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Synergic action between tumor necrosis factor and endotoxins or poly(A·U) on cultured bovine endothelial cells*

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Summary. In order to investigate whether direct effects on tumor vasculature may contribute to induction of necrosis of solid tumors in vivo, agents and combinations with an established different capacity to induce tumor necrosis were studied for their effects on endothelial cells in vitro. Tumor necrosis serum caused a marked inhibition of [^{3}H]thymidine incorporation by bovine umbilical cord endothelial cells after 4 h coincubation. Endotoxin was less inhibitory, whereas detoxified endotoxin and recombinant human tumor necrosis factor (rTNF) were hardly active in concentrations that can be achieved in vivo. Combinations of rTNF and (detoxified) endotoxin caused synergic inhibition. By 24 h effects of the separate agents and synergic effects of the combinations were much stronger. The nontoxic dsRNA, poly(A·U), also had inhibitory activity, and acted synergistically with rTNF. Morphologically, a combination of endotoxin and rTNF but not the separate constituents induced marked cell detachment by 24 h, an indication of cell death. Whereas both endotoxin and rTNF inhibited DNA synthesis of human endothelial cells, the agents did not act synergistically on these cells. The ability of the agents and the combinations to affect endothelial cells in culture appeared to be well in line with their capacity to induce tumor necrosis. Data suggest that direct (synergic) effects on endothelium may contribute to the induction of vascular damage in tumors by (combinations of) the agents. The fact that endothelial cell death is only induced by the combinations and not by the separate agents in vivo, may be a cause of the greater therapeutic activity of the combinations in vivo. The synergy between rTNF and the other agents indicates that the agents act by different mechanisms.

Introduction

The capacity of endotoxin to induce necrosis of solid tumors within 24 h of administration is well known [15, 26]. Besides endotoxin, several other agents possess tumor-necrotizing activity to some extent. These include the vasoactive agents epinephrine, serotonin and histamine [7, 8], synthetic double-stranded RNAs [6, 26], tumor necrosis serum (TNS) [9], and tumor necrosis factor (TNF) [27]. The underlying mechanism is still a matter of discussion. It is generally agreed that the action of endotoxin is host-mediated. TNF, elicited by endotoxin, may be a direct tumoricidal mediator, since the factor was shown to have rather selective cytostatic and cytotoxic activity against various malignant cell lines in vitro [14, 25, 31].

Involvement of indirect mechanisms, however, is likely, since induction of tumor necrosis by TNF appeared not to be restricted to tumors with sensitivity to TNF in vitro [1, 4, 11]. Histological studies revealed that strong inducers of necrosis of solid Meth A tumors in mice caused marked hyperemia, congestion and vascular damage in the tumor within 4 h of injection [18, 21, 29], in contrast to agents that only have a weak capacity to induce tumor necrosis, such as chemically detoxified endotoxin [23] and the nontoxic dsRNA, poly(A·U) [6]. Vascular effects might be due to direct activity against endothelium, since both endotoxin and TNF were shown to cause morphological changes and inhibition of DNA synthesis of cultured endothelial cells [17, 28].

Recently, recombinant TNF (rTNF) has been shown to act synergistically with endotoxin against solid murine Meth A tumors in vivo [4, 10], demonstrating that TNF is not the sole mediator of endotoxin-induced antitumor activity. Moreover, combinations of rTNF with detoxified endotoxin or poly(A·U) were much more potent inducers of tumor necrosis and regression than the separate constituents [4].

The purpose of this study was to investigate whether the differential antitumor action of the agents in vivo can be related to direct activity against endothelial cells in vitro. Therefore, changes in morphology and DNA synthesis of cultured bovine and human endothelial cells, induced by (combinations of) the agents, were studied.

Materials and methods

Materials. Polyadenylic·polyuridylic acid [poly(A·U)] was obtained from Miles Laboratories Inc. (Elkhart, Ind, USA). Endotoxin from *Salmonella typhimurium* Re mutant and a chemically detoxified (monophosphoryl) preparation of the same molecule were obtained from Ribi ImmunoChem Inc. (Hamilton, Mont, USA). Lyophilized *Propionibacterium acnes* (strain VPI 0009) was a gift from Dr. C. S. Cummins (Virginia Polytechnic Institute, Blacksburg, Va, USA). Suspensions of formalin-killed *P. acnes*

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were prepared as described previously [5]. Recombinant human TNF (specific activity 1.5×10^7 U/mg protein) was kindly provided by Biogen SA (Geneva, Switzerland) and Knoll/BASF (Ludwigshafen, FRG).

Preparation of sera. Tumor necrosis serum was prepared according to Green et al. [16]. Briefly, Swiss mice, bred and maintained at the Laboratory of Microbiology (Utrecht, The Netherlands), were injected intravenously with 1 mg *P. acnes* and 2 weeks later with 10 μg endotoxin. Mice were bled 90 min later, and serum was prepared. Normal mouse serum was prepared from blood of untreated mice. Sera were stored at −20° C, and prior to use heated at 56° C for 10 min.

Isolation and culture of endothelial cells. Bovine endothelial cells were isolated from umbilical cord arteries, and human endothelial cells from umbilical cord veins. The isolation procedure was performed as described by Vandenbroucke-Grauls et al. [33] with some minor modifications. Briefly, cord arteries or veins were rinsed with phosphate-buffered saline, and subsequently filled with 0.05% trypsin/0.02% EDTA (Gibco Biocult Ltd., Paisley, UK). After 40 min (bovine cords) or 20 min (human cords) incubation in a 37° C water bath the trypsin solution containing detached endothelial cells was collected and centrifuged (10 min, 200 *g*). Supernatant was discarded and cells were resuspended in medium. Finally, cells were plated in 25-cm^2 (bovine cells) or 75-cm^2 (human cells) tissue-culture flasks (Costar, Cambridge, Mass, USA), which had been previously coated with fibronectin (50 μg/cm^2; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). Bovine cells were grown in medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 20% (v/v) heat-inactivated (30 min, 56° C) newborn calf serum, penicillin/streptomycin, Hepes (0.02 M) and $NaHCO_3$ (2 g/l). Human cells were cultured in the same medium with 20% heat-inactivated pooled human serum instead of newborn calf serum. Cells were cultured at 37° C in a 5% CO_2 humidified atmosphere until confluence. Cells were then trypsinized (0.25% trypsin) and used for assays or further culture. The cells were identified as endothelial cells by cell morphology and growth pattern. For assays, passages 4–12 (bovine cells) or 1–2 (human cells) were used.

Measurement of [^{3}H]thymidine incorporation. Cells (5×10^4 cells in 0.1 ml medium) were plated in 96-well flat-bottom tissue-culture plates (Costar), and cultured for 24 h to obtain adherent monolayers. After addition of desired concentrations of the (combined) agents in 0.1 ml medium the plates were incubated for 4 h or 24 h. Four hours before termination of the incubation 0.5 μCi [^{3}H]thymidine (specific activity 5 Ci/mmol, Amersham International, UK) in 50 μl medium was added to each well. Finally, cells were detached by trypsinization, and harvested with a multiple cell-culture harvester. Incorporated radioactivity was measured with the aid of a liquid scintillation counter. All tests were performed in six-fold.

Morphology studies. For morphology studies bovine cells were plated in 6-well flat-bottom plates (1.6×10^7 cells in 3 ml medium/well; Costar), and cultured for 24 h. After a subsequent 4-h or 24-h coincubation with rTNF (10^4 U/ml), endotoxin (1 μg/ml) or the combination cells were fixed with 4% (v/v) formalin and stained with toluidine blue.

Data handling and interaction analysis. Data have been presented as mean percentage inhibition of [^{3}H]thymidine incorporation ± SEM as compared to that of controls, cultured in medium. Synergic interactions were calculated using an algebraic expression of the isobologram method, as described by Berenbaum [2]. In brief, if the combined agents A and B in the concentrations $[A_{comb}]$ and $[B_{comb}]$ produce exactly the same quantitative effect as the separate agents in the concentrations $[A_s]$ and $[B_s]$, respectively, and

$$\frac{[A_{comb}]}{[A_s]} + \frac{[B_{comb}]}{[B_s]} = 1$$

the agents act additively. If the sum of the fractions is less than or greater than 1, the agents act synergistically or antagonistically, respectively. In the case of no equi-effective concentrations being found, interactions were calculated as in the following example. In Table 2, the combination of 10^3 U rTNF and 1 μg endotoxin/ml caused 38% inhibition of [^{3}H]thymidine incorporation by bovine cells after 4 h. Equi-effective concentrations of endotoxin and rTNF were $> 10^4$ U/ml and > 10 μg/ml, respectively. Substitution of these values into the equation shows that the sum of the fractions $(10^3/>10^4)+(1/>10)$ is less than 0.2, so the agents act synergistically.

Results

Inhibition of bovine and human endothelial cell DNA synthesis by TNS

Comparison of bovine and human cells cultured in medium revealed that the bovine cells incorporated about 25 times more [^{3}H]thymidine than the human cells. TNS caused a distinct, concentration-dependent reduction of thymidine uptake as compared to the controls (Table 1). A 20% and 73% inhibition of bovine cells was induced by

Table 1. Ability of tumor necrosis serum (TNS) to inhibit [^{3}H]thymidine incorporation by bovine (BEC) and human (HEC) endothelial cells[a]

Agent	Concentration (%)	Time of incubation (h)	Inhibition (%) in BEC	Inhibition (%) in HEC
TNS	0.1	4	8±8[b]	12± 6
	1	4	20±6	34± 2
	10	4	73±2	47±11
NMS[c]	10	4	−2±6	15± 4
TNS	0.1	24	−28±4	14± 2
	1	24	56±2	41± 2
	10	24	85±2	84± 2
NMS	10	24	11±3	30± 2

[a] Radioactivity (dpm) of controls: BEC (4 h), 84900±200; BEC (24 h), 14300±3100; HEC (4 h); 3380±190, HEC (24 h), 5540±160

[b] Mean inhibition ± SEM as compared to [^{3}H]thymidine incorporation of controls

[c] NMS, normal mouse serum

Table 2. Inhibition of [^{3}H]thymidine incorporation by bovine endothelial cells caused by combinations of rTNF and endotoxin[a]

Time of incubation (h)	Concentration of endotoxin (μg/ml)	Inhibition (%) with rTNF concentrations (U/ml) of					
		0	1	10	10^2	10^3	10^4
4	0	0±2[b]			5±2	1±3	3±3
	10^{-1}	1±3			17±1	19±3	
	1	24±5			26±2	38±2[c]	
	10	27±4			37±2		
24	0	0±1	5±1	27±2	42±1	59±1	58±1
	10^{-3}	7±2	2±2	40±3	49±1	45±3	
	10^{-2}	13±2	11±3	43±3	63±1[c]	64±2[c]	
	10^{-1}	52±1	50±2	76±2[c]	87±1[c]	89±1[c]	
	1	64±2	37±2	58±2	87±1[c]	97±0[c]	
	10	60±1					

[a] Radioactivity (dpm) of controls: 4 h, 68 800±900; 24 h, 59 900±500
[b] Mean inhibition ± SEM as compared to [^{3}H]thymidine incorporation of controls
[c] Synergic interaction

Table 3. Combined inhibitory effect of rTNF and detoxified endotoxin (Detox) on [^{3}H]thymidine incorporation by bovine endothelial cells[a]

Time of incubation (h)	Concentration of Detox (μg/ml)	Inhibition (%) with rTNF concentrations (U/ml) of			
		0	10^2	10^3	10^4
4	0	0±2[b]	10±2	6±2	10±2
	0.1	−6±1	9±2	5±6	
	1	0±1	6±1	16±1[c]	
	10	6±2			
24	0	0±2	65±1	69±2	72±1
	0.1	−3±3	62±0	72±1	
	1	18±2	72±1	80±1[c]	
	10	54±1			

[a] Radioactivity (dpm) of controls: 4 h, 14 600±290; 24 h, 47 000±900
[b] Mean inhibition ± SEM as compared to [^{3}H]thymidine incorporation of controls
[c] Synergic interaction

1% and 10% (v/v) TNS, respectively. After 24 h incubation these figures were 56% and 85%. The incorporation of radioactivity by human endothelial cells had been decreased with 34% and 47% after 4 h, and 41% and 84% after 24 h incubation with 1% and 10% TNS, respectively. The inhibitory activity of normal mouse serum appeared to be much weaker (Table 1).

Inhibitory effects of combinations of endotoxin and rTNF on bovine cells

Culture of bovine cells for 4 h in the presence of 10^2–10^4 U/ml rTNF did not affect their thymidine incorporation (Table 2). Endotoxin had no effect at a concentration of 0.1 μg/ml, and caused a slight but significant inhibition in concentrations of 1 μg/ml and 10 μg/ml. The inhibition caused by combinations of endotoxin and rTNF was stronger.

After 24 h incubation rTNF inhibited in a concentration-dependent manner up to 10^3 U/ml (Table 2). A maximum reduction of DNA synthesis in bovine endothelial cells of 59% was measured at 10^3 U rTNF/ml, which did not increase upon addition of a tenfold higher concentration of rTNF. Inhibitory effects of endotoxin (10 ng–10 μg/ml) were similar to rTNF, having a maximum of 64% at 1 μg/ml. Combinations of appropriate concentrations of the agents reduced bovine cell thymidine incorporation more than 85%.

Table 4. Inhibition of incorporation of [^{3}H]thymidine by bovine endothelial cells caused by 24 h coincubation with rTNF combined with poly (A · U)[a]

Concentration of poly (A · U) (μg/ml)	Inhibition (%) with rTNF concentrations (U/ml) of			
	0	10^2	10^3	10^4
0	0±3[b]	63±1	75±1	86±1
10	44±2	95±0[c]	90±0[c]	
100	66±1			

[a] Radioactivity (dpm) of control: 47 800±1200
[b] Mean inhibition ± SEM as compared to [^{3}H]thymidine incorporation of controls
[c] Synergic interaction

Table 5. Inhibition of [^{3}H]thymidine incorporation by human endothelial cells caused by 24 h coincubation with combinations of rTNF and endotoxin[a]

Concentration of endotoxin (μg/ml)	Inhibition (%) with rTNF concentrations (U/ml) of				
	0	10^2	10^3	10^4	10^5
0	0±5[b]	52± 6	49±5	46±7	55±4
0.1	25±4	52± 6	55±5	53±6	
1	27±5	52±10	54±6	61±7	
10	37±5	57± 5	61±7	58±8	

[a] Radioactivity (dpm) of control: 5050±760
[b] Mean inhibition ± SEM as compared to [^{3}H]thymidine incorporation of controls, data from five separate experiments have been combined

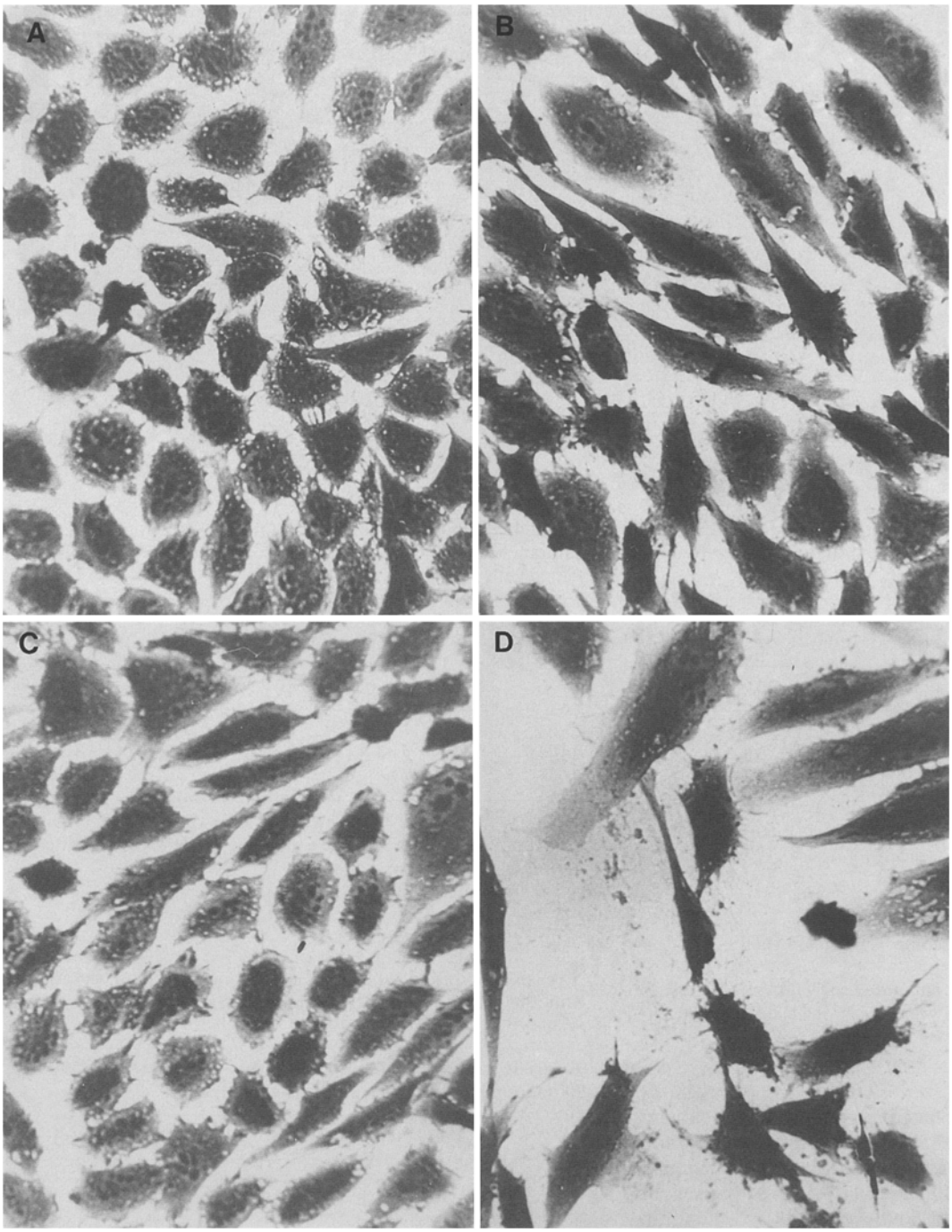

Fig. 1. Morphological alterations of bovine endothelial cells, induced by 24 h coincubation with rTNF, endotoxin and the combination. **A** control; **B** rTNF (10^4 U/ml); **C** endotoxin (1 μg/ml); **D** rTNF (10^4 U/ml)+ endotoxin (1 μg/ml)

Interaction analysis showed that several combinations of endotoxin and rTNF caused synergic inhibition of bovine endothelial cell DNA synthesis by 4 h and 24 h.

Morphological changes of bovine endothelial cells induced by endotoxin and rTNF

No distinct changes in morphology of the bovine cells were observed after 4 h incubation with 1 μg/ml endotoxin, 10^4 U/ml rTNF, or the combination. By 24 h the separate agents caused marked elongation of the cells (Fig. 1). Many cells were fibroblast-shaped, but gaps in the monolayer, indicating detachment of cells, were not seen. After 24 h incubation with the combination, however, many empty spaces between cells were observed, suggesting that a major portion of the cells had detached. Most of the remaining cells appeared extremely spindle-like, and hyperbasophilic.

Inhibitory activity of detoxified endotoxin and rTNF on bovine endothelial cells

Detoxified endotoxin inhibited DNA synthesis of the bovine cells, but about 100 times higher concentrations were needed to equal the effect of the toxic preparation (Tables

2 and 3). A marked inhibition was only seen after 24 h incubation with the highest concentration of detoxified endotoxin tested (10 μg/ml). The agent acted synergistically with rTNF on bovine cells by 4 h and 24 h.

Inhibition of bovine endothelial cell DNA synthesis by poly(A · U) and rTNF

The effect of poly(A · U) on thymidine uptake by bovine cells after 24 h incubation is shown in Table 4. At concentrations of 10 μg/ml and 100 μg/ml 44% and 66% inhibition of thymidine incorporation was measured respectively. A marked synergy was seen upon combination of 10 μg poly(A · U) and 10^2 or 10^3 U rTNF/ml. The combinations caused 90%–95% inhibition of the bovine cells.

Ability of endotoxin and rTNF to inhibit [^{3}H]thymidine incorporation by human endothelial cells

Effects of endotoxin and rTNF on human cells were less pronounced than effects on bovine cells. After 4 h incubation with 10^2–10^5 U/ml rTNF, 0.1–10 μg endotoxin/ml, or the combinations, no effect on thymidine uptake was seen (data not shown). By 24 h rTNF caused a 46%–55% reduction of incorporated radioactivity, but the effect seemed not to be concentration-dependent in the range studied (Table 5). Endotoxin caused moderate inhibition of DNA synthesis in human endothelial cells. A maximum inhibition of 37% was observed in the presence of 10 μg endotoxin/ml. Combinations of rTNF and endotoxin showed no synergy (Table 5).

Discussion

The observation that endotoxin and rTNF inhibited DNA synthesis of cultured endothelial cells confirms earlier reports by others [17, 28]. In the present study over 50% inhibition of DNA synthesis in bovine cells was found after 24 h incubation with 0.1 μg endotoxin or 10^3 U rTNF/ml (Table 2). Doses of at least 10 μg endotoxin or 3×10^4 U rTNF appeared necessary to induce marked vascular effects and necrosis of murine Meth A tumors upon systemic administration [4, 34], so the observed effects on endothelial cells in vitro may have relevance in vivo. In comparison with endotoxin, much higher concentrations of detoxified endotoxin and poly(A · U) were required to induce the same degree of inhibition of bovine endothelial cells (Tables 2–4). These observations are well in line with previous data showing that systemic injection of 10 μg detoxified endotoxin or 100 μg poly(A · U) failed to induce significant necrosis of Meth A tumors [4, 23]. Concentrations of the agents achieved in vivo may have been too low to cause significant direct effects on endothelium. Effective concentrations, however, are likely to be achieved upon addition of rTNF as a result of the synergic interaction.

Whereas on a quantitative basis the efficacy of the combinations against endothelial cells in vitro correlated very well with their previously observed potent tumor-necrotizing capacity [4, 10], tumor necrosis is probably not solely the consequence of a direct interaction between agents and endothelium. Both endotoxin and rTNF appeared rather cytostatic than cytolytic for endothelial cells in vitro (Fig. 1), while the morphology of tumors undergoing necrosis revealed distinct injury of the endothelial cells [21, 34]. Moreover, the vascular effects in Meth A tumors could already be seen within 4 h of treatment, whereas the activity of the agents against endothelial cells in vitro was very moderate or absent at that time. Notably, the rather late action of rTNF against endothelial cells questions whether direct activity against endothelium is involved in induction of tumor necrosis, in the light of the reversibility of the effects of rTNF on endothelial cells in vitro [28, 30] and its short half-life in vivo [3]. The reported angiogenic action of TNF in vivo [24] adds further doubt to the relevance of the observed cytostatic effects of rTNF on endothelial cells in vitro. However, it is probably not at variance with induction of vascular damage in vivo, since it has been characterized as a hemorrhagic angiogenesis.

The question remains: why does systemic injection of tumor-necrotizing agents at therapeutic doses selectively induce necrosis of tumor tissue? It is possible that tumor vasculature possesses enhanced susceptibility to the agents. The more prominent effects of the agents on bovine compared to human endothelial cells suggests that properties of the endothelial cells themselves define their sensitivity. Since proliferation rates of bovine cells appeared about 25 times higher than those of the human cells, highly mitotic endothelial cells may be more vulnerable to the cytostatic action of tumor-necrotizing agents. The observed cell-cycle-dependence of the cytostatic action of TNF [12, 32] is consistent with this notion. Enhanced susceptibility of proliferative endothelium would also provide an explanation for the exquisite vulnerability of tumor vessels to tumor-necrotizing agents, because proliferation rates of endothelium appeared very high in neoplastic tissue and very low in normal tissues [13]. Also data showing that the placenta, another tissue with highly proliferative endothelium [13], appeared to be very sensitive to induction of hemorrhage by endotoxin [35] may be explained in this way. Effects of the agents on tumor vasculature may be further enhanced by aberrant conditions in tumors, such as low pH and oxygen pressure, and the poor repair capacity [13].

Besides the enhanced vulnerability of tumor vasculature host-mediated effects may contribute to induction of endothelial injury and necrosis in the tumor. There is ample evidence that polymorphonuclear cells are involved [1, 19, 22, 33]. Our observations with TNS suggest that cytotoxins other than TNF, and induced by the agents in vivo, are at play. TNS appeared to cause a marked and prompt inhibition of cultured endothelium (Table 1). Synergy between TNF and residual endotoxin present in TNS can only partially explain its strong activity, because the effects of TNS on bovine endothelial cells after 4 h far surpassed those of optimal combinations of rTNF and endotoxin (Tables 1 and 2). Other cytotoxins reported to be present in TNS [20] are probably involved and may be implicated in the strong synergic activity of rTNF and endotoxin against Meth A tumors [4, 10].

In conclusion, present data suggest that direct (synergic) effects on endothelium may contribute to the previously observed vascular effects in vivo. The synergy between rTNF and (detoxified) endotoxin or poly(A · U) suggests that the agents act by different mechanisms on bovine cells. The prompt and marked effects of TNS on cultured endothelial cells are possibly due to interactions of TNF with other agents, such as endotoxin, present in TNS. Elucidation of these interactions may be important for the understanding of the mechanisms that underlie induction of tumor necrosis in vivo.

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