Virchows Arch [Pathol Anat] (1984) 403:377–389

Virchows Archiv A

Haemorrhagic tumour necrosis following endotoxin administration

I. Communication: morphological investigation on endotoxin-induced necrosis of the Methylcholanthrene (Meth A) tumour in the mouse

N. Freudenberg [1], K. Joh [2], O. Westphal [3], CH. Mittermayer [4], M.A. Freudenberg [3], and CH. Galanos [3]

[1] Pathologisches Institut der Universität Freiburg/Br., Albertstraße 19, D-7800 Freiburg
[2] Pathologisches Institut der JIKEI Medizinischen Hochschule Tokyo, Japan, Stipendiat des Deutschen Akademischen Austauschdienstes
[3] Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg
[4] Abteilung Pathologie der RWTH Aachen, Goethestraße 27–29, D-5100 Aachen

Summary. Endotoxin induced necrosis of the Meth A mouse tumour has been investigated using macroscopic, histological and ultrastructural examination methods.

On the 8th day after tumour cell transplantation, the animals received a relatively non-toxic dose of the Salmonella abortus equi endotoxin intravenously. The natural history of the tumour necrosis took the following course:

1. The earliest morphological changes could be seen with the electron microscope 90 min after administration of the endotoxin, and were seen as an interstitial oedema with separation of the tumour cells.

2. Haemmorrhagic necrosis of the tumour was complete 4 hours after injection, and could be easily recognized with the naked eye.

3. Rejection of the necrotic malignant tumour was complete two weeks after LPS administration. Only minor residual scarring of the belly-wall remained.

Haemorrhagic tumour necrosis due to endotoxin can be compared with the localized Shwartzman reaction and probably involves tumour necrotizing factor (TNF). For complete destruction of a tumour by haemorrhagic necrosis the size of the tumour is critical. Certain regression after endotoxin administration depends upon additional T-cell-mediated immunity (provided the tumour is immunogenic).

In contrast to the haemorrhagic necrosis, BCG-induced tumour regression is accompanied by granulomatous inflammation, which may be responsible for destruction of the tumour.

Key words: Meth A mouse tumour – Endotoxin – Haemorrhagic tumour necrosis

Offprint requests to: N. Freudenberg at the above address

It has long been known that bacterial infection can inhibit the induction of a tumour or that it may lead to the regression of a tumour already in existence. The regression of a tumour apparently due to a bacterial infection in a human being was reported by Busch 1866 and by Coley in 1896. These original observations led to the investigation of the role of bacterial toxins in the inhibition of tumour development. This inhibition is particularly associated with bacillus Calmette-Guerin (BCG; Old et al. 1959; Weiss et al. 1966; Lemperle 1966). The histologically demonstrable BCG-mediated granulomatous reaction has been suggested as a possible cause for the tumour regression (Hanna et al. 1972).

Various endotoxins have been proved to cause necrosis of a large number of experimental tumours including sarcomas (Old et al. 1961; Tripoldi et al. 1970), carcinomas (Mizuno et al. 1968; Grohsman and Nowotny 1972; Berendt et al. 1978) hepatomas (Ribi et al. 1975/76) and the cells of multiple myeloma (Bober et al. 1976). Green et al. (1976) reported the regression of a cell line from human melanoma brought about by lipopolysaccharide (LPS).

Although much information is available on LPS induced tumour necrosis, there is practically no morphological description of the phenomenon in the literature. In the limited number of morphological studies carried out on haemorrhagic tumour necrosis induced by bacterial toxins a role for LPS may be assumed, although in such studies ill-defined bacterial extracts, such as "bacterial filtrates" (Andervont 1936; Diller 1947) or "mixed bacterial toxins" (Donnelly et al. 1958) were employed, and the necrosis observed may not be a pure LPS-induced effect.

In the present study we investigated the alteration in the Meth A mouse tumour brought about by standardized endotoxin preparations, using macroscopic, histological and ultrastrucutral examination methods.

Materials and methods

Experimental animals

10-week-old female BALB/c-mice were bred under specific pathogen free conditions until required (animal house of the Max-Planck-Institut für Immunbiologie, Freiburg).

Tumour transplantation

5×10^5 cells from the Methylcholanthrene-induced tumour were cultivated in the ascites fluid of CBF_1 mice, and then injected intradermally into the abdominal wall of BALB/c animals (OLD et al. 1961).

Lipopolysaccharide (LPS)

The Salmonella abortus-equi S (smooth) form LPS was isolated from bacteria by means of the phenol-water-method (Westphal et al. 1952) and purified by means of the phenol-chloroform-petroleum-ether method (Galanos et al. 1969). Single doses (20 μg per mouse) of LPS dissolved in sterile and pyrogen-free PBS were injected intravenously at different times after transplantation (5^{th}, 8^{th} and 10^{th} day).

Table 1. Times of investigation of the Meth A mouse tumour and methods of morphological investigation employed

Times of investigation after LPS administration (30 min – 2 weeks) and after tumour cell transplantation (6 weeks)													
Methods of investigation	30 min	60 min	90 min	2 hours	4 hours	7 hours	1 day	2 days	4 days	6 days	9 days	2 weeks	6 weeks
Macroscopy							+	+	+	+	+	+	+
Histology	+	+	+	+	+	+	+	+	+	+	+	+	
Electron microscopy	+	+	+	+	+								

Morphological investigations

The morphological observations extended over a period of 6 weeks following transplantation. In Table 1 the times at which each of the methods (direct observation, histology and electron microscopy) were used are seen. Necrosis of the tumour was observed for 2 weeks after the LPS-administration.

1. Direct observation. The macroscopic exposures were made under artificial light with an 1.6-fold enlargement.

2. Histology. The tissue was fixed in formalin and embedded in paraffin in the standard way: 5 µm sections were cut and stained with one or more of the following stains: H. & E., PAS, Berlin-blue, and TIBOR PAP.

3. Electron microscopy. A piece of tissue approximately 1 mm in diameter was removed from the tumour and immediately fixed at 4° C for 4 h in 2.5% glutaraldehyde, buffered at pH 7.2 with cacodylate. Thereafter the tissue was stained for 2 h in 1% buffered osmium tetroxide at 4° C, dehydrated in ethanol, propylene oxide and embedded in EPON 812. The ultrathin sections were counterstained with uranyl acetate and lead citrate and examined under ZEISS EM 9 S-2 electron microscope at a primary magnification of × 1,300.

Results

Natural history of the transplanted Meth A mouse tumour

1. Growth

The intradermal injection of the tumour cells produces the development of a solid tumour in the subcutaneous tissue, which can already be seen 3 days after the transplantation.

By the 8th day this tumour is about 8 mm in diameter (see Fig. 4a) and replaces the entire thickness of the subcutaneous tissue and the underlying abdominal musculature as far as the parietal layer of the peritoneum (see Fig. 4b).

From the 8th day onwards one finds an extensive infiltration of the tumour into the overlying epidermis, with small areas of necrosis and haemorrhage in the neighbourhood. At about the same time spontaneous necroses

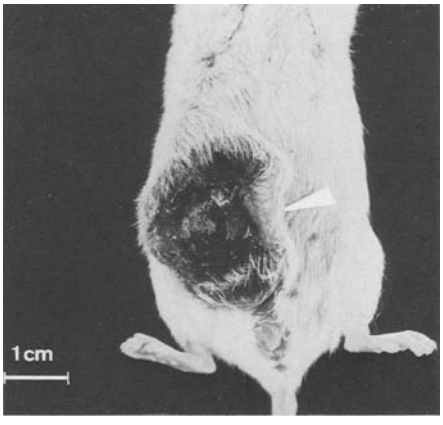

Fig. 1. Meth A mouse tumour (◀), 4 weeks after intradermal transplantation of tumour cells. Macroscopic photograph

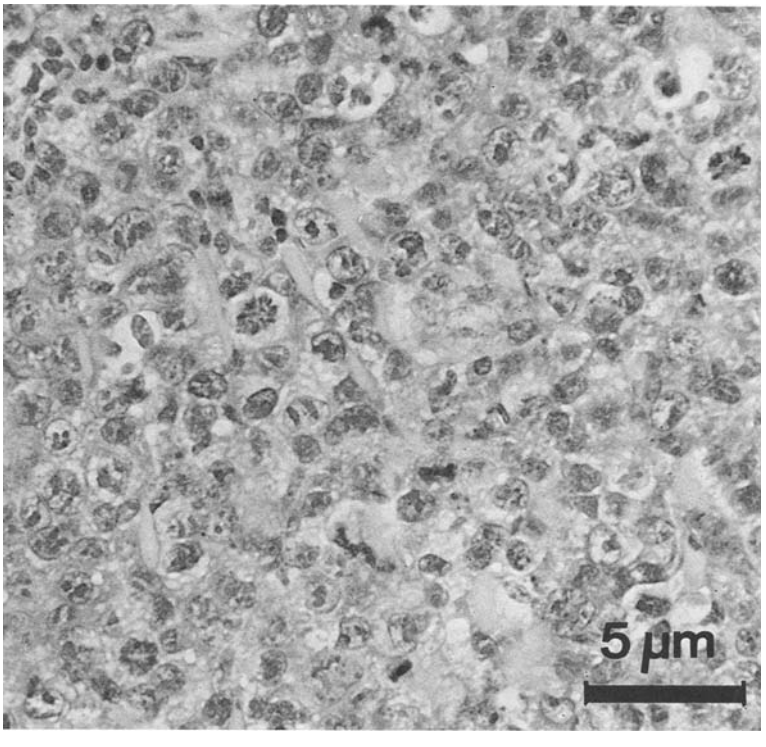

Fig. 2. Histology of the Meth A mouse tumour, 8 days after transplantation. H. & E. staining

can be seen in the deep part of the tumour where it lies against the peritoneum.

Up to the 4th or 5th week after transplantation the tumour growths extensively and brings about massive ulceration of the skin (Fig. 1), often invading the abdominal cavity to produce ascites. By the 6th week after transplantation at the latest the mouse dies.

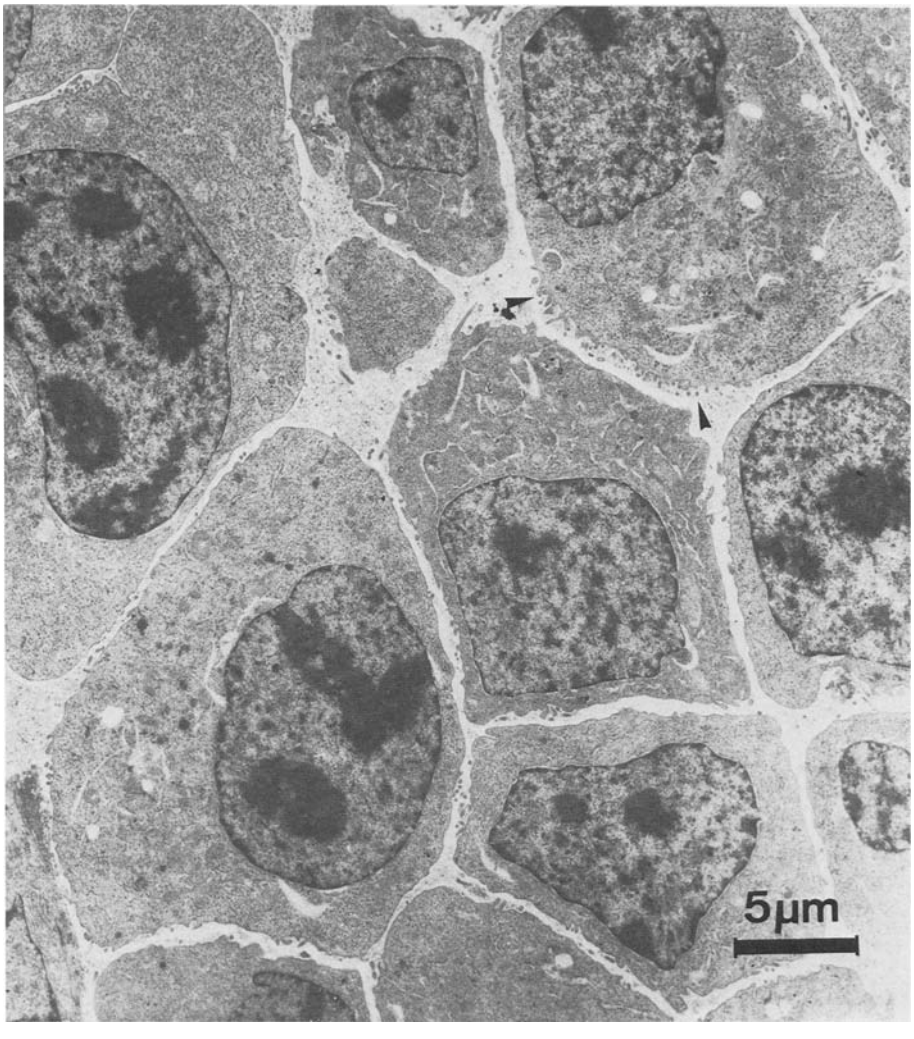

Fig. 3. Ultrastructural appearance of the Meth A mouse tumour, 8 days following transplantation. Note the microvilli (*arrows*) of the tumour cells

2. Morphology

a. Histology. Histologically one sees a highly cellular tumour infiltrating the local structures diffusely (Fig. 2). The tumour shows an increased cell population with nuclei between 17–27 µm in diameter surrounded by a moderate amount of cytoplasm. These nuclei are obviously polymorphic and present a slight hyperchromasia, which is coarsely granular. The nucleoli are particularly striking and the majority greatly enlarged and polymorphic. The cytoplasm of the cells shows moderate acidophilic staining with H. & E. and isolated vacuoles are seen. With silver staining a moderately thick network of fibres can be seen, with fine irregular reticular fibres winding around

Table 2. Dependence of the degree of tumour necrosis upon (i) time elapsed since transplantation of tumour cells, (ii) time elapsed since endotoxin injection

Time after LPS injection (hours)	Time after tumour cell transplantation (days)		
	5	8	10
4	+	+++	++
7	++	+++	+++
24	+++	+++	+++

+ = slight tumour necrosis; ++ = moderate tumour necrosis; +++ = marked tumour necrosis

individual cells. The tumour is PAS negative and shows no deposition of haemosiderin.

b. Ultrastructure. The ultrastructural appearance of the Meth A tumour is shown in Fig. 3. This tumour is composed of closely packed cells between which a narrow interstitial space can be seen. Intercellular connections such as desmosomes cannot be demonstrated. The appearance of the nuclei under the light microscope is confirmed by the electron microscope (moderate polymorphy, moderate increase in heterochromatin and also strikingly malignant nucleoli). The cytoplasm is of average volume and shows a remarkably electron dense matrix, and organelles which cannot easily be differentiated. Quite a number of cisterns of the RER are seen. In addition to this, single indistinct cytosomes can be recognized. The plasmalemma shows distinct focal particularities in the form of microvillus like bulges.

Against a finely granulated, moderately electron dense background single bundles of collagen fibres are seen lying between the cells.

Time of appearance of the most effective tumour necrosis following endotoxin administration

Table 2 shows that, out of all times after LPS-injection which were investigated (namely 5th, 8th and 10th day after transplantation) the 8th day presents the most impressive result, with the tumour necrosis having brought about complete healing of the lesion. On this day maximal necrosis has already appeared 4 h after LPS administration. The exact time of the appearance of the most effective necrosis will be investigated further.

Morphology of the necrosis after endotoxin administration

1. Naked eye and low magnification

The series of pictures (Figs. 4–6) shows the appearances of the tumour at different times after intravenous injection of endotoxin in the entire animal (figures on left) and under low magnification (figures on right).

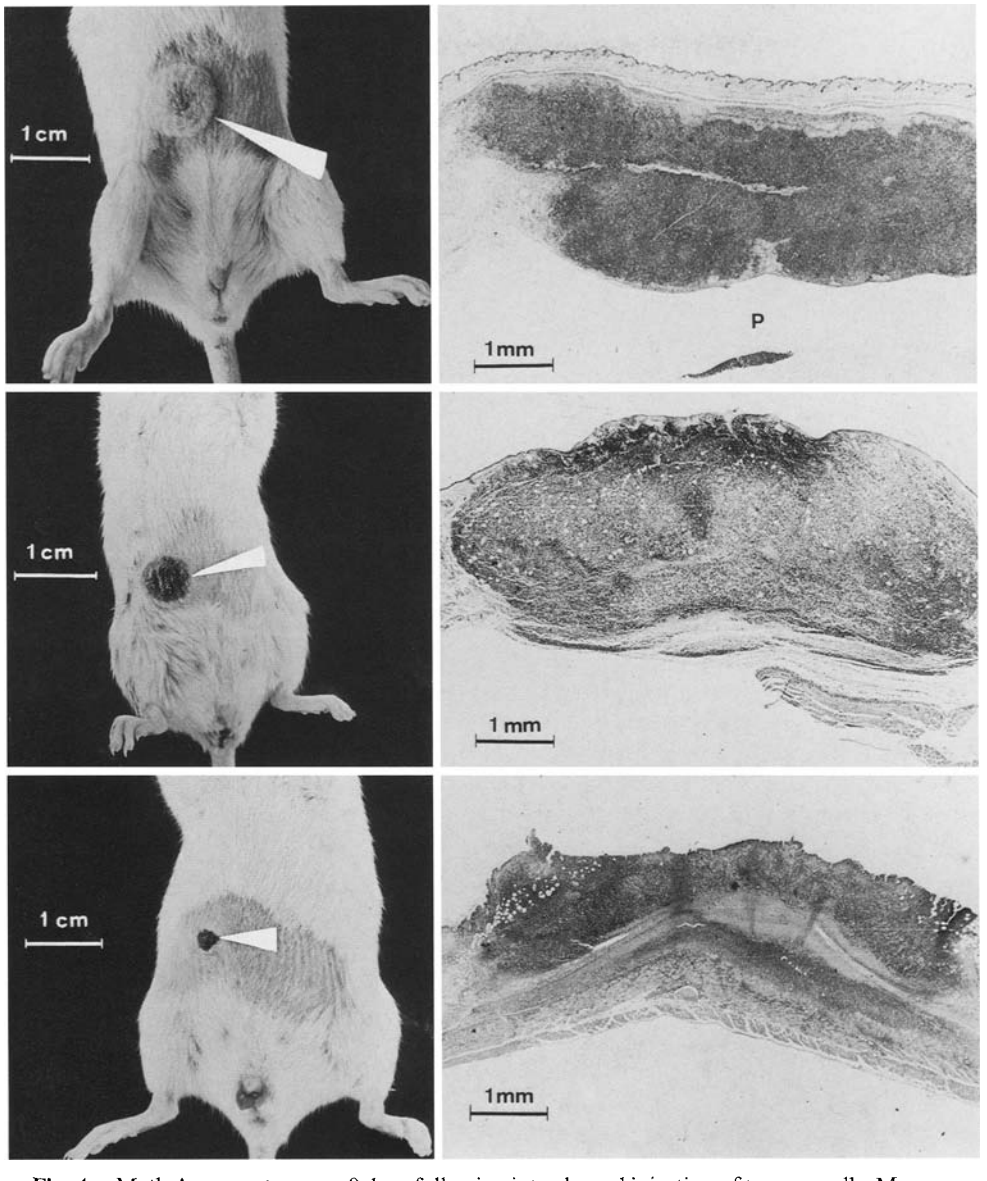

Fig. 4. a Meth A mouse tumour, 8 days following intradermal injection of tumour cells. Macroscopic photograph. **b** Meth A mouse tumour, 8 days following intradermal injection of tumour cells. *P* = peritoneal cavity. Magnifying glass magnification

Fig. 5. a 8 days old Meth A mouse tumour, 4 h after intravenous injection of 20 μg *Salmonella* abortus equi endotoxin, showing a marked haemorrhagic necrosis (*arrow*) in the centre of the tumour. Macroscopic photograph. **b** The same experimental situation as in **a** under the magnifying lens. Note the haemorrhagic necrosis in the apical third of the tumour. H. & E. staining

Fig. 6. a Advanced stage of tumour rejection on 4th day after endotoxin treatment. Note the small defect of the abdominal wall tissue (*arrow*). Macroscopic photograph. **b** Meth A mouse tumour on 4th day after endotoxin injection under the magnifying glass. Note the ulcer, which contains necrotic tumour tissue. H. & E. staining

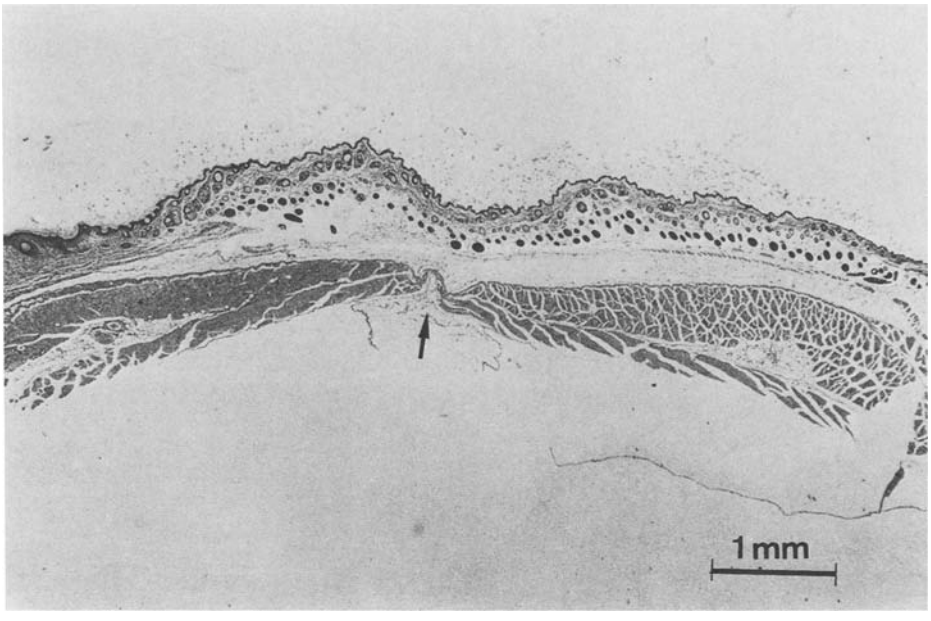

Fig. 7. Transversal section through the abdominal wall in the region of the former Meth A tumour, 14 days following endotoxin treatment. Note the dimple in the skin surface and the deficiency of abdominal musculature (*arrow*). Magnifying glass magnification. H. & E. staining

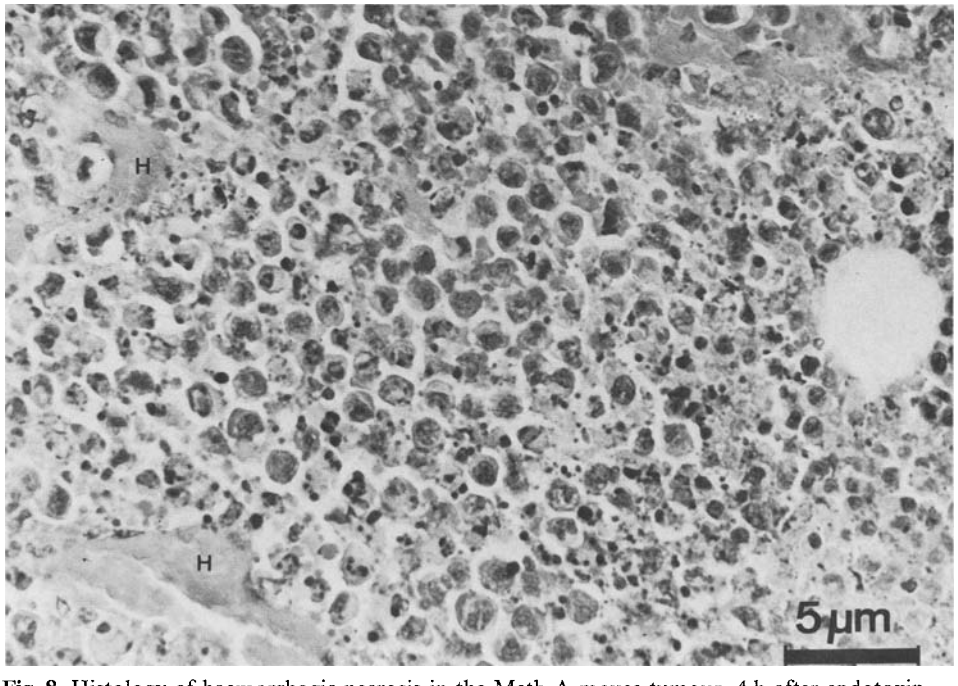

Fig. 8. Histology of haemorrhagic necrosis in the Meth A mouse tumour, 4 h after endotoxin application. (H = hyperaemia) H. & E. staining

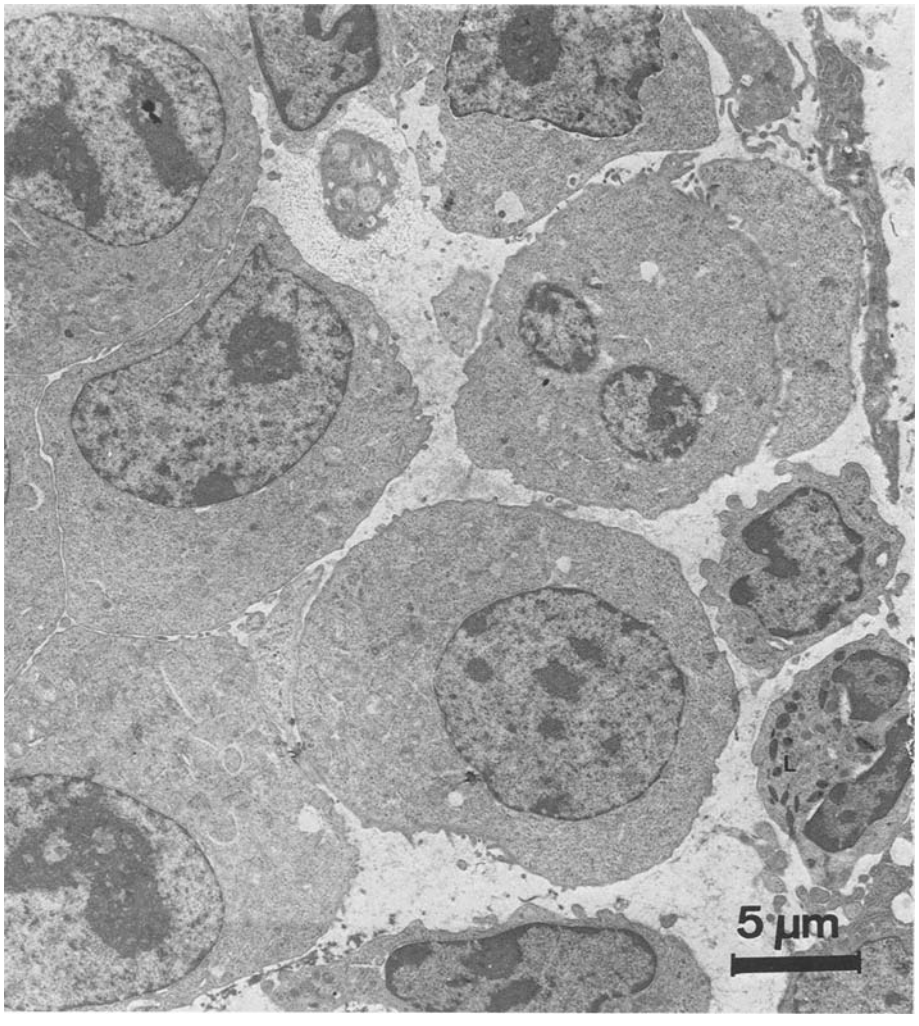

Fig. 9. Ultrastructural appearance of Meth A mouse tumour, 90 min after intravenous injection of 20 µg Salmonella abortus equi endotoxin. Note the interstitial oedema, separated tumour cells and single leukocyte infiltrations (*L*)

In Fig. 4a an untreated Meth A mouse tumour on the belly-wall is displayed. It is approximately 8 mm in diameter and is significantly raised above the level of the surrounding skin. Under the magnifying glass (Fig. 4b) one can see the highly cellular tissue of the tumour that extends through the subcutaneous layers and the underlying abdominal muscles to reach the peritoneum. The appearance of the neoplastic tissue is homogeneous and dark. In this case spontaneous tumour necrosis did not occur.

Figure 5a shows the tumour 4 h after endotoxin injection. Even with the naked eye, an extensive central haemorrhage, which leaves only a narrow intact rim of neoplastic tissue at the periphery can be recognized. The low

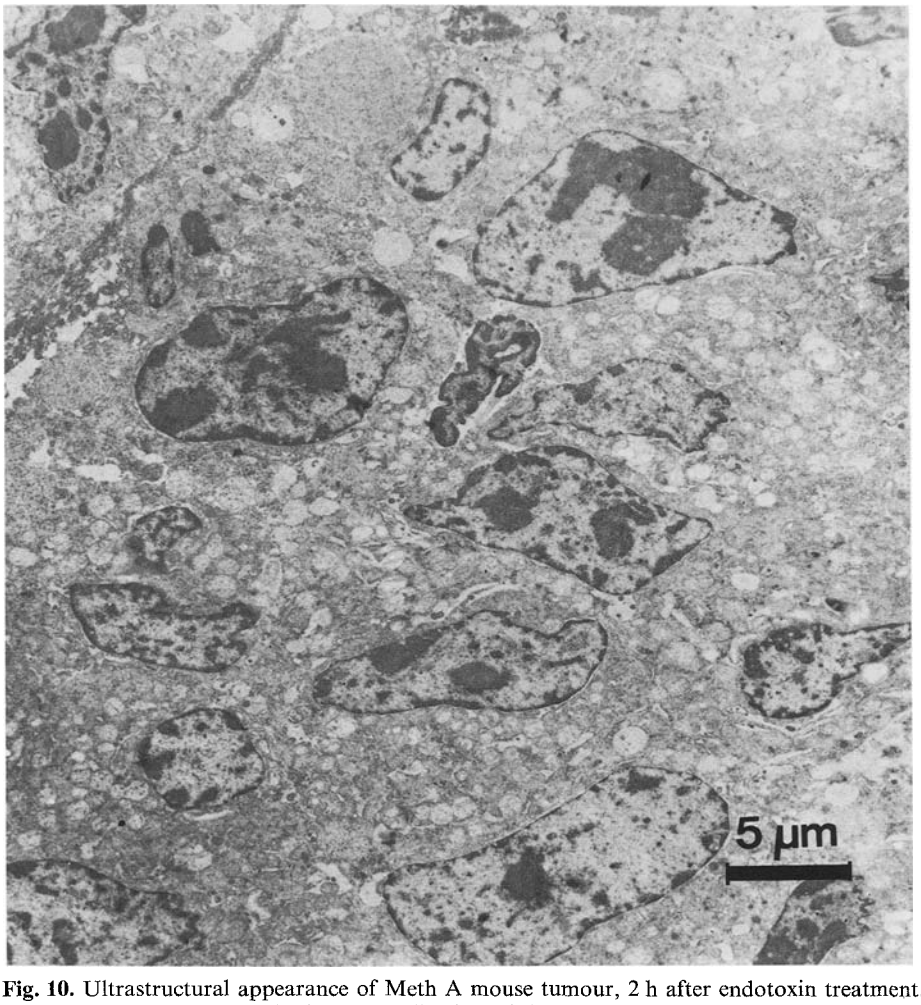

Fig. 10. Ultrastructural appearance of Meth A mouse tumour, 2 h after endotoxin treatment. The majority of tumour cells show vacuolization of the cytoplasmic organelles and a marked reduction in the cellular matrix

magnification (Fig. 5b) makes the apical necrosis and haemorrhage in the superficial third of the tumour quite obvious. The changes here include tissue fragmentation and a dark coloration due to local haemorrhage.

Figure 6a and b were taken from day 4 after the beginning of endotoxin treatment. Necrosis is far advanced and the greater part of the tumour has been destroyed. In the macroscopic picture (Fig. 6a) one can only see a gross defect in the skin about 2 mm in diameter, the corresponding Fig. 6b shows an ulcer consisting almost entirely of necrotic neoplastic tissue – and this necrosis extends as far as the abdominal musculature.

Figure 7 shows the site of the original tumour under low magnification 14 days after the beginning of LPS treatment. The original cutaneous defect has been reduced to a mere dimple, beneath which a deficiency of the ab-

dominal musculature remains. Round about the destroyed and necrotic tumour newly developed skin with a delicate epidermis and a dermis poor in collagen fibres can be seen.

2. Histology

Figure 8 shows the situation 4 h after the injection of the endotoxin. The individual tumour cells are widely dissociated and show degenerative changes. Interstitial oedema with detritus and hyperaemia of the vessels running through the tumour are striking.

3. Electron microscopy

The first ultrastructural changes occur 90 min after LPS has been given. The tumour cells have been forced apart by interstitial oedema, and the separated cells show a tendency to become circular. Single leukocytes can be seen here and there in the tumour (Fig. 9).

Only 30 min later (120 min after LPS injection) the necrosis is much advanced. Among single cells with pyknotic nuclei other cells can be seen with marked degenerative changes in the cytoplasm, mostly consisting of the appearance of vacuoles (Fig. 10). The intercellular spaces are now packed with the remnants of dead cells.

Discussion

The hemorrhagic tumour necrosis which a relatively non toxic dose of endotoxin brings about, is an impressive example of one advantageous use of LPS.

The mechanism of this necrosis is largely speculative. We know now that a "tumour necrotizing factor" (TNF, Carswell et al. 1975) plays a part and that this factor has been demonstrated in the serum of mice infected with BCG, subsequently subjected to endotoxin administration (Carswell et al. 1975). TNF produces massive haemorrhagic necrotizing inflammation in a solid tumour; a response which Shwartzman and Michailovsky (1932) and Apitz (1933) interpreted as a localized Shwartzman reaction. If this be so, the presence of the tumour itself corresponds to the first injection of the classical experiment, and it is on the tumour that some unidentified substance presumably acts. Stetson (1961) was able to prove that solid tumours treated with endotoxin reacted with a marked but short-lived vascular disturbance. Our own observations on the severe degree of hyperaemia in the vessels of the tumour after LPS administration confirm this finding. In spite, however, of the dramatic destruction of the central tissue of the tumour by haemorrhagic necrosis, it is said that only in a relatively small number of cases does the remainder of the neoplastic tissue regress completely (Berendt et al. 1978). So far as the size of the tumour is a decisive factor for complete regression after haemorrhagic necrosis, we agree with Shear (1943); Donelly et al. (1958); Parr et al. (1973) and Berendt et al. (1978).

Only in the case of an 8-day-old tumour of 8 mm in diameter were we able to bring about complete destruction with LPS.

In contrast to the haemorrhagic necrosis, the tumour regression induced by endotoxin is dependent upon a T-cell mediated anti-tumour immunity that is only produced against immunogenic tumours. Berendt et al. (1978) were able to show that also in this case the size of the tumour is critical for anti-tumour immunity to be effective. Since the Meth A sarcoma investigated by us must be reckoned to be an immunogenic tumour (Berendt et al. 1978), it is likely that also in our experiments T-cell-mediated regression played its part in bringing about the destruction of the tumour.

Duration as well as morphological differences distinguish endotoxin induced necrosis from the tumour regression which follows treatment with BCG. Within 90 min of LPS administration degenerative tissue changes can be demonstrated in the tumour and after only 4 h maximal necrosis has occurred. However, after treatment with BCG a latent period of 24 h must elapse before an inflammatory reaction eventually destroys the neoplasm (Hanna et al. 1972).

Histologically the distinction between the responses of the tumour to endotoxin and BCG lies in this: after LPS administration haemorrhagic necrosis accompanied by moderate infiltration with leukocytes appears, whereas with BCG a granulomatous reaction with heavy infiltration of mononuclear cells is seen. These differences, in both kinetics and histology, suggest strongly that different mechanisms are involved in the two reactions.

It therefore seems that the haemorrhagic necrosis is a type of local Shwartzman reaction in which the TNF plays a part. Under certain circumstances (an immunogenic tumour of just the right size) a complete regression due to T-cell mediated immunity can follow the original haemorrhagic necrosis. In the case of tumour necrosis brought about by BCG it is very probably a matter of granulomatous inflammation with the production of a massive cellular reaction which destroys the growth.

Acknowledgments. We express our thank to our colleague Dr. F. Steel for discussions on the preparation of the English manuscript. We are grateful to U. Pein, K. Bandara and U. Wiehle for expert technical assistance.

References

Andervont HB (1936) The reaction of mice and of various mouse tumours to the injection of bacterial products. Am J Cancer 27:77–83

Apitz K (1933) Über Blutungsreaktionen am Impfcarcinom der Maus. Zeitschr Krebsforsch 40:50–70

Berendt MJ, Mezrow GF, Saluk PH (1978) Requirement of a radiosensitive lymphoid cell in the generation of lipopolysaccharide-induced rejection of a murine tumour allograft. Infect Immun 21:1033–1035

Bober LA, Kranepool MJ, Hollander VP (1976) Inhibitory effect of endotoxin on the growth of plasma cell tumour. Cancer Res 36:927–929

Busch (1866) Verhandlungen ärztlicher Gesellschaften. Berl Klin Wochenschr 3:245–247

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumours. Proc Nat Acad Sci USA 72:3666–3670

Coley WB (1986) Further observations upon the treatment of malignant tumours with the toxin of erysipelas and bacillus prodogiosus, with a report of 160 cases. Bull Johns Hopkins Hosp 65:157–162

Diller IC (1947) Degenerative changes induced in tumour cells by Serratia marcescens polysaccharide. Cancer Res 7:605–626

Donnelly AJ, Havas HF, Groesbeck ME (1958) Mixed bacterial toxins in the treatment of tumours. II. Gross and microscopic changes produced in sarcoma 37 and in mouse tissues. Cancer Res 18:149–161

Galanos C, Lüderitz O, Westphal O (1969) A new method for the extraction of R lipopolysaccharides. Eur J Biochem 9:245–249

Galanos C, Lüderitz O, Westphal O (1979) Preparation and properties of a standardized lipopolysaccharide from Salmonella abortus equi (Novo Pyrexal): Zbl Bakt Hyg I. Abt Orig A 243:226–244

Green S, Dobrjansky A, Carswell EA, Kassel RL, Old LJ, Fiore N, Schwartz MK (1976) Partial purification of a serum factor that causes necrosis of tumours. Proc Natl Acad Sci USA 73:381–385

Grohsman J, Nowotny A (1972) The immune recognition of TA3 tumours, its facilitations by endotoxin, and abrogation by ascites fluid. J Immunol 109:1090–1095

Hanna MG, Zbar B, Rapp HJ (1972) Histopathologie of tumour regression after intralesional injection of mycobacterium bovis. I. Tumour growth and metastasis. J Nat Cancer Inst 48:1441–1455

Lemperle G (1966) Reticulo Endothelial System. J Reticul Soc 3:385

Mizuno D, Yoshioka O, Akamatu M, Kataoka T (1968) Antitumour effect of intracuteneous injection of bacterial lipopolysaccharide. Cancer Res 28:1531–1537

Old LJ, Clarke DA, Benacerraf B (1959) Effect of Bacillus Calmette-Guérin (BCG) infection on transplanted tumours in the mouse. Natur (London) 184:291–293

Old LJ, Benacerraf B, Clarke DA, Carswell A, Stockert E (1961) The role of the RES in the host reaction to neoplasia. Cancer Res 21:1281–1300

Parr I, Wheeler E, Alexander P (1973) Similarities of the antitumour actions of endotoxin, lipid A and double-stranded RNA. Br J Cancer 27:370

Ribi EE, Granger DL, Millner KC, Strain SM (1975) Brief communication: tumour regression caused by endotoxin and mycobacterial fractions. J Nat Cancer Inst 55:1253–1257

Ribi EE, Takayama K, Milner K, Gray GR, Goren M, Parker R, Mc Laughlin C, Kelly M (1976) Regression of tumors by an endotoxin combined with trehalose mycolates of differing structure. Cancer Immunol Immunether 1:265

Shear MJ (1943) Chemical treatment of tumours. IX. Reactions of mice with primary subcutaneous tumours to injection of a hemorrhagic-producing bacterial polysaccharide. J Nat Cancer Inst 4:461

Shwartzman G, Michailowsky N (1932) Phenomenon of local skin reactivity to bacterial filtrates in the treatment of mouse sarcoma 180. Proc Soc Exper Biol Med 29:737–741

Stetson C (1961) Vascular effects of endotoxins. Bull NY Acad Sci 101:80

Tripoldi D, Hollenbeck L, Pollack W (1970) The effect of endotoxin on the implantation of a mouse sarcoma. Int Arch Allerby 37:575–585

Weiss DW, Bonhag RS, Leslie P (1966) Studies on the heterologous immunogenicity of a methanol-insoluble fraction of attenuated tubercle bacilli (BCG) II. Protection against tumour isografts. J Exp Med 124:1039–1065

Westphal O, Lüderitz O, Bister F (1952) Über die Extraktion von Bakterien mit Phenol/Wasser. Z Naturforsch 76:148–155

Yang C, Nowotny A (1974) Effect of endotoxin on tumour resistance in mice. Infect Immun 9:95–100

Accepted March 13, 1984