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Tumour Regression after Treatment with Aminated β1-3D Polyglucose Is Initiated by Circulatory Failure

R. SELJELID

Institute of Medical Biology, University of Tromsø, Norway

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Meth A sarcoma grew progressively when inoculated intradermally in CB6 mice. When the mice were treated on day 7 after inoculation with 10 mg aminated polyglucose (AG) [Bogwald, J., Hoffman, J. & Seljelid, R. *Carbohydrate Res.* **148,** 101, 1986], the tumours regressed completely in over 90% of the cases. During the first hours after AG treatment, tumour thymidine incorporation decreased, adenosine triphosphate (ATP) content decreased, and there were indications of circulatory disturbance as shown by decreased deposition of dye (trypan blue) in the tumour tissue after intravenous injection. Histological examination demonstrated a conspicuous thickening of the walls of small tumour vessels, statis of red blood cells, and perivascular collections of mononuclear cells only hours after AG treatment. In thymectomized animals, where regression does not occur after AG treatment [Seljelid, R. *Bioscience Reports* **6,** 845, 1986], there was no evidence of circulatory failure, no tumour diameter reduction, and no decrease in colouring following intravenous injection of trypan blue. On the basis of these findings, we conclude that the early phase of events after AG treatment leading to tumour regression involves a vascular phenomenon that causes circulatory disturbance and necrosis. The data also indicate that this initial circulatory failure requires the involvement of functional T cells.

Rolf Seljelid, IMB, P.O. Box 977, 9001 Tromsø, Norway

It was recently reported that systemic treatment with aminated β1-3D polyglucose (AG) causes total regression of two different transplantable sarcomas in mice [15]. In T cell-depleted animals there was no corresponding regression. Apart from this observation, and the previous finding of macrophage stimulation in vitro by AG [2, 16], not much is known about the mechanisms underlying the regression. In fact, not much is known generally about tissue processes related to the regression of established solid tumours. The rather dramatic effect induced by treatment with AG, involving macroscopic and histological signs of tumour tissue injury only hours after a single dose of AG, suggests a vascular mechanism. This study was carried out to investigate the mechanisms underlying the necrotization of the tumour. The observations support the hypothesis that tumour necrosis is induced via vascular mechanisms.

MATERIALS AND METHODS

Specific pathogen-free CB6 F_1 (BALB/c × C57 BL/6) female mice were purchased from Bomholtgaard Ltd, Ry, Denmark, and from Charles River, Margate, Kent, UK.

Some mice were made T cell-deficient (T × B) at 3 weeks of age by thymectomy followed by 950 rad whole body irradiation. They were then injected with 10^7 syngeneic bone marrow cells intravenously and used in experiments 4–5 weeks later.

The Meth A fibrosarcoma, syngeneic in BALB/c mice, was grown in ascites form in the CB6 hybrid. For each experiment the cells were harvested, washed once in phosphate-buffered saline, and injected intradermally at 10^6 cells per animal midventrally in naive mice about 8 weeks of age. Tumour diameters (mean of transversal and longitudinal diameters) were measured every 2–3 days with calipers. Experimental groups consisted of five animals, and experiments were performed 2–5 times. Results were recorded as means of tumour diameters within the group, plus/minus standard deviation (SD).

Water-soluble AG was prepared as reported [2]. The

nitrogen content was determined at 1.3% by elemental analysis, the amino groups being attached mainly at C.6.

The endotoxin content of the AG stock solution (10 mg/ml) was assayed by thromboplastin activation [12] at 10 ng/ml, which was not significantly different from the endotoxin content of the control isotonic saline. The AG solution—in the case of the controls, the saline—was injected intraperitoneally.

To assay thymidine incorporation in tumour tissue, 0.5 μCi tritiated thymidine (New England Nuclear, Dreieich, FRG) was injected intraperitoneally. At specific times, tumour tissue was removed and homogenized, and DNA extracted by sequential solubilization in 0.1 N NaOH followed by precipitation in 0.5 N perchloric acid. The radioactivity incorporated in acid precipitable material was determined with a liquid scintillation spectrometer (Packard Instruments, Downers Grove, Ill., USA) and the DNA content of the same material assayed as described [4]. Protein was assayed by the amino schwarz reaction [14] and ATP by a luminogenic method [8].

Trypan blue (Fluka AG, Buchs SG, Switzerland) was dissolved in isotonic saline at 1% and injected in a tail vein. The animals were killed by neck dislocation exactly 15 min after the injection, and the tumour tissue was dissected free of skin and subcutaneous tissue and homogenized/extracted with 70% ethanol in a Potter-Elvehjem homogenizer for 3 min. After centrifugation for 30 min at 12,000 *g*, the extracts were read at 585 nm in a Hitachi (Tokyo, Japan) Model 100-40 spectrophotometer. In some experiments—not reported here—the trypan blue was replaced with the isomeric Evans blue, which gave fainter colouring of tissues but otherwise identical results.

Histological examination of the tissues was done after fixation in 5% buffered formaldehyde and paraffin embedding. Tissue for plastic embedding was fixed by immersion of thin slices in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7, with 0.1 M sucrose. The tissue

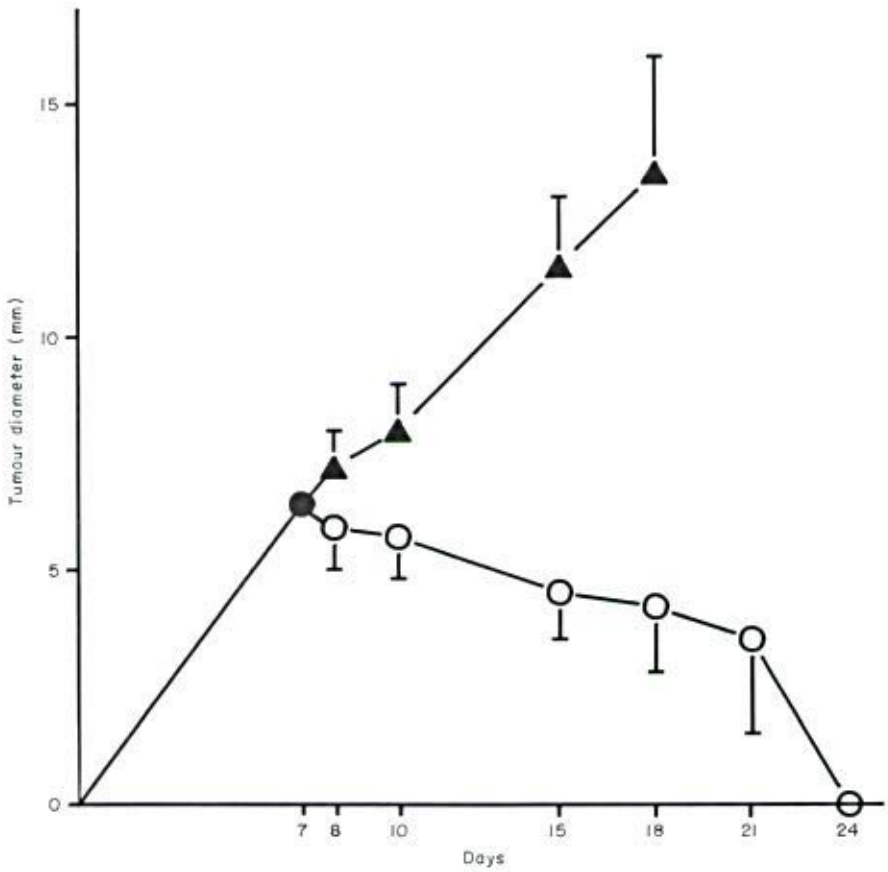


FIG. 1. A typical display of the growth of Meth A sarcoma in the skin of CB6 mice. Tumours in control animals treated on day 7 with saline intraperitoneally (▲); tumours in mice treated on day 7 with 10 mg AG (○). Results are given as means of tumour diameters ± SD.

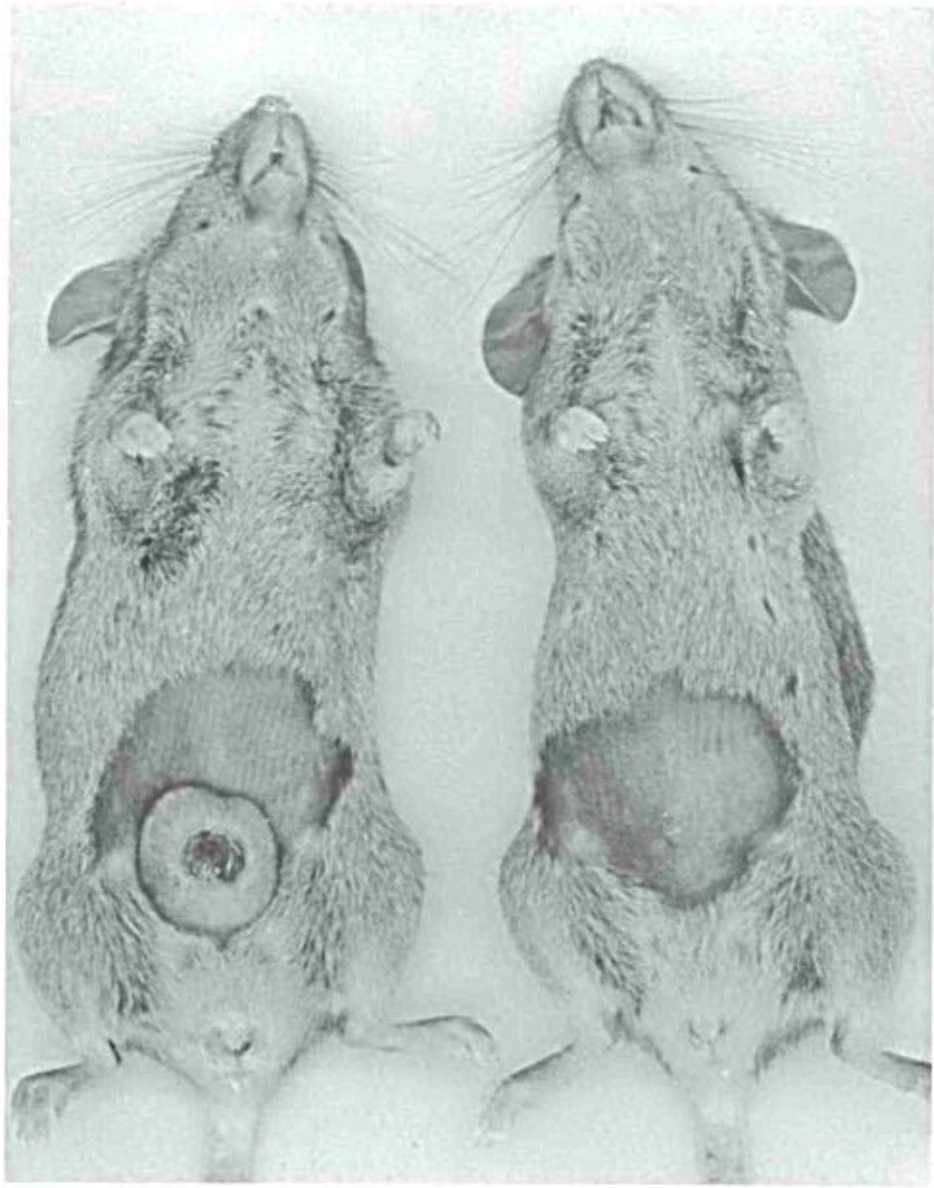


FIG. 2. To the left an untreated mouse on day 28 after the inoculation of 10^6 Meth A sarcoma cells intradermally. A tumour is clearly visible in the lower abdomen. To the right a mouse on day 28 after the inoculation of Meth A sarcoma and treated on day 7 with 5 mg of AG by intraperitoneal injection. No tumour can be seen.

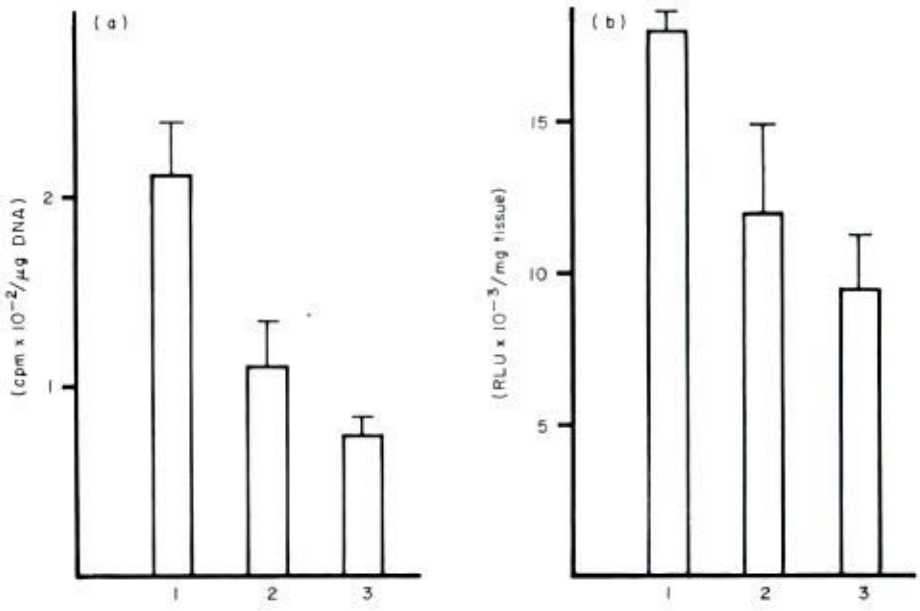


FIG. 3. (a) The incorporation of radioactive thymidine per μg DNA in tumour tissue 7 days after inoculation. Column 1: tumours from control animals, treated with saline 8 h previously; column 2: tumours from animals treated with AG 8 h previously; column 3: tumours from animals treated with AG 24 h previously. Data are given as mean ± SD. Column 1 versus 2 or 3: $P < 0.01$. (b) ATP content of tumour tissue 7 days after inoculation. Columns as for Fig. 3(a). Data are given as mean ± SD. Column 1 versus 2: $P < 0.05$, column 1 versus 3: $P < 0.01$.

pieces were then rinsed in buffer and postfixed in 2% OsO_4 in the same buffer. After dehydration in alcohol, the material was embedded in Epon-Araldite (Serva, Heidelberg, FRG). One-micrometre sections were prepared with a Reichert OmU3 ultramicrotome (C. Reichert, Vienna, Austria), and stained with toluidine blue.

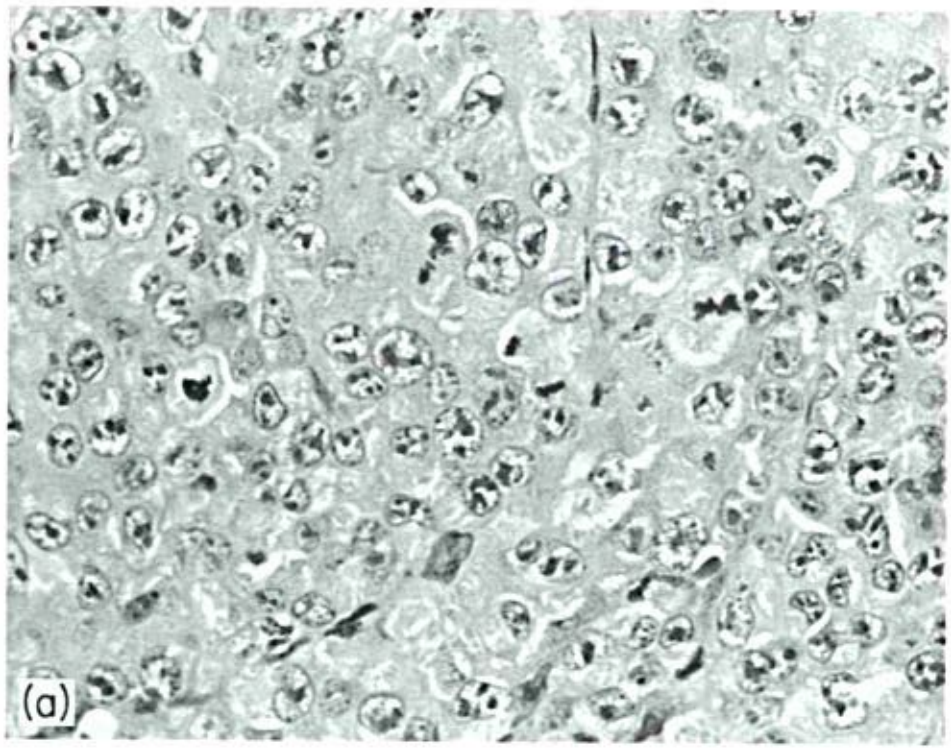
RESULTS

Tumour growth

In untreated control animals the sarcoma grew progressively from inoculation on day 0 until the animals were killed, usually around day 20.

When 10 mg AG was injected intraperitoneally on day 7, there was a characteristic darkening and loss of turgor of the skin overlying the tumour after about 4–6 h. The treatment was well tolerated, with no obvious signs of distress in the animals. In more than 9 out of 10 cases tumour diameters decreased significantly ($P < 0.01$) until day 8 (Fig. 1). Thereafter there was a slow decrease until days 16–21, after which most of the tumours regressed completely (Fig. 2), while some resumed growth. The percentage of regressing tumours depended on the mode of AG administration as well as on the properties of the particular batch of AG. Typically, after one dose of 5 mg AG on day 7 about half the tumours regressed completely; with increasing amounts (up to 10 mg) or number of daily doses (up to 3) of AG, the percentage of regressing tumours increased to over 90. There appeared to be a correlation between the extent of shrinkage from day 7 to 8 and the final outcome. Tumours that did not decrease markedly in size from day 7 to 8, invariably resumed growth some days later. Animals whose tumours had regressed after treatment, lived in apparent good health for weeks

FIG. 4. (a) Tumour tissue from untreated control animal on day 8 after inoculation. Viable pleomorphic tumour cells with numerous mitoses are seen (× 1000). (b) Tumour tissue from control animal on day 8 after inoculation. To the left is the subcutaneous tissue with scattered leucocytes, to the right the tumour tissue (× 200).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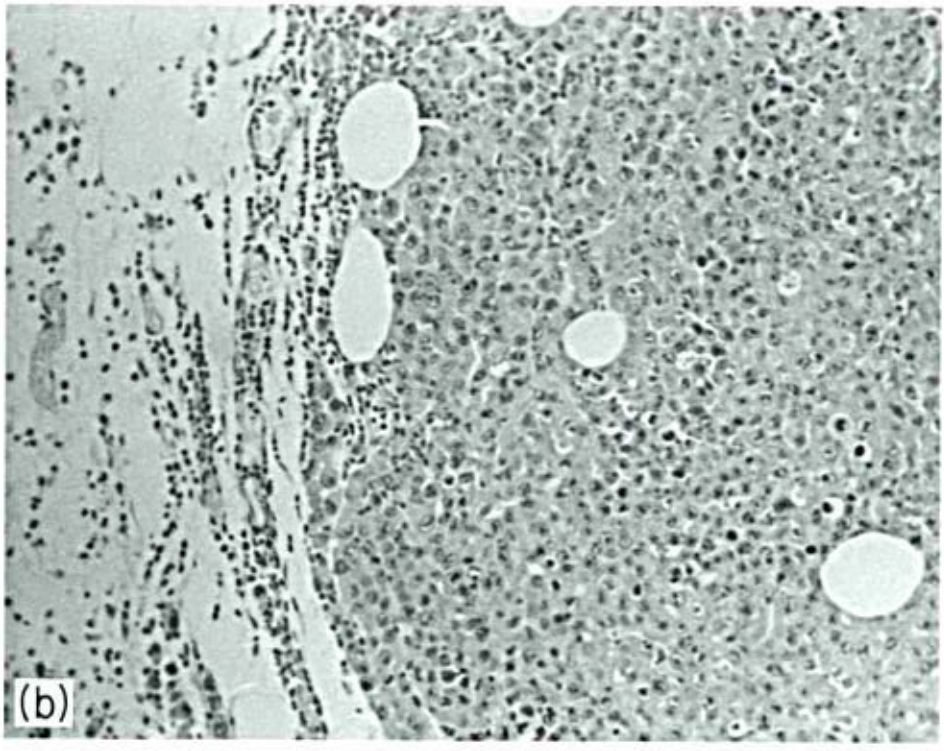
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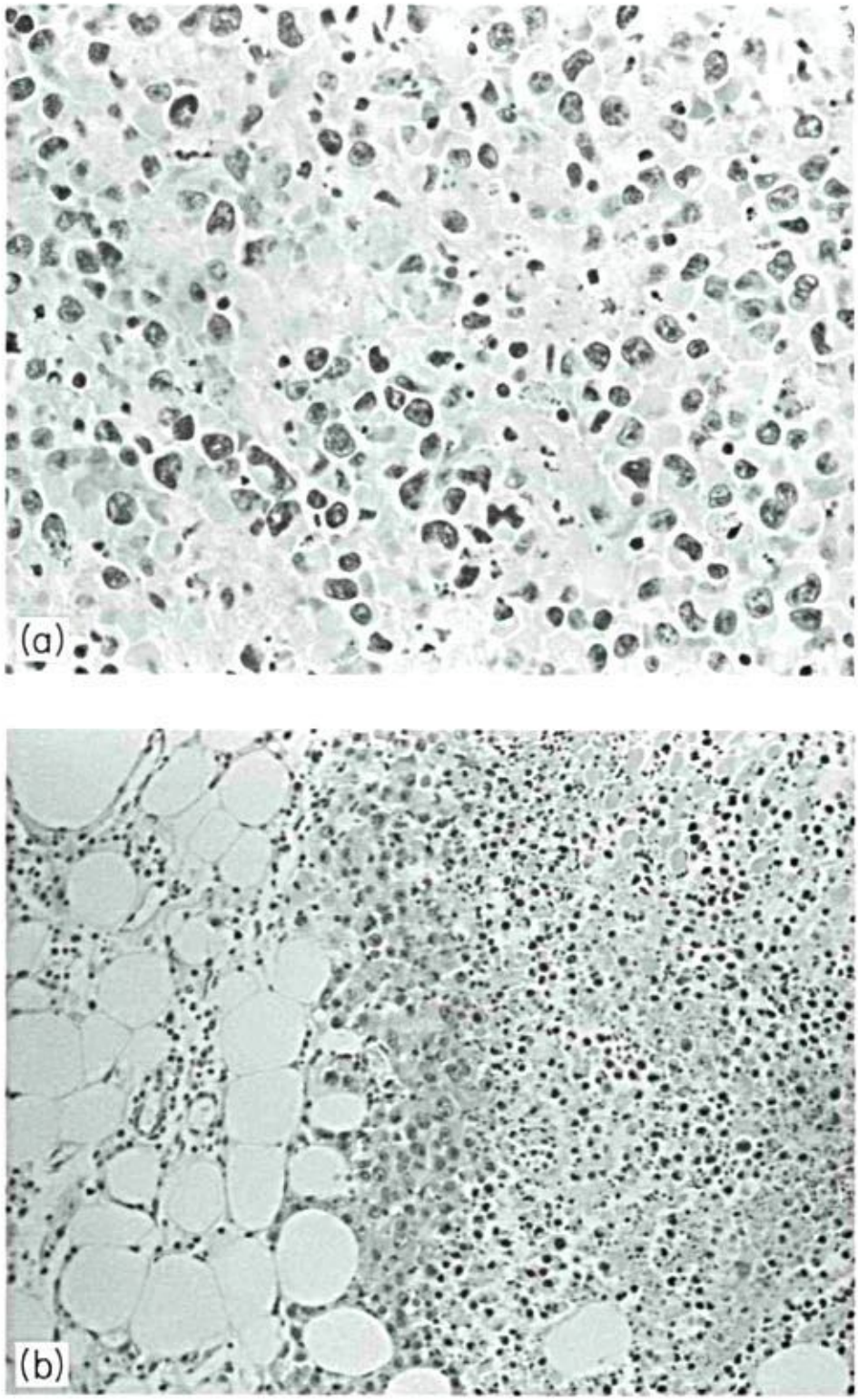
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after the experiments had been finished. Autopsy of these animals revelaed no pathological changes apart from scar tissue in the abdominal skin.

The decrease in size of the tumour from day 7 to 8 was accompanied by a decrease in tumour DNA synthesis, as demonstrated by reduced incorporation of tritiated thymidine, already significant ($P<0.01$) 8 h after the administration of AG and even more pronounced after 24 h (Fig. 3a).

Furthermore. the amount of ATP per weight unit of tumour tissue was significantly reduced 8 ($P<0.05$)–24 ($P<0.01$) h after AG administration (Fig. 3b).

Histology

Histological examination of control tumours on days 7–8 revealed great pleomorphism of the sarcoma cells, and numerous mitoses (Fig. 4a). There was a scant stroma within the tumour, with thin-walled blood capillaries and venules. In some tumours there was a small superficial necrosis with ulceration of the skin, and also several small (10–20 cell diameters wide) necrotic areas throughout the tumour mass. In, and immediately around the tumours, there was a small amount of inflammatory exudate (Fig. 4b), mainly with mononuclear cells, but scattered granulocytes were also seen.

In tumours from animals given AG 8 h previously there was no definite change in tumour cell structure that could be observed in the paraffin-embedded material, and no overall invasion of inflammatory cells. However, several small vessels with stasis of red cells and conspicuously thickened walls were seen, especially at the border zone between the tumour and the underlying subcutaneous fat or skeletal muscle.

Twenty-four hours after injection of AG, the tumours contained large, centrally located necrotic areas (Fig. 5a). At the periphery, however, apparently viable tumour tissue was seen. The AG-treated animals also had a small amount of inflammatory exudate at the periphery (Fig. 5b).

Examination of 1 μm sections of Epon-embedded material from tumour tissue fixed as early as 8 h after AG treatment, revealed early degenerative changes of tumour cells, mainly in the form of clumping of the chromatin and vacuolization of the cytoplasm. There was no significant increase in the number of inflammatory cells among the tumour cells as compared with control tumours (Fig. 6a, b). Also, in tumours from AG-treated animals there was a conspicuous number of small vessels with thickened walls, and dense infiltrates of mononuclear cells in the immediate vicinity (Fig. 7a, b). These changes were most pronounced along the deep borders of the tumours, and absent or less pronounced towards the epidermal side.

Observations on the tumour circulation

The results described above, especially the rapid onset of a centrally located necrosis and the lack of histological evidence for an invasion of potentially cytotoxic leucocytes into the tumour prior to the necrosis, suggested that a circulatory mechanism is responsible for the necrosis. Against this background, the following experiments were performed.

Intravenous injection of 0.5 ml 1% trypan blue (or Evan's blue) in saline was well tolerated by the mice. Fifteen minutes after the injection, the skin and tumours were uniformly coloured. Alcohol extracts of tumour tissue from control animals were vividly blue. Preliminary experiments established that the colouring of tumour tissue reached a maximum after 15 min. In animals treated with 5–10 mg AG and subjected to the same injection of dye 4, 8, or 24 h later, there was a slight reduction in colouring in the 4-h group (not statistically significant) and a significant reduction after 8 or 24 h ($P<0.01$) (Fig. 8).

Similar, but less pronounced effects were found when ^{125}I-labelled human albumin was injected intravenously and the tissues removed 5 min later: there was a small but significant reduction in radioactivity in tumours from animals treated with AG 8 or 24 h previously (data not shown).

FIG. 5. (a) Tumour tissue from animal 8 days after inoculation and 24 h after treatment with 5 mg AG intraperitoneally. Necrotic tumour cells can be clearly seen (× 1000). (b) Tumour tissue from animal 8 days after inoculation and 24 h after AG treatment. To the left is the subcutaneous tissue, with scattered leucocytes. In the centre of the micrograph is a rim of apparently viable tumour tissue, to the right the centrally located massive necrosis (× 200).

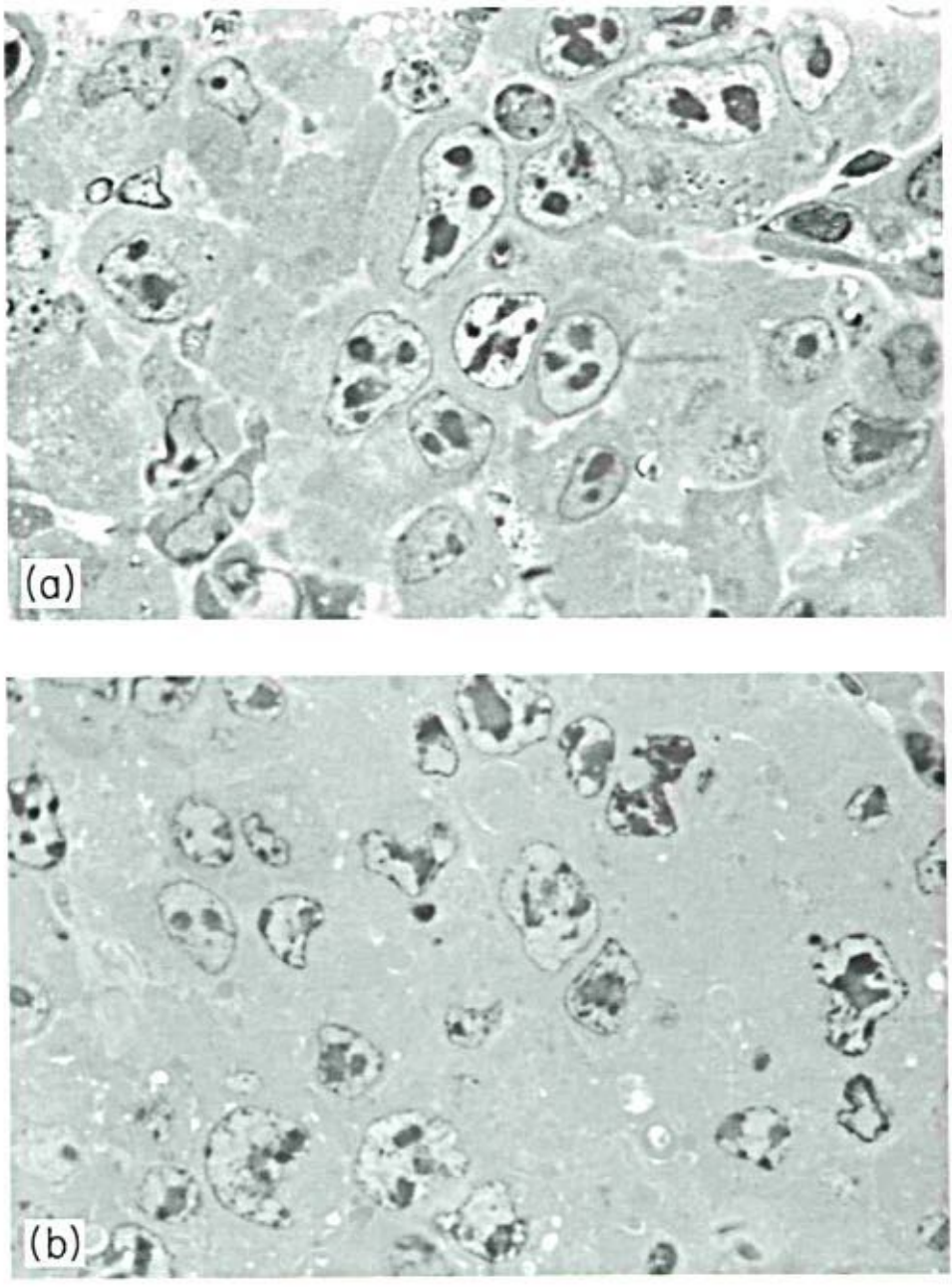

FIG. 6. (a) One micrometre Epon section of representative, central part of tumour from untreated control animal 7 days after inoculation. Viable pleomorphic tumour cells and a scant stroma can be seen (×1200). (b) One micrometre Epon section of representative central part of tumour 7 days after inoculation and 8 h after AG treatment. Tumour cells are vacuolated with clumping of chromatin. No leucocytes can be seen (×1200).

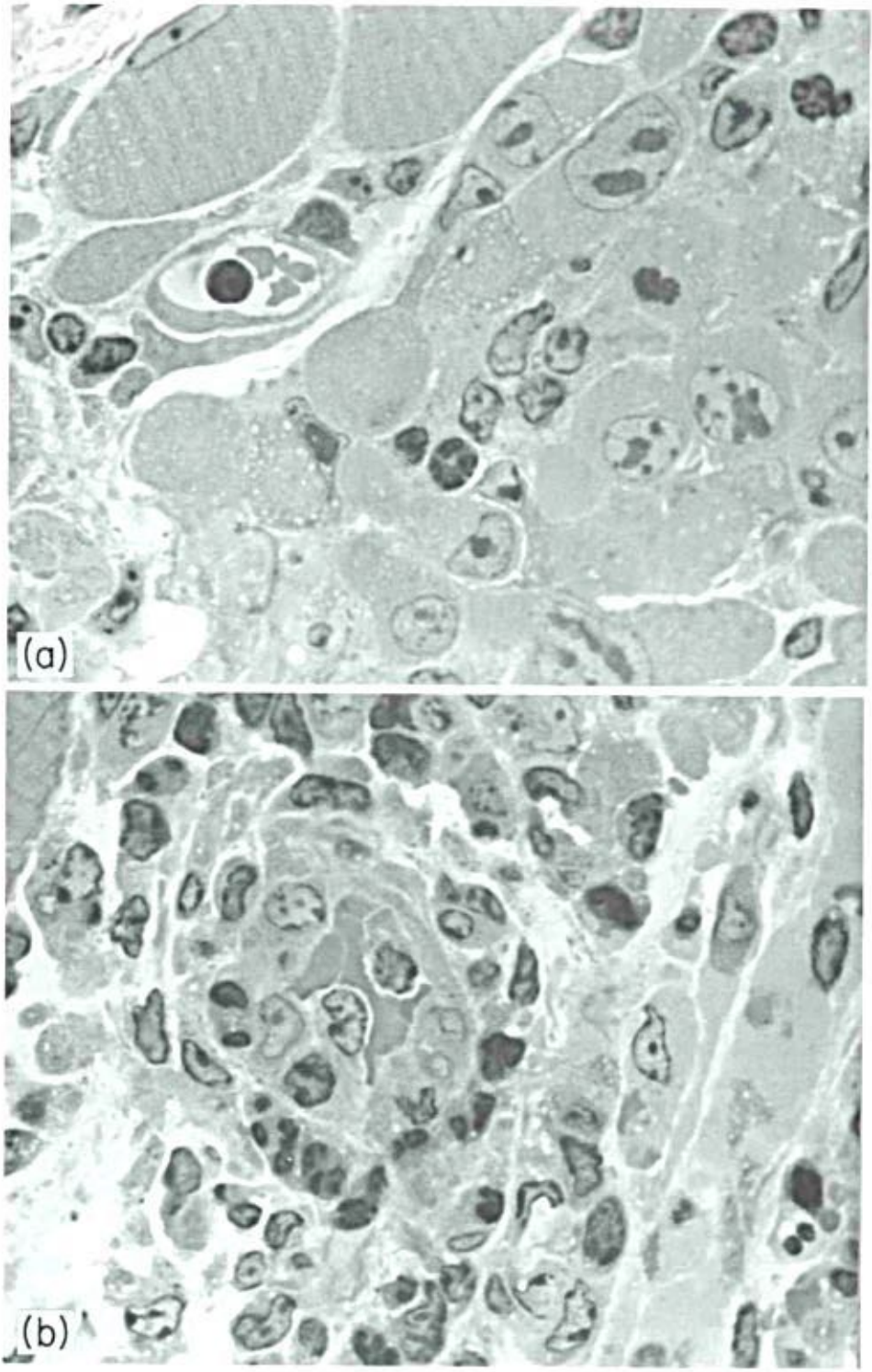

FIG. 7. (a) Micrograph of 1 μm Epon section of the central part of an untreated control tumour on day 7 after inoculation. Tumour cells are seen to the right. One small vessel and fibres of skeletal muscle are seen, as well as scattered leucocytes ($\times$ 1200). (b) Micrograph of 1 μm Epon section of the periphery of a tumour on day 7 after inoculation and 8 h after AG treatment. Tumour cells are seen to the right. One small vessel is at the centre of the micrograph. Stasis of red cells, thickening of the vessel wall, and a conspicuous intra- and perivascular accumulation of inflammatory cells can be seen ($\times$ 1200).

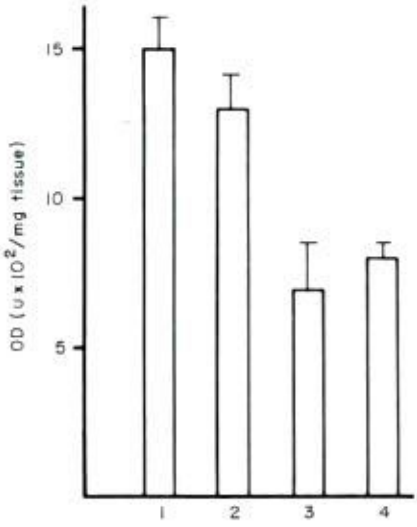

FIG. 8. Spectrophotometric determination of blue colour (read as optical density at 585 nm) in alcohol extracts from tumour tissue 15 min after the intravenous injection of trypan blue. Column 1: tumours from control animals treated with saline 8 h previously; column 2: tumours from animals treated with AG 4 h previously; column 3: tumours from animals treated with AG 8 h previously; column 4: tumours from animals treated with AG 24 h previously. Mean ± SD. Column 1 versus 2: not significant, column 1 versus 3 or 4: $P < 0.01$.

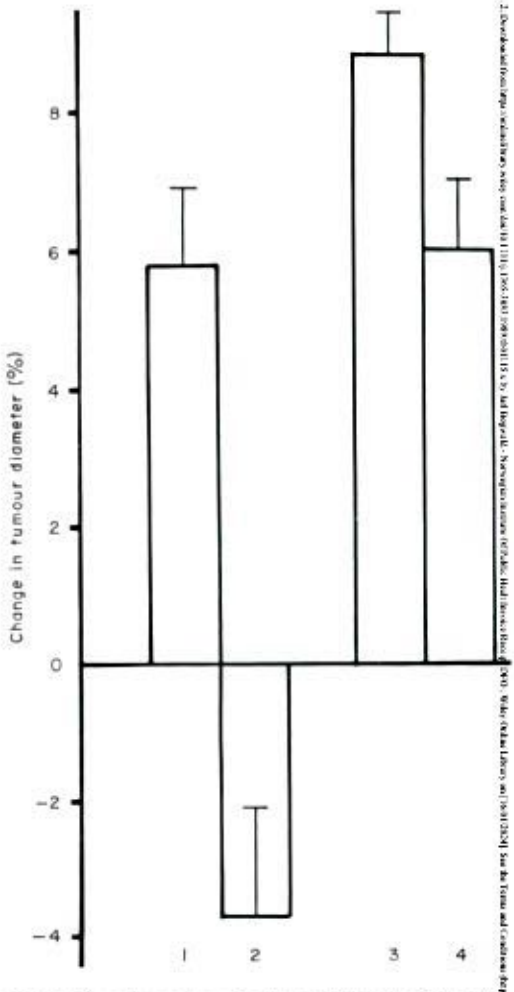

FIG. 9. Percentage change in tumour diameter, from day 7 to 8. Positive figures mean growth, negative figures mean reduction. Column 1: tumours in normal untreated animals; column 2: tumours in normal animals treated with AG on day 7; column 3: tumours in T × B animals, not otherwise treated; column 4: tumours in T × B animals treated with AG on day 7. The data are given as mean ± SD. Column 1 versus 2: $P < 0.01$, column 3 versus 4: not significant.

Tumour regression does not occur after AG treatment of T × B animals [14]. It was therefore pertinent to investigate whether there were any signs of a shutdown of circulation in these animals after AG treatment. As can be seen from Figs 9 and 10 however, there was no significant diameter reduction from day 7 to 8 nor any reduction in trypan blue colouring after AG treatment in T × B animals.

DISCUSSION

This study confirms our previously published finding [15] that systemic administration of AG leads to complete regression of transplanted Meth A sarcoma in syngeneic mice.

Even though full regression normally took 3–4 weeks, distinct changes in the tumours could be observed only hours after the treatment. At 24 h after AG injection, there was significant reduction in tumour diameters. During the same period of time, DNA synthesis, as evidenced by thymidine incorporation, was slower. This indicated that the reduction in tumour size during the first 24 h of treatment was not merely an effect of altered fluid content of the tumour tissue, but that the reduction was related to a decreased proliferation of tumour cells.

The regression of Meth A sarcoma following AG treatment is reminiscent of what has been reported with natural polysaccharides [5] as well

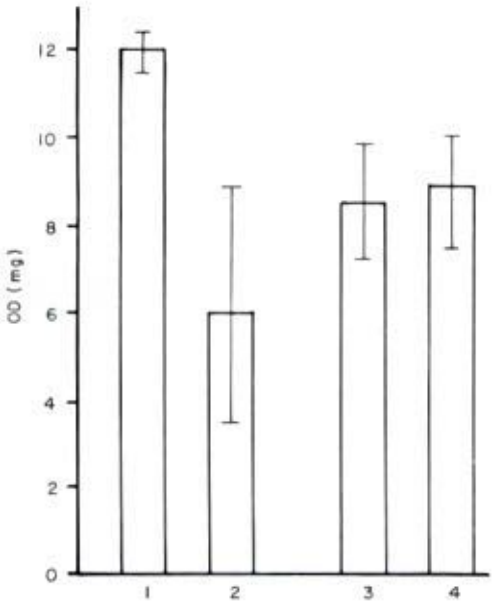

FIG. 10. Spectrophotometric determination of blue colour (read at optical density 585 nm) in alcohol extracts from tumour tissue on day 8 of tumour growth and 15 min after the intravenous injection of trypan blue. Columns as in Fig. 9. Data are given as mean ± SD. Column 1 versus 2: $P < 0.05$, column 3 versus 4: not significant.

as with endotoxin [13]. Little is actually known about the mechanism underlying the regression in these instances. It is established that immune cells accumulate in the Meth A sarcoma during its development, and that the relative number of the various subclasses of T cells is correlated with the susceptibility to regression of the tumour following treatment with lipopolysaccharide (LPS) [10]. It is not known, however, how these cellular events are related to the actual damage to the tumour. It has recently been suggested that tumour necrosis caused by treatment with endotoxin (LPS) or tumour necrosis factor (TNF) may be mediated through modulation of endothelial cell homeostatic properties [9, 16]. No actual measurements of tumour tissue circulation after LPS or TNF treatment appear to have been performed, however.

The accumulated evidence from work on tumour cell cytotoxicity in vitro, as well as several reports on the cellular composition of tumours, seem to indicate that direct cytotoxic action by immune cells on tumour cells is a major component of host defence against malignancy and that it is the underlying mechanism in regression following treatment with immunopotentiators [1, 3, 6, 7].

We found no histological evidence of significant tumour cell–host immune cell interactions during the first hours after AG treatment. In fact, the early reduction in ATP levels in tumour tissue, and the reduced accumulation of intravenous dye in tumour tissue well before the development of visible necrosis, indicated circulatory failure as the underlying mechanism.

In accordance with this we found striking histological changes in tumour vasculature 8 h after AG injection: stasis of red cells, thickening of the endothelium, and perivascular accumulation of inflammatory cells. It is easy to envisage that changes like these could lead to reduced circulation with ensuing decreased oxygenation of the tumour tissue and subsequent necrosis. Our observations so far do not allow us, however, to tell which comes first: pathological changes in the cells of the tumour vessels or the invasion of inflammatory cells into the vessel walls.

We do not know how AG triggers these events. Previous studies on AG have given no evidence of a direct cytotoxic effect on tumour cells at the relevant concentrations [2]. In a subsequent publication it will be reported that AG is a poor stimulant of TNF release but a very strong stimulant of IL-1 release. The question of how these facts are linked to the rapid circulatory failure in the tumour is now under investigation.

There is no good explanation for necrosis only occurring in the tumour, and not in the entire organism. The fact that tumour regression is not seen in T cell-deficient animals treated with AG [15] and that there is also no circulatory failure under these circumstances, appears to implicate an immunological component targeting the unknown factor causing vascular damage to the tumour vessels. The possibility that tumour vessels are especially sensitive to whatever noxious factor AG treatment releases, and that the requirement for T cells does not imply an immunologically specific event but rather a requirement for a co-factor cannot be excluded at this stage.

ACKNOWLEDGMENTS

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