenalectomized mice (C. Galanos, personal communication in Green et al., 1977). Further, induction of TNF and especially interferon could be inhibited by α-adrenoceptor antagonists (Bloksma et al., 1982b),

while administration of adrenaline shortly before endotoxin inhibited endotoxin-induced release of interferon (Jensen, 1969) and TNF (Bloksma et al., 1982b), suggesting tachyphylaxis. As TNF and interferon could not be elicited by adrenaline alone, this hormone may be needed to give a kind of permissive signal.

In conclusion, data of this and other papers provide only indirect evidence for a role of vasoamines in the induction of hyperemia and hemorrhagic necrosis by endotoxin. Non-toxic vasoactive agents such as serotonin may be useful adjuvants to other tumor therapies.

CHAPTER 4

EFFECTS OF ENDOTOXIN-TREATMENT ON INFLAMMATORY CELL INFILTRATES IN MURINE METH A SARCOMA

C. Frieke Kuper, Nanne Bloksma, Joost P. Bruyntjes, Frans M.A. Hofhuis and Gerrit Wolterink

SUMMARY. The effect of intravenously injected endotoxin on inflammatory cells within solid Meth A tumors was studied at 4, 24 and 48 h respectively. The effects were studied in semithin sections and cytocentrifuge preparations of the tumors. The inflammatory cell reaction evoked by the tumors in untreated animals was relatively slight. It was located predominantly around the lateral margins of the tumors and only a few inflammatory cells were found inside the tumor. Prominent effects of endotoxin were a transient increase of mononuclear inflammatory cells in the centre of the tumor by 4 h and a reduction of the influx of lymphocytes, observed in and around the margin of control tumors, by 48 h. Mast cells formed an important part of the inflammatory cell infiltrate, but no distinct changes in number and appearance was observed in time or due to treatment. Total host cell numbers within tumors did not increase significantly upon endotoxin-treatment. Results suggest that a direct cytotoxic action of host cells cannot account for the extensive tumor damage observed. Rather, endotoxin-induced regression seems to be related to decreased lymphocyte numbers.

INTRODUCTION

Among the many effects of endotoxin are the induction of necrosis and, in a limited number of cases, complete regression in transplanted tumors, as was already described in 1931 (Gratia and Lintz, 1931)). There is, however, no

general agreement as to the mode of action of endotoxin, except that the antitumor effects are mediated by the host. One of the main controversies is the role of inflammatory cells in tumor destruction (Parr et al., 1973; North, 1986; Kodama et al., 1982).

A previous macroscopic study with transplanted Meth A sarcomas showed that intravenously injected endotoxin caused clear hyperemia by 4 h followed within 24 h by central necrosis. In general, completely regressing tumors began to collapse by 48 h (Bloksma et al., 1983). Morphological studies at these times, using paraffin-embedded sections, showed numerous dilated blood vessels exclusively in the centre of the tumors by 4 h. The vessels were plugged by erythrocyte debris by 24 h. At this time, necrosis could be observed in the tumors except for a small rim of vital tumor tissue at the margins aside. No clear accumulation of inflammatory cells was present, although extensive necrosis could be observed in these tumors (Chapter 2; Bloksma et al., 1984). The aim of the present study was to analyse in more detail the effects of endotoxin on the nature of inflammatory cell infiltrates within and directly around transplanted Meth A sarcoma using semithin sections and cytocentrifuge preparations.

MATERIALS AND METHODS

Animals and tumor . Female BALB/c inbred mice (Laboratory of Microbiology, State University of Utrecht, Utrecht, the Netherlands) were used at the age of 12 weeks (weight about 20 g). The syngeneic Meth A fibrosarcoma (Clinical Research Centre, Harrow, Middlesex, England) was maintained as ascites tumor by serial intraperitoneal passage in BALB/c mice. Tumor cells from 5 to 6 day old transplants were harvested in Eagle's minimal essential medium (MEM; Flow Laboratories, Irvine, Scotland) and washed at least 3 times by centrifugation at low gravity to remove erythrocytes. Viability was determined by trypan blue exclusion.

Tumor assays. Mice received a subcutaneous injection with 3×10^5 viable Meth A cells in the abdomen; 9 days later they were injected intravenously

with 1.25 mg endotoxin (LPSw from *E. coli* 0111:B4; Difco Laboratories, Detroit, Michigan, USA)/kg body weight in 0.5 ml saline, 0.5 ml saline alone or they were left untreated. Tumors were removed with a substantial amount of surrounding host tissue 4, 24 or 48 h after endotoxin injection. The peritoneum underlying the tumor was removed together with the tumors used for microscopical examination but was not included in the tissue used for the cytocentrifuge preparations.

Histology. Tumors were fixed in 4% phosphate-buffered formalin. Glycol methacrylate-embedded sections (1 - 2 μm), made in the dorso-ventral direction from the central part of the tumor, were stained with haematoxylin-basic fuchsine or toluidine blue and examined microscopically.

For cytocentrifuge preparations, tumors were divided in a central and marginal part (Fig.1). The parts were cut in small blocks and desintegrated by incubation in enzyme solution for 40 min at 37°C under gently shaking. The enzyme solution consisted of Eagle's MEM with 0.05 % collagenase (Boehringer, Mannheim, Germany), 0.025 % collagenase/dispase (Boehringer, Mannheim, Germany), 0.0002 % DNA-se (Sigma Chemical Company, St. Louis, Missouri, USA), 10 % heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland) and 0.05 M sucrose. Suspended cells were washed with Eagle's MEM. Cytocentrifuge preparations were made and 1 slide/tumor part was stained with May-Grünwald Giemsa for differential analysis. In addition, 2 slides/tumor part were stained for α-naphtylbutyrate esterase (Koski et al., 1976) and counterstained with haematoxylin for analysis of cells of the mononuclear phagocytic system. When possible, twice a total of 100 cells with intact nuclei/slide were counted.

Statistical analysis. The data obtained from the cell counts of the cytocentrifuge preparations were evaluated by analysis of variance (Cohen and Holliday, 1979).

Sections of tumors from saline-treated mice showed a solid tumor mass between the epithelium of the ventral skin and the abdominal wall as schematically depicted in Fig.1. As histology of the tumor itself has been described earlier, attention was focussed on inflammatory cell infiltrates.

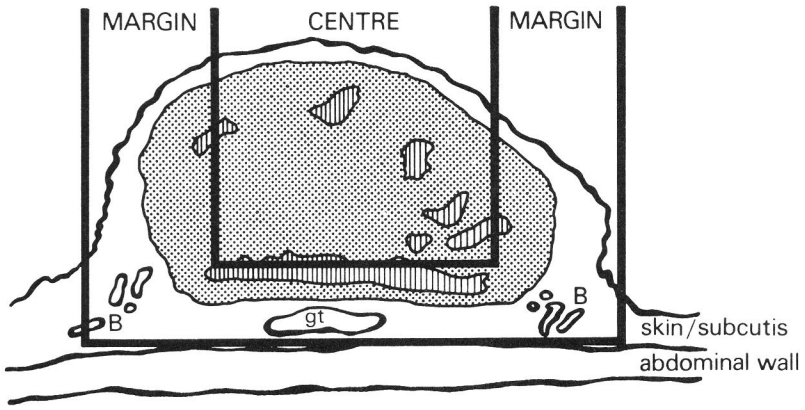
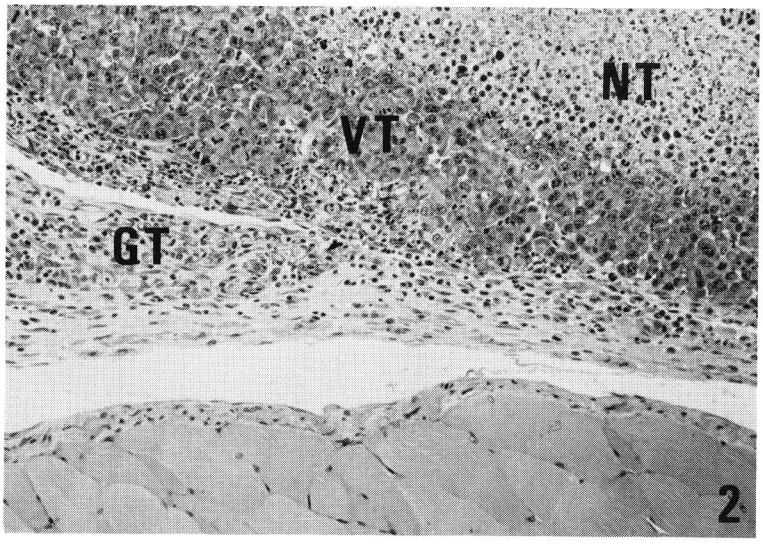


Fig.1. Schematic survey of a Meth A sarcoma section (⁘) with areas of necrosis (||||) from an untreated animal. Thick lines indicate division in central and marginal part; large bloodvessels (B) and a compact area of granulation tissue (gt) are pictured.

A compact and distinct area of granulation tissue could be observed between the abdominal wall and the base of the tumor (Fig. 2). In the centre of the tumors only a few host cells could be observed predominantly located intra- and perivascularly. Most inflammatory cells were found inside and around the lateral margins of the tumors where large blood vessels had been transsected (Figs. 3 and 4). These vessels also contained increased numbers of inflammatory cells. Inflammatory infiltrates observed inside and around the tumors consisted mainly of polymorphonuclear cells and mast cells. The latter cells were also abundantly present in the papillary and superficial reticular dermis directly overlying the tumor (Fig. 5). Especially at these sites



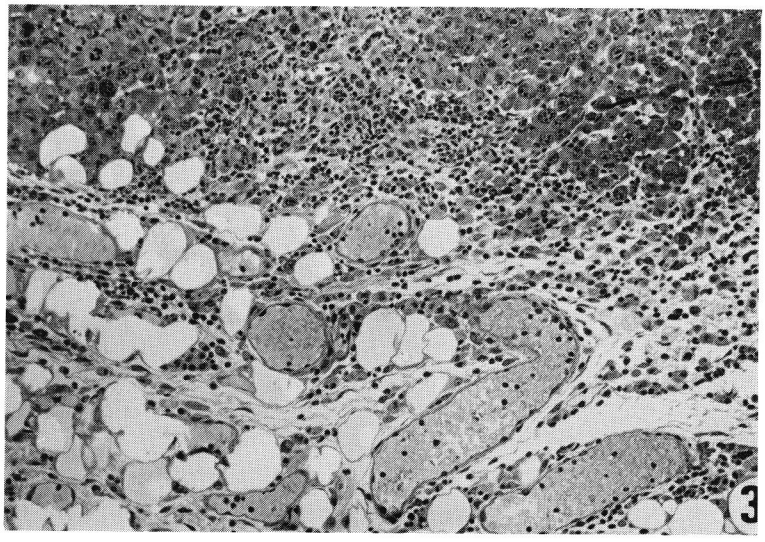
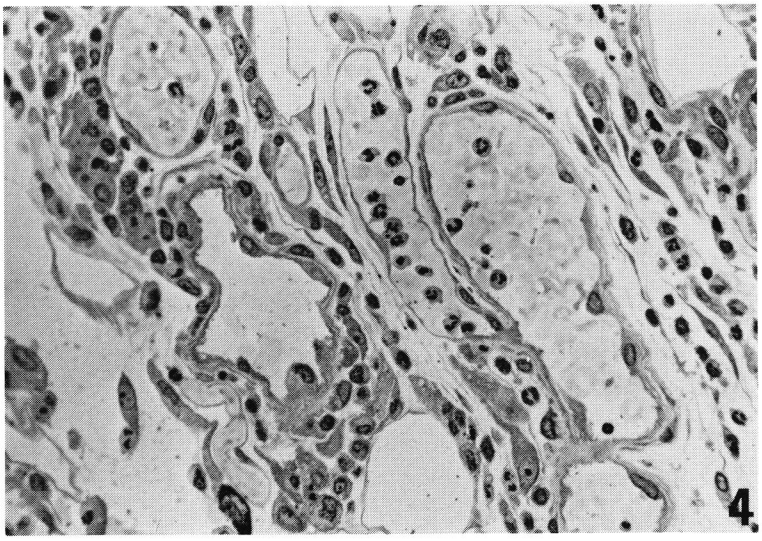


Fig.2. Highly vascularised granulation tissue between a 9-day-old Meth A sarcoma and the abdominal wall of a saline-treated animal. A necrotic area and a vital area of the tumor is shown. GT: granulation tissue; NT: necrotic tissue; VT: vital tissue

Fig.3. Lateral margin of a 9-day-old Meth A sarcoma from a saline-treated animal with a few large bloodvessel transsections. Inflammatory cells infiltrate the lateral margin of the tumor.



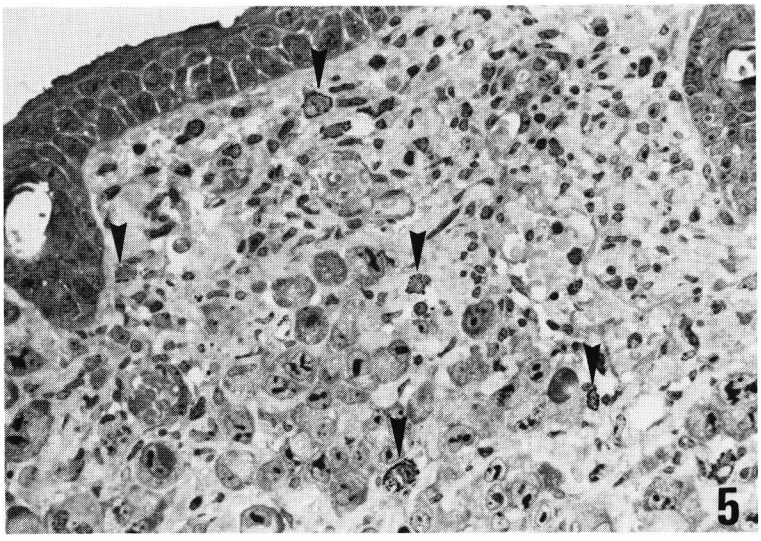


Fig.4. Detail of bloodvessel transsections around the lateral margin of a 9-day-old Meth A sarcoma. Numerous inflammatory, mainly polymorphonuclear, cells are located in and directly around the vessels.

Fig.5. Poorly granulated mast cells (▼) in and around a 9-day-old Meth A sarcoma of a saline-treated animal.

mast cells contained few and frequently enlarged granules.

Examination of the cytocentrifuge preparations showed that mast cells were completely absent, which is probably due to the fragility of these cells. Number and type of other host cells in disaggregated tumors of saline-treated controls appeared to change with time and differed as to the part of the tumor investigated (Figs. 6A and B). The centre of these tumors contained 19, 11 and 34% host cells by 4, 24 and 48 h respectively. Polymorphonuclear cells, mainly neutrophilic leukocytes, were predominant at all times. The increased ratio of host cell numbers by 48 h was due to increased numbers of these cells, lymphocytes and macrophages. Within the tumor margins of saline-treated mice, host cells formed 29, 26 and 60 % of the total cell numbers at the respective observation times. By 4 and 24 h the ratio between polymorphonuclear and mononuclear inflammatory cells was about 3 : 1. The ratio changed to 1 : 1 by 48 h. The increase of mononuclear inflammatory cells was mainly due to an increase of lymphocytes. The inflammatory cell infiltrate by 48 h was comparable with the infiltrate of tumors of the same age from untreated animals (Fig. 7).

Distinct endotoxin-induced changes in the centre of the tumors were only observed by 4 h as compared to saline-treated controls (Fig. 6A), namely a significant increase of lymphocytes, monocytes and macrophages. Polymorphonuclear cell numbers changed hardly. In the margins endotoxin induced only a small but significant increase of macrophages by 4 and 24 h (Fig. 6B). By 48 h, however, the relative number of host cells was significantly lower compared to treatment with saline (38 versus 60 % respectively). This was almost exclusively due to a considerably smaller number of lymphocytes.

DISCUSSION

Examination of sections and cytocentrifuge preparations of Meth A tumors of saline-treated mice showed that host cell numbers within the centre of the tumors were relatively small at all observation times. Higher numbers of

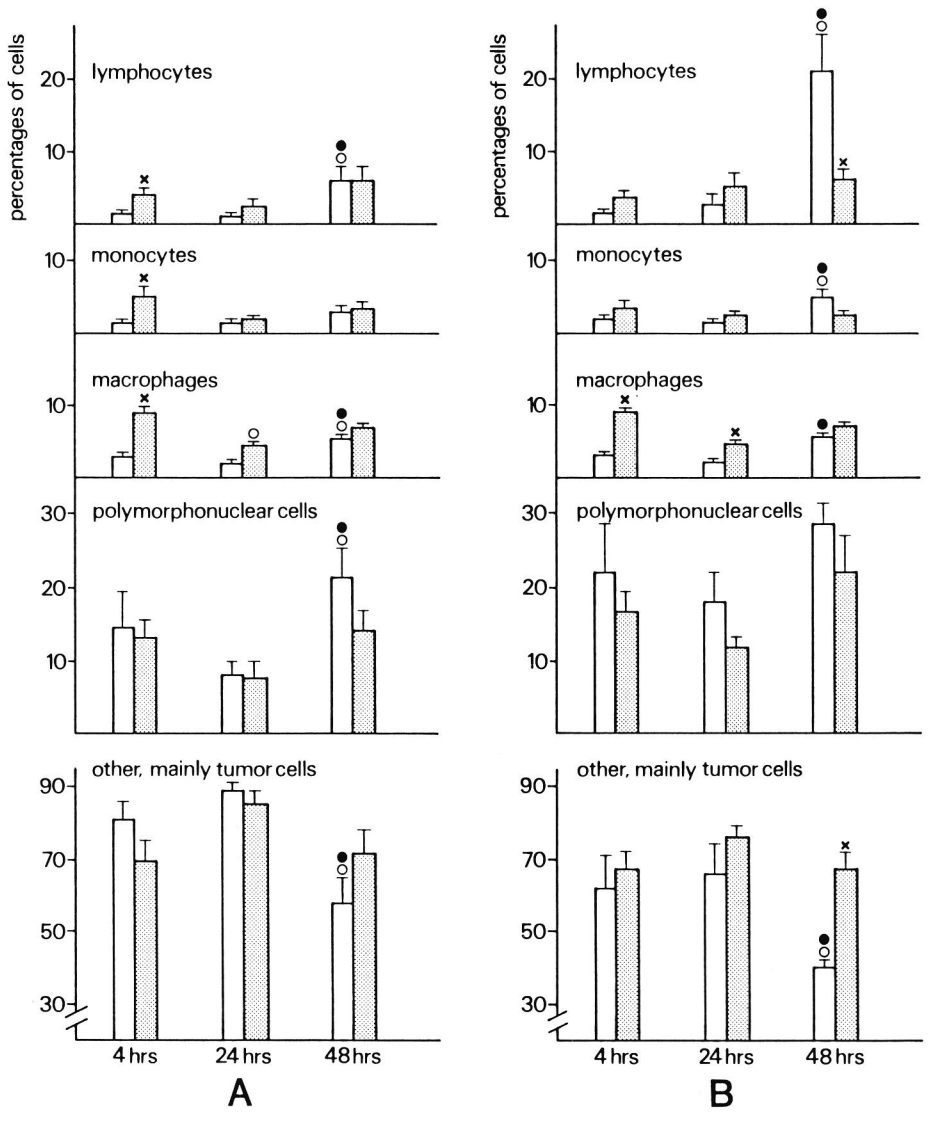


Fig.6. Cellular composition of cytocentrifuge preparations (mean of six animals ± SEM) of the central (A) and marginal (B) parts of Meth A tumors at different times after i.v. treatment with endotoxin (▨) or saline (□).

× $p < 0.05$ for differences with saline-treated controls at the same time.

○ $p < 0.05$ for differences with the same treatment group at 4 h.

● $p < 0.05$ for differences with the same treatment group at 24 h.

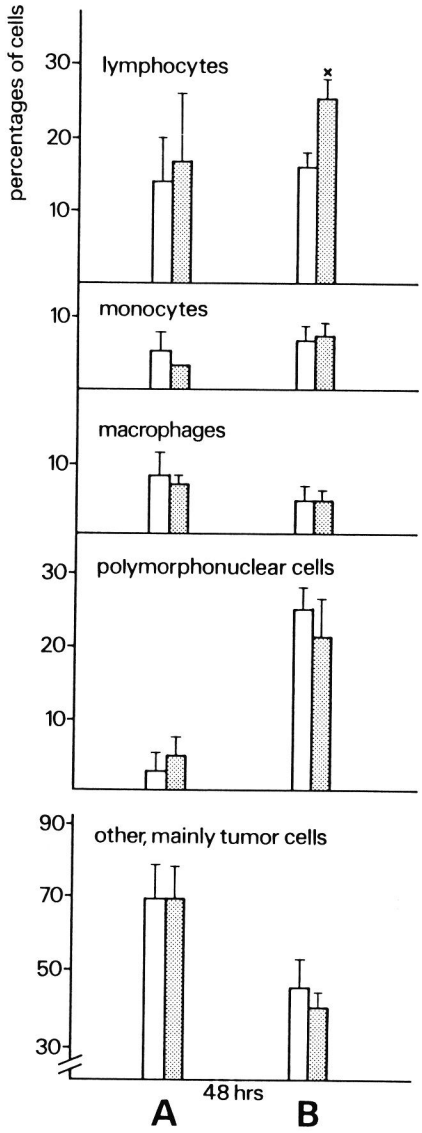


Fig.7. Cellular composition of cytocentrifuge preparations (mean of three animals ± SEM) of central (A) and marginal parts (B) of Meth A tumors, 48 h after injection of saline (□) or untreated tumors of the same age (▨).

inflammatory cells were present inside and around the lateral margins of the tumors especially by 48 h. Polymorphonuclear cells and mast cells formed the

major part of the infiltrate at all times, irrespective of the site of observation. In the margins, however, substantial numbers of lymphocytes were observed in addition, by 48 h. This lymphocytic infiltrate could not be attributed to the treatment with saline, as untreated tumors of the same age showed the same phenomenon.

The only prominent differences induced by endotoxin were a small, transient but significant increase of mononuclear phagocytes in the centre of the tumors and the absence of a strong lymphocytic infiltrate in the margins by 48 h. Despite increased mononuclear phagocyte numbers in the centre, the ratio between these cells and tumor cells was still 1 : 5 at most. Although mononuclear phagocytes have well known tumoricidal activity after contact with endotoxin, the observed effector-target cell ratio is too insufficient to assume that cell-mediated cytotoxicity can account for the extensive necrosis observed at this site by 24 h. The locally increased mononuclear phagocyte infiltrate, however, coincides very well with the hyperemia induced exclusively at this site. Histologically similar hyperemia and subsequent necrosis was observed after injection of serum with tumor necrosis factor (TNF). As TNF is thought to originate from activated macrophages upon contact with endotoxin (Taramelli and Varesio, 1981), effects might be due to the local release and action of this factor, but this needs to be confirmed.

The observed differences in composition and number of host cells in and around the lateral margins of tumors 2 days after treatment with saline or endotoxin are interesting in view of the following data. The ability of endotoxin to induce complete tumor regression is known to depend on the age (size) of the tumor (Berendt et al., 1978). Meth A tumors older than 9 days were shown to become rapidly refractory to this effect which has been related to the loss of T-lymphocyte-mediated concomitant immunity to this tumor and the appearance of specific suppressor T lymphocytes in the spleen (North, 1981). It is therefore tempting to hypothesize that the high number of lymphocytes in the margins of 11-day-old control tumors are suppressor T cells. Attempts to further analyse these cells by various immunohistochemical means, however, have failed up till now because of various non-specific reactions of, especially necrotic, tumor cells. Such interference has been described by others too (Santer et al., 1980; Seeger et al., 1982). Data,

however, showed that endotoxin interfered somehow with the influx of lymphocytes and this might be related to its ability to induce definite regression. Further study is needed to get a better insight in these phenomena.

A serious drawback of cell analysis with the aid of cytocentrifuge preparations is shown with respect to the mast cells. They could only be detected occasionally and then largely degranulated in the preparations, but were found in large numbers in the semithin sections. The granulation of the cells seemed to be dependent on the localisation: the cells were poorly granulated at the ventral side of the tumor where hyperemia and early necrosis take place after endotoxin-treatment; at the lateral margins and at the base of the tumor they were more richly granulated. Although mast cells are a source of vasoactive agents and could as such play a role especially in the early phase of tumor destruction, no distinct changes in cytoplasmic granulation were observed after endotoxin-treatment.

In conclusion, endotoxin-induced tumor damage probably involves other mechanisms than a direct interaction of cytocidal cells and tumor cells. An interference by endotoxin with the onset of suppressor activity of lymphocytes may favour ultimate regression induced by this agent.

CHAPTER 5

ANTITUMOR EFFECTS OF ENDOTOXIN AGAINST SOLID MURINE METH A TUMORS OF DIFFERENT AGES. I. QUANTITATIVE HISTOLOGY OF THE TUMORS AND REGIONAL LYMPH NODES

C. Frieke Kuper, Nanne Bloksma, Joost P. Bruyntjes and Frans M.A. Hofhuis

SUMMARY. Age-dependent induction of tumor necrosis by intravenously injected endotoxin was studied histologically in murine Meth A sarcoma, 3, 6, 9 or 15 days after transplantation in the abdominal skin. In addition, the draining lymph nodes were examined. In all tumors, early hyperemia and prolonged mitotic arrest were induced. Hyperemia tended to be most marked at day 9 and 15. Mitotic arrest was strongest at day 9. Macroscopically, endotoxin induced tumor necrosis in 9- and 15-day-old tumors, which was more extensive in the latter. Histological study, however, showed the opposite and indicated that 6-day-old tumors were also susceptible to induction of tumor necrosis. Coagulation necrosis was predominant. Three-day old tumors appeared resistant to induction of tumor necrosis. Only in 9- and 15-day-old tumors, a small area of hemorrhagic necrosis was observed at the skin side, including skin tissue. This necrosis was characterized by parallel layers of degenerating tumor cells with elongated, hyperbasophilic nuclei, extensive hemorrhage and erythrocyte destruction. Only in this area, mast cell depletion occurred, although at a rather late stage. Mast cells were most numerous in and around 9-day-old tumors.

The tumor evoked an extensive reaction in the regional lymph nodes. Initially in the paracortex but later on almost exclusively in the B-cell-dependent areas. Already at day 7 after tumor transplantation, tumor cells were observed in the subcapsular sinuses of these lymph nodes. Endotoxin had no effect on the disseminated tumor cells and the lymph node reaction.

Data indicate that endotoxin induced very extensive necrosis only in 9-day-old tumors. The extent might be of pivotal importance for induction of

complete regression which is known to occur almost exclusively in tumors of about this age. Hyperemia and mitotic arrest are probably contributing to the induction of tumor necrosis, but appeared not conclusive per se. Other factors among which possibly mast cells must be involved. It has been suggested that the initial T-cell reaction followed by a very pronounced B-cell stimulation in the regional lymph nodes resembles a chronic graft-versus-host reaction, contributing possibly to the decay of concomitant immunity. The observation that the morphology of the lymph nodes and the tumor cells located in these nodes were not affected by endotoxin treatment suggests that this agent does not induce cytotoxic lymphocytes and/or tumoricidal factors in effective amounts.

INTRODUCTION

The susceptibility of solid tumors to endotoxin-induced tumor necrosis and complete regression in mice has been shown to be restricted by various conditions. Tumor necrosis could only be induced when tumors had grown beyond a given size. Incidence and extent increased with the size of the tumor (Bloksma et al., 1984e; North, 1984; Freudenberg et al., 1984). Immunogenicity of the tumor and the T-cell dependent immune status against it were not essential (Parr et al., 1973; North, 1981; Kodama et al., 1982). On the other hand, necrosis appeared not to be followed, in most cases, by complete regression. Parr et al. (1973) have shown that T-lymphocytes were involved in endotoxin-induced regression. Further experiments by North and coworkers (reviewed by North, 1984) have pointed out that cures were only seen when endotoxin was injected during a state of concomitant immunity to the tumor, which was mediated by specific T lymphocytes. Generation and decay of concomitant immunity to a given immunogenic tumor could be associated with defined tumor sizes. The decay has been related to a progressive acquisition of suppressor T lymphocytes detected in the spleens of tumor-bearing mice.

The Meth A fibrosarcoma used in this study is a strongly immunogenic tumor in syngeneic BALB/c mice and has a very low frequency of metastases (North, 1984). Concomitant immunity to intradermal Meth A in the belly region appeared optimal when their diameter was 7 - 8 mm. Below and

especially beyond that size, concomitant immunity was low or absent. Intravenous injection of endotoxin (*E. coli* 0111:B4) in mice with 9-day old Meth A tumors (diameter about 7 mm) induced necrosis in and cures of about 90 and 30 percent of the tumors, respectively (Bloksma et al., 1982b; 1984e; 1984a). Smaller tumors (day 5 and day 7) were almost resistent to both effects, while larger tumors (day 15) showed extensive necrosis without definite regression.

To get more information about the factors involved in the susceptibility of solid tumors to endotoxin-induced necrosis and the onset of definite regression, we studied histological events in Meth A tumors of different ages and in their draining lymph nodes upon intravenous injection of endotoxin.

MATERIALS AND METHODS

Mice and tumor. BALB/c mice were bred and maintained at the Laboratory of Microbiology (Utrecht, The Netherlands). Female mice were used at the age of 11 weeks (approximate weight 20 g). The Meth A sarcoma of BALB/c origin was obtained from the Clinical Research Centre (Harrow, Middlesex, UK) and maintained in the ascites form by serial intraperitoneal passage.

Material. Endotoxin (LPSw from *E. coli* 0111:B4) was obtained from Difco Laboratories, Detroit, Michigan. Endotoxin was dissolved in saline. It was injected i.v., in a total volume of 0.5 ml.

Experimental design. Mice received a s.c. injection in the ventral skin with 3×10^5 viable Meth A cells and were treated i.v. with endotoxin (25 μg) or saline 3, 6, 9 or 15 days later. One group was left untreated. Diameter of the tumor and incidence and extent of macroscopically observed necrosis were recorded from mice, just before and 24 h after endotoxin or saline injection. Mice were killed at the times indicated and tumors were excised, together with a considerable amount of surrounding host tissue including the abdominal wall.

Histology. Tumors (n=4, for each treatment at each time) and the regional inguinal lymph nodes (n=3 pair, for each treatment at each time) were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.35, osmolality = 500 mOsm) at room temperature during 24 h. Glycol methacrylate-embedded sagittal sections (1 - 2 μm) of the central part of the tumors, and central sections of the lymph nodes, were stained with toluidine blue or May-Grünwald Giemsa and examined microscopically without previous knowledge of the treatment. Combined hemoglobin-hemosiderin staining was performed according to Puchtler and Sweat (1963) and bilirubin staining according to Gmelin (described by Pearse, 1968).

Criteria for observation of the tumor have been described previously (Chapter 2). Hyperemia was expressed as the number of vessel transsections with a diameter of > 20 μm/square mm vital tumor tissue in the section. Counts surmounting 60 were indicated with > 60. Dividing tumor cells were counted on 10 predetermined sites, with an area of 0.06 square mm each, representing all parts of the section. In addition, mast cells were counted at 13 predetermined sites (0.06 square mm/site). Four of the sites were situated in the lateral margins of the tumor, and 3 sites each at the skin side, centre and peritoneal side of the tumor, respectively.

In the lymph node sections, the numbers of germinal centres were counted, and the degree of plasmacytosis, sinus histiocytosis and mast cell accumulations were determined semiquantitatively. The morphometric analysis was performed with the aid of a computerized graphical tablet (MOP II, Kontron Messgerät GmbH, Munich).

Statistics. When appropriate, data have been expressed as mean $\pm$ SEM. The morphometric data of the tumors were evaluated by analysis of variance using the standard statistical package 'Genstat' (Lawes Agricultural Trust, version 1984; Rothamsted Experimental Station; Alvey et al., 1982). Treatment of the mice and age of the tumor were used as factors; time after treatment, endotoxin against saline and their mutual interaction were used as contrasts within treatments of the mice. Untreated and saline-treated groups were used both as separate control groups and together as one control group. When appropriate, this is mentioned explicitly in the results. In addition, data of the lymph nodes were analysed with the Mann-Whitney U-test (Siegel, 1959).

RESULTS

Gross observations.

Tumors of untreated and saline-treated mice grew expansively, with diameters of about 1 mm at day 3 (estimated after excision with the aid of a stereo-microscope at 5 x magnification) to about 11 mm at day 15.

Endotoxin-treatment of mice with 6-day-old tumors induced a clear growth inhibition by 24 h (Table 1). Treatment at day 9 caused a marked regression as judged by the negative growth index whereas the effect of treatment of mice with 15-day old tumors was slightly less.

Table 1 - Antitumor effects of endotoxin[a]

Treatment	n	Tumor age (days)	Diameter of tumor (mm)[b]	Growth index (%)[c]	Necrosis Incidence(%)[d] light	dark	Extent[e]
Endotoxin	5	6	5.7± 0.1				
		7	5.7± 0.2	-0.4±0.2	0	0	-
	5	9	7.1± 0.2				
		10	6.5± 0.2	-8.8±1.6	20	60	54±4
	3	15	10.4± 0.8				
		16	9.8± 1.0	-6.4±2.3	33	67	65±7
Saline	5	6	5.6± 0.1				
		7	6.0± 0.1	8.2±1.3	0	0	-
	5	9	7.4± 0.2				
		10	7.9± 0.3	7.4±2.2	0	0	-
	3	15	10.5± 0.7				
		16	11.2± 0.7	6.4±1.0	67	33	26±4

a. BALB/c mice with Meth A sarcoma (6-, 9- or 15-day-old) on the abdomen, were injected i.v. with 25 μg endotoxin or saline. Observations were made at the time of injection and 24 h later. Data of 3-day-old tumors are not included, as these tumors could not be examined properly by macroscopic observation.

b. Mean ± SEM

c. Mean ± SEM of: 100x (tumor diameter 24 h after injection/tumor diameter at time of injection)-100

d. Dark- and light-stained necrosis have been scored separately

e. Ratio of the diameters of necrotic area and tumor area x100. Mean ± SEM

Tumor necrosis was not apparent after endotoxin treatment of mice with 3- and 6-day-old tumors (Table 1; data of day 3 not included). Similar treatment of mice with 9- and 15-day-old tumors caused extensive necrosis by 24 h, which was most pronounced in the oldest tumors. Fifteen-day-old control tumors showed already some spontaneous ulcerating necrosis by 4 h (data not included in the table), which was quite considerable in 16-day-old control tumors.

Tumor histology.

A. Untreated and saline-treated animals

The Meth A sarcoma transplant was situated between the abdominal wall musculature and the epidermis, with the bulk of tumor cells in the subcutis. Growth occurred predominantly into the surrounding subcutis and dermis, and only from day 9 onwards into the abdominal wall musculature and the epidermis (Fig. 1). The tumor formed highly cellular sheets of anaplastic cells, which were, especially at the lateral margins of the tumor, rounded off. Inflammatory cell infiltrate within the tumor was scarce. Around the tumor, however, a considerable number of mainly polymorphonuclear cells was observed from day 6 onwards (see Chapter 6). In tumors of untreated and saline-treated mice, numerous mitoses were present (Fig. 2).

The mean mitotic index of these control groups of mice taken together, increased with tumor age (Fig. 3). A number of small capillary transsections was apparent throughout 3-day-old tumors, suggesting vascularization of the tumor already at that time. Despite the presence of these small bloodvessels, a small cluster of necrotic tumor cells, was observed at the abdominal wall side of the tumor (Fig. 1). This cluster of necrotic cells increased in size with the age of the tumor forming a broad area of coagulation necrosis at the base of the tumor. Except for the basally located necrosis, scattered necrotic areas were observed in tumors of 6 days and older. In 15-day-old tumors, about 45 percent of the tumor appeared vital (Figs. 1 and 2). In the centre and around the base of the tumor, only a few mast cells were observed (data not shown). At the lateral margins and the skin side however, numerous

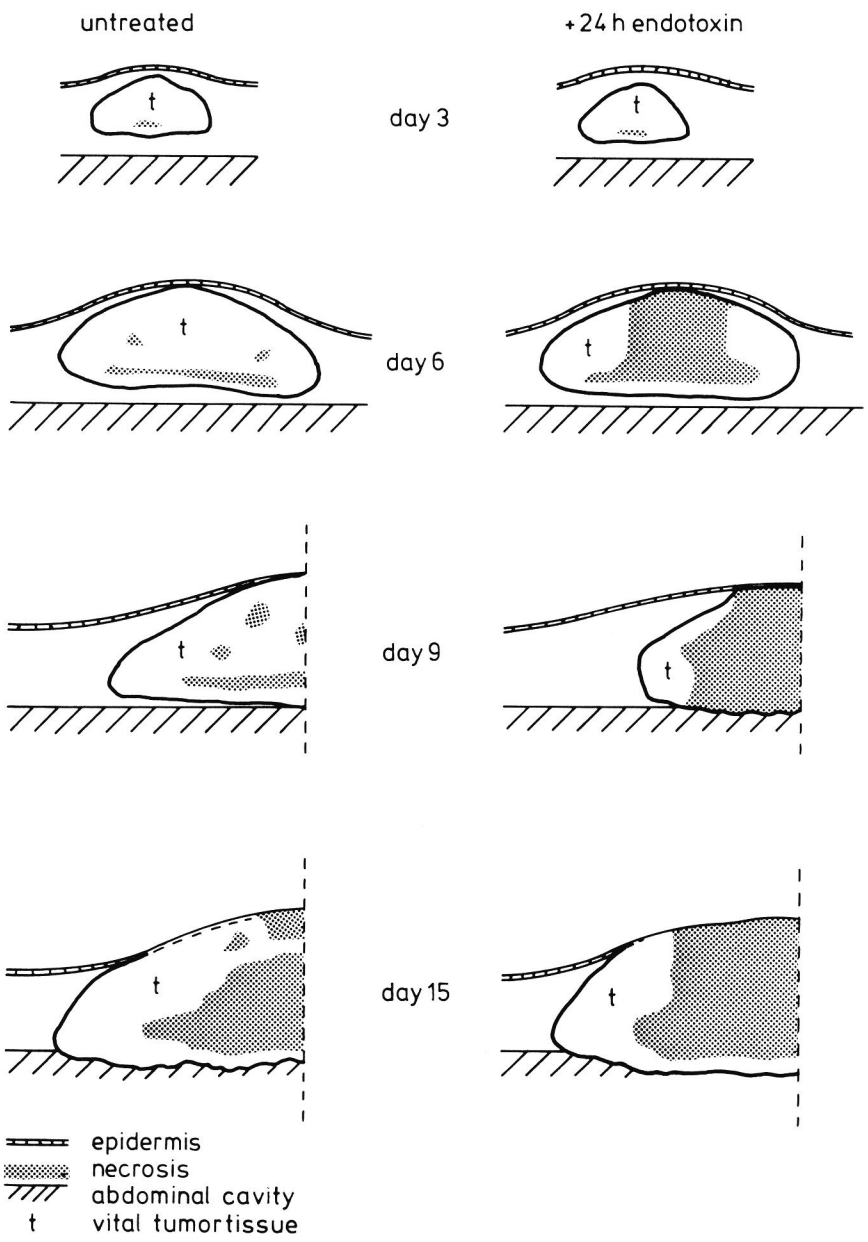


Fig. 1. Schematic representation of sections of Meth A sarcoma of different ages. Figures give an impression of tumors from mice after i.v. injection of endotoxin (right) or which were left untreated (left).

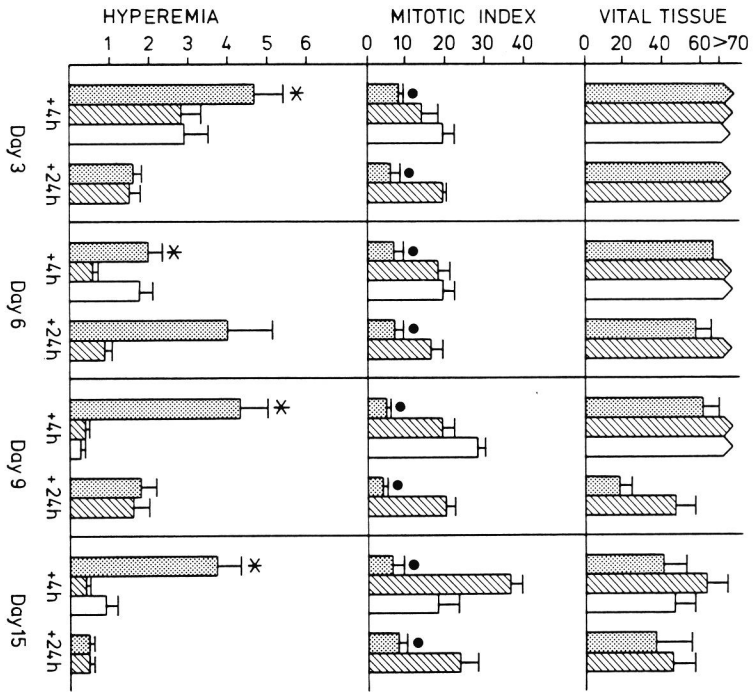


Fig. 2. Histology of Meth A sarcoma after treatment with endotoxin of *E. coli*. BALB/c mice with Meth A sarcoma (3-, 6-, 9- or 15-days-old) on the abdomen, were injected i.v. with endotoxin (⊡), saline (▨) or left untreated (□). Hyperemia is indicated as the number of vessel transsections with a diameter over 20 µm/ square mm of vital tumor tissue. Mitotic index is indicated as the number of dividing cells/0.06 square mm tumor section. Vital tissue is indicated as the area relative to the whole tumor section; areas of vital tissue covering > 70 percent of the section were not quantified in detail. Bars indicate mean ± SEM. * $p < 0.04$ as compared to controls (untreated and saline-treated groups together) and endotoxin-treated groups at 24 h. ● $p < 0.001$ as compared to controls (untreated and saline-treated groups together).

mast cells were present (Table 2). When the data of untreated and saline-treated mice were taken together, the number of mast cells in the lateral margins and the skin sites increased with the age of the tumor, till day 9. The number of mast cells in the margins at day 15 were comparable with those of day 9, but were decreased at the skin sites.

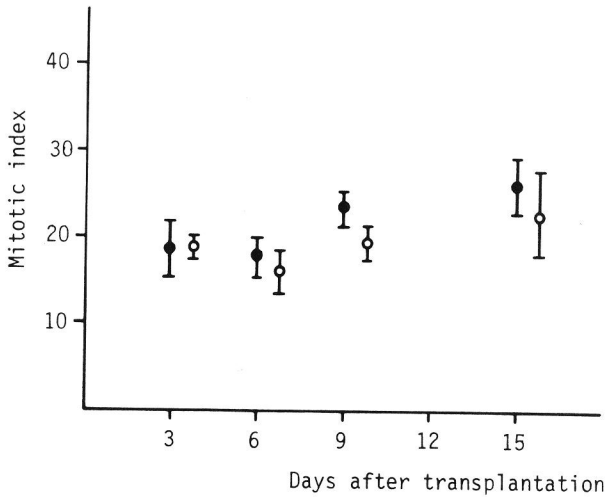


Fig. 3. Mitotic index of control tumors with age. The mitotic index is indicated as the number of dividing cells/0.6 square mm tumor section (mean ± SEM). ● = data of tumors from untreated and saline-treated mice at 4 h together; o = data of tumors from saline-treated mice at 24 h. The mitotic index was increased with tumor age ($p = 0.011$, Analysis of variance).

B. Endotoxin-treated mice

Endotoxin induced extensive dissociation and signs of degeneration of the tumor cells in the core of tumors of 6 days and older. Parts of the tumors were edematous. A few erythrocytes were seen between the tumor cells. In addition, marked hyperemia, seen as increased number of widened vessel transsections, was observed by 4 h, also in 3-day-old tumors (Fig. 2). The endotoxin-induced hyperemia as compared to the matched controls, tended to be more extensive in older tumors although this was not significant. By 24 h, hyperemia was generally no longer evident in tumors of endotoxin-treated mice, except, seemingly, at day 6. The high value at day 6 by 24 h was however, not statistically significant in the test used. The number of mitoses was less than one-third of that in controls by 4 h after endotoxin-treatment, regardless the age of the tumors (Fig. 2). This persisted by 24 h. Six-day-old and older tumors exhibited extensive areas of necrosis by 24 h after endotoxin treatment, leaving an area of vital-looking tumor tissue at the lateral and bottom margins of the tumors (Figs. 1 and 2). The area of vital tissue was smallest in 9-day-old tumors. Necrosis was characterized

Table 2 - Mast cell numbers at the lateral margins and skin sites of Meth A sarcoma after treatment with endotoxin[a]

Treatment i.v.	n	Tumor age (days)	Time after treatment (h)	Mast cell numbers Marginal sites	Skin sites
Endotoxin	3	3	4	5 ± 1	5 ± 2
			24	3 ± 1	3 ± 1
	4	6	4	3 ± 1	13 ± 1
			24	6 ± 3	8 ± 4
	4	9	4	7 ± 3	21 ± 3
			24	13 ± 3	11 ± 4
	3	15	4	9 ± 1	8 ± 2
			24	11 ± 3	3 ± 1
Saline	4	3	4	2 ± 1	3 ± 1
			24	4 ± 4	4 ± 2
	4	6	4	5 ± 1	13 ± 1
			24	9 ± 1	19 ± 3
	4	9	4	8 ± 2	18 ± 3
			24	16 ± 4	20 ± 3
	3	15	4	10 ± 4	4 ± 1
			24	10 ± 2	21 ± 2
Untreated	4	3		3 ± 1	3 ± 2
	4	6		6 ± 1	11 ± 3
	3	9		11 ± 4	23 ± 8
	3	15		17 ± 3	8 ± 2

a. BALB/c mice with Meth A sarcoma (3-, 6-, 9- or 15-day old) on the abdomen, were injected i.v. with 25 μg endotoxin, saline or left untreated. At 4 or 24 h after treatment, mast cell numbers were counted at various pre-determined sites of the tumor sections, in 4 sites at the lateral margins and 3 sites at the skin side. Data have been expressed as mean sum of 4 (margins) or 3 (skin sites) ± SEM. The number of mast cells in the margins and at the skin sites of the control tumors (saline-treated and untreated groups together) was increased with time. The numbers did not increase after day 9 ($p < 0.002$, Analysis of variance). Endotoxin treatment reduced mast cell numbers at the skin sites as compared with the saline treated controls ($p = 0.045$).

by pyknosis, karyorrhexis and cytoplasmic eosinophilia. In 9- and 15-day-old tumors, so-called hemorrhagic necrosis could be distinguished in addition at the ventral side of the tumor, where hyperemia was most overt. It was

apparent in 9-day-old tumors already by 4 h, and in 15-day-old tumors by 24 h. This type of necrosis was characterized by parallel layers of degenerating tumor cells with elongated hyperbasophilic nuclei and yellow staining of the blood vessel content and slightly of the surrounding tissue, which was also visible in unstained sections, and was very weakly iron-positive and weakly bilirubin-positive. This is indicative for erythrocyte destruction. Hemorrhagic necrosis was seen in a single 10- and 16-day-old tumor of saline-treated mice. The contribution of hemorrhagic necrosis to overall necrosis was very small (< 2 percent of the section areas). Endotoxin-induced necrosis was not associated with an increase of inflammatory cells. The number of mast cells, however, decreased at the skin-side 24 h after treatment with endotoxin (Table 2).

Lymph node histology.

The total area of the regional (inguinal) lymph nodes of untreated mice showed a progressive enlargement with increasing tumor age (Table 3), at first primarily due to an expansion of the paracortical area. From day 6 onwards plasmacytosis (Fig. 4) and germinal centre formation were very prominent. Sinus histiocytosis was apparent at day 3, decreasing slightly during further tumor growth. Mast cells were present, mainly in the subcapsular sinuses, but their numbers did not change considerably in time (data not shown).

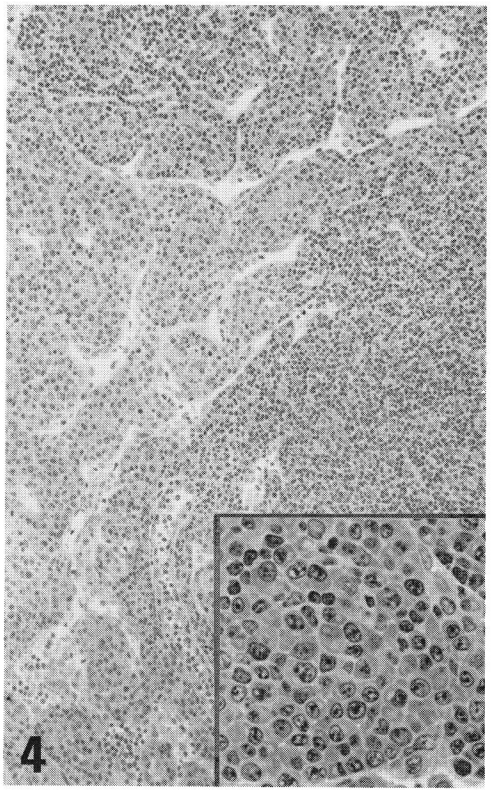
Lymph node histology of saline- and endotoxin-treated mice was essentially comparable with that of untreated mice (Table 3). Generally, however, treatment with either saline or endotoxin tended to decrease the number of plasma cells at 24 h. On the other hand, the number of small lymphocytes was increased. In the nodes of saline-treated mice, the latter effect was slightly less pronounced.

The earliest occurrence of tumor cells in the regional lymph nodes was observed in an animal with a 7-day old tumor (Table 3; 24 h after saline injection), although already 4 h after endotoxin treatment at day 3, a tumor cell was observed in a lymph vessel. At day 9, 1 or 2 animals per group had tumor-positive lymph nodes. One day after treatment with saline or endotoxin, 2 or 3 mice had positive nodes. At day 15, the nodes of all mice were tumor-positive, irrespective of treatment. The tumor cells were located in

Table 3 - Histology of inguinal lymph nodes of Meth A tumor-bearing mice after treatment with endotoxin[a]

Treatment i.v.	Tumor age (days)	Time after treatment (h)	Lymph node area (mm^2)[b]	Number of germinal centres	Mean presence of plasma cells[c]	Mean sinus histiocytosis[c]	No. of animals with tumor-positive lymph nodes
Endotoxin	3	4	1.8 ± 0.4	1.5 ± 0.6	1.0	2.0	0
		24	1.9 ± 0.3	0.5 ± 0.3	1.0	2.0	0
	6	4	3.2 ± 0.6[d]	5.3 ± 0.9[d]	1.3	2.0	0
		24	3.2 ± 0.6[d]	6.1 ± 0.8[d]	1.3	2.0	0
	9	4	5.6 ± 0.6[d]	7.5 ± 0.5[d]	2.0	2.0	1
		24	4.7 ± 0.7[d]	5.0 ± 1.0[d]	1.3	2.0	3
	15	4	6.0 ± 0.5[d]	6.4 ± 0.4[d]	2.7	1.0	3
		24	6.0 ± 0.6[d]	5.3 ± 0.8[d]	1.5	2.0	3
Saline	3	4	2.1 ± 0.3[d]	0.0 ± 0.0	1.0	3.0	0
		24	2.3 ± 0.4[d]	0.0 ± 0.0	1.0	1.0	0
	6	4	3.5 ± 0.7[d]	4.3 ± 0.6[d]	2.0	2.0	0
		24	3.2 ± 0.6[d]	6.0 ± 0.9[d]	1.3	1.7	1
	9	4	5.5 ± 0.7[d]	3.5 ± 0.5[d]	2.7	1.7	2
		24	4.4 ± 0.5[d]	8.0 ± 2.0[d]	1.3	2.0	2
	15	4	5.3 ± 1.0[d]	6.2 ± 0.9[d]	3.0	1.0	3
		24	5.7 ± 0.7[d]	8.3 ± 0.7[d]	2.7	2.0	3
Untreated	3		1.8 ± 0.3	1.3 ± 0.0	1.0	3.0	0
	6		3.1 ± 0.6[d]	3.5 ± 0.4[d]	1.7	2.0	0
	9		4.4 ± 0.7[d]	6.3 ± 0.7[d]	3.0	2.0	1
	15		6.7 ± 0.7[d]	9.1 ± 1.1[d]	3.0	1.0	3

a. BALB/c mice (n = 3 for each treatment at each time) with Meth A sarcoma (3-, 6-, 9- or 15-day-old) on the abdomen, we injected i.v. with 25 μg endotoxin, saline or left untreated
b. Mean + SEM of individual lymph nodes (n = 6)
c. 1 = <10 cells, 2 = 10-30 cells, 3 = >30 cells, scored per individual lymph node (n = 6)
d. $p < 0.05$ compared to the same treatment at day 3 (U-test)



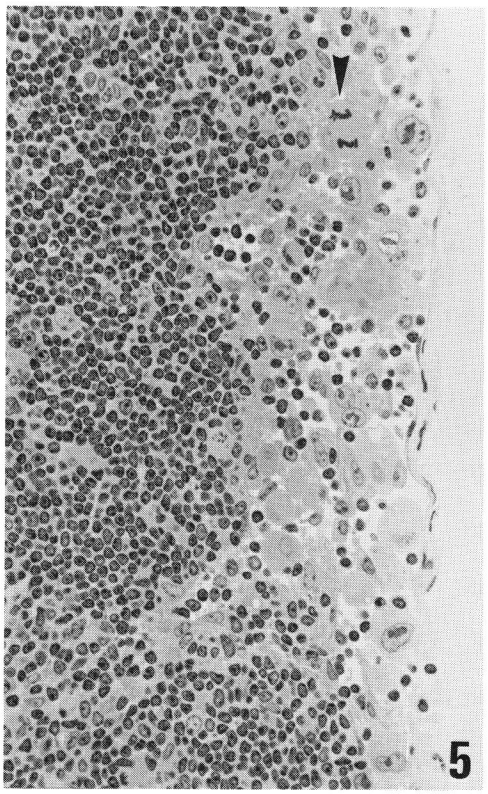


Fig. 4. Inguinal lymph node of untreated BALB/c mouse with a 9-day-old Meth A sarcoma. The medullary cords are closely packed with plasma cells. x 100. Inset: detail of medullary cord with plasma cells. x 325

Fig. 5. Inguinal lymph node of an untreated BALB/c mouse with a 9-day-old Meth A sarcoma. Tumor cells are seen in the subcapsular sinuses. Arrowhead indicates a dividing tumor cell. x 300

the subcapsular sinuses and most appeared vital (Fig. 5). Some were in mitosis, but there were no established metastases. Endotoxin had no effect on their number or their morphology.

DISCUSSION

The macroscopic effects of endotoxin against Meth A tumors of different ages indicated that this agent could induce tumor necrosis by 24 h, when injected into mice with 9- and 15-day old tumors. This observation is in line with previous experiments (Bloksma et al., 1984e). Necrosis was accompanied by a reduction of tumor size within 24 h, while tumors of saline-treated mice showed a marked enlargement during this period. Tumors of mice treated with endotoxin at day 6 did not show regression by 24 h, but this treatment prevented tumor growth during the observation period. Three-day-old tumors could not be followed macroscopically. Microscopic data indicated that the macroscopically measured extent of necrosis induced by endotoxin gave a very inaccurate impression of the real extent of necrosis (Table 1, Fig. 1). Intact 7-day-old tumors did not show necrosis one day after treatment with endotoxin, but histological data indicated that considerable necrosis was induced (Fig. 1). The absence of superficial hemorrhagic necrosis and/or the still intact (epi)dermal layer above these tumors might explain why necrosis was not observed with the naked eye. More essential is the observation that in intact 10- and 16-day-old tumors of endotoxin-treated mice necrosis appeared to be more extensive in the older tumors, while sections of the tumors showed just the opposite. In fact, histological data indicated that 16-day old tumors of endotoxin-treated mice were hardly more necrotic than similar tumors of saline-treated mice (Fig 2), and that induced necrosis was almost confined to the superficial area of the tumor (Fig 1). The overt difference in their macroscopical appearance is thus very deceitful.

It has been suggested that endotoxin-induced prompt necrosis would facilitate subsequent complete regression, which would be mediated by concomitantly immune cells (North, 1981). Our recent experiments with 9-day-old Meth A tumors have shown that 25 μg of endotoxin from *E. coli* 0111:B4, also used in this study, induced complete regression of only 3 out of 10 necrotic tumors on an average (Bloksma et al., 1983a; 1984a; 1984e). The small vital tumor mass left after endotoxin-treatment of mice with these tumors (Fig. 1) might indicate that the immune cells can cope with a very small vital tumor mass only. Tumor burdens such as found 24 h after endotoxin-treatment in 7- and 16-day-old tumors are probably far too large to be eradicated by concomitantly immune cells. All data together suggest that the optimal

susceptibility of 9-day-old Meth A tumors to endotoxin-induced complete regression is highly defined by the degree of prompt necrosis inflicted upon the tumor within 24 h.

The mechanisms leading to tumor necrosis are still largely unknown. In various studies with 9-day-old Meth A tumors, the observed degree of hyperemia, seen as increased number of widened vessel transsections, and mitotic arrest correlated well with the extent of necrosis (Chapters 2, 3, 4 and 7). The present study shows that endotoxin caused hyperemia in tumors of all ages and that this was always accompanied by mitotic arrest. As hyperemia frequently leads to a decreased energy supply of the affected tissue, mitotic arrest is well in line with energy shut off. The observation, however, that all tumors of endotoxin-treated mice exhibited these phenomena, while only 9-day-old tumors appeared susceptible to induction of extensive necrosis as judged histologically, suggests that additional factors leading to necrosis must be involved. These factors are not clearly apparent from the data obtained in the present study. Mast cells at the skin side of the tumor might somehow be involved, as these cells were most abundant in 9-day old tumors (Table 3). Although these cells appeared morphologically intact by 4 h after treatment with endotoxin, this does not exclude release of certain mediators at that time, as has been demonstrated recently for serotonin (Van Loveren et al., 1984). Serotonin could induce slight hyperemia, mitotic arrest and necrosis of Meth A tumors after i.v. administration (Chapter 3). Moreover, this agent is known to potentiate the action of various vasoactive agents and to have a more potent action in hypoxic tissue (Makabali et al., 1982; Douglas, 1975). Mast cells were also shown to have a cytotoxic action against murine and rat fibrosarcoma cells in vitro (Farram and Nelson, 1980a; 1980b). These data together prompt us to further studies on the possible role of mast cell mediators in endotoxin-induced tumor necrosis. A role of circulating tumoricidal or tumoristatic factors such as tumor necrosis factor and interferons, which are induced by endotoxin in vivo, in the necrosis observed, is not very likely. Both factors were shown to be cytostatic for Meth A cells in vitro, but this effect was not detectable before 24 h of in vitro incubation (Carswell et al., 1975), whereas strong mitotic arrest in Meth A tumors was already apparent by 4 h after endotoxin treatment (Fig. 2). Moreover, the Meth A cells in the regional inguinal lymph nodes remained unaffected in endotoxin-treated mice

despite a marked necrotic reaction of the primary Meth A tumor.

A small area of the necrosis induced at the skin side of 9- and 15-day-old tumors was histologically different from the main necrotic area, and was designated as hemorrhagic necrosis. Growth of the tumor into the papillary dermis, which occured apparently between day 6 and 9, might be related to induction of hemorrhagic necrosis. Superficial blood vessels in the papillary dermis are regarded to be anatomically and functionally distinct from vessels located at the junction of papillary and reticular dermis (Dvorak et al., 1976; Willms-Kretschmer et al., 1967). Upon histamine and immunologically mediated hypersensitivity reactions, edema and intravascular compaction of erythrocytes appeared to be dominant features in the superficial region, whereas perivascular lymphocyte cuffing and endothelial cell activation and damage were mainly observed in the deeper dermis. The importance of hemorrhagic necrosis in tumor destruction, however, is doubtful and might be related to the toxic properties of endotoxin, because tumor necrosis induced by highly therapeutic nontoxic combination treatments was not attended with hemorrhagic necrosis (Chapter 7).

The Meth A sarcoma is regarded as a hardly metastasizing tumor (North, 1984; Reichert et al., 1985). A low frequency of metastases has been explained by host immune mechanisms against the tumor such as concomitant immunity, which would prevent dissemination, establishment and outgrowth of detached tumor cells (Gorelik, 1983). Although Meth A is known to induce concomitant immunity, tumor cells did disseminate to regional lymph nodes. These tumor cells appeared vital and capable to divide. No evidence of tumor cell necrosis could be observed in the lymph nodes. Nevertheless, these tumor cells failed to form established metastases, which might be related to their incapacity to adhere to surfaces as observed in vitro (Carswell et al., 1975). Endotoxin had no significant effect on dissemination or appearance of these tumor cells, which confirms early observations that it has no direct tumoricidal action (Carswell et al., 1975; Green et al., 1977).

The regional lymph nodes of tumor-bearing mice had a distinct reaction to the tumor. Their size increased proportionally with the tumor size, initially due to stimulation of the T-cell dependent paracortex but from day 6 on mainly due to increased reactivity of the B-cell dependent areas. Comparable changes were described by others and were associated with the development of a state of concomitant immunity by some (Edwards et al., 1971;

Nelson and Kearney, 1976), while Van De Velde et al. (1978) failed to show a relation between lymph node response and immunogenicity of the tumor. It is very unlikely that the B-cell response represents a specific antibody response to the Meth A cells as production of antisera against this tumor was shown to be very difficult even in xenogeneic animals (Livingston et al., 1985). The B-cell reactivity rather resembles that seen in chronic graft-versus-host reactions, where prolonged specific activation of helper T-lymphocytes would cause polyclonal activation of B-lymphocytes (Gleichmann et al., 1984). Such reactions are known to be attended with marked non-specific immune suppression (Shearer and Levy, 1983; Maier et al., 1985; Mendes et al., 1985).

Endotoxin did not cause significant changes in the lymph nodes within one day, as compared to controls. This supports evidence indicating that induction of tumor necrosis is not an immune-mediated event.

The present study has shown that histology is needed to allow a reliable estimate of the extent of necrosis induced by endotoxin in small as well as large tumors. Whereas ample circumstantial evidence in literature has shown that the therapeutic activity of endotoxin requires a state of concomitant immunity, the present data indicate that susceptibility to induction of very extensive prompt tumor necrosis, as only observed in 9-day-old tumors, is probably an indispensable condition. The mechanisms determining tumor age-dependent susceptibility to induction of tumor necrosis are still far from clear. Induction of hyperemia and mitotic arrest are probably important but not sufficient. The observation that endotoxin did not affect tumor cells in the lymph node may confirm that the agent is not tumoricidal itself and indicates that it does not generate cytotoxic lymphocytes or tumoricidal factors effective during the observation period.

CHAPTER 6

ANTITUMOR EFFECTS OF ENDOTOXIN AGAINST SOLID MURINE METH A TUMORS OF DIFFERENT AGES II. ULTRASTRUCTURE OF VASCULAR AND PERIVASCULAR EVENTS

C. Frieke Kuper, Nanne Bloksma and Evert G.J. Hendriksen

SUMMARY. The ultrastructure of blood vessels and perivascular tissue of 3-, 6-, 9- and 15-day-old Meth A tumors and changes induced by intravenously injected endotoxin were studied to get a better insight in the tumor age-dependent antitumor effects of this agent as described in Chapter 5. In control tumors vascular injury increased with the age of the tumor up till day 9. The neovasculature in the tumor lacked smooth musculature and vessel lumina were compacted with polygonal erythrocytes. Their leukocyte content varied with age. It was low at day 3. At day 6 and 9 high numbers of mononuclear and polymorphonuclear cells were seen respectively. The more moderate infiltrate at day 15 consisted of an equal mixture of both. Few leukocytes were observed outside the vessels. Endotoxin enhanced vascular disintegration at the skin side of 9- and 15-day-old tumors markedly, already by 4 h. Hemorrhagic necrosis was observed in this area. In the remaining part of the tumor, with exception of the margins, extensive edema and coagulation necrosis, followed by vascular injury were observed. Development and ultimate aspect of tumor cell necrosis in both areas were quite different. Signs of early intravascular coagulation were only apparent in 15-day-old tumors. Host cell infiltrates were not significantly changed by treatment with endotoxin.

Data support the hypothesis that endotoxin-induced tumor necrosis is mainly the result of its effects on the tumor vasculature. The regionally different vascular effects are probably related to the different types of tumor necrosis induced. The lack of vascular homeostatic control and the more pronounced preexistent vascular defects in larger tumors, predominantly

localized in the tumor core, might explain why induction of tumor necrosis is restricted to the cores of larger tumors. Vascular injury and subsequent necrosis might be potentiated by the presence of large numbers of polymorphonuclear cells only observed in 9-day-old tumors.

INTRODUCTION

In the previous chapter we have described the antitumor effects of endotoxin against Meth A tumors of different ages by light microscopy. Tumor necrosis was most marked in 9-day-old tumors. It was preceded by hyperemia and hemorrhage which occurred earlier than in younger and older tumors. These and other data indicate that the tumor vasculature is a prime target of the antitumor actions of endotoxin (Parr et al., 1973; Bloksma et al., 1984d; 1984f; Chapters 2 and 5). How vascular alterations would be induced is still a matter of controversy. The oldest theory on endotoxin-induced tumor necrosis, still favoured by some investigators at present, suggested that endotoxin would cause a Shwartzman reaction within the tumor. Local intravascular coagulation would result in hypoxic ischemia and cell death (Gratia and Lintz, 1931; Parr et al., 1973; Kodama et al., 1982). Polymorphonuclear leukocytes are thought to play a main role in it. Anticoagulants, however, did not influence endotoxin-induced necrosis of Meth A tumors (Bloksma et al., 1984c) and endotoxin-induced fibrin deposition and polymorphonuclear cell infiltrates appeared to be moderate or even absent in Meth A tumors undergoing necrosis (Chapters 2 and 4). Another theory suggested that necrosis would be mainly caused by an abnormal reaction of the tumor vasculature to vasoactive agents released by endotoxin, leading to congestion, stasis of blood flow and subsequent hypoxic cell death (Bloksma et al., 1984c; 1984d; 1984f).

In immunologic reactions like delayed type hypersensitivity, allograft rejection and Arthus reactions, tissue destruction has been attributed to the consequences of vascular damage rather than to direct contact of individual target cells and cytotoxic leukocytes or their cytotoxic products (Dvorak et al., 1976; Bogman et al., 1984). Because the Meth A tumor used in this study is immunogenic, immunologically mediated vascular damage has to be considered too, although many data indicate that tumor necrosis induced

by endotoxin within 24 to 48 h is not dependent on T-cell mediated immune reactions (Parr et al., 1973, Kodama et al., 1982; North, 1984).

To get a better insight in the mechanisms leading to tumor age-dependent vascular reactions within Meth A sarcoma we did an electron microscopical study of the vascular and perivascular morphology within and around tumors of different ages before and after administration of endotoxin by electron microscopy.

MATERIALS AND METHODS

The animals used in this study were part of the experiment described in Chapter 5. The materials and methods are described in that chapter, except the procedures for the light and electron microscopic examination. Glutaraldehyde-fixed tumor pieces were postfixed in 1% OsO4, buffered in 0.1 M cacodylate (pH 7.35) overnight, dehydrated by graded water-aceton mixtures and embedded in an Epon-Araldite mixture. Semithin sagittal sections (1-2 μm) and sections perpendicular to that direction from the central part of the tumor and surrounding host tissue were stained with toluidine blue. Semithin and ultrathin sections, from representative sites (Fig.1), were cut

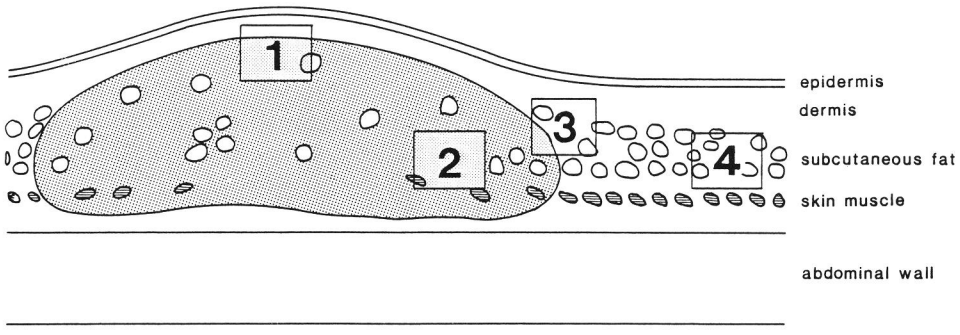


Fig. 1. Schematic presentation of the sampling for electron microscopical examination of Meth A sarcoma. Level 1 is situated at the skin-side, level 2 at the deep margin and levels 3 and 4 at the border and at some distance of the tumor respectively.

using the mesa technique. Mesa 1 was situated at the skin-side of the tumor (indicated as level 1), mesa 2 was situated in the margin of the tumor (level 2), mesa 3 and 4 were situated at the border (level 3) and at some distance (level 4) of the tumor. The ultrathin sections were stained with magnesium uranyl acetate and lead citrate, and studied with a Philips electron microscope, type EM 201G at 60 kV.

RESULTS

The tumor.

Ultrastructural examination revealed sparse bundles of collagen fibres between the Meth A sarcoma cells (Fig. 2). The tumor cells were rich in polyribosomes and had a considerable number of mitochondria, which were centered around the nucleus in the cells in the periphery of the tumor. Some mitochondria were swollen. The surface of the cells that were not too closely packed, showed microprojections. At the base of 3-day-old tumors, possibly at the inoculation site, an area of tumor cells with fatty change was observed. This area was clearly necrotic at day 6, and did expand with the age of the tumor, while cells with fatty change around these areas indicated progressive decay. In the vital parts individual cell necrosis was found from day 6, characterized by severe mitochondrial damage and compact polygonal nuclei, without fatty degeneration. Sometimes, lymphocytes were seen in close proximity of these cells. At day 9 and 15, also scattered areas of necrosis were found throughout the tumor. These were not accompanied by marked inflammatory cell infiltrates. At day 15, ulceration of the skin was observed.

The most rapidly occurring effects of endotoxin were observed in 9-day-old tumors (Table 1). Two patterns of tumor cell necrosis could be distinguished. In the upper part of level 1, the hemorrhagic necrosis (HN) area, tumor cells had elongated nuclei with chromatin clumping by 4 h. No fatty degeneration was seen. By 24 h, the cytoplasm was devoid of recognizable organelles and only some electron-lucent vacuoles could be observed (Fig. 3). Tumor cells in the lower part of level 1 and in level 2 were rounded off and had prominent microprojections by 4 h after endotoxin (Fig. 4).

Table 1. Effects of endotoxin on vascular and perivascular morphology at 4 different levels in and outside Meth A sarcoma, 3, 6, 9 or 15 days after transplantation[a]

Tumor age (days)	3		6		9		15	
Time after treatment (h)	4	24	4	24	4	24	4	24
LEVEL 1 (skin-side)								
Hemorrhagic necrosis (HN) area								
HN present	0	0	0	0	±	+	0	+
Endothelial cell damage	0	0	0	+	+	++	+	+
Hemorrhage	0	0	0	0	+	nm[b]	+	nm
Platelet aggregates with fibrin	0	0	0	0	0	+	++	++
Coagulation necrosis (CN) area								
CN present	0	0	0	+	±	+	±	+
Fatty change	0	+	0	+	+	nm	0	+
Interstitial edema	0	±	0	±	+	+	+	+
Platelet aggregates without fibrin	0	0	±	±	±	+	++	++
Endothelial cell damage	0	0	0	0	0	+	0	+
LEVELS 2 and 3 (deep margin and lateral border inside the tumor)								
Coagulation necrosis (CN) area								
CN present	0	0	0	±	+	+	+	+
Fatty change	0	+	0	+	+	+	0	+
Interstitial edema	0	±	0	±	+	+	+	+
Hemorrhage	0	0	0	±	+	+	+	+
Endothelial cell damage	0	0	0	±	±	+	+	+
Platelet aggregates with fibrin	0	0	0	±	0	0	0	++
LEVEL 3 (lateral border outside the tumor)								
Compaction of vessels with erythrocytes	+	+	+	+	+	0	+	+
Mononuclear inflammatory cells	0	0	−	−	−	−	0	0
Echinocyte-like erythrocytes	+	0	0	0	0	±	+	0
LEVEL 4 (at some distance of the tumor)								
Echinocyte-like erythrocytes	+	0	+	+	0	±	±	0

a Effects of endotoxin are expressed insofar as there were differences with control tumors: − = decreased; 0 = absent or comparable with controls; ±, +, ++ = slightly, markedly, very markedly increased as compared with controls, or present.

b nm = could not be examined properly because of extensive necrosis

The interstitium was edematous (Table 1). Most cells showed already extensive fatty change at that time (Table 1), but mitochondrial damage was not consistent. By 24 h, signs of irreversible nuclear damage such as chromatin clumping, and more advanced cytoplasmic damage accompanied the fatty change (Fig. 5). Tumor cells in level 3 were hardly affected by endotoxin. Changes in 3-day-old tumors were very moderate. Slight fatty degeneration was observed in most cells by 24 h and some edema around a few large vessel transsections (Table 1). In 6-day-old tumors HN was not observed, but other changes were similar to those seen in 9-day-old tumors, be it that effects were less prominent and delayed. Fifteen-day-old tumors showed the same pattern of tumor cell degeneration as 9-day-old tumors (Table 1).

Vasculature, platelets and fibrin deposits.

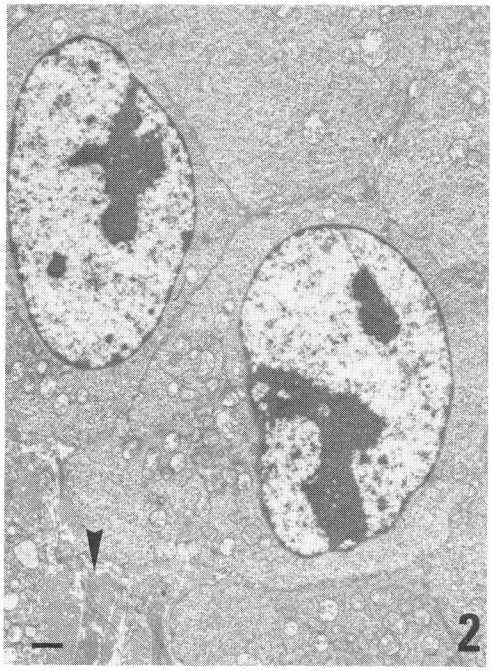
Large, probably preexistent vessels with a size over 40 μm in diameter, were located at level 1 of 3-day-old tumors. Only a few very small vessels were found throughout the tumors. Outside the tumor, at level 3, clusters of small vessels were observed. Tumors older than day 3 were supplied by numerous small vessels. Few of these had a diameter over 20 μm. Remarkably,

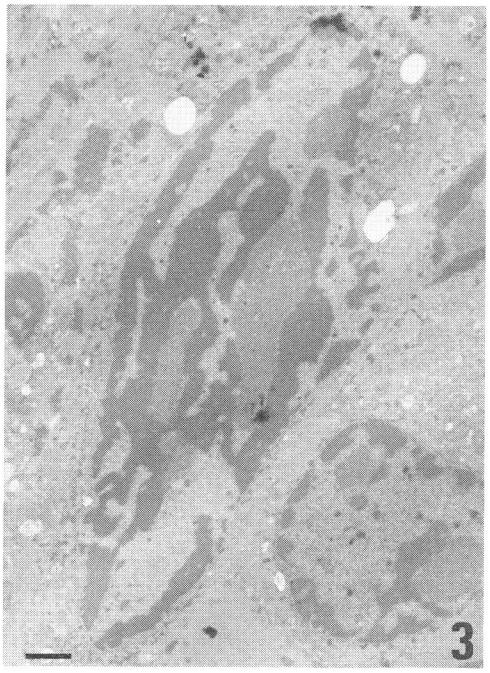
Fig. 2. Six-day-old tumor of untreated (control) mouse. Meth A sarcoma cells with intercellular bundles of collagen fibres (arrowhead) (bar = 1 μm).

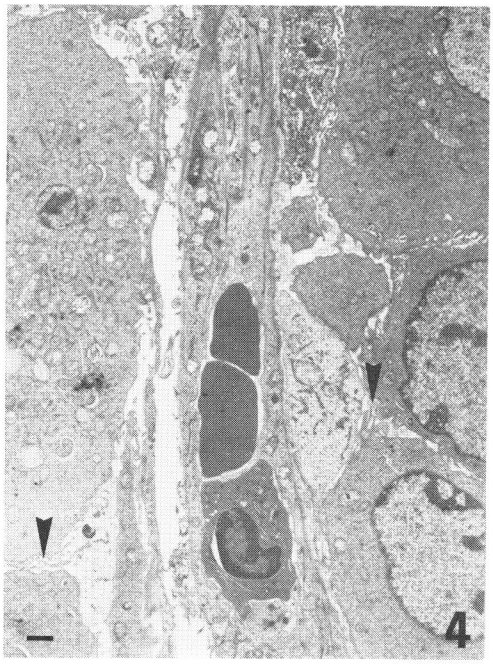
Fig. 3. Tumor cell in the HN area at level 1, showing the virtual absence of cytoplasmic organelles and the presence of electron-lucent vacuoles, 24 h after endotoxin injection in a mouse with a 9-day-old tumor (bar = 1 μm).

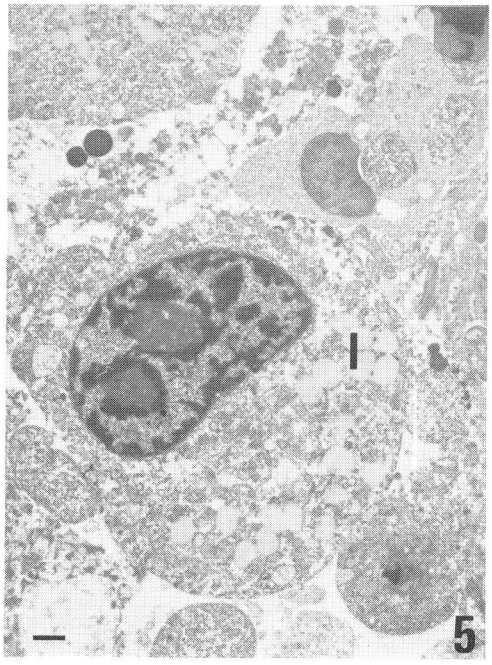
Fig. 4. Tumor cells in the coagulation necrosis area at level 2, 4 h after endotoxin injection in a mouse with a 9-day-old tumor. Slight interstitial edema and rounding off of tumor cells, showing numerous microprojections (arrowheads) (bar = 1 μm).

Fig. 5. Tumor cell in the coagulation necrosis area at level 2, 24 h after endotoxin injection in a mouse with a 9-day-old tumor. Extensive cell damage and cytoplasmic lipid droplets (l) (bar = 1 μm).





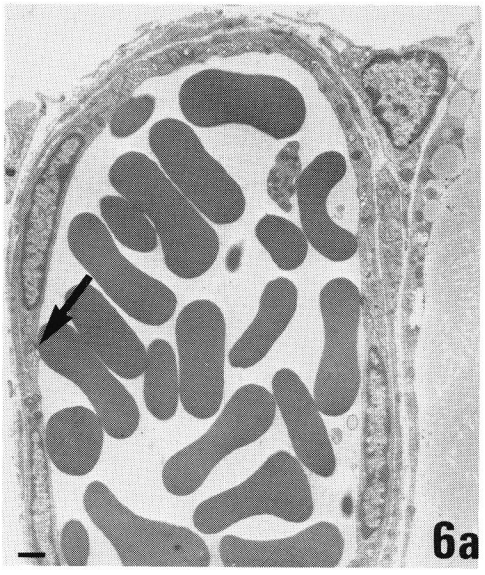




vessels were also found within areas of necrosis. At level 3, outside tumors of day 6 and older, vessels with larger diameters than usually present in the skin were observed. All intratumoral vessels were markedly compacted with polygonally-shaped erythrocytes; immediately outside all tumors, however, no compaction was found, except in tissues outside 6-day-old tumors. The non-preexistent intratumoral vessels consisted of a sheet of continuous, occasionally plump, endothelial cells, often surrounded by pericytes. Smooth muscle cells were absent and the basal lamina was slightly thickened. The endothelial cells were not fenestrated, and had less pinocytotic and micropinocytotic vesicles than endothelium of normal skin vessels (Figs. 6a-b and 7). Some of the mitochondria were swollen. Degenerated endothelial cells showing lucent cytoplasm and nuclear blebs were often found, both in obviously preexistent and non-preexistent vessels. Edema and hemorrhage, suggestive of vascular disintegration, were observed in tumors of days 9 and 15 (Fig. 8). Vessels immediately outside 6- and 9-day-old tumors and some 3-day-old tumors had a thickened basal lamina too. The endothelial cells varied in thickness. Most endothelial cells lining vessels outside the tumor at level 3 of 3- and 15-day-old tumors, and at levels 3 and 4 of 6- and 9-day-old tumors were plump. Occasionally, pericytes were plump too. Intraluminal flaps at the margins of the endothelial cell junctions were only found in vessels outside the tumor. The number of platelets in intratumoral vessels was higher than normally found in peripheral blood, probably due to increased sloughing of blood within the tumor. Single platelets adhered to the vessel wall. At day 6 and 15, platelet aggregates were observed at level 1 (Fig. 9). These platelets showed granules with

Fig. 6a-b. Bloodvessel in the skin of a tumor-free mouse. a. Overview of vessel with disc-shaped erythrocytes and a normal platelet. b. Detail of endothelial cells, indicated in 6a by arrow. Numerous pinocytotic bodies (arrowhead) are present (bar = 1 μm).

Fig. 7. Blood vessel at level 2 of a 6-day-old tumor of an untreated mouse. The vessel is clogged by erythrocytes and leukocytes. The endothelial cells (e) are plump and show few cytoplasmic organelles. The basal lamina is thickened (see also Fig 8) (bar = 1 μm).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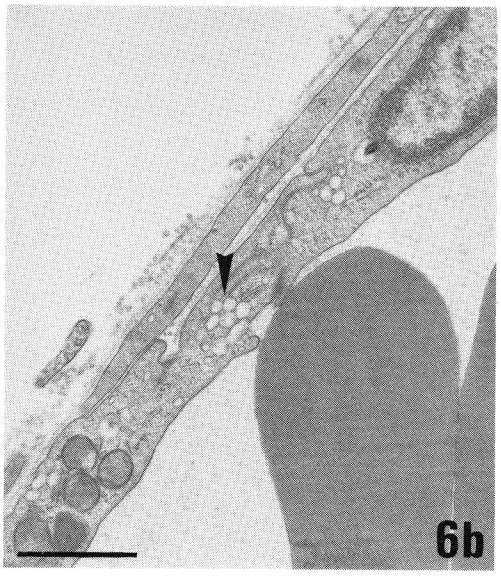
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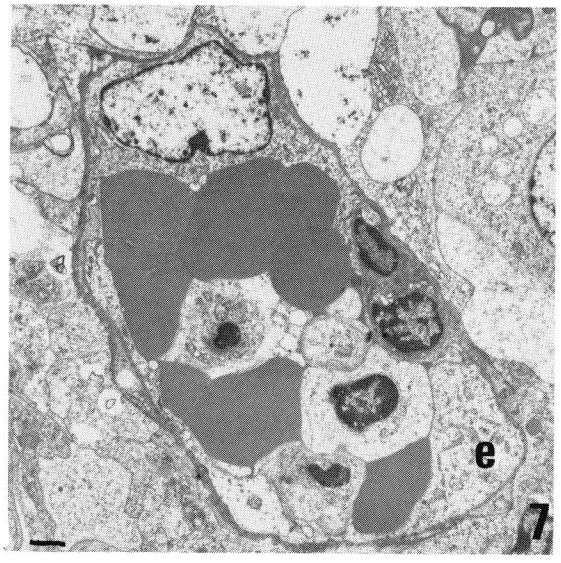
e
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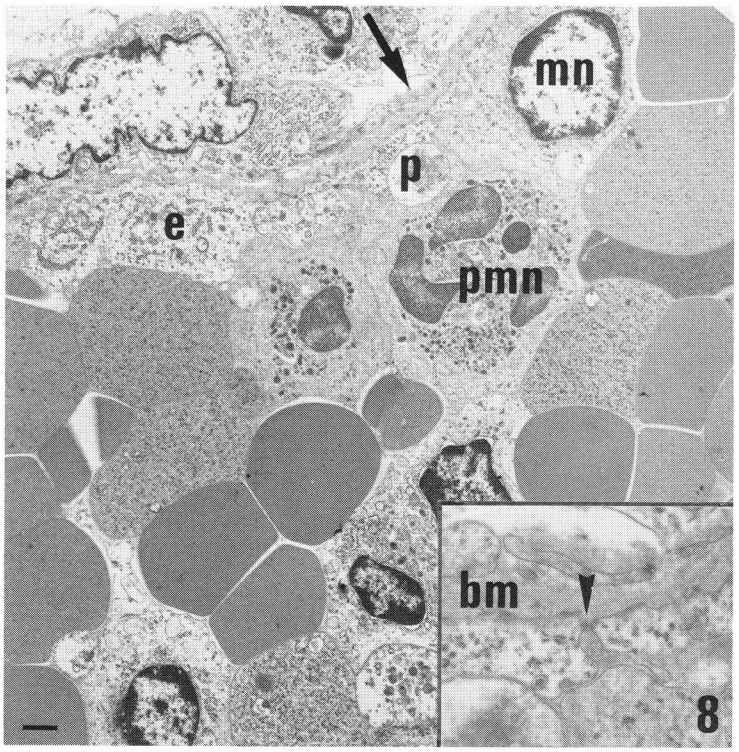
different degrees of vacuolization. Adherent platelets or platelet aggregates were not observed in vessels outside the tumor, but some platelets had pseudopods. At day 15, an increased number of normally granulated, disc-like platelets was found in and around the tumor at level 3. Despite endothelial cell damage, fibrin deposits were not observed.

By 4 h after endotoxin injection into mice with 9-day-old tumors, already some endothelial cells were severely damaged in the HN area of level 1 (Table 1; Figs. 11a-b and 12) and increased erythrocyte extravasation was seen. Some endothelial cells exhibited large vacuole-like structures, open to the basal lamina; others showed irregularly dilated rough endoplasmic reticulum. The underlying basal lamina had lost its compactness. This was more marked by 24 h. At that time also aggregates of degranulated platelets with fibrin were observed in some vessel transsections, especially the larger ones (Table 1; Fig. 10). Smaller vessels appeared less affected. In the coagulation necrosis area at levels 1 and 2 extensive edema was found by 4 h (Table 1). Endothelial cell damage became apparent by 24 h. At level 1, some platelet aggregates without fibrin were found. Increased hemorrhage was confined to the edge of the basal necrotic area. At level 3 outside the

Fig. 8. Blood vessel at level 1 of a 9-day-old tumor of an untreated mouse. Aggregate of mononuclear (mn) and polymorphonuclear (pmn) leukocytes and platelets (p) adhering to an endothelial cell (e) sheet. Some edema is present. Inset: Detail of the endothelial sheet, in the cytoplasm many fragments of rough endoplasmic reticulum, indicated by the arrow in the main figure. The basement membrane (bm) is markedly thickened. The arrowhead indicates a gap at the endothelial cell junction or in the cytoplasm by fusion of pinocytotic bodies (bar = 1 μm).

Fig. 9. Platelet aggregate in a bloodvessel at level 1 of a 6-day-old tumor of an untreated mouse. The platelets have lost their normal shape and are to some extent degranulated. Arrowhead indicates fusion of vacuoles (bar = 1 μm).

Fig. 10. Platelet-fibrin aggregate in a bloodvessel in the HN area at level 1, 24 h after endotoxin injection in a mouse with a 9-day-old tumor. Most platelets (p) are completely devoid of granules (bar = 1 μm).


mn
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bm
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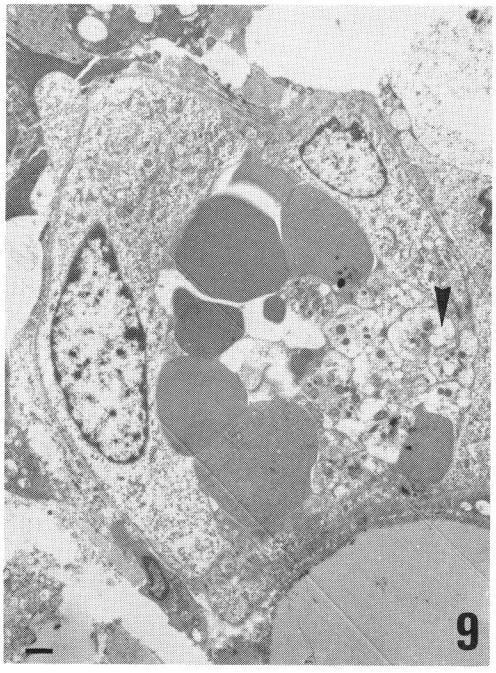
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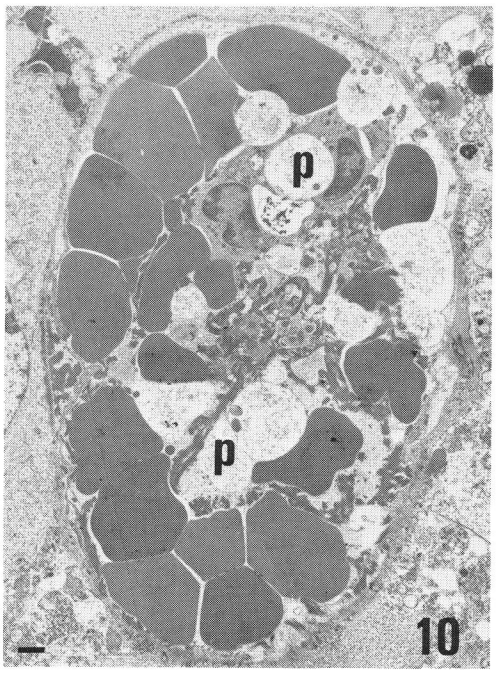
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tumor, vessels were compacted with erythrocytes by 4 h (Table 1). This had disappeared by 24 h. A few echinocyte-like erythrocytes were observed at levels 3 and 4 by 24 h (Table 1). In 3-day-old tumors, the centre of the tumor was slightly edematous by 24 h. Compaction of erythrocytes in vessels at level 3 was more extensive and prolonged than in the 9-day-old tumor. Also, more echinocytelike erythrocytes were found. Six-day-old tumors showed the same effects. In addition, endothelial cell damage in the HN area at level 1 was found. Platelet aggregates with some fibrin were observed in the coagulation necrosis area at level 2 (Table 1). Effects in 15-day-old tumors were com-parable with those of 9-day-old tumors, except that the number of platelet aggregates with or without fibrin and endothelial cell damage at levels 1 and 2 were more marked.

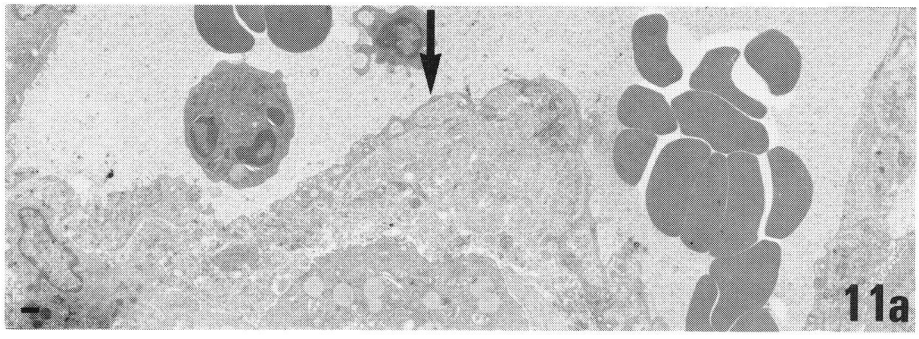
Compaction of erythrocytes in vessels just outside the tumor was also observed after saline injection into mice with 9- or 15-day-old tumors, although by 24 h instead of 4 h as seen with endotoxin (Table 1).

Inflammatory cell infiltrate.

Relatively few inflammatory cells were observed in the central part (levels 1 and 2) of all tumors. Incidentally cells had migrated into the tumor mass. Inflammatory cells were most numerous at level 3 of the tumors. At day 3, a few polymorphonuclear and mononuclear cells were observed in and around the tumor. At day 6, however, a marked intravascular accumulation of inflammatory cells consisting of lymphocytes, monocytes, neutrophils and an occasional eosinophil, was found at levels 2 and 3 of the tumor. These cells

Fig. 11a-b. Nine-day-old tumor, 4 h after endotoxin injection. a. Overview of a vessel at the border between HN area and CN area at level 1. Endothelial cells are extensively damaged. b. Detail of an endothelial cell (arrow in Fig 11a) with large cytoplasmic vacuoles, open to the basement membrane. The basement membrane has lost its compactness (bar = 1 μm). Bm: basement membrane

Fig. 12. Nine-day-old tumor, 4 h after endotoxin injection. Endothelial cell with marked irregular dilation of the rough endoplasmic reticulum (bar = 1 μm).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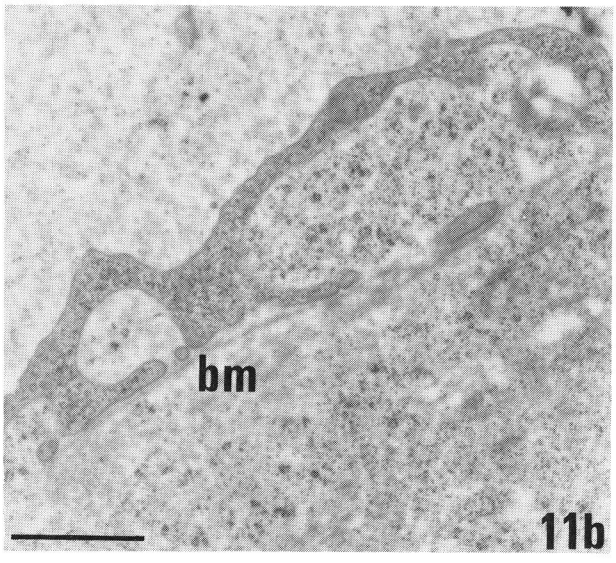
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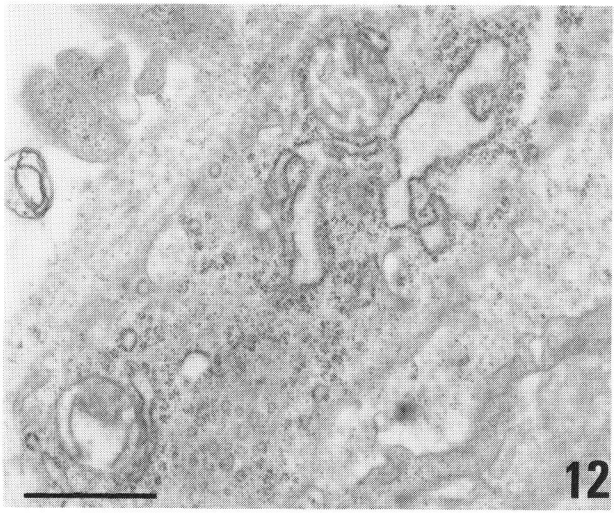
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clogged some of the vessels almost completely (Figs. 7 and 8). The influx had probably started with mononuclear inflammatory cells, as 4-day old tumors (3-day old tumors, 24 h after saline injection) showed some accumulation of mononuclear cells in the margins of the tumor. At day 9, a considerable and predominantly polymorphonuclear cell accumulation was found. Fifteen-day old tumors showed less inflammatory cells, consisting of about equal numbers of mononuclear and polymorphonuclear cells. The inflammatory cells sometimes adhered to both injured and intact endothelial cells.

Numbers and type of inflammatory cells in and around the vessels of all tumors were not changed by endotoxin. Outside 6- and 9-day-old tumors at level 3, the numbers of mononuclear inflammatory cells were slightly decreased (Table 1).

DISCUSSION

Vascular and perivascular morphology within and around Meth A tumors of different ages was studied in control and endotoxin-treated mice in order to shed more light on the age-dependent susceptibility of tumors to the antitumor effect of endotoxin. Three-day-old control tumors (diameter about 1 mm) showed already neovascularization. This is consistent with data of Folkman and Cotran (1976). Neovascularization kept pace with tumor growth. Intratumoral blood flow seemed not optimal as evidenced by the marked intraluminal compaction. This might be related to the lack of adequate mechanisms for homeostatic control of these vessels. The frequently observed degenerating endothelial cells also, do not promote undisturbed blood flow. The innate lack of homeostatic control together with the increased deterioration of vascular condition with age, especially in the tumor core might be related to the extent of spontaneous necrosis. Vascular deterioration has been attributed to the action of inflammatory cells (Dvorak and Dvorak, 1982; Galli et al., 1982). Number and composition of inflammatory cells within Meth A varied with age. The marked inflammatory cell accumulation in 6-day-old tumors beared good resemblance to a delayed type hypersensitivity reaction to polysaccharides, found to be attended by vascular injury and fibrin deposition (Crowle and Hu, 1967). Polymorphonuclear cells, known for their potential to induce vascular damage (Issekutz, 1981; Issekutz and Movat,

1982; Issekutz et al., 1983; Movat and Wasi, 1985) were observed in high numbers in 9-day-old tumors. However, the most abundant host cell accumulation, both inside and outside the vessels, was always found at level 3. The relatively good vascular condition and the absence of substantial necrosis in this area raises the question whether inflammatory cells contribute to the induction of spontaneous necrosis, either by causing vascular damage or by direct tumoricidal action.

The observed decrease of cell infiltrates in 15-day-old tumors as compared to 9-day-old tumors is remarkable because an accumulation of scavenger cells would be expected due to the massive spontaneous necrosis in tumors of that age. A decrease of infiltrates in older Meth A tumors was not found by Reichert et al. (1985). Polymorphonuclear and mononuclear cell infiltrates remained high or even increased till about 20 days after transplantation in the abdominal skin of semi-syngeneic mice. The hybrid resistance of semi-syngeneic mice (Marsili et al., 1986) and other differences in experimental conditions might account for this.

The antitumor effects of endotoxin could be best characterized by an acceleration and/or amplification of naturally occurring events during tumor growth. Antitumor effects were most prominent in 9- and 15-day-old tumors and consisted of a premature onset of hemorrhagic necrosis and an accelerated extension of coagulation necrosis. Hemorrhagic and coagulation necrosis could be clearly discerned in their development. It is not unlikely that hemorrhagic necrosis would primarily be due to a severe loss of vascular integrity causing a rather abrupt and irreversible hypoxic damage. Coagulation necrosis might be the consequence of enhanced pressure within the tumor due to the interstitial edema which might increasingly hamper oxygen and nutrient supply by the already highly compacted blood vessels. Our data do not indicate that intravascular coagulation would play a pivotal role in the induction of tumor necrosis. Only 15-day-old tumors showed marked early fibrin deposition (Table 1), while the extent of induced necrosis was greater in the younger tumors (Chapter 5). A Shwartzman reaction as cause of tumor necrosis (Kodama et al., 1982) is therefore not applicable to this tumor.

In the lateral border (level 3) of the tumor, cells generally survived endotoxin treatment. Endotoxin did not cause severe blood vessel damage in this area and interstitial edema was less prominent than observed in

level 2. These regional, relatively favourable, conditions might explain the tumor cell survival seen in level 3. Nine-day-old tumors showed the lowest marginal tumor cell survival. Even in this instance tumor regrowth from the margins was frequently observed (Bloksma et al., 1984e). Therefore, a better understanding of the mechanisms determining marginal tumor cell survival after endotoxin-treatment is a clue for further research and design of additional treatment strategies.

Data obtained from this study do not provide clear cut indications why 9-day-old tumors showed the most extensive necrosis after endotoxin treatment. Qualitatively, necrosis was very similar in 9- and 15-day-old tumors. An already poor condition of the blood vessel function seems to contribute to induction of tumor necrosis, although only to a certain degree. Probably, supplies for counteracting or recovery of vascular damage are more favourable in older tumors. The observation that Meth A tumors show exponential tumor growth with age (North, 1984) supports this. The composition of the tumor cell infiltrate at the time of treatment also might more or less define the extent of necrosis. The predominance of polymorphonuclear cells within and immediately outside 9-day-old tumors might be significant. These cells and their products are well-known for their potential to induce edema, hemorrhage and endothelial damage (Issekutz, 1981; Issekutz and Movat, 1982; Issekutz et al., 1983; Movat and Wasi, 1985) and were shown to be cytotoxic for a variety of tumor cells in vitro (Korec et al., 1980, Dvorak and Dvorak, 1982). Moreover, endotoxin-induced vascular injury has been frequently attributed to activation of these cells (Shwartzman and Michailovsky, 1932; Urbaschek and Urbaschek, 1977; Kopaniak et al., 1980). The importance of these cells in tumor damage induced by endotoxin might be stressed by the observations that endotoxin from *Salmonella typhimurium* Re is a much more potent antitumor agent than *E. coli* 0111:B4 endotoxin (Bloksma et al., 1984a). Only the first agent induced an increase of polymorphonuclear cells into Meth A tumors (Chapter 7).

In brief, data from this study indicate that the susceptibility of Meth A tumors to endotoxin-induced tumor necrosis is defined by the condition of the tumor vasculature at the time of treatment. A poor condition would enhance the vascular effects of endotoxin. The spontaneous increase of vascular damage with tumor age and its predominant localization in the tumor core might explain tumor age-dependent and core-defined induction of tumor

necrosis by endotoxin. Other factors, such as number and composition of inflammatory cells within the tumor at the time of treatment might amplify the effects of endotoxin. The survival of tumor cells in the margins has been related to the relatively good vascular condition and to recovery mechanisms of the vasculature. The latter might be more adequate in very large tumors, which showed already extensive spontaneous necrosis of the tumor core at the time of treatment.

CHAPTER 7

QUANTITATIVE HISTOLOGY OF MURAMYL DIPEPTIDE-POTENTIATED INDUCTION OF TUMOR NECROSIS BY ENDOTOXINS

C. Frieke Kuper, Nanne Bloksma, Joost P. Bruyntjes and Frans M.A. Hofhuis

SUMMARY. The present study confirmed that combinations of muramyl dipeptide (MDP) and toxic or detoxified endotoxin induced far more necrosis and subsequent cures of solid Meth A tumors in syngeneic mice than toxic endotoxin alone, whereas MDP and detoxified endotoxin had negligible antitumor effects of their own. Histological observations were well in agreement with these data. Neither MDP nor detoxified endotoxin induced significant changes in and around the tumor by 4, 24, and 48 h after intravenous administration as compared to treatment with saline. MDP amplified various effects of toxic endotoxin such as induction of hyperemia, mitotic arrest, mast cell depletion, non-hemorrhagic necrosis and reduction of lymphocyte infiltrates, but not hemorrhagic necrosis and influx of polymorphonuclear cells. The combination of MDP and detoxified endotoxin lacked the latter two effects, but the other effects were similar to those induced by the toxic combination, although slightly less.

Because the degree of hyperemia was very proportional to the degree of subsequent non-hemorrhagic necrosis, it was suggested that MDP would potentiate necrosis by enhancing mechanisms leading to hyperemia. The role of mast cell mediators in the latter effect was discussed. Further, lymphocyte influx and therapeutic outcome might be somehow related, as exclusively therapeutic treatments reduced the influx of these cells.

INTRODUCTION

Toxic endotoxins can induce necrosis and complete regression of solid immunogenic animal tumors after systemic administration (North, 1981;

Bloksma et al., 1984a; 1984b). A frequently used tumor model to study the antitumor effects of endotoxins is the Meth A sarcoma transplanted subcutaneously in syngeneic BALB/c mice. This immunogenic tumor is optimally sensitive to the action of endotoxins when having a diameter of 7-8 mm. After injection of an optimal dose of endotoxin generally all tumors show dark extensive necrosis of the core by 24 to 48 h. On an average, 20-40% of these tumors undergo definitive regression (Berendt et al., 1978a; Galanos et al., 1979; Bloksma et al., 1984a; 1984b). Microscopical studies of Meth A tumors after injection of toxic endotoxin of *E. coli* 0111:B4 have shown a characteristic sequence of histopathological changes, i.e. hyperemia and mitotic arrest by 4 h followed by hemorrhagic and non-hemorrhagic necrosis by 24 h (Chapters 2 and 4). The total number of inflammatory cells inside and around the tumor was not increased, but their composition showed several changes. Essentially similar effects on Meth A tumors, induced by toxic endotoxin of *Salmonella abortus equi S*, have been reported (Freudenberg et al., 1984), be it that effects were observed earlier in time.

Recently, Bloksma et al. (1984a; 1984b; 1985) found that muramyl dipeptide (MDP) markedly potentiated necrosis and subsequent cures of Meth A tumors, induced by various toxic endotoxins after combined i.v. injection in saline. In addition it appeared that MDP combined with detoxified endotoxins had about the same antitumor potential as combinations with toxic endotoxins, although neither MDP nor detoxified endotoxins had appreciable effects of their own. The mechanism(s) underlying MDP-induced potentiation are not known till now. MDP was shown to enhance toxicity of toxic endotoxins but not of detoxified endotoxins (Ribi et al. 1982, Chedid et al., 1982; Bloksma et al., 1984a). A study using various MDP analogues has shown that the capacity of MDP to enhance specific cellular and humoral immunity and non-specific antimicrobial resistance is probably not involved (Bloksma et al., 1985). Recently it was shown that MDP did not enhance the production of in vitro active antitumor cytotoxins, among which probably tumor necrosis factor (TNF), by human monocytes (Kildahl-Andersen and Nissen-Meyer, 1985). Also, in vivo induction of TNF by toxic endotoxin, as measured by the capacity of serum of treated mice to necrotize solid tumors after intravenous injection, appeared not to be enhanced upon admixture of MDP. The same study showed that detoxified endotoxin alone or combined with MDP, failed to induce TNF (Bloksma et al., 1984c).

We therefore investigated whether histopathological changes in solid Meth A tumors could shed some light on the mechanism(s) by which MDP would potentiate induction of tumor necrosis by endotoxins. For this purpose we used a highly refined toxic endotoxin of the heptose-less (Re) mutant of *Salmonella typhimurium*, consisting of diphosphoryl lipid A and KDO and a chemically detoxified preparation of the same molecule, consisting of monophosphoryl lipid A (Ribi et al., 1982).

MATERIALS AND METHODS

Animals and Methods. About 11-weeks-old female BALB/c inbred mice (Laboratory of Microbiology, State University of Utrecht, Utrecht, The Netherlands) were used. The Meth A sarcoma of BALB/c origin was obtained from the Clinical Research Centre (Harrow, Middlesex, UK) and maintained in the ascites form by serial intraperitoneal passage.

Materials. N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP) was obtained from the Institut Pasteur Production (Marnes-la-Coquette, France). The refined toxic and detoxified endotoxin preparations from the heptose-less (Re) mutant of *Salmonella typhimurium* were prepared as described by Ribi et al. (1982) and were obtained from Ribi ImmunoChem Research Inc. (Hamilton, Montana, USA). MDP was dissolved in pyrogen free saline. Endotoxins were dissolved in 0.5% triethylamine (2 mg endotoxin/0.4 ml) and diluted in saline. Agents were injected i.v., alone or mixed, in a total volume of 0.5 ml. Controls received saline. The doses were chosen on the basis of information from previous experiments (Bloksma et al., 1984a). Ten μg toxic endotoxin alone was very effective against Meth A sarcoma. Since 10 μg toxic endotoxin mixed with 30 μg MDP was frequently lethal, most of the experiments were carried out with 3 μg toxic endotoxin mixed with 30 μg MDP.

Tumor assay. Viable Meth A cells (3×10^5) were injected s.c. in the abdomen. Mice were treated 9 days later (tumor diameter $\pm$ 7 mm). Part of the treated mice was used for determination of tumor necrosis and incidence of complete cures. Tumors of the remaining mice were excised by 4, 24 and 48 h, after

measurement of macroscopically visible necrosis. For preparation of sections, tumors were excised with a considerable amount of surrounding host tissue, including the underlying abdominal wall. Cytocentrifuge preparations were made of tumors excised without the abdominal wall.

Histology. Tumors (n=3, for each treatment at each time) were fixed in 4% phosphate-buffered formalin. Glycol methacrylate-embedded sagittal sections (1-2 μm), from the central part of the tumor, were stained with hematoxylin-basic fuchsine or toluidine blue and examined microscopically without previous knowledge of the treatment. Criteria for observation have been described previously (Chapter 2). Hyperemia was expressed as the number of vessel transections with a diameter of >40 μm per section. Counts surmounting 60 were indicated with >60. Dividing tumor cells were counted on 10 predetermined sites each, with an area of 0.01 square mm representing all parts of the section. The morphometric analysis was performed with the aid of a computerized graphical tablet (MOP II, Kontron Messgerät GmbH, Munich, Germany).

In addition, mast cells were counted in toluidine blue stained sections, at 13 predetermined sites, by means of an ocular square grid at 400x magnification (an area of 0.06 square mm at that magnification). Four sites were situated in the lateral margins of the tumor, and 3 sites each at the skin side, centre and peritoneal side of the tumor.

The procedure for the manufacture of cytocentrifuge preparations has been described before (Chapter 4). In short, tumors (n=4, for each treatment) were divided in a central and marginal part. The parts were chopped into small blocks and desintegrated by incubation at 37°C for 40 min in a solution of Eagle's MEM, collagenase (Boehringer, Mannheim, Germany), collagenase/ dispase (Boehringer, Mannheim, Germany), DNAse (Sigma Chemical Company, St Louis, Missouri, USA), heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland) and sucrose. One cytocentrifuge preparation/ tumor was stained for α-naphtylbutyrate esterase (Koski et al., 1976) and counterstained with toluidine blue and one preparation/tumor was stained with May-Grünwald Giemsa. When possible, 200 cells with intact nuclei/slide were counted under code. Mast cells were not included in the count, because previous experiments had shown that, probably due to membrane fragility,

numbers of mast cells in these preparations differed considerably with numbers of mast cells observed in the sections (Chapter 4).

Statistics. When appropriate, data have been expressed as mean ± SEM. Significance analysis of the morphometric data was done by the Mann-Whitney test (Siegel, 1959); P-values over 0.05 were considered not significant. Data obtained from the cytocentrifuge preparations were evaluated by analysis of variance using the standard statistical package 'Genstat' (Lawes Agricultural Trust, version 1984; Rothamsted Experimental Station; (Alvey et al., 1982). This analysis was of the split-plot type with the mouse as the whole plot unit. For analysis of tumor cell numbers, percentages were used. Inflammatory cell numbers were analysed using the log percentages. Log percentages were used because of the heterogeneity of the variances.

RESULTS

Macroscopic observations.

The antitumor potential of the treatments used in this study against 9-day-old Meth A tumors is shown in Fig. 1. Necrosis has been scored 48 h after treatment, because at that time necrosis was most prominent. MDP did not induce significant necrosis as compared with saline-treated controls. Toxic endotoxin (10 μg and 3 μg) alone induced a dose-dependent necrosis. Detoxified endotoxin (10 μg) had a negligible effect.
Necrosis induced by MDP combined with either endotoxin was very pronounced. The combination of MDP and 10 μg toxic endotoxin was most effective, but also very toxic as judged by lethargy and diarrhoea. The incidence of subsequent complete cures paralelled the extent of necrosis very well.

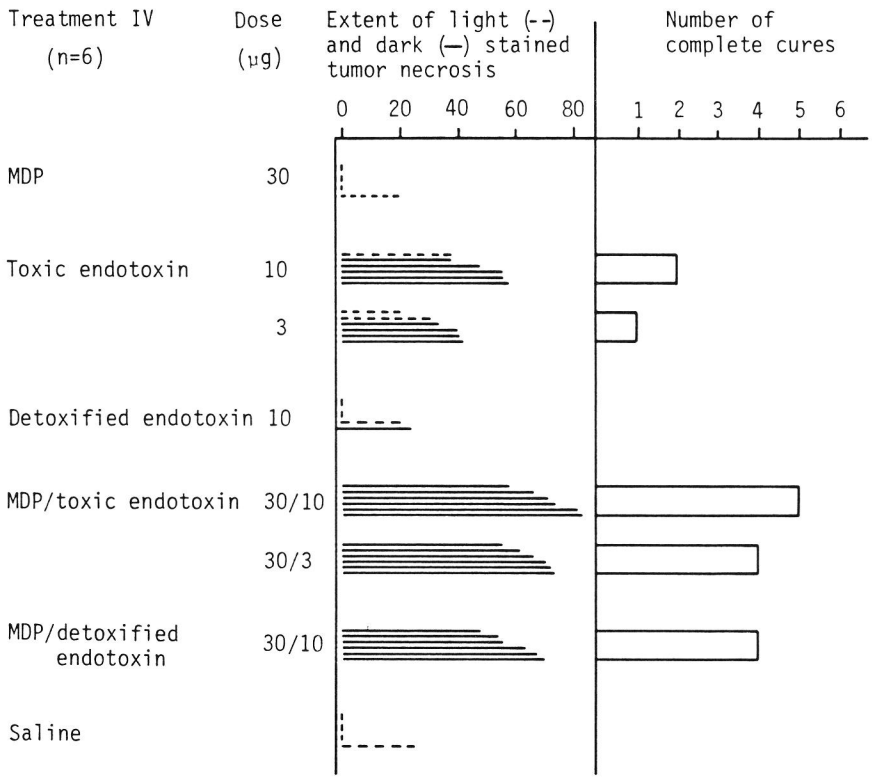


Fig. 1. Tumor necrosis and complete cures induced by MDP and/or endotoxins in BALB/c mice with 9-day-old Meth A tumors. Tumor necrosis was measured on day 11. Extent was expressed as 100x the ratio of necrotic area and tumor area. Each line represents the extent of necrosis of an individual mouse. N = 6, except for treatment group MDP/ toxic endotoxin (30/10 μg) in which of 9 mice treated, 3 mice died within 24 h.

Necrosis induced in tumors excised for histological examination is given in Table 1. By 4 h after treatment none of the agents induced necrosis (data not shown). Necrosis observed by 48 h after treatment was usually more extensive than that observed by 24 h and was very similar to that seen in Fig. 1.

Table 1. Necrosis induced by toxic and detoxified endotoxin alone or with MDP in Meth A tumors excised by 24 h and 48 h for overall histology[a]

Treatment	Dose (μg)	n	Time (h)	Necrosis Incidence (%)[b] light	dark	Extent[c]
MDP	30	6	24	33	0	30±2
		3	48	33	0	33
Endotoxin	10	6	24	17	83	47±7
		3	48	0	100	55±4
	3	3	24	66	34	42±4
Detoxified endotoxin	10	6	24	0	17	38
		3	48	0	33	46
MDP/endotoxin	30/10	6	24	0	100	68±4
	30/ 3	6	24	0	100	65±3
		3	48	0	100	70±5
MDP/detoxified endotoxin	30/10	6	24	17	83	51±3
		3	48	0	100	62±4
Saline	-	6	24	0	0	-
		3	48	33	33	28±5

a. BALB/c mice with 9-day-old Meth A sarcoma on the abdomen, were injected i.v. with endotoxins alone or mixed with MDP in saline.

b. Dark- and light-stained necrosis have been scored separately.

c. Mean ± SEM of 100x the ratio of necrotic area and tumor area.

Microscopic observations.

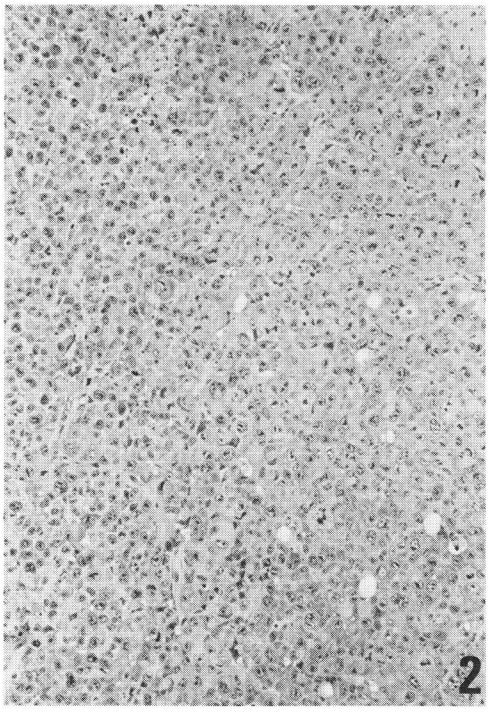
Sections of tumors from saline-treated mice showed a solid tumor mass with high mitotic activity, supplied by numerous small bloodvessels. Only a few had a diameter over 40 μm (Fig. 2; Table 2). Inflammatory cells, mostly polymorphonuclear cells and mast cells, were observed especially in the margins of the tumors.

Tumor sections of mice treated with 10 μg toxic endotoxin showed a distinct hyperemia in the centre and especially at the skin side of the tumor by 4 h, and a marked reduction of the mitotic activity all over the tumor (Table 2). By 24 h, hyperemia had almost returned to control values, while the mitotic arrest was still present. All tumors of mice treated with 10 μg toxic endotoxin exhibited hemorrhagic necrosis at the skin side of the tumor where hyperemia was most overt by 4 h and extensive areas of coagulation necrosis in the remaining part of the tumor. Hemorrhagic necrosis was characterized by parallel layers of degenerating tumor cells, diffuse hemorrhage and blood vessels plugged by erythrocyte debris (Fig. 3). Hemorrhagic necrosis covered 7 and 22% of the total area of the tumor sections by 24 h and 48 h respectively. The relative increase by 48 h was largely due to severe collapse of the tumor, as absolute areas of hemorrhagic necrosis differed hardly at both times. A small area of vital-looking tumor tissue was still present at the lateral and bottom margins of the tumor. Injection of 3 μg toxic endotoxin induced hyperemia and mitotic arrest by 4 h, but incidence and degree of hemorrhagic and non-hemorrhagic necrosis were far less by 24 h as compared with 10 μg toxic endotoxin. The histology of tumors of animals treated with detoxified endotoxin alone did not differ significantly from control tumors. MDP alone induced a slight mitotic arrest at 4 and 24 h, but no other changes. When MDP was given in combination with 3 μg toxic or 10 μg detoxified endotoxin, all effects on the tumor tissue by 48 were comparable with those induced by 10 μg endotoxin alone, except that hemorrhagic necrosis was virtually absent. Instead of hemorrhagic necrosis, the ventral side of the tumor showed strong coagulation necrosis as seen in the remaining part of the tumors (Fig. 4; Table 2).

Fig. 2. S.c. transplanted Meth A sarcoma in mice, 24 h after i.v. injection of saline. Skin-side is at the left (x 100)

Fig. 3. S.c. transplanted Meth A sarcoma in mice, 24 h after i.v. injection of 10 μg toxic endotoxin. Area of hemorrhagic necrosis (HN) with bloodvessels plugged by erythrocyte debris (▼). Skin-side is at the left (x 100)

Fig. 4. S.c. transplanted Meth A sarcoma after i.v. injection of 30 μg MDP mixed with 3 μg toxic endotoxin. Skin-side is at the left (x 100)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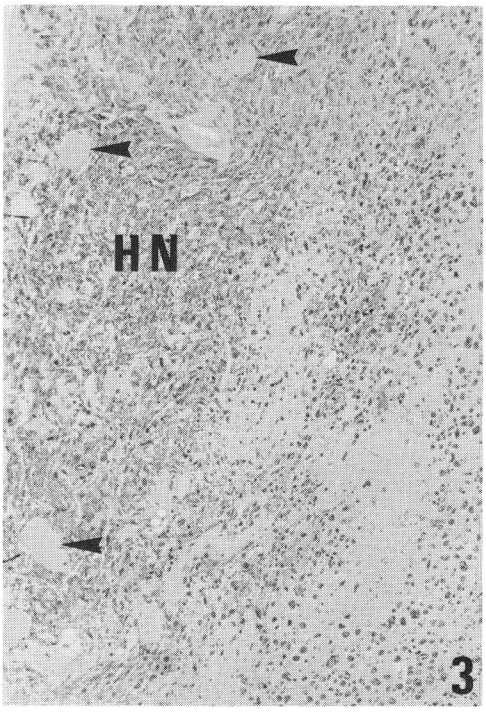
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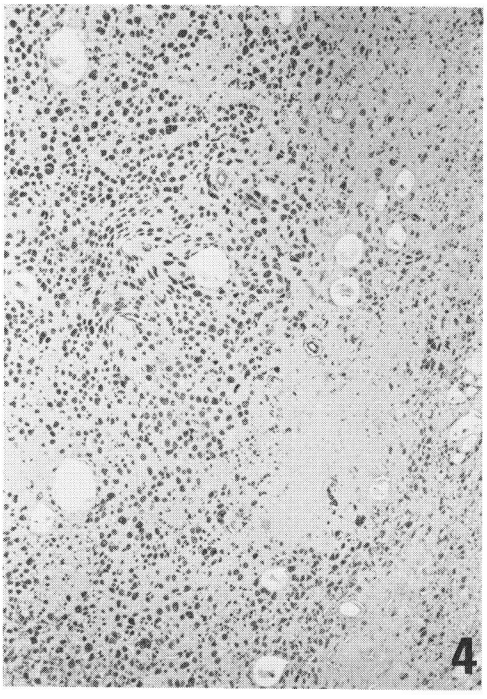
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Table 2 - Histology of Meth A sarcoma after treatment with toxic and detoxified endotoxin alone or combined with MDP[a]

Treatment i.v.	Dose (μg)	Time h	Hyperemia[b] (mean ± SEM)	Mitotic Index[c] (mean ± SEM)	Haemorrhagic necrosis Incidence	Haemorrhagic necrosis Extent[d]	Vital tissue[e]
MDP	30	4	3 ± 1	5.3 ± 1.5[f]	0/3	-	>50 ± 0
		24	4 ± 3	4.5 ± 0.5	0/3	-	>48 ± 2
		48	5 ± 2	6.7 ± 1.2	0/3	-	>47 ± 3
Endotoxin	10	4	>31 ± 9[f]	0.0 ± 0.0[fg]	0/3	-	>50 ± 0
		24	6 ± 1	0.7 ± 0.3[fg]	3/3	7 ± 3	5 ± 3[f]
		48	2 ± 1	0.0 ± 0.0[fg]	3/3	22 ± 6	< 5 ± 4[f]
	3	4	27 ± 8[f]	2.2 ± 0.3[f]	0/3	-	>50 ± 0
		24	8 ± 4	3.6 ± 1.8	1/3	6	>43 ± 7
Detoxified endotoxin	10	4	7 ± 4	8.3 ± 3.0	0/3	-	>50 ± 0
		24	3 ± 2	6.3 ± 1.3	0/3	-	>50 ± 0
		48	4 ± 1	7.0 ± 1.2	1/3	3	>50 ± 0
MDP/Endotoxin	10/30	24	4 ± 1	0.1 ± 0.1[fg]	3/3	15 ± 5	< 1 ± 0
	3/30	4	>50 ± 5[f]	1.3 ± 0.7[fg]	0/3	-	>50 ± 0
		24	5 ± 2	0.0 ± 0.0[fg]	0/3	-	< 2 ± 1[fi]
		48	9 ± 7	2.3 ± 1.2[fg]	1/3	2	< 4 ± 2[f]
MDP/Detoxified endotoxin	10/30	4	35 ± 5[fh]	3.0 ± 1.7[fg]	0/3	-	>50 ± 0
		24	3 ± 1	3.3 ± 1.2	2/3	3 ± 2	18 ± 2[f]
		48	3 ± 2	0.3 ± 0.3[fg]	1/3	2	< 4 ± 2[f]
Saline	-	4	10 ± 3	11.0 ± 1.5	0/3	-	>50 ± 0
		24	2 ± 0	8.3 ± 3.2	0/3	-	>50 ± 0
		48	4 ± 0	7.7 ± 2.0	0/3	3	>50 ± 0

a. BALB/c mice (n = 3, for each treatment at each time) with 9-day-old Meth A sarcoma on the abdomen, were injected i.v. with toxic or detoxified endotoxin of *S. typhimurium* Re alone or mixed with MDP
b. Number of dilated vessel transsections/section
c. Number of dividing cells/mm^2 tumor section
d. Mean [(necrotic area/tumor area) x 100] ± SEM
e. Area relative to whole tumor section (mean ± SEM). Areas <1% or >70% were not quantified in detail
f. $P < 0.05$ compared with saline group by the same time after treatment (U-test)
g. $P < 0.05$ compared with MDP group by the same time after treatment (U-test)
h. $P < 0.05$ compared with corresponding treatment without MDP (U-test)
i. $P < 0.05$ compared with detoxified endotoxin/MDP group by the same time after treatment (U-test)

The induction of maximal necrosis by detoxified endotoxin combined with MDP, however, required clearly more time in comparison with 10 μg toxic endotoxin and MDP combined with 3 μg toxic endotoxin. MDP combined with 10 μg toxic endotoxin had slightly better activity than the same dose of toxic endotoxin alone.

No significant changes in inflammatory cell infiltrates could be observed in the sections except for the mast cells. The number of these cells was decreased at the skin side of the tumor by 24 h and 48 h after treatment with 10 μg toxic endotoxin alone or 3 μg combined with MDP (Table 3). Similar effects were observed 24 h after treatment with detoxified endotoxin combined with MDP. The decrease observed by 48 h after this combination was not significant. Mast cell numbers at all other sites of the tumor were relatively low and were not changed by any treatment. Mast cells at the skin side were poorly granulated in contrast to mast cells at the other sides. Their morphology was not clearly affected by any treatment by 4 h and 24 h. The number and composition of host cells in disaggregated tumors of saline-treated mice differed as to the part of the tumor investigated. The centre of the tumors contained approximately 30% host cells, predominantly macrophages and polymorphonuclear cells (Fig. 6). The margins contained about 40% host cells. Compared to the centre, numbers of monocytes, lymphocytes and polymorphonuclear cells were higher.

None of the agents investigated, changed the number of macrophages and monocytes significantly compared to tumors of saline-treated mice. Polymorphonuclear cells were only increased by preparations containing toxic endotoxin. Toxic endotoxin (10 μg) alone increased polymorphonuclear cell numbers in both parts, whereas 3 μg toxic endotoxin combined with MDP caused an increase in the margin only. The number of lymphocytes in the centre was not changed by toxic endotoxin or both combinations, although MDP and detoxified endotoxin alone increased lymphocyte numbers markedly. In the margin both combinations and toxic endotoxin alone decreased lymphocyte numbers significantly. MDP and detoxified endotoxin alone had no effect.

Table 3 - Mast cell numbers at different sites of Meth A sarcoma after treatment with toxic and detoxified endotoxin alone or combined with MDP[a]

Treatment i.v.	Dose (μg)	Mast cell numbers by 4 h: Lateral margins	4 h: Skin-side	4 h: Centre	4 h: Peritoneum side	24 h: Lateral margins	24 h: Skin-side	24 h: Centre	24 h: Peritoneum side	48 h: Lateral margins	48 h: Skin-side	48 h: Centre	48 h: Peritoneum side
MDP	30	2±1	17±2	2±1	2±1	6±3	19±3	1±1	2±1	6±3	18±2	1±0	3±1
Endotoxin	10	4±3	21±7	1±0	1±1	4±2	7±1[c]	1±0	2±2	2±2	2±1[c]	0±0	4±1
	3	5±2	18±4	1±0	2±1	4±2	13±5	1±0	1±0				
Detoxified endotoxin	10	3±2	23±2	2±1	2±2	4±3	18±4	1±1	0±0	7±1	18±6	1±1	2±1
MDP/endotoxin	30/ 3	7±2	13±3	1±1	2±1	5±1	4±2[c]	0±0	0±0	1±1	0±0[c]	0±0	3±2
MDP/Detoxified endotoxin	30/10	9±3	24±2	1±0	3±2	3±4	3±3[c]	0±0	2±0	5±3	6±4	0±0	4±2
Saline	-	6±3	24±5	1±0	2±1	6±3	21±1	1±0	3±2	4±2	13±2	2±1	2±1

a. BALB/c mice with 9-day-old Meth A sarcoma on the abdomen, were injected i.v. with toxic or detoxified endotoxin of *S. typhimurium* Re alone or mixed with MDP. At different times after treatment mast cell numbers were counted at various predetermined sites of the tumor sections, 4 sites in the lateral margins and 3 sites in each of the other areas. Data have been expressed as mean ± SEM

b. N = 3, for each treatment at each time

c. $P < 0.05$ compared to the saline group by the same time at the same site (U-test)

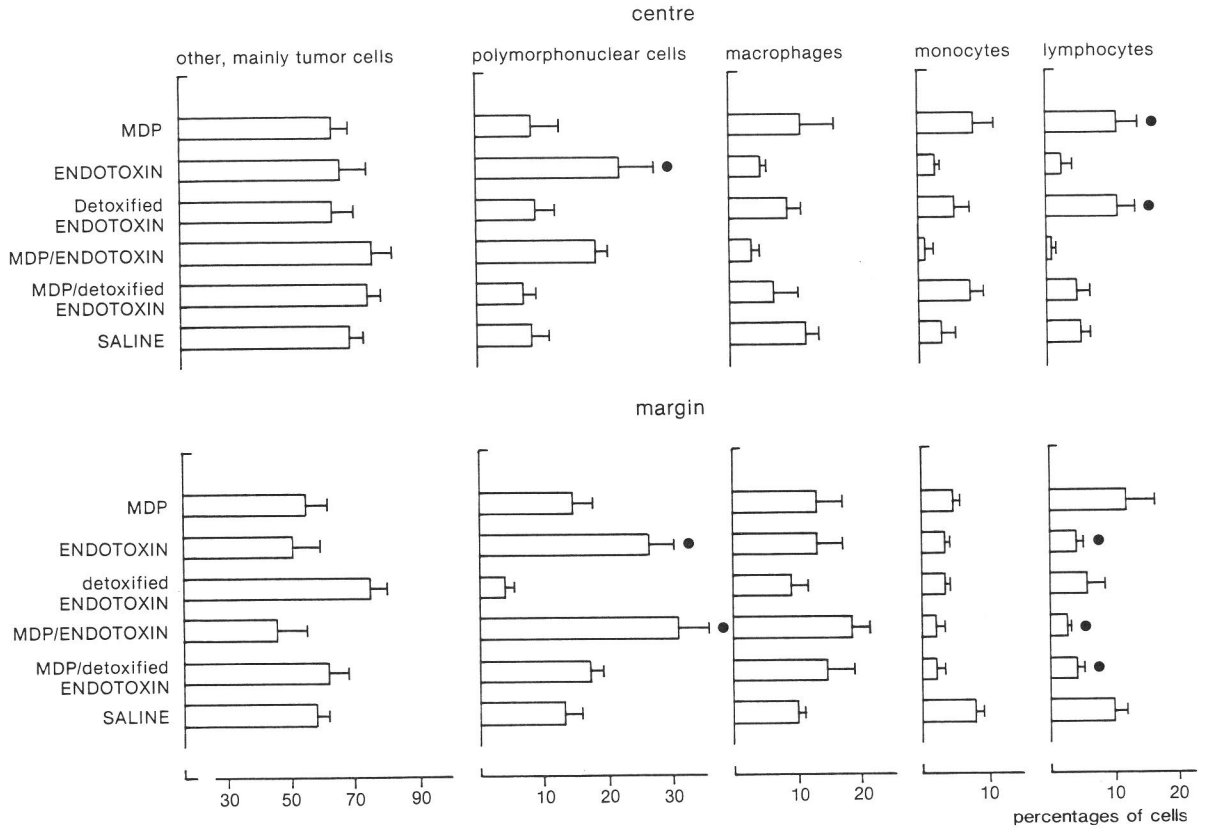


Fig. 5. Cellular composition of cytocentrifuge preparations (mean of four animals ± SEM) of the central and marginal parts of transplanted Meth A tumors in mice, 24 h after treatment with MDP (30 μg), endotoxin (10 μg), MDP/endotoxin (30 μg/3 μg), MDP/detoxified endotoxin (3 μg/10 μg) or saline. ●, P <0.05 compared to treatment with saline.

DISCUSSION

Incidence, intensity and extent of macroscopically observed tumor necrosis induced by agents used in this study diverged widely between the treatment groups (Fig. 1; Table 1). The most potent necrotizing treatments were toxic endotoxin (10 and 3 μg) combined with MDP, followed in effectivity by detoxified endotoxin (10 μg) with MDP and toxic endotoxin (10 μg) alone. Toxic endotoxin (3 μg) alone induced more necrosis than observed in controls. MDP as well as detoxified endotoxin alone had no significant effects. Histopathologically scored necrosis was well in line with the macroscopical observations, be it that the latter gives a considerable under-estimation of the real extent of necrosis. Comparison of the necrotizing potential of the different treatments with their therapeutic effect suggests that the severity of the prompt tumor necrosis is highly decisive for complete regression to occur.

Endotoxin-induced tumor necrosis has always been characterized as a hemorrhagic necrosis (Gratia and Linz, 1931; Shwartzman and Michailovsky, 1932; Carswell et al., 1975; Freudenberg et al., 1984). Hemorrhagic necrosis, however, was only a minor component of total necrosis induced by different toxic endotoxins (Freudenberg et al., 1984; Chapters 2 and 4; Table 2). Moreover, the incapacity of MDP to potentiate hemorrhagic necrosis indicates that this is not the mechanism by which MDP would amplify tumor necrosis. In a previous study (Chapter 2) a relation between hyperemia and hemorrhagic necrosis was suggested, because both were observed in the same tumor area and because an α-adrenoceptor blocking agent reduced both properties of toxic endotoxin. This study showed that MDP potentiated hyperemia only. Apparently extensive hyperemia does not inevitably lead to early massive extravasation of erythrocytes. Hyperemia, however, was always accompanied by a marked reduction of mitotic activity and subsequent non-hemorrhagic necrosis, suggesting a relation. Hyperemia can be accompanied by slowing down or even complete stasis of the blood flow leading to hypoxic necrosis. Thus hypoxia might be an important cause of the tumor necrosis observed.

The mechanism by which MDP would potentiate endotoxin-induced hyperemia might be related to mast cell changes, exclusively observed in this area.

Both MDP and detoxified endotoxin failed to induce hyperemia and mast cell depletion, whereas the combination was very active (Tables 2 and 3). In addition, MDP markedly potentiated both effects induced by 3 μg toxic endotoxin. The observation that hyperemia was observed at a time that the mast cells were still morphologically intact, does not exclude a mechanistical relation, as Van Loveren et al. (1984) have recently shown that murine mast cells could selectively release serotonin from their granules without degranulation. Serotonin might be a mediator of the induction of hyperemia. This vasoamine could cause hyperemia in Meth A tumors upon i.v. injection (Chapter 3) and preliminary experiments have shown that the selective serotonin receptor antagonists ketanserin and pirenperone were potent inhibitors of the induction of hyperemia and subsequent necrosis and regression by MDP combined with toxic endotoxin. Whether serotonin is selectively released from mast cells shortly after administration of the combinations, will be subject of further investigation.

Examination of cytocentrifuge preparations of Meth A tumors allowed a better evaluation of other types of host cells infiltrating the tumor. Toxic endotoxin alone or combined with MDP increased the numbers of polymorphonuclear cells in the tumor (Fig. 6). Similar effects with toxic endotoxins have been reported by others (Shwartzman and Michailovsky, 1932; Kodama et al., 1982), who suggested that polymorphonuclear cells would cause tumor necrosis. This is not very likely, because toxic endotoxin of *E. coli* 0111:B4, which induces tumor necrosis, did not enhance polymorphonuclear cell infiltrates in the Meth A tumor (Chapter 4) and also MDP combined with detoxified endotoxin did not do so (Fig. 6). Increase of polymorphonuclear cells might be rather a side-effect of some toxic endotoxins, as the endotoxin of *S. typhimurium* Re appeared to be far more toxic than endotoxin of *E. coli* 0111:B4 as measured by diarrhoea, lethargy and LD50 (Bloksma et al., 1984a). The number of monocytes and macrophages was not significantly changed by any treatment. This and the observation that the majority of macrophages was observed in the margin of the tumor, where the only vital tissue can be observed after treatment with the combinations, indicate that these cells do not play a major role in the induction of tumor necrosis.

An interference with the infiltration of lymphocytes might be related to the therapeutic potential. Only curative treatments reduced lymphocyte numbers, as compared to control tumors. The same effect has been observed

after treatment with *E. coli* 0111:B4 endotoxin (Chapter 4). It is tempting to hypothesize that this reduction is due to a decrease of suppressor T cells, which have been found in spleens of mice bearing 9-day-old Meth A tumors and did increase rapidly afterwards (North, 1981). This has to be studied further. Another interesting, but enigmatic observation is that both MDP and detoxified endotoxin increased lymphocyte numbers in the centre of the tumor by 4 h. The combination, however, did not influence lymphocyte numbers as compared to controls.

In conclusion, data showed that the capacity of MDP to potentiate tumor necrosis and regression cannot be attributed to specific effects of this agent on its own. MDP potentiated, however, various effects innate to toxic endotoxins, such as induction of hyperemia, mitotic arrest, mast cell depletion, non-hemorrhagic necrosis and reduction of lymphocyte infiltrates. This potentiation was also seen upon combination with detoxified endotoxin, which lacked any effect of its own. Hemorrhagic necrosis and increased influx of polymorphonuclear neutrophils caused by toxic endotoxin was not increased by MDP.

Data suggest that the capacity of MDP to potentiate the induction of hyperemia might be a mechanism by which it amplifies induction of tumor necrosis. Mast cell mediators might be involved in the induction of hyperemia, while the reduced influx of lymphocytes seems to be associated with the onset of definite regression.

CHAPTER 8

IN VIVO EFFECTS OF TOXIC AND DETOXIFIED ENDOTOXIN ALONE OR IN COMBINATION WITH MURAMYL DIPEPTIDE ON LYMPHOID AND NON-LYMPHOID CELLS IN THE SPLEEN OF METH A SARCOMA-BEARING MICE

C. Frieke Kuper, Paul H.P. Groeneveld and Nanne Bloksma

SUMMARY. In this study we confirmed that combinations of toxic or detoxified endotoxin with muramyl dipeptide (MDP) induced much more necrosis of transplanted Meth A sarcoma in mice than toxic endotoxin alone. Detoxified endotoxin and MDP alone had little antitumor effects. We investigated whether these divergent antitumor effects could be related to histopathological changes in the white pulp of the spleen of Meth A sarcoma-bearing mice. Toxic endotoxin reduced the T:B cell compartment ratio in the splenic white pulp by increasing the size of the B-cell compartment while leaving the size of the T-cell dependent inner PALS unaffected. The number of the T-lymphocytes in this area, however, was reduced. The border of B-lymphocytes in the marginal zone was strongly narrowed and the number of marginal metallophils along the inner border of the marginal sinus was decreased. None of these changes were observed after treatment with detoxified endotoxin or MDP. Addition of MDP to either endotoxin did not change their effects.

The histopathological changes in the lymphoid and non-lymphoid compartments of the splenic white pulp are apparently exclusively induced by toxic endotoxin. As the antitumor activity of both toxic and detoxified endotoxin combined with MDP are about equal and more powerful than the activity of toxic endotoxin alone, it is concluded that these antitumor effects cannot be related to changes in the white pulp of the spleen.

INTRODUCTION

Toxic endotoxins are well-known inducers of necrosis and complete regression of solid animal tumors (Gratia and Lintz, 1931; Shear et al., 1943; Nowotny et al., 1971; Parr et al., 1973). Recently, it was found that effects of intravenously injected toxic endotoxins against immunogenic Meth A sarcoma transplanted in syngeneic mice were markedly potentiated by admixture of muramyl dipeptide (MDP), although this agent had no significant antitumor effects of its own (Bloksma et al., 1984a, 1984b). Furthermore, MDP converted detoxified endotoxins, devoid of significant antitumor activity, into very potent antitumor agents without enhancing the toxicity.

There is still no consensus on how toxic endotoxins induce necrosis and regression, but it is generally agreed that effects are mediated by the tumor-bearing host (Shapiro, 1940; Männel et al., 1979). Various data indicate that endotoxin-induced tumor necrosis is independent of the immune system, while subsequent complete regression would require specific T-cell immunity (Parr et al., 1973; Berendt et al., 1978a, 1978b). Specific suppressor T-lymphocytes were shown to interfere with endotoxin-induced tumor regression (North et al., 1976; North, 1981). Histopathological examination of Meth A tumors has shown that treatment could change lymphocyte accumulation around the tumor (Chapters 4 and 7). Toxic endotoxin markedly reduced the influx of lymphocytes into the outer margins of the tumor already at 24 h after treatment. The combinations of MDP and toxic or detoxified endotoxin were more effective in this respect, but detoxified endotoxin alone had no effect and MDP alone even enhanced lymphocyte influx. Further characterization of these lymphocytes by histo-chemical means has been unsuccesful so far, due to intense staining of the necrotic tumor cells. This problem is not encountered in lymphoid organs of tumor-bearing mice.

North and Bursuker (1984) have shown that spleens of mice with curable 9-day-old Meth A tumors harbour already demonstrable numbers of specific suppressor T-lymphocytes. Their numbers increased rapidly in older tumors. Such cells might be the source of the lymphocyte infiltrate observed in untreated tumors. As toxic endotoxin is known to induce marked changes of lymphoid and non-lymphoid cells in the splenic white pulp of tumor-free mice (Groeneveld et al., 1983a, 1983b, Groeneveld and Van Rooijen, 1984), we studied whether we could relate the divergent antitumor action of toxic and

detoxified endotoxin of *Salmonella typhimurium* Re alone or combined with MDP to effects on the splenic white pulp of Meth A sarcoma-bearing mice.

MATERIALS AND METHODS

Mice and tumor. Female BALB/c mice, 11 weeks old and weighing about 20 g, bred and maintained at the Laboratory of Microbiology (Utrecht, The Netherlands), were used. The Meth A sarcoma of BALB/c origin was obtained from the Clinical Research Centre (Harrow, Middlesex, UK) and maintained in the ascites form by serial intraperitoneal passage.

Drugs. N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP) was obtained from the Institut Pasteur Production (Marnes-la-Coquette, France). Refined toxic and detoxified endotoxin from the heptose-less (Re) mutant of *Salmonella typhimurium* were prepared as described by Ribi et al., (1982) and obtained from Ribi ImmunoChem Inc. (Hamilton, MT, USA). MDP was dissolved in pyrogen-free saline. Endotoxins were dissolved in 0.5 % triethylamine (2 mg endotoxin/0.4 ml) and further diluted in saline. Agents were injected intravenously, alone or mixed, in a total volume of 0.5 ml.

Experimental design. Viable Meth A cells (3×10^5) were injected s.c. in the abdomen of mice. The mice were treated 9 days later (tumor diameter $\pm$ 7 mm) with MDP (30 μg), toxic or detoxified endotoxin (10 μg) or MDP (30 μg) mixed with toxic (3 μg) or detoxified (10 μg) endotoxin. The combination of 30 μg MDP with 3 μg toxic endotoxin was used preferentially because addition of MDP to 10 μg of this endotoxin was frequently lethal (Bloksma et al., 1984a). Twenty-four h later, the mice were killed (3 animals/group) and their spleens removed and prepared for the immunoperoxidase and enzymehistochemistry technique. Tumor necrosis was measured and expressed as 100 times the ratio of the mean diameters of necrotic area and tumor (extent).

Enzymehistochemistry and immunoperoxidase technique. These methods were done as described previously (Groeneveld et al., 1983b; Groeneveld and Van Rooyen, 1984).

For enzymehistochemistry, blocks of fresh splenic tissue were frozen and stored in liquid nitrogen. Cryostat sections of 8-10 μm thickness were picked up on glass slides, air-dried and fixed in Baker's formol for 10 min at 4°C. Strong non-specific esterase (NSE) activity was used as a marker for marginal metallophils (Eikelenboom, 1978). The enzyme activity was demonstrated with α-naphtyl-acetate as a substrate (Pearse, 1972). The substrate for demonstrating acid phosphatase (AP) activity was naphthol-AS-BI-phosphate (Pearse, 1968) with hexazotized pararosaniline as diazonium salt for both reactions (Davis and Ornstein, 1959). Both enzyme substrates were obtained from Sigma (St Louis, MO, USA).

For immunoperoxidase methods, cryostat sections of 8-10 μm were fixed in acetone for 10 min and air-dried for at least 30 min. The sections were washed in 0.01 M phosphate-buffered saline (PBS) and subsequently incubated for 30 min with a suitable dilution of a monoclonal anti-mouse-anti-Thy-1 rat-antibody (59-AD-22, 1:5, kindly provided by Dr W. van Ewijk, Rotterdam, The Netherlands) or a monoclonal anti-mouse-anti-heavy-chain IgM rat-antibody (33-24-12, 1:5, kindly provided by Dr G. Kraal, Amsterdam, The Netherlands). Thereafter, the sections were covered with peroxidase-conjugated rabbit-anti-rat immunoglobulin (Dako, Copenhagen, Denmark) for 30 min. The conjugate was diluted 1:75 in PBS/bovine serum albumin (Poviet, The Netherlands). The slides were then stained for peroxidase activity with 3,3'-diaminobenzidine-tetra-hydrochloride (DAB; Sigma, St Louis, MO, USA). All slides were lightly counterstained with haematoxylin.

Morphometry and statistical analysis. Areas of B-cell compartments (outer PALS with follicles) and T-cell compartments (inner PALS) were measured with the aid of a computerized graphical tablet (MOP II, Kontron Messgerät GmbH, Munich, Germany) on 3 sections/spleen. When possible, 10 transsections of each compartment/spleen section were measured. The same transsections were used for determination of the width of the B-lymphocyte border at 4 representative sites in the marginal zone. Strongly NSE-positive cells (marginal metallophils) along the inner border of the marginal sinus were counted per field of vision at 250 x magnification.

When appropriate, data have been expressed as means $\pm$ SEM. Analysis for significance was performed by the Mann-Whitney U-test (Siegel, 1956). P-values < 0.05 were considered statistically significant.

RESULTS

The antitumor activity of endotoxins alone or combined with MDP.

Injection of 10 μg toxic endotoxin of *S. typhimurium* into BALB/c mice with 9-day-old Meth A tumors induced 75 % incidence of central necrosis by 24 h, detoxified endotoxin induced no necrosis at all (Table 1). MDP alone induced 25 % incidence of necrosis. However, when given in combination with toxic or detoxified endotoxin, respectively 100 and 75 % incidence of necrosis was induced. Tumors from animals injected with MDP in combination with toxic endotoxin were, already by 24 h, smaller than from animals injected with saline, MDP or detoxified endotoxin alone.

Table 1 - Antitumor effect of toxic or detoxified endotoxin alone or combined with MDP[a]

Treatment	Dose (μg)	Time (h)	Necrosis Incidence (%)	Necrosis Extent[b]	Tumor diameter[c] (mm)
MDP	30	4	0	-	
		24	25	32	7.2±0.3
Toxic endotoxin	10	4	0	-	
		24	75	47±7	6.2±0.3
Detoxified endotoxin	10	4	0	-	
		24	0	-	7.0±0.2
MDP/toxic endotoxin	30/ 3	4	0	-	
		24	100	64±2	6.0±0.3[d]
MDP/detoxified endotoxin	30/10	4	0	-	
		24	75	51±3	6.5±0.2
Saline	0/ 0	4	0	-	
		24	0	-	7.0±0.1

a. BALB/c mice with 9-day old Meth A sarcoma on the abdomen, were injected i.v. with endotoxins alone or mixed with MDP in saline. N=4, for each treatment at each time.

b. Mean ± SEM of 100x the ratio of the mean diameter of necrotic area and tumor area.

c. Mean ± SEM.

d. $P < 0.05$ compared with saline, MDP or detoxified endotoxine alone (U-test).

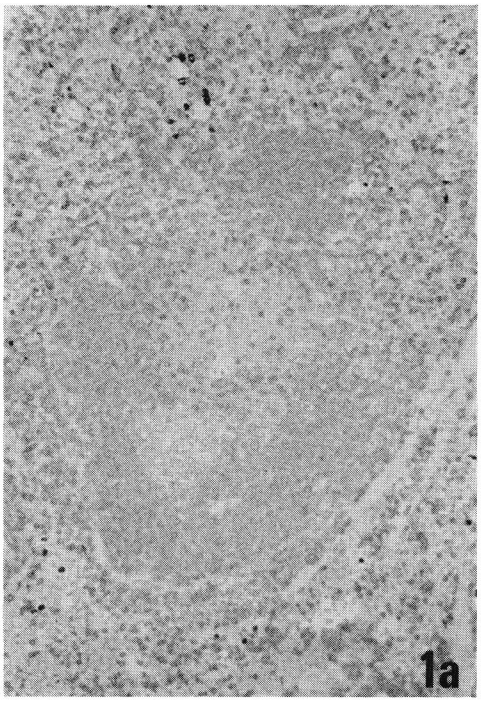
Histopathological changes in the spleen induced by endotoxins alone or combined with MDP.

In the white pulp of spleens from saline-treated tumor-bearing mice, IgM-positive cells were mainly located in the outer PALS, the follicles and in the entire marginal zone (Fig. 1a), while Thy-1-positive lymphocytes were predominantly found in the inner PALS (Fig. 2a). A considerable number of IgM-positive and Thy-1-positive cells were seen throughout the red pulp. NSE-enzymehistochemistry showed that marginal metallophils were located predominantly at the periphery of the white pulp along the inner border of the marginal sinus with most of their cell processes oriented towards the centre of the white pulp. A significant number of strongly NSE-positive cells was also located more centrally, in the follicles (Fig. 3a). Strong AP-positive, weakly NSE-positive cells were observed mainly in the red pulp and the marginal zone. Also, AP-positive cells were found in the other white pulp compartments (Fig. 4a).

Treatment with toxic endotoxin caused histopathological changes in all compartments of the spleen (Table 2). The total area of the white pulp was increased, which was due to an increase of B-lymphocyte numbers leading to enlargement of the B-dependent follicular area and outer PALS (Table 2; Fig. 1b). The reticulum of the marginal zone, however, was depleted from its B-lymphocyte population, leaving a small border of B-cell-populated reticulum near the marginal sinus (Table 2). The area of the T-dependent inner PALS was not changed (Table 2), although it was less densely populated with Thy-1-positive cells than the inner PALS of saline-treated controls (Fig. 2b). The red pulp was depleted of both IgM-positive and Thy-1-positive

Fig. 1 a-b. Staining for IgM in the spleen of a mouse bearing a 9-day-old Meth A sarcoma, 24 h after treatment (x 30). a. Saline. B-lymphocytes are found in the marginal zone, outer PALS, follicles and red pulp. b. Toxic endotoxin (10 μg). Depletion of marginal zone and red pulp lymphocytes and enlargement of the B-dependent follicular area.

Fig. 2 a-b. Staining for Thy-1 in the spleen of a mouse bearing a 9-day-old Meth A sarcoma, 24 h after treatment (x 30). a. Saline. T-lymphocytes are seen predominantly in the inner PALS and the red pulp. b. Toxic endotoxin (10 μg). Reduction in the number of T-lymphocytes in the red pulp and the inner PALS.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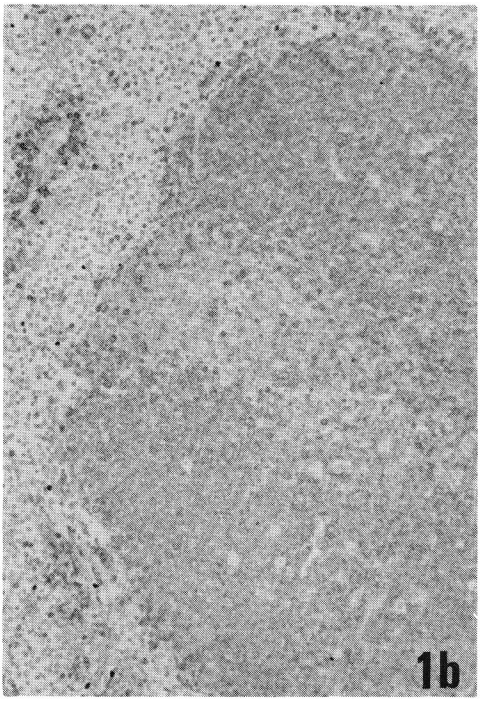
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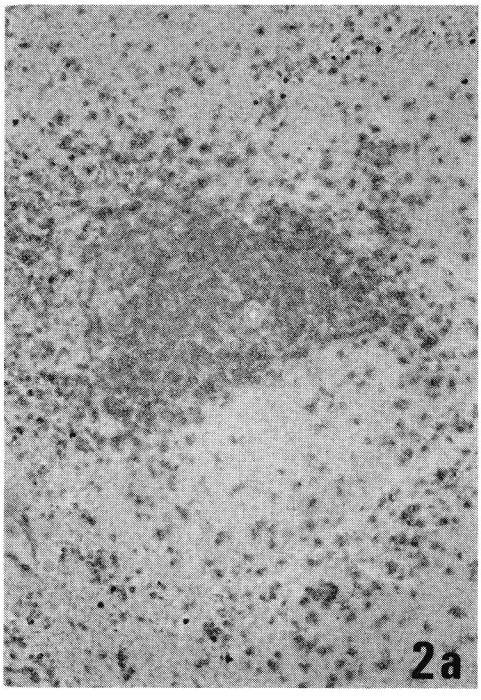
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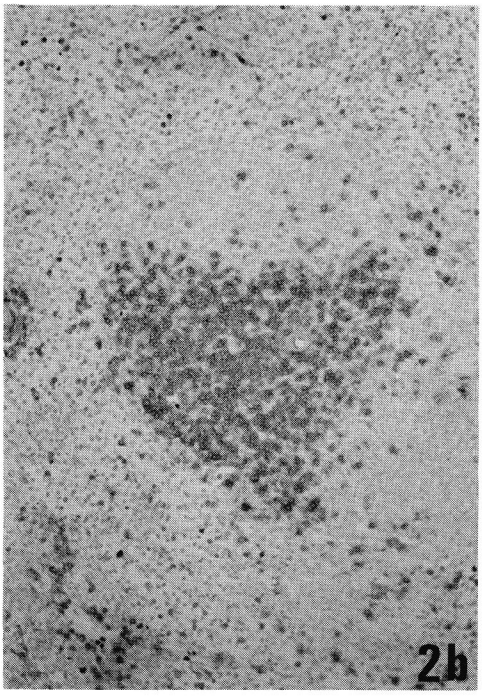
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cells after treatment with toxic endotoxin. The number of strongly NSE-positive cells (the marginal metallophils) was decreased along the inner border of the marginal sinus and in the centre of the follicles (Fig. 3b). The number of intensely AP-staining, weakly NSE-positive cells increased in the inner PALS in the spleen of 2 out 3 mice (Fig. 4b).

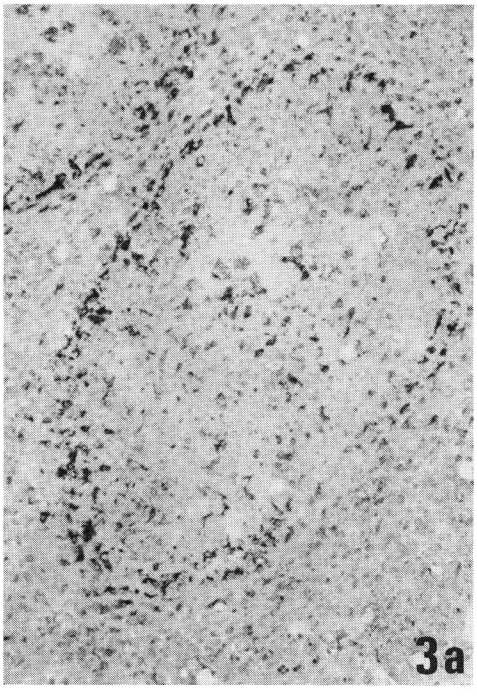
Neither MDP nor detoxified endotoxin caused clear morphological changes in the spleen. (Table 2). Addition of MDP to toxic or detoxified endotoxin does not modify the effects of either endotoxin (Table 2).

DISCUSSION

Recent histopathological studies suggested that the tumor-therapeutic activity of toxic endotoxin alone or combined with MDP and detoxified endotoxin combined with MDP might be related to an interference with lymphocyte traffic to the tumor (Chapters 3 and 7). While lymphocytes started to accumulate around 10-day-old Meth A tumors of untreated mice, treatment with the agents mentioned above on day 9, prevented this accumulation completely. Treatment with MDP or detoxified endotoxin alone or saline did not prevent the lymphocyte accumulation. Spleens of mice with 9-day old Meth A tumors were shown to contain significant numbers of suppressor T-lymphocytes which increased very rapidly with the age of the tumor (North and Bursuker, 1984; Bursuker and North, 1984). We investigated, therefore, the effect of therapeutic and non-therapeutic treatments on the overall lymphocyte distribution in the

Fig. 3 a-b. NSE activity in the spleen of a mouse with a 9-day-old Meth A sarcoma, 24 h after treatment (x 30). a. Saline. Strongly NSE-positive cells are found along the inner border of the marginal sinus (marginal metallophils) and in the follicular area of the white pulp. b. Toxic endotoxin (10 μg). Reduction in the number of strongly NSE-positive cells along the inner border of the marginal sinus and in the follicles.

Fig. 4 a-b. AP activity in the spleen of a mouse with a 9-day-old Meth A sarcoma, 24 h after treatment (x 30) a. Saline. AP-activity is found mainly in the red pulp and marginal zone, and occasionally in the other white pulp compartments.
b. Toxic endotoxin (10 μg). Increase in the number of AP positive cells around the central arteriole.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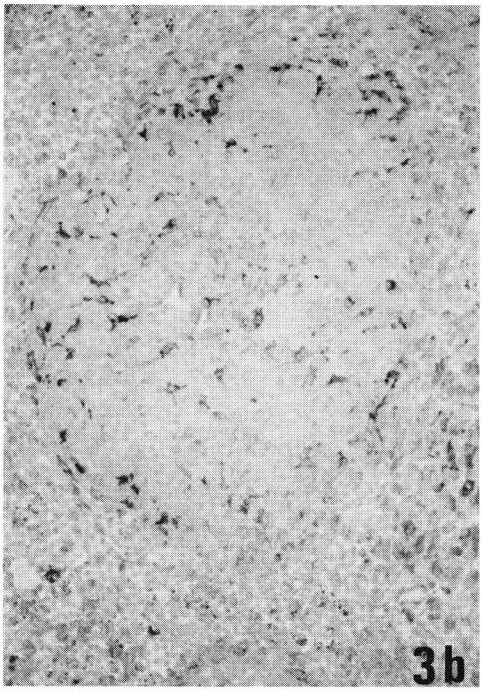
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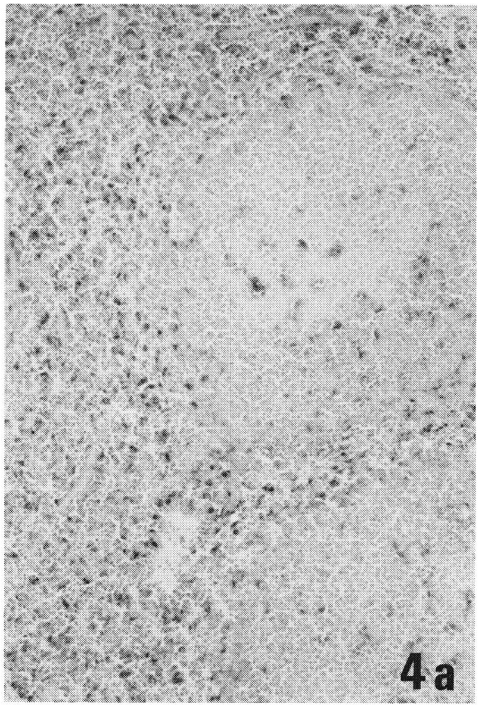
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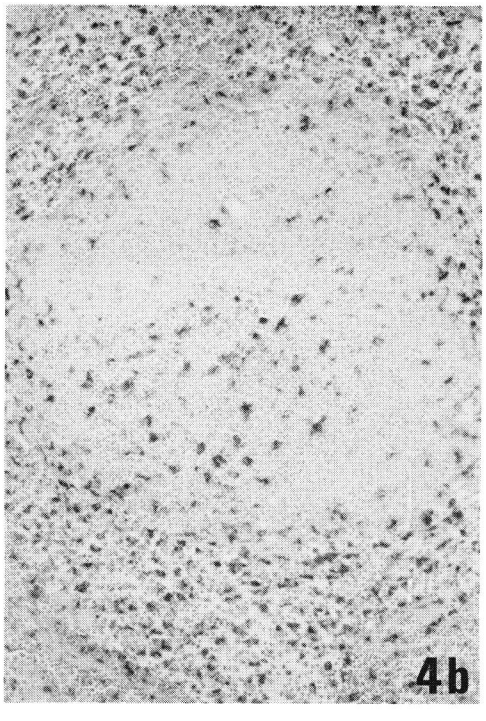
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Table 2 - Histology of different compartments in the splenic white pulp of Meth A sarcoma-bearing mice, 24 h after treatment with endotoxins alone or combined with MDP[a].

Treatment	Dose (μg)	Area of the B-cell dependent PALS with follicles (mm^2)	Area of the T-cell dependent PALS (mm^2)	Ratio T : B	Width marginal zone(mm)[b]	Number of marginal metallophils[c]
MDP	30	0.7±0.1	0.2±<0.1	0.3±0.1	0.4± 0.1	54± 4
Toxic endotoxin	10	1.4±0.3[d]	0.3± 0.1	0.2±0.1[d]	0.1±<0.1[d]	32± 7[d]
Detoxified endotoxin	10	0.9±0.1	0.4± 0.1	0.4±0.2	0.4± 0.1	54±10
MDP/toxic endotoxin	30/3	1.2±0.2[d]	0.3±<0.1	0.2±0.1[d]	0.1±<0.1[d]	33±10
MDP/detoxified endotoxin	30/10	0.8±0.1	0.4± 0.1	0.5±0.2	0.4± 0.1	54± 4
Saline	-	0.7±0.1	0.3± 0.1	0.5±0.2	0.4±<0.1	57± 4

a. BALB/c mice with 9-day-old Meth A sarcoma on the abdomen were injected i.v. with endotoxins alone or mixed with MDP. N=3, for each treatment. Data have been expressed as mean ± SEM.

b. The width of the B-lymphocyte-containing border in the marginal zone was measured.

c. Number of marginal metallophils along the inner border of the marginal sinus/field of vision at 250x magnification.

d. $P \leq 0.05$ compared with saline (U-test).

spleens of Meth A-bearing mice. Spleens of saline-treated mice had a T:B ratio which was very similar to that of spleens of tumor-free mice (Table 2; Groeneveld and Van Rooijen, 1984). This is surprising because of the large antigenic load in tumor-bearing animals.

Treatment of tumor-bearing mice with toxic endotoxin induced a marked reduction of the number of Thy-1 positive cells in the inner PALS. Similar effects have been observed in spleens of tumor-free mice (Groeneveld and Van Rooijen, 1984). The combination of toxic endotoxin and MDP had the same effect on splenic Thy-1 positive cells as toxic endotoxin alone, although the antitumor effects of both treatments diverged considerably (Bloksma et al., 1984a). These data suggest that the MDP-induced potentiation cannot be related to the depletion of Thy-1 positive cells in the inner PALS. The observation that the highly effective antitumor combination of detoxified endotoxin and MDP did not have any effect on splenic T-cell numbers even indicates that T-cell depletion is not necessary for the therapeutic activity of the agents. The same holds for the role of the B-cells in the antitumor activity, as identical changes of the splenic B-cell compartments were found after administration of toxic endotoxin alone or combined with MDP whereas detoxified endotoxin alone or combined with MDP had no effect at all. Therefore, the prior observation that both combinations as well as toxic endotoxin alone inhibited lymphocyte accumulation around Meth A tumors cannot be attributed to a distinct effect on splenic lymphoid compartments. Our data, however, cannot exclude selective effects on different subsets of B and T-lymphocytes, leaving their overall numbers unchanged.

Concerning the non-lymphoid cells in the different compartments of the spleen, we found a significant number of strongly NSE-positive cells in the follicular area of the white pulp of all tumor-bearing mice. Strong NSE positivity, together with a localization along the inner border of the marginal sinus, is indicative for so-called marginal metallophils (Eikelenboom, 1978). In normal mice, strongly NSE-positive cells were found to be absent in the follicular area, but were present in this compartment after a single injection of toxic endotoxin (Groeneveld et al., 1983b). As this coincided with a decrease of marginal metallophils along the border, it was suggested that endotoxin induced migration of marginal metallophils from the border into the follicular area (Groeneveld et al,. 1983b). The present

experiments showed that treatment of tumor-bearing mice with toxic endotoxin depleted the number of strong NSE-positive cells along the inner border of the marginal sinus with a concomitant decrease of these cells in the follicular area. It is not clear whether the strong NSE-positive cells in the follicular area of untreated, tumor-bearing mice are marginal metallophils or activated macrophages that acquired strong NSE activity.
Spleens of Meth A-bearing mice were shown to have enhanced macrophage activity as measured by their capacity to kill Listeria monocytogenes (Bloksma et al., 1984e). If the strongly NSE-positive cells in the follicular area are activated macrophages, their disappearance might be explained by the observation that activated macrophages are very sensitive to the toxicity of endotoxins (Peavy et al., 1979). Because addition of MDP did not influence the effect of toxic endotoxin on marginal metallophils and because detoxified endotoxin combined with MDP had no effect on the behaviour of these cells, it is not likely that they play an essential role in the antitumor activity of these agents. Another population of macrophages, characterized by weak NSE-positivity and strong AP-positivity showed no distinct changes upon treatment with either agent. Groeneveld et al., (1985) have found that relatively high doses of toxic endotoxin reduced the number of these cells in the splenic marginal zone of normal mice. They have related the effect to the toxicity of endotoxin for these cells. Although the LD50 of toxic endotoxin was found to be much lower in tumor-bearing mice than in normal mice (Berendt et al., 1980; Muirhead et al. 1981), which has been related to the state of macrophage activation, the dose applied in our experiments might still have been too low to affect this macrophage population.

The toxicity and most beneficial effects of endotoxins have been attributed to the lipid A moiety of these molecules (Morrison and Ulevitch 1978; Morrison and Ryan, 1979). In our studies we have used a highly refined toxic endotoxin consisting of diphosphoryl lipid A and KDO and a chemically detoxified derivative consisting of monophosphoryl lipid A (Ribi et al., 1982). Only the toxic preparation had significant antitumor activity (Bloksma et al. 1984a), suggesting a relation between toxicity and antitumor activity. This is supported by data of Männel et al., (1979) showing that solid tumors in C3H/HeJ mice, which are very resistant to the lethal effects of endotoxins, are not susceptible to the antitumor effects of endotoxins unless lymphoreticular cells of endotoxin-susceptible mice are transferred.

However, the relation between toxicity and antitumor activity seems more complicated, as addition of MDP to detoxified endotoxin did not clearly increase its toxicity (Ribi, 1984; Bloksma et al., 1984a) but endowed this molecule with potent antitumor activity.

The relationship between toxicity and induction of splenic changes seems more consistent. C3H/HeJ mice did not show splenic changes upon injection of toxic endotoxin (Groeneveld et al., 1985) and detoxified endotoxin alone or combined with MDP caused no splenic changes in endotoxin-sensitive BALB/c mice (Table 2). However, addition of MDP to toxic endotoxin, which markedly increases its toxicity (Ribi, 1984; Bloksma et al., 1984a) did not increase the splenic changes observed. Further experiments with synthetic endotoxins are needed to shed more light on the relation between toxicity and the various effects of endotoxins.

In conclusion, we have found that only toxic endotoxin, whether or not combined with MDP, induced distinct changes in the spleens of tumor-bearing mice. Furthermore, we could not find a consistent relation between the antitumor activity of endotoxins alone or combined with MDP and their potential to induce changes in the spleens of tumor-bearing mice.

CHAPTER 9

GENERAL DISCUSSION

The experiments described in this thesis deal with the histopathological aspects of antitumor action of endotoxins. The significance of the results obtained have been discussed in the respective chapters. In this chapter data will be discussed in a broader scope, with special reference to the main theories on endotoxin-induced tumor necrosis, which have been briefly outlined in Chapter 1.

Histology of tumor cell damage.

Endotoxin-induced necrosis has been described as hemorrhage with necrosis or hemorrhagic necrosis (reviewed by Shear and Perrault, 1943; Parr et al., 1973; Freudenberg et al., 1984). Our studies showed that substantial hemorrhage was present but always restricted to a small area in the upper core of the tumor (Chapters 2, 3, 5, 6 and 7). Moreover, we have obtained ample evidence that endotoxin induces probably 2 different kinds of necrosis. A distinction was made between hemorrhagic necrosis and non-hemorrhagic necrosis, because the α-adrenergic receptor antagonist, phenoxybenzamine, prevented almost exclusively the induction of tumor hemorrhage and the typical parallel lining of the necrotic tumor cells by endotoxin in that region (Chapter 2). In addition, isoproterenol, adrenaline and histamine induced almost exclusively hemorrhagic necrosis (Chapter 3; Bloksma et al., 1984f). Electron microscopical studies ultimately provided strong evidence that pathogenesis as well as ultimate aspect of tumor cell degeneration in the hemorrhagic area were quite distinct from those observed in the remaining part of the tumor (Chapter 6). Both kinds of tumor cell necrosis can be considered as different expressions of coagulation necrosis. For ease of distinction, the former will be indicated as hemorrhagic necrosis (HN) and the latter as coagulation necrosis (CN). A compilation of the differences between HN and CN in solid Meth A tumors has been given in Table 1.

Table 1 - Differences of hemorrhagic necrosis and coagulation necrosis induced in solid Meth A tumors[a]

	Hemorrhagic necrosis	Coagulation necrosis
1. Inducers	- Toxic endotoxin - Serotonin - Isoproterenol - Adrenaline - Histamine	- Toxic endotoxin - Detoxified endotoxin with MDP - Concanavalin A - Serotonin
2. Extensive hemorrhage	Present	Absent
3. Fatty change prior to tumor cell necrosis	Absent	Present
4. Necrosis as judged by light microscopy	Elongated, parallel-lining tumor cells. Hyperemia. Vessels with yellow pigment.	Rounded-off tumor cells with interstitial edema.
5. Ultrastructure of necrosis	Early endothelial cell damage. Early loss of tumor cell organelles.	Morphology fitting coagulation necrosis type.
6. Location in the tumor	Upper core	Centre
7. Dependence on age of Meth A tumor	Absent in 3- and 6-day-old tumors; present in 9- and 15-day-old tumors.	very slight in 3-day-old tumors; moderate in 6-day-old tumors; marked in 9- and 15-day-old tumors.
8. Effect of addition of MDP to toxic endotoxin	None	Potentiation
9. Inhibition by α-adrenoceptor blockade	Marked	Moderate

[a] All data included in the table are compiled from this study, except data on histamine (Bloksma et al., 1984f).

The confinement of endotoxin-induced HN and CN to distinct areas of the tumor might be related to local differences in the respective tumor areas, like blood pressure and condition of the vasculature. Such conditions might predispose to dissimilar reactions in response to a single stimulus. Otherwise, distinct effector mechanisms triggered by endotoxin, might account for the diverse effects. Local conditions within the tumor apparently do not intrinsically determine induction of HN, because concanavalin A (Bloksma et al., 1983a) and MDP/detoxified endotoxin (Chapter 7) induced merely CN within the tumor, including the upper core. Therefore, it is likely that toxic endotoxins possess an additional property, not innate to the agents mentioned above, which would cause HN. Supportive evidence for the existence of 2 different effector mechanisms provoked by toxic endotoxin, is the observation that MDP potentiated only the induction of CN by toxic endotoxin (Chapter 7) and that phenoxybenzamine interfered very selectively with the induction of HN by toxic endotoxin (Chapter 2). As HN was always restricted to the upper core of the tumor, irrespective of the eliciting agent and as susceptibility of Meth A tumors to this phenomenon appeared related to the age of the tumor (Chapters 5 and 6), local conditions within the tumor seem to determine the elicitation of this phenomenon as well. Possible mechanisms will be discussed in the next sections.

Tumor cells as target of endotoxin-induced tumor necrosis.

Two theories on endotoxin-induced tumor damage regard the tumor cells as direct target. One theory states that the tumor cells are killed by tumor necrosis factor (TNF) released from macrophages and monocytes upon contact with endotoxin (Carswell et al., 1975; Ruff and Gifford, 1981). The other theory postulates that tumor cell damage would be due to a prompt interference with the energy supply by non-esterified fatty acids, catecholamines or prostaglandins (Jones, 1979). A direct tumoricidal action by specifically immune cells or their nonspecific effector mechanisms as a cause of tumor necrosis has been excluded because induction of necrosis appeared independent of the immune system, in contrast to induction of complete regression (Parr et al., 1973; North, 1981; 1984).

The TNF theory implicates that injection of endotoxin would result in the release of effective amounts of TNF as well as direct cytotoxic action of the factor. Bloksma et al. (1984c; 1984g) have already listed ample indirect evidence which doubts both. Data from literature since that time (see below) and the experiments described in the previous chapters provide additional evidence. Although pure recombinant TNF (rTNF) was recently found to be capable to induce necrosis of solid Meth A tumors in semi-syngeneic (Pennica et al., 1984) and syngeneic mice (Bloksma, Hofhuis and Willers, to be published), the dose required to induce about the same effect as i.v. injection of *E. coli* endotoxin appeared to be very high*. Earlier studies have shown that sera of endotoxin-injected Meth A sarcoma-bearing mice contained ineffective amounts of TNF, while this could not be attributed to absorption of the factor to the tumor cells (Bloksma, 1982; Bloksma et al., 1983b). Local production of TNF, however, might be marginal to sufficient as calculated from the estimated number of mononuclear phagocytes in 9-day-old Meth A tumors (Chapter 4) and the potential amount of TNF produced per macrophage upon exposure to endotoxin**. In addition, observations of Bloksma, Hofhuis and Willers (to be published) suggest that even ineffective amounts of TNF are able to contribute to the induction of tumor necrosis, because suboptimal doses of rTNF could cause extensive necrosis upon addition of submicrogram amounts of endotoxin, that are ineffective on their own. Such a synergistic mechanism might occur in endotoxin-injected tumor-bearing mice. Whether TNF is indispensable to induction of tumor necrosis, however, seems doubtful, because MDP/detoxified endotoxin induced extensive tumor necrosis (Chapter 7), without inducing endotoxic side effects, such as hypothermia, diarrhea, weight loss and lethargy (Bloksma et al., 1984a), which have been attributed to TNF (Beutler and Cerami, 1986). Moreover, the nontoxic

* In the syngeneic system, 3×10^4 units i.v. or 10^4 units i.t. are needed.

** Estimated volume of 9-day-old Meth A sarcoma is 0.18 ml ($4/3$ pi x 0.35^3 cm). Minus 45% necrosis, reticulum and skin gives 0.10 ml cell volume, containing 10% macrophages. The number of macrophages in the tumor is calculated as minimally 3×10^6 producing together 3×10^3 units TNF, when the following premises are emanated from: a). the mean diameter of the mononuclear phagocytes is 12 μm, b) all other cells are tumor cells with a mean diameter of 20 μm, c) the TNF production of the mononuclear phagocytes is comparable with that of a murine macrophage cell line of which 10^8 cells produced 10^5 units within 22 h upon stimulation with endotoxin (Beutler et al., 1985a).

combination did not induce HN, while toxic endotoxin (Chapter 7) and rTNF (Van de Wiel, Bloksma, Kuper and Willers, to be published) induced HN in a dose-dependent way. This suggests that induction of HN is associated with the toxic effects of endotoxins. Whether TNF is the toxic mediator causing HN after injection of toxic endotoxin can only be determined in experiments studying the antitumor effects of endotoxin in mice treated with neutralizing antibodies to TNF.

The observation that tumor-bearing mice displayed endotoxic side effects upon treatment with toxic endotoxin, irrespective of tumor age (FMA Hofhuis, personal communication) might allow the conclusion that TNF is induced in these mice. Nevertheless, only mice with 9- and 15-day-old Meth A tumors showed extensive tumor necrosis upon endotoxin-treatment (Chapters 5 and 6). In this respect the refractoriness of 6-day-old tumors is striking. Namely extensive tumor necrosis has to be expected theoretically in these tumors, because only tumors of this age contained numerous mononuclear phagocytes, which should guarantee a high local production of TNF and because TNF injected locally appeared far more effective against Meth A cells than TNF administered systemically (Pennica et al., 1984; Bloksma, Hofhuis and Willers, to be published). These data suggest that either the production of TNF is insufficient in these mice or that necrosis of tumor cells is not the result of a direct cytocidal effect of TNF. The latter suggestion seems the most likely because tumor cells disseminated to the regional lymph nodes did not show cytostasis and necrosis upon endotoxin-treatment in mice which reacted with extensive necrosis of the primary tumor (Chapter 5). In vitro experiments provide further evidence for an indirect tumoricidal effect of TNF in vivo. Incubation of Meth A cells with 10^5 units of rTNF during 3 days was reported to cause '25% or more cytotoxicity' (Sugarman et al., 1985). Similar experiments of Van de Wiel, Bloksma and Willers (to be published) failed to show any harmful effect of rTNF against Meth A in vitro. This is in sharp contrast to its in vivo activity, where i.v. injection of 3×10^4 units killed the majority of tumor cells within 24 h. One of the indirect effects of TNF in vivo might be the induction of fatty change observed after treatment of tumor-bearing mice with endotoxin (Chapter 6). The fatty change might be related to the action of cachectin, a recently discovered macrophage hormone released by endotoxin and soon afterwards identified to be identical to TNF (Beutler et al., 1985a). Cachectin was shown to derange

the fat metabolism by suppressing the production of lipoprotein lipase. Hyperlipemia as a cause of a defective triglyceride clearance is the result. Tumor cells can take up triglycerides (Damen et al., 1984), which frequently leads to intracellular lipid accumulation (Spiegel et al., 1982). Regarding this, it is not unlikely that the lipid accumulation observed in Meth A cells is the result of increased triglyceride ingestion from the circulation. As its onset was most rapid in 9-day-old tumors, which were most susceptible to endotoxin-induced tumor necrosis, fatty change might be related to the extent of subsequent necrosis. However, fatty change per se is considered not to be lethal to a cell, although it is often observed prior to cell death (Robbins and Cotran, 1979). Lipid accumulation might impair cell function, especially in hypoxic cells. The hypoxic state might aggravate the fatty change because in the absence of oxygen, triglycerides cannot be degraded. Hypoxia is likely to be prevalent in the cores of 9-day and 15-day-old tumors after treatment with endotoxin (Chapters 5 and 6). Hypoxia would also cause an accumulation of lactic acid and inorganic phosphate as a consequence of anaerobic glycolysis. These products do not cause irreversible cell damage either, provided conditions are improved within due time (Robbins and Cotran, 1979). The endotoxin-induced disturbance of blood supply within the tumor cores, however, will probably not enable improvement. Only young tumors and tumor margins, which are generally regarded the most well-nourished (Robbins and Cotran, 1979), and which retained a relatively intact vascular supply after treatment with endotoxin (or are independent of tumor vasculature) might escape from irreversible damage by adequate removal of metabolic waste products and supply of oxygen and nutrients.

In view of the above discussion it is not very likely that a selective uncoupling of the oxydative phosphorylation within the tumor cells (Jones, 1979) contributes significantly to endotoxin-induced tumor cell death. The hypoxic state within the tumor already hampers oxidative processes. Moreover, if this mechanism would occur, it is difficult to explain why only tumors of a certain age and why only tumor cores would be affected.

An extensive direct tumoricidal effect by inflammatory cells as a cause of endotoxin-induced tumor necrosis could be excluded. The majority of host cells were located inside the vessels and endotoxin did not augment their extravasation (Chapter 6). Moreover, most host cell infiltrates were found

in the margins. The absence of substantial necrosis in this region upon injection of endotoxin, indicates that either this agent virtually lacks the capacity to activate these cells to a tumoricidal state or that the host cells in this region are intrinsically unable to exert cytocidal activity. As therapeutic activity correlated with a decrease of host cells in that region, it has been suggested that a considerable part of the inflammatory infiltrate in the margins has suppressor activity (Chapters 4 and 7). The observation that agents with therapeutic but without toxic activity did not induce changes in the spleen, indicates that activated splenic cells are not necessarily involved in the endotoxin-induced antitumor effects (Chapter 8).

Tumor vasculature as indirect target of endotoxin-induced tumor necrosis

Various data obtained in this study point to the tumor vasculature as indirect target of endotoxin-induced tumor necrosis. The first macroscopic antitumor effect that could be observed after injection of toxic endotoxins in mice with intradermal Meth A tumors susceptible to endotoxin-induced tumor necrosis was a red discoloration of the tumor core (Chapter 2), also noticed in other tumors (Parr et al., 1973). In Meth A tumors this discoloration could be attributed to an increased number of dilated vessels in the upper tumor core (Chapters 2, 3, 5, 7). Furthermore, α-adrenergic receptor blockade appeared to modulate endotoxin-induced hyperemia and to prevent induction of HN (Chapter 2), while vasoactive agents like serotonin and adrenaline, known to be released upon administration of endotoxin, appeared capable to induce hyperemia and necrosis of Meth A (Chapter 3). In addition, the capacity of MDP to potentiate endotoxin-induced tumor necrosis seemed to be related to its capacity to potentiate hyperemia (Chapter 7). Finally, morphological studies on the tumor age-dependent susceptibility of Meth A to induction of necrosis by endotoxin indicated that it was determined by the condition of the tumor vasculature prior to injection and that overt vascular damage and edema were precursors of HN and CN respectively (Chapter 6).

The ability of endotoxins to interfere with host mediation systems, which in their turn change vascular functions and integrity, is well known (Morrison and Ulevitch, 1978). Moreover, endotoxin is thought capable of

direct interactions with blood vessels (reviewed by Semeraro, 1980). Some of the host factors which might be involved in endotoxin-induced tumor damage have been summarized in Table 2. Their possible contribution to induction of tumor necrosis will be discussed. In addition, features of the tumor vasculature that might define the enhanced susceptibility of tumor tissue to endotoxin-induced necrosis will be evaluated.

The oldest theory on endotoxin-induced tumor necrosis suggested that the reaction of the tumor was similar to a local Shwartzman reaction; tumor blood supply would be obliterated by fibrin thrombi (Gratia and Lintz, 1931). Thrombi as hallmark of such a reaction, however, were not prominent in Meth A tumors. If present, they seemed a consequence rather than a cause of tumor necrosis (Chapter 6). A vascular phenomenon which was always observed at the onset of necrosis and correlated very well with the extent of endotoxin-induced necrosis, appeared to be vasodilation designated as hyperemia (Chapters 2, 3, 5, 7). This effect might be mediated by vasoamines, like adrenaline, serotonin and histamine, which were themselves capable of inducing hyperemia (Chapter 3; Bloksma et al., 1984f). Moreover, agents antagonizing their action, interfered with induction of hyperemia and necrosis (Chapter 2; Bloksma et al., 1982b; 1984f). The mode of action of vasoamines, however, is still not clear. Although tumor blood vessels were surrounded by pericytes, the absence of smooth muscle cells around the blood vessels in the tumor (Chapter 6) makes a vasoconstrictor response of these vessels less likely. It might explain why a potent peripheral vasoconstricting agent like adrenaline does not induce vasoconstriction within the tumor. In addition, an abnormal reaction of the tumor vasculature to vasoactive agents may be a reflection of the paucity of micropinocytotic vesicles in the endothelium (Chapter 6). It has been suggested that these organelles play a role in vascular homeostasis and in the maintenance of microvascular integrity (Ryan et al., 1985; Shepro and Hechtman, 1985). The relation of hyperemia with subsequent necrosis is not clear either. Hyperemia was always restricted to the upper core of the tumor, irrespective of the inducing agent. Whether HN or CN developed in that particular area depended on the inducing agent. HN was observed after i.v. injection of toxic endotoxins (Chapters 2, 3, 5, 7), crude TNF (Bloksma et al., 1983a), rTNF (Van De Wiel, Bloksma, Kuper and Willers, to be published) or vasoactive agents (Chapter 3), whereas CN was seen after similar treatment with concanavalin A (Bloksma

Table 2 - Host factors that might mediate changes of vascular functions and integrity in Meth A tumors of endotoxin-treated mice

Factor	Relevant Source	Vascular effects	Effects observed in Meth A
Serotonin	Platelets, mast cells, several tissues including spleen[1]	Increased permeability; smooth muscle contraction[1]	Hyperemia, mitotic arrest, HN and CN[2]
Histamine	Mast cells and basophils[1]	Increased permeability, vasodilation[1]	Macroscopically observed necrosis[3]
Adrenaline	Adrenal medulla[4]		Hyperemia, mitotic arrest, HN[2]
PMN-granule factors	Polymorphonuclear cells	Injury of basement membrane and endothelial cells[5]	
TNF/cachectin	Mononuclear phagocytes[6,7]	Synthesis of procoagulant activity by endothelial cells[11]. Enhanced neutrophil adherence and degranulation[12]	Hyperemia, vasculitis, mitotic arrest, HN and CN[8,9]
IL-1	Mononuclear phagocytes[10] Endothelial cells, neutrophils[14]	Synthesis of procoagulant activity, PGE2 and prostacyclin by endothelial cells[10,13] Adhesiveness of endothelium for leukocytes[10]	
Activated Hagemann factor	Plasma[4]	Fibrin formation [4]	

1. reviewed by Emerson Jr., 1985;
2. Chapter 3;
3. Bloksma et al., 1984f;
4. reviewed by Robbins and Cotran, 1979;
5. Movat and Wasi, 1985;
6. Carswell et al., 1975;
7. Beutler et al., 1985a;
8. Bloksma et al., 1983a;
9. Van De Wiel et al., unpubl.;
10. Bevilacqua et al., 1985.
11. Nawroth and Stern (1986)
12. Nathan et al., (1985)
13. Albrightson et al., (1985)
14. Oppenheim et al., (1986)

et al., 1983a) or MDP/detoxified endotoxin (Chapter 7). As already touched upon in the previous section, development of HN might reflect a toxic effect, possibly a vasotoxic effect. It might act at blood vessels with predisposition to it, thereby causing a prompt endothelial cell damage as observed at the onset of HN (Chapter 6). Various data are in support of this view. Phenoxybenzamine, an antagonist of effects of adrenaline and in higher doses also of serotonin and histamine (Weiner, 1980b) was shown to diminish the toxicity of endotoxins (Filkins, 1979) and to prevent the induction of HN by endotoxin (Chapter 2). Whether these effects are only due to an interference of phenoxybenzamine with the vascular effects of the vasoamines released by endotoxin seems doubtful. The capacity of phenoxybenzamine to reduce the toxicity as well as the antitumor effects of endotoxin might be as much due to an inhibition of the release of TNF by endotoxin (Bloksma et al., 1982a), while crude TNF by itself induced, just like endotoxins, strong hyperemia (Bloksma et al., 1983a). It was therefore suggested that TNF would act by a direct or indirect interaction with the vasculature. Recent literature on rTNF suggests that TNF might have a direct action at blood vessels in vivo. This is indicated by its capacity to induce hypotension (Beutler and Cerami, 1986) as well as its influence on endothelial cell functions in vitro (Nawroth and Stern, 1986; Nathan et al., 1985; Table 2). Moreover, intratumoral injection of rTNF induced hyperemia, endothelial cell damage and vasculitis all over the tumor (Van De Wiel, Bloksma, Kuper and Willers, to be published). Data obtained till now, however, do not allow a conclusion on the relative contribution of vasoamines and rTNF to the vascular effects observed in Meth A after injection of endotoxin.

The observed adherence of platelets and leukocytes to the lining of Meth A blood vessels (Chapter 6), might well be mediated by endotoxin-induced IL-1 and rTNF, which were shown to increase the adhesiveness of endothelial cells to leukocytes in vitro (Bevilacqua et al., 1985; Table 2). Other effects like prior existence of endothelial cell damage and diminished blood flow as a consequence of induced vasodilation are likely to amplify adherence. Such effects might explain why adherence was less prominently observed in younger tumors and in the periphery of older tumors and hardly at all immediately outside the tumors (Chapter 6). It is very likely that leukocyte adherence contributes to the antitumor effects of endotoxin through a further disturbance of vascular functions. Especially, polymorphonuclear neutrophils have

to be suspected as mediators of vascular damage. Lysosomal constituents of these cells, known to be released by endotoxins, were shown to enhance vasopermeability and to cause hemorrhage after intradermal injection (Movat and Wasi, 1985). In addition, endotoxins and TNF were reported to release oxygen radicals from these cells (Weiss and Lo Buglio; 1982; Shalaby et al., 1986), which might effect endothelial cell damage directly or indirectly by the generation of oxidized triglycerides, shown to have potent cytotoxic effects on endothelial cells (Cathcart et al., 1985). The capacity of neutrophils to cause vascular damage seems to relate very well to endotoxin-induced tumor necrosis. The highest number of intravascular polymorphonuclear cells, mainly neutrophils (Chapter 6) was observed in 9-day-old Meth A tumors and coincided with an optimal susceptibility to endotoxin-induced tumor necrosis (Bloksma et al., 1984e; Chapter 5). Also Shalaby et al. (1986) have recently suggested that TNF-activated neutrophils participate in the necrosis and eventual regression of the tumor. Although these data stress an important role of polymorphonuclear cells in the induction of endotoxin-induced tumor necrosis, an essential contribution of other host cells within the tumor capable to produce IL-1 (monocytes, macrophages, endothelial cells), TNF (monocytes, macrophages) and vasoamines (platelets, mast cells) in the overall effects seems obvious.

Despite strong evidence that endotoxin-induced necrosis of solid tumors requires the presence of endotoxin-responsive lymphoreticular cells (Männel et al., 1979), it cannot be excluded that a direct effect of endotoxins on blood vessels contributes to endotoxin-induced tumor necrosis. The above-mentioned capacity of endotoxin to induce the production of IL-1 and procoagulant activity by endothelial cells is probably conducive to an optimal antitumor effect. Further, the property of endotoxin to cause a disarray of the endothelial glycocalyx might be a cause of edema by permitting the extravasation of larger molecules (Ryan et al., 1985; Demling et al., 1984).

Concluding remarks.

Data obtained in this thesis combined with data from literature allow the following main conclusions:

- I.v. injected toxic endotoxins induce two distinct types of necrosis in solid Meth A tumors within 24 h: hemorrhagic necrosis and coagulation necrosis. Detoxified endotoxin lacks the capacity to induce any tumor necrosis at all
- Hemorrhagic necrosis is exclusively seen in a small superficial part of the tumor, that shows already extensive vascular damage at the time of treatment. Amplification of vascular damage precedes hemorrhagic necrosis and is considered as a main cause of this phenomenon.
- Induction of hemorrhagic necrosis seems attributable to toxic properties of endotoxin, possibly its potential to release vasoactive agents and/or TNF.
- Endotoxin-induced vascular dilation and hemorrhagic necrosis, although sequentally observed in the same region, are not intimately related, because agents which cause coagulation necrosis in this region induce vasodilation as well.
- Coagulation necrosis is the most extensive form of necrosis that is induced by toxic endotoxin, and was always preceded by edema and fatty change of the tumor cells. Only cells in the tumor margins escaped from this effect of endotoxin.
- Congestion, lack of smooth musculature and aberrant endothelial cells are aspects in which tumor blood vessels differ from blood vessels in normal tissues. These differences might largely explain the selective susceptibility of tumor tissue to induction of necrosis after injection of a moderate dose of endotoxin. Disturbance of vascular functions by endotoxin and/or endotoxin-induced mediators, like interleukin-1 and TNF, in an already malfunctioning vasculature will facilitate the development of hypoxia and accumulation of metabolic waste products, which more or less contribute to necrosis.
- The inability of toxic endotoxin to induce necrosis of young tumors and of the margins of older tumors may mainly be the consequence of the relatively unaffected condition of the blood vessels in these tissues

- Tumor necrosis cannot be attributed to distinct changes in the number of host cells within the tumor or be related to morphological changes in spleen and regional lymph nodes
- The moderate infiltrate within the tumor and its localization indicate that direct host cell-mediated tumor cell killing does not play a crucial role in tumor necrosis
- Host cells within tumor blood vessels may contribute to tumor necrosis by aggravation of vascular damage. Especially polymorphonuclear cells may be involved, because 1. abundant numbers of these cells were exclusively observed in tumors which were the most susceptible to induction of tumor necrosis; 2. the difference in capacity of two distinct toxic endotoxins to induce tumor necrosis (and complete regression) could be related to their ability to induce an influx of polymorphonuclear cells; 3. these cells are well-known inducers of vascular injury upon contact with endotoxins or its mediators
- Mast cell mediators are possibly involved in vasodilation induced by tumor-necrotizing agents
- Killing of tumor cells by tumoricidal factors, locally and systemically released by toxic endotoxins, seems not to be a major cause of tumor necrosis, since young tumors, margins of older tumors and disseminated tumor cells in the regional lymph nodes were not affected
- The ability of MDP to potentiate the tumoricidal action of endotoxins is related to its capacity to potentiate many morphological effects of toxic endotoxin and to elicit these effects exclusively in combination with detoxified endotoxin. The potentiation cannot be attributed to any particular morphological effect of this agent alone on tumor and/or spleen
- Therapeutic activity of toxic endotoxin is directly correlated to the extent of early induced necrosis in Meth A sarcoma and, according to data from literature, to the degree of specific immunity to the tumor. As both phenomena are highly defined by the tumor size (age) and are optimal in Meth A of the same size, it is very likely that they act in concert with each other to effect complete cures
- Only tumor necrotizing (combinations of) agents which prevent lymphocyte accumulation around the tumor, are capable to induce complete regression

In brief: therapeutic activity of toxic endotoxins is the result of a cascade of complex events. The condition of the tumor and vasculature, existing defense mechanisms against the tumor at the time of treatment and humoral and cellular reactions of the host upon challenge with endotoxin, many of which are directed at the tumor vasculature, determine together the therapeutic outcome.

SAMENVATTING

Endotoxinen, de lipopolysacchariden uit de celwand van gram-negatieve bacteriën, hebben vele biologische effecten, waaronder een sterke antitumorwerking. De effectiviteit van de antitumorwerking is afhankelijk van het type en de grootte van de tumor, de manier van toediening van het endotoxine en de immuunstatus van het dier. Toediening van endotoxine in de bloedbaan van muizen met een subcutaan getransplanteerde bindweefseltumor veroorzaakt roodkleuring (hyperemie) van de tumor binnen 4 uur en donkerbruine tot zwarte verkleuring (necrose) binnen 24 uur. Dit wordt soms gevolgd door het volledig verdwijnen (regressie) van de tumor.

Het induceren van necrose en volledige regressie berust waarschijnlijk op verschillende mechanismen. Necrose kan opgewekt worden in zowel immunogene als niet-immunogene tumoren en is uitgebreider naarmate de tumor groter is. Bovendien kan necrose optreden in tumoren bij dieren met een onvolledig T-cel systeem. Gegevens uit de literatuur wijzen erop dat de necrose voor een belangrijk deel veroorzaakt wordt door een abnormale reactie van de tumorbloedvaten op vasoactieve stoffen zoals serotonine en adrenaline, die in een verhoogde mate worden aangetroffen in het bloed van dieren na inspuiting van endotoxine. De abnormale vaatreactie leidt tot een verminderde doorbloeding van de tumor en mogelijk zelfs tot een volledige stilstand van het bloed, waardoor de tumorcellen dood kunnen gaan door gebrek aan zuurstof en ophoping van afvalstoffen. Volledige regressie treedt vrijwel alleen op in immuuncompetente dieren met immunogene tumoren die een diameter tussen 6 en 8 mm hebben. Het vereist bovendien, op het tijdstip dat endotoxine wordt toegediend, een toestand van 'concomitant immunity', d.w.z. het vermogen van het tumordragende dier om een tweede transplantaat van dezelfde tumor af te stoten.

Endotoxine is te toxisch om toe te passen bij de mens. Tot voor kort heerste de mening dat dit omzeild zou kunnen worden door "tumor necrosis factor" (TNF) te gebruiken. Dit is een factor die door mononucleaire fagocyten in sterk verhoogde mate gevormd en uitgescheiden wordt als reactie op toxische endotoxinen en die evenals toxische endotoxinen in staat is om necrose en regressie van solide tumoren te induceren. Recent onderzoek heeft echter aangetoond dat TNF beschouwd moet worden als een van de belangrijkste

mediatoren van de toxische effecten van endotoxinen. Toepassing van gedetoxificeerd endotoxinen biedt ook geen uitkomst, daar deze vrijwel geen activiteit tegen solide tumoren bezitten. Onlangs is echter gevonden dat een combinatie van muramyldipeptide (MDP) en gedetoxificeerd endotoxine een sterkere antitumorwerking bezit dan toxisch endotoxine alleen, hoewel deze combinatie niet de ongewenste neveneffecten van toxische endotoxinen induceert. Toevoeging van MDP aan toxische endotoxinen resulteerde daarentegen niet alleen in een versterking van de therapeutische maar ook van de toxische werking. Waarop de potentiërende werking van MDP berust is nog niet duidelijk.

Het doel van de, in dit proefschrift beschreven, experimenten was de vroege werking van intraveneus toegediende toxische endotoxinen tegen solide tumoren in de muis morfologisch nader te onderzoeken. Daartoe werden de effecten van toxisch endotoxine op tumoren van verschillende grootte (en met uiteenlopende gevoeligheid) en de regionale lymfeklieren morfologisch onderzocht, werd de antitumorwerking van toxisch endotoxine vergeleken met die van vasoactieve stoffen, gedetoxificeerd endotoxine en endotoxinen in combinatie met MDP, werd het effect van een remmer van vasoactieve stoffen op de antitumorwerking van toxisch endotoxine onderzocht en werd het effect van endotoxinen met of zonder MDP op de milt van tumordragende muizen nagegaan. Als tumor werd het oorspronkelijk met methylcholantreen in de BALB/c muis opgewekte Meth A fibrosarcoom gebruikt. Solide tumoren werden verkregen door Meth A cellen onder de buikhuid van BALB/c muizen te spuiten. Ondanks het feit dat de tumor sterk immunogeen is, groeit de tumor ongestoord in syngene en semisyngene muizen, maar metastaseert vrijwel niet. Uit macroscopische waarnemingen is gebleken dat vanaf dag 7 na transplantatie necrose kan worden opgewekt in de tumor en dat tussen dag 8 en dag 11 de tumor gevoelig is voor het induceren van volledige regressie.

In hoofdstuk 2 zijn de microscopisch waarneembare effecten van een toxisch endotoxine op de tumor beschreven. Daarnaast is het effect van adrenerge receptor blokkade op de antitumorwerking van endotoxine onderzocht. Vier uur na toediening van endotoxine werd een sterke vermindering van het aantal mitosen in de gehele tumor gevonden. Bovendien werd op datzelfde tijdstip sterke vaatverwijding (hyperemie) gezien, met name aan de huidzijde van de tumor. Deze was 24 uur na behandeling vrijwel verdwenen. In

plaats daarvan werd necrose van het daar gesitueerde tumorweefsel waargenomen, gekarakteriseerd door evenwijdig gerangschikte, langwerpige dode tumorcellen, bloedvaten gevuld met geel materiaal en diffuse bloeding. Deze necrose werd daarom aangeduid met hemorragische necrose. Hemorrhagisch necrose bleek slechts een gering deel van de totale necrose uit te maken. Een veel groter, centraal gelegen deel van de tumor vertoonde necrose, zonder uitgebreide hemorragie en met afgeronde dode tumorcellen, benoemd als de niet-hemorragische of coagulatienecrose. De cellen in de randen van de tumor bleven meestal vitaal, hoewel ook daar na 24 uur nog steeds vrijwel geen mitosen werden aangetroffen.

Eenmalige toediening van de alpha-adrenerge receptorblokkerende stof fenoxybenzamine (die ook de werking van serotonine en histamine tegengaat) kort voor behandeling met endotoxine, verminderde het vermogen van endotoxine om na 4 uur vaatverwijding te veroorzaken. Na 24 uur waren de bloedvaten echter sterker verwijd dan 4 uur na toediening van endotoxine alleen. Fenoxybenzamine voorkwam het optreden van hemorragische necrose en groeiremming. Beta-adrenerge receptorblokkade met propranolol verminderde de vaatverwijding 4 uur na toediening van endotoxine en verhinderde de groeiremming. Zowel fenoxybenzamine als propranolol stimuleerden de tumorgroei, wanneer ze werden toegediend zonder endotoxine.

Aangezien fenoxybenzamine het optreden van hemorragische necrose voorkwam, maar niet de coagulatienecrose en de mitoseremming, werd gesuggereerd dat met name de inductie van de hemorragische necrose afhankelijk was van de aanwezigheid van intacte receptoren voor adrenaline en mogelijk ook serotonine en histamine.

In hoofdstuk 3 is de antitumor werking van endotoxine vergeleken met die van de vasoactieve aminen serotonine, isoproterenol en adrenaline. Mede op grond van de resultaten, beschreven in hoofdstuk 2, is een rol van deze aminen in de antitumor werking van endotoxine aannemelijk. De vasoactieve aminen veroorzaakten net als endotoxine duidelijk waarneembare necrose en een tijdelijke remming van de tumorgroei, maar alleen endotoxine was in staat volledige tumorregressie te induceren. Histologisch onderzoek toonde aan dat alle stoffen hyperemie en de oppervlakkig gelokaliseerde hemorragische necrose induceerden. Alleen serotonine veroorzaakte daarnaast de meer centraal gelegen coagulatienecrose doch in mindere mate dan endotoxine.

De vasoactieve stoffen misten het vermogen van endotoxine om aanzienlijke hoeveelheden TNF, hitte-stabiele cytostatische factoren en interferon te induceren in serum van met *Corynebacterium parvum* (*Propionibacterium acnes*) voorbehandelde, tumor-vrije muizen.

De resultaten ondersteunen het vermoeden dat vasoactieve stoffen een rol spelen bij de inductie van vaatverwijding en hemorragische necrose door endotoxine. De rol van de hyperemie blijft echter onduidelijk, maar lijkt verschillend te zijn voor hemorragische en coagulatienecrose. Het opwekken van hemorragische necrose is waarschijnlijk niet direct afhankelijk TNF synthese.

In hoofdstuk 4 zijn de effecten van endotoxine op het ontstekingsinfiltraat in en rondom 9-dagen oude tumoren onderzocht, 4, 24 en 48 uur na toediening. Hiertoe werden preparaten gemaakt van celsuspensies, verkregen door de cellen van de tumoren enzymatisch te scheiden. Centrum en randen van de tumoren werden apart verwerkt omdat de reacties in deze gebieden zoals boven beschreven sterk van elkaar verschilden. Ontstekingscellen werden bij dieren die met fysiologisch zout behandeld waren, vooral gevonden in en rondom de randen van de tumoren. Opmerkelijk was dat daar tussen 24 en 48 uur het aantal lymfocyten sterk toenam. Omdat deze toename in de tijd samenviel met een in de literatuur beschreven afname van de 'concomitant immunity' en een toename van lymfocyten met suppressoractiviteit in de milt, werd verondersteld dat de lymfocyten in de randen van de tumor suppressoractiviteit zouden bezitten. Het bleek echter niet mogelijk om de cellen nader te karakteriseren met behulp van immuuncytochemische technieken, omdat een deel van de tumorcellen zelf sterk meekleurden.

Endotoxine beïnvloedde het totaal aantal ontstekingscellen niet. Vier uur na behandeling werd een voorbijgaande, geringe toename van monocyten en macrofagen en een afname van polymorfkernige cellen in het centrum van de tumor waargenomen. Het sterkste effect was een afname van lymfocyten tussen 24 en 48 uur in de randen van de tumor. Een belangrijk deel van het infiltraat aan de randen en de bovenzijde van de tumor bestond uit mestcellen maar endotoxine leek lichtmicroscopisch geen invloed te hebben op het aantal en de morfologie.

De verkregen resultaten suggereren dat de tumorschade door endotoxine niet het gevolg is van een direct cytotoxische werking van ontstekingscellen en dat de door endotoxine geïnduceerde regressie in verband lijkt te staan met een afname van het aantal lymfocyten.

In de hoofdstukken 5 en 6 werd onderzocht waarom de werkzaamheid van endotoxine tegen Meth A tumoren afhankelijk is van de tumorleeftijd. Daartoe werden 3-, 6-, 9- en 15-dagen oude tumoren en de regionale lymfklieren, voor en na behandeling van de muizen met endotoxine, licht- en electronenmicroscopisch onderzocht. Ogenschijnlijk bleek endotoxine pas bij 9 dagen oude tumoren necrose te kunnen induceren en was de necrose bij 15 dagen oude tumoren uitgebreider. Microscopie van onbehandelde tumoren toonde reeds een klein basaal gelegen gebied met spontane coagulatienecrose op dag 3. Deze necrose breidde zich met de leeftijd concentrisch uit en was zeer aanzienlijk in de oudste tumoren. Zichtbare spontane vaatschade, zoals endotheelbeschadiging en hemorragie was vooral aanwezig in het centrum van de tumoren en nam eveneens toe met de leeftijd tot dag 9. De bloedvaten in de gehele tumor weken sterk af van de vaten in normale huid. Glad spierweefsel was afwezig en het endotheel bezat minder pinocytotische blaasjes. De vaten waren bovendien altijd volgepakt met erythrocyten. Het aantal leucocyten in de vaten varieerde met de leeftijd. Het was gering op dag 3. Op dag 6 en 9 werden respectievelijk grote aantallen mononucleaire en polymorfonucleaire cellen waargenomen. Op dag 15 was het aantal leucocyten weer afgenomen en bevatte ongeveer evenveel van beide celtypen. Slechts weinig leucocyten bleken de tumormassa binnengedrongen te zijn. Mestcellen werden vooral in 9 en 15 dagen oude tumoren waargenomen aan de huidzijde en aan weerszijden van de tumor.

Endotoxine veroorzaakte vaatverwijding en aanhoudende mitoseremming in alle tumoren. De vaatverwijding was het meest uitgebreid op dag 9 en 15. De mitoseremming was het sterkst bij 9 dagen oude tumoren. Endotoxine vergrootte de vaatschade aan de huidzijde van 9 en 15 dagen oude tumoren waar later hemorragische necrose werd waargenomen. In het overige deel van deze tumoren, de randen uitgezonderd, trad sterk oedeem op gevolgd door coagulatienecrose en zichtbare vaatschade. De geïnduceerde necrose was relatief het sterkst bij 9 dagen oude tumoren, hetgeen dus in tegenstelling is met de macroscopische waarnemingen. De mate van oedeem en coagulatie-necrose bij

6 dagen oude tumoren was geringer dan bij de oudere tumoren, terwijl de jongste tumoren alleen oedeem vertoonden. Tekenen van een vroege intravasculaire stolling, die aan een Shwartzman-reactie doen denken, werden slechts in de oudste tumoren waargenomen. Endotoxine had geen invloed op de samenstelling en het uiterlijk van de intravasculaire leucocyten, maar verhoogde wel hun hechting aan het endotheel. Er werd geen verhoogde uittreding van deze cellen waargenomen. De aan de huidzijde aanwezige mestcellen bleken echter binnen 24 uur na toediening van endotoxine volledig gedegranuleerd te zijn bij 9 en 15 dagen oude tumoren.

De regionale lymfeklieren vertoonden een duidelijke reactie op de transplantatie van tumorcellen, aanvankelijk in het T-celafhankelijke gebied, daarna vooral in de B-celafhankelijke gebieden. Vitale tumorcellen werden reeds 7 dagen na transplantatie in de subcapsulaire sinussen waargenomen, maar metastasen bleven uit. Behandeling met endotoxine had geen invloed op deze tumorcellen noch op de lymfklierreactie.

De gegevens leiden tot de conclusie dat de vroege antitumorwerking van endotoxine grotendeels het gevolg is van diverse effecten op de tumorbloedvaten en dat zijn vermogen om tumornecrose te induceren sterk afhankelijk is van de conditie van de bloedvaten in de tumor ten tijde van behandeling. De aanwezigheid van polymorfkernige cellen en mestcellen draagt waarschijnlijk in belangrijke mate bij aan de inductie van necrose. De relatief goede conditie van de bloedvaten in de randen van oude Meth A tumoren verklaart mogelijk voor een groot deel hun geringe gevoeligheid voor inductie van necrose door endotoxine. Tumornecrose is duidelijk niet het gevolg van een uitgebreide directe cytotoxische activiteit van het infiltraat tegen individuele tumorcellen. Inductie van zeer snelle en uitgebreide necrose, uitsluitend waargenomen in 9 dagen oude tumoren, is mogelijk van doorslaggevend belang voor inductie van volledige regressie, die slechts bij tumoren van deze leeftijd wordt gezien.

In hoofdstuk 7 en 8 is nagegaan of histopathologische veranderingen in de Meth A tumor en de milt aanwijzingen konden geven omtrent het vermogen van MDP de antitumor werking van zowel toxische als niet-toxische endotoxinen te potentiëren. In deze experimenten werd gebruik gemaakt van het zeer zuivere toxische endotoxine van *Salmonella typhimurium* Re en een chemisch gedetoxificeerd preparaat hiervan. De histologie van de tumor kwam zeer goed overeen

met de macroscopische waarnemingen dat alleen toxisch endotoxine met of zonder MDP en gedetoxificeerd endotoxine met MDP in staat waren om tumornecrose en volledige regressie te bewerkstelligen. MDP noch gedetoxificeerd endotoxine was in staat duidelijke morfologische veranderingen in de tumor te veroorzaken. Toevoeging van MDP aan hetzij toxisch, hetzij gedetoxificeerd endotoxine resulteerde echter in een uitgebreidere necrose dan waargenomen werd na toediening van toxisch endotoxine alleen. MDP versterkte de door toxisch endotoxine veroorzaakte vaatverwijding, mitoseremming, mestceldegranulatie, coagulatienecrose en remming van lymfocytenaccumulatie, maar niet de hemorragische necrose en de influx van polymorfkernige cellen. Al deze effecten op de laatste twee na werden ook waargenomen na toediening van MDP in combinatie met gedetoxificeerd endotoxine, zij het in iets mindere mate.

Toxisch endotoxine veroorzaakte sterke veranderingen in de milt van Meth A dragende muizen binnen 24 uur na toediening. De afmeting van en het aantal B-lymfocyten in het B-afhankelijke compartiment binnen de marginale sinus werden veel groter, doch de hoeveelheid B-cellen in de marginale zone nam sterk af. Het T-cel afhankelijke compartiment veranderde niet van grootte, maar bevatte veel minder T-lymfocyten. Voorts nam het aantal metallofiele cellen langs de binnenkant van de marginale sinus sterk af. Toevoeging van MDP had geen enkele invloed op al deze effecten. Geen van deze veranderingen werd waargenomen na toediening van gedetoxificeerd endotoxine, MDP of MDP met gedetoxificeerd endotoxine.

Conclusies uit deze onderzoekingen zijn dat MDP op zich geen duidelijke morfologische effecten vertoont op tumor en milt waaruit zijn vermogen om de antitumoreffecten van endotoxinen te potentiëren verklaard kan worden. Het vermogen van MDP om de door toxisch endotoxine geïnduceerde hyperemie in de tumor te versterken en om uitsluitend in combinatie met gedetoxificeerd endotoxine sterke hyperemie te veroorzaken, draagt waarschijnlijk in hoge mate bij aan de daaropvolgende tumornecrose en -regressie. Mestcellen, als bron van vasoactieve aminen, spelen hier mogelijk een belangrijke rol. De overige door MDP gepotentiëerde antitumoreffecten, zoals mitoseremming en coagulatienecrose, dragen eveneens bij aan versterkte antitumorwerking, maar of deze effecten het gevolg zijn van de gepotentiëerde hyperemie dan wel op zich staande effecten zijn, is niet duidelijk geworden. Een directe relatie tussen inductie van hyperemie en hemorragische necrose is onwaarschijnlijk.

Hemorragische necrose is mogelijk het gevolg van een neveneffect van toxische endotoxinen en lijkt niet wezenlijk bij te dragen aan het uiteindelijke antitumoreffect. Het feit dat alleen therapeutische behandelingen een vermindering van de lymfocytenaccumulatie na 24 uur aan de randen van de tumor veroorzaakten, doet vermoeden dat dit effect bij-draagt aan de uiteindelijke volledige regressie van de tumor. De waarnemingen dat van alle behandelingen alleen toxisch endotoxine duidelijke effecten op de milt sorteerde, suggereert dat deze gerelateerd zijn aan de toxische neveneffecten van dit molecuul en dat effecten op de milt niet wezenlijk bijdragen aan het antitumoreffect, met name de reductie van de lymfocyten-accumulatie.

Op grond van de in deze studie verkregen resultaten en de gegevens uit de literatuur kunnen de volgende hoofdconclusies getrokken worden.

- Toxische endotoxinen induceren binnen een dag na i.v. toediening twee duidelijk onderscheidbare vormen van necrose in solide Meth A tumoren: hemorragische necrose en coagulatienecrose. Gedetoxificeerd endotoxine mist het vermogen om enige vorm van tumornecrose te induceren.
- Hemorragische necrose treedt slechts in een klein, oppervlakkig gelegen, deel van de tumor op, namelijk daar waar op het tijdstip van behandeling reeds uitgebreide spontane schade van de bloedvaten waarneembaar is. Een versterking van de vaatschade door endotoxine gaat vooraf aan en is een oorzaak van hemorragische necrose.
- De inductie van hemorragische necrose lijkt toegschreven te kunnen worden aan de toxische eigenschappen van endotoxine, mogelijk zijn vermogen om vasoactieve stoffen en/of TNF vrij te maken.
- De kort na toediening van toxisch endotoxine optredende verwijding van de bloedvaten in dat deel van de tumor waar later hemorragische necrose optreedt leidt niet per definitie tot hemorragische necrose, daar een dergelijke vaatverwijding ook werd waargenomen na toediening van stoffen die coagulatienecrose in dat deel veroorzaken.
- Coagulatienecrose is de meest uitgebreide vorm van tumornecrose die door toxisch endotoxine geinduceerd wordt. Deze vorm van necrose beslaat onder optimale condities vrijwel de gehele tumor met uitzondering van de uiterste randen en het deel met hemorrhagische necrose. Coagulatienecrose wordt voorafgegaan door oedeem en vetstapeling in de tumorcellen.

- Sterke congestie, het ontbreken van spierweefsel rond de bloedvaten en afwijkend endotheel zijn aspecten waarin de bloedvaten in de tumor afwijken van die in normale weefsels. Dergelijke afwijkingen verklaren waarschijnlijk in hoge mate waarom uitsluitend tumorweefsel na toediening van een geringe dosis endotoxine met necrose reageert. Verstoring van de vaatfunctie door endotoxine en/of door endotoxine geinduceerde mediatoren (interleukine-1, TNF) in een reeds slecht functionerende vaatbed zal gemakkelijk leiden tot zuurstofgebrek en ophoping van afvalstoffen, hetgeen (mede)bepalend is voor de necrose.
- Het onvermogen van toxisch endotoxine om necrose te induceren in jonge tumoren en de randen van oudere tumoren is mogelijk grotendeels het gevolg van de relatief gezonde toestand van de bloedvaten in deze weefsels.
- Geen van beide vormen van necrose kan worden toegeschreven aan een duidelijke verandering van het aantal gastheercellen in de tumor of in verband gebracht worden met morfologische veranderingen in milt en regionale lymfklieren.
- Op grond van het geringe infiltraat in de tumor en de localisatie ervan moet de bijdrage van tumoricide gastheercellen aan de necrose als uiterst gering beschouwd worden.
- Gastheercellen in de tumorbloedvaten dragen zeer waarschijnlijk bij aan de tumornecrose middels een verergering van de vaatschade. Deze rol lijkt met name toebedacht te kunnen worden aan polymorfkernige granulocyten, omdat 1. deze cellen slechts in grote hoeveelheden voorkwamen in de bloedvaten van die tumoren die het meest gevoelig waren voor inductie van tumornecrose; 2. het verschil in vermogen van twee verschillende toxische endotoxinen om necrose (en volledige regressie) te veroorzaken weerspiegeld werd in hun vermogen om al dan niet het aantal polymorfkernige granulocyten in de tumor te doen toenemen; 3. deze cellen volgens de literatuur in reactie op endotoxinen en zijn mediatoren tot sterke vaatbeschadiging in staat zijn.
- Mestcelmediatoren zijn mogelijk betrokken bij de vaatverwijding.
- Doding van tumorcellen door tumoricide factoren, die lokaal en systemisch na toediening van toxisch endotoxine vrijkomen, als belangrijke oorzaak van de tumornecrose is onwaarschijnlijk daar jonge tumoren, de randen van oudere tumoren en uitgezaaide tumorcellen in de regionale lymfklieren onaangetast bleven.

- Het vermogen van MDP om de tumoricide werking van endotoxinen te potentiëren kan niet toegeschreven worden aan directe morfologische effecten van deze stof op tumor en milt, maar wel aan zijn vermogen om vele morfologische effecten van toxisch endotoxine in de tumor te versterken en deze effecten uitsluitend in combinatie met gedetoxificeerd endotoxine op te roepen
- Therapeutische activiteit van toxisch endotoxine tegen Meth A tumoren is direct gecorreleerd met de uitgebreidheid van de vroeg geïnduceerde necrose. Deze wordt in hoge mate bepaald door de leeftijd (grootte) van de tumor. Gegevens in de literatuur hebben aangetoond dat therapeutische activiteit van endotoxine tegen Meth A gecorreleerd is met leeftijdsafhankelijke specifieke immuniteit tegen de tumor. Daar de meest uitgebreide necrose werd gevonden in Meth A tumoren van die grootte, die ook de sterkste immunologische afweer oproepen, is het zeer waarschijnlijk dat beide processen tezamen de genezing bewerkstelligen
- Alleen tumornecrotiserende (combinaties van) stoffen die een ophoping van lymfocyten rond de tumor verhinderen, blijken in staat om voledige regressie te veroorzaken.

In het kort samengevat kan gezegd worden dat de therapeutische werking van toxische endotoxinen tegen solide tumoren als een zeer complex gebeuren beschouwd moet worden. De conditie van de tumor en zijn bloedvaten, de ten tijde van de behandeling bestaande afweerprocessen tegen de tumor en de uiteenlopende humorale en cellulaire reacties van de gastheer op endotoxine, die vooral gericht lijken te zijn tegen de tumorbloedvaten, bepalen tezamen de therapeutische uitkomst.

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CURRICULUM VITAE

Frieke Kuper werd op 26 juli 1950 geboren te Elsenerbroek. Zij behaalde in 1968 het diploma gymnasium β aan het Murmellius Gymnasium te Alkmaar. In hetzelfde jaar begon zij met de studie biologie aan de Rijksuniversiteit te Utrecht. In 1971 werd het kandidaatsexamen B1 behaald en in 1976 het doctoraal examen met als hoofdvakken fytopathologie en biologische toxicologie en de nevenrichting didaktiek van de biologie. Sinds 1977 is zij werkzaam als wetenschappelijk medewerkster op de afdeling Biologische Toxicologie van het Instituut CIVO-Toxicologie en Voeding TNO te Zeist.

S. 15.109

STELLINGEN

1. Het is gevaarlijk om de functie van een ontstekingscel alleen af te leiden uit zijn expressie van membraanmarkers.
   North en Bursuker, J. Exp. Med., 159, 1984, 1295

2. Bij de diagnostiek van solide tumoren dient de morfologie van de bloedvaten in de tumor betrokken te worden.
   Rofstad en Brustad, Cancer Res., 46, 1986, 355

3. Daar tumoren vooral op hogere leeftijd manifest worden en zodoende geen bedreiging vormen voor het in stand houden van het genetisch materiaal is het, vanuit evolutionair standpunt bekeken, niet te verwachten dat het lichaam hiertegen een specifieke afweer heeft ontwikkeld.

4. Het voorkomen van "clear vesicles" in intraepitheliale axonen van het trilhaarepitheel van de neus en de aanwezigheid van acetylcholine esterase in de trilhaardragende cellen vormen een sterke aanwijzing voor een regulatie van de trilhaarslag door het centraal zenuwstelsel.
   Graf en Stockinger, Z. Zellforsch., 72, 1966, 184
   Monteire-Rivière en Popp, Am. J. Anat., 43, 1984, 43

5. De borstelcel in het trilhaarepitheel van de neus dient, naar analogie van de "M cell" in het darmepitheel, beschouwd te worden als antigeen-presenterende cel.
   Tenner-Rácz et. al., Lab. Invest., 41, 1979, 106

6. De thymus speelt een rol bij de leeftijdsafhankelijke afname van de vruchtbaarheid van vrouwelijke ratten.
   Pierpaoli en Besedowsky, Clin. Exp. Immunol., 20, 1975, 323
   Kuper et. al., Vet. Pathol., 23, 1986, 270

7. In de toxicologie zou meer gebruik gemaakt moeten worden van patroon-herkenning om verborgen verbanden tussen gegevens op te sporen.

8. Bij het interpreteren van de resultaten van toxicologisch onderzoek zijn gegevens over de achtergrondspathologie van de gebruikte species van groot belang voor het beoordelen van de kans op "vals-negatieve" en "vals-positieve" uitkomsten.

9. Het stellen van maximaal aanvaarde concentratie (MAC) waarden voor werkzame verbindingen in bestrijdingsmiddelen levert nauwelijks een serieuze bijdrage aan arbeidsbeschermend beleid voor de agrarische sector.

Utrecht, 25 november 1986 C.F. Kuper