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METASTASIS AND THE RETICULOENDOTHELIAL SYSTEM. II. EFFECT OF TRIAMCINOLONE ACETONIDE ON ORGAN RETENTION OF MALIGNANT CELLS IN ENDOTOXIN-TREATED MICE

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The lung retention patterns of B16 melanoma cells were determined after intravenous injection of [^{125}I]dUrd-labelled tumor cells into B16 melanoma-bearing mice. Experiments were performed to assess the effects of a synthetic glucocorticoid, triamcinolone acetonide, on the retention of B16 melanoma cells arrested in the lungs of mice with endotoxin-induced reticuloendothelial system hyperfunction. Lung clearance of malignant cells was greatly accelerated in mice treated with endotoxin alone but was markedly inhibited in mice with only triamcinolone acetonide. In mice treated with both endotoxin and subsequently triamcinolone acetonide after tumor-cell arrest processes had occurred, the endotoxin-induced increase in clearance was nullified. These results are discussed in terms of the mutually antagonistic activity of both pharmacologic agents upon the reticuloendothelial system and the role of the latter in regulating organ retention of disseminated malignant cells.

This study is part of a series on the role of the reticuloendothelial system (RES) in modifying the arrest and retention of circulating cancer cells in the organs of experimental animals. In the previous experiments (Glaves, 1980) it was observed that endotoxin-induced stimulation of the RES was associated with diminished organ retention of intravenously injected tumor cells. Endotoxin treatment results in a number of modifications in host physiology, many of which are antagonized by steroids. Although the effects of endotoxin and a synthetic glucocorticoid, triamcinolone acetonide, on organ retention of melanoma cells have been described separately (Glaves, 1980; Fidler and Lieber, 1972) we now describe the combined effects of both agents for the first time.

MATERIAL AND METHODS

Animals

Syngeneic male C57BL/6Ja mice, 20–25 g, were used for all experiments. All mice carried solid B16 melanomas induced by intramuscular inoculation of 1×10^5 viable B16 cells from tissue culture 14–18 days prior to each experiment. Experiments were made at least in duplicate and data points represent results from 9–14 animals per group.

Tumor

The B16 melanoma cells were originally obtained from Dr. M. Goldrosen (Surgical Oncology, Roswell Park Memorial Institute) and were maintained in plastic tissue culture flasks (Falcon, Oxnard) in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were routinely subcultured by treatment of monolayers with calcium- and magnesium-free Hanks' balanced salt solution (HBSS), containing 0.25% trypsin and 0.25% EDTA, for 2 minutes at 37°C. Single-cell suspensions were washed with culture medium and seeded into tissue culture flasks or washed with HBSS and inoculated into mice as described above.

Radiolabel

Subconfluent cultures of B16 cells were inoculated with 0.03 μCi [^{125}I]-5-iodo-2^1-deoxyuridine [^{125}I]dUrd (Amersham Searle, Arlington Heights) per ml of culture fluid. After 24 h, cell monolayers were rinsed twice with HBSS and cells detached by exposure to trypsin-EDTA as described above. Detached cells were washed three times in HBSS and finally resuspended in HBSS containing 1% normal C57BL/6Ja mouse serum. Any cell clumps were removed by filtration through a 400-gauge stainless steel mesh and cell suspensions were adjusted to contain 1×10^7 cells/ml. Cell viability was routinely greater than 95% as assessed by trypan blue exclusion tests. The suitability of [^{125}I]dUrd as a stable, little re-utilized label for *in vivo* tracing of malignant cells has previously been validated in depth (Weiss and Glaves, 1976).

Retention of radiolabelled cells

Groups of treated or control B16 melanoma-bearing mice were given 1×10^6 radiolabelled B16 cells in 0.1 ml vehicle via the lateral tail vein. At subsequent intervals, animals were anesthetized and exsanguinated by cardiac puncture, and their major organs were placed in 70% ethanol. Organs were counted in a gamma-spectrometer (Beckman 8000) for 10 min and washed three times with 70% ethanol over a period of 3 days to remove radiolabel not associated with intact cells, then gamma counts were repeated. Results were expressed as percentage recovery of the total radioactivity injected.

Agents

Endotoxin (lipopolysaccharide W from *S. typhosa;* Difco, Detroit) at 10 μg/mouse was administered i.v. in 0.1 ml of HBSS containing 1% normal mouse serum. Control mice received vehicle alone and animals were dosed 72 h (t-72 h) prior to injection of radiolabelled cells. Suspensions of triamcinolone acetonide (TA; Sigma, St. Louis) were made by dissolving 5 mg TA in 0.6 ml absolute ethanol and subsequent by adding phosphate-buffered saline, pH 7.3 (PBS) to produce a fine precipitate of TA. Mice received an intraperitoneal injec-

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tion of 0.5 ml of this suspension, or vehicle alone, 5 min after injection of radiolabelled cells. For certain experiments, B16 melanoma cells were cultured in the presence of TA *in vitro* prior to retention studies. In these cases, subconfluent monolayers of B16 cells were inoculated with [^{125}I]dUrd 24 h before experiments as described above and treated with TA or vehicle 6h before cells were harvested for injection. TA used for *in vivo* studies was in the form of a suspension; therefore, to obtain a sterile solution of TA for *in vitro* use, we used dimethylsulfoxide as the vehicle. TA was dissolved at a concentration of 5 mg/ml dimethylsulfoxide and this stock solution was added to cultures to give a final TA concentration of 0.1 mg/ml culture fluid. Control cultures received an equivalent volume of vehicle only. At the end of the incubation period, cells were harvested as described above and used in retention studies. No loss of cell viability could be detected.

Statistics

Results of experiments were evaluated using Student's *t*-test.

2 h and t-6 h but this effect had disappeared 24 h after cells were injected. In endotoxin-treated animals, acceleration of clearance from the lung was evident by 2 h post injection so that 61% fewer cells were retained than in vehicle-treated controls. This effect was even more apparent at t-6 h when there was a 92% reduction in retention and at t-24 h when there were 90% fewer cells retained than in the lungs of internal control mice.

In a second group of experiments, TA or its vehicle were given 5 min after injection of radiolabelled cells so that tumor cell arrest processes had already occurred and the effects of TA on retention of arrested cells were being examined. Again, the vehicle (ethanol-PBS) had its own effects on melanoma cell clearance as reflected in this case by a 55% decrease in lung retention at t-6 h, a rate which increased to give 73% fewer cells than in untreated controls by t-24 h. However, when the results from TA-treated animals were compared with their own controls, it can be seen that TA had opposite effects from endotoxin on melanoma cell retention.

TABLE I

EFFECT OF TRIAMCINOLONE ACETONIDE ON LUNG RETENTION OF B16 MELANOMA CELLS IN RES-STIMULATED MICE

	% Total radiolabel retained (±SE)						
Time after cells[1]	Overall controls	Endotoxin$_{t\text{-}72\,h}$	Vehicle$_{t\text{-}72\,h}$	TA[2]$_{t\text{-}5\,min}$	Vehicle$_{t\text{-}5\,min}$	Endotoxin$_{t\text{-}72\,h}$: TA$_{t\text{-}5\,min}$	Endotoxin$_{t\text{-}72\,h}$: Vehicle$_{t\text{-}5\,min}$
$t_{10\,min}$	81.8(±4.0)	79.8(±2.9)	78.7(±4.3)	82.3(±1.6)	78.1(± 1.8)	78.7(±3.6)	79.5(±5.4)
$t_{2\,h}$	49.8(±1.6)	22.4(±2.4)[3]	58.2(±3.8)	66.9(±8.2)	65.0(±10.8)	40.3(±4.6)	29.2(±4.5)
$t_{6\,h}$	19.8(±1.2)	2.2(±0.6)[3]	29.6(±2.2)	26.3(±3.5)[3]	8.9(± 1.6)	7.4(±2.5)[3]	2.0(±0.7)
$t_{24\,h}$	3.0(±0.5)	0.4(±0.1)[4]	4.2(±1.4)	6.6(±2.6)[4]	0.8(± 0.5)	2.3(±0.5)[4]	0.5(±0.1)

[1] 1 × 10^6 B16 melanoma cells given i.v. at t_0. – [2]TA, triamcinolone acetonide. – [3]$0.05 > p > 0.01$ compared with vehicle control. – [4]$0.0005 > p > 0.0001$ compared with vehicle control.

RESULTS

The results listed in Table I summarize retention experiments and show that, 10 min after i.v. injection of radiolabelled B16 melanoma cells, approximately 80% of the injected dose was retained in the lungs of otherwise untreated tumor-bearing mice. Two hours later, 50% of cancer cells could be detected and by 24 hours only 3% of the injected cells remained. Results for other major organs are not presented since there was little relocalization of radiolabel in organs other than the lungs and these organs play little role in supporting the growth of spontaneous metastases from B16 tumors or the growth of nodules after i.v. injection of melanoma cells. In studies on the effects of endotoxin pretreatment on lung retention, summarized in Table II, it can be seen that the vehicle alone produced a slight modification of the basic retention pattern of B16 melanoma cells so that the clearance of radiolabelled cells was slower than in untreated animals between t-

These effects were slower to appear but by t-6 h the numbers of radiolabelled cells retained in the lungs of TA-treated mice were higher than those in vehicle controls by a factor of 2 and by t-24 h there were approximately eight times more cells in the lungs of test animals compared with vehicle controls.

TABLE II

EFFECT OF TRIAMCINOLONE ACETONIDE TREATMENT *IN VITRO* ON LUNG RETENTION OF B16 MELANOMA CELLS

Time after cells[1]	% Total radiolabel retained (±SE)	
	TA-treated	Vehicle-treated
$t_{10\,min}$	78.9(±7.2)	79.1(±5.6)
$t_{2\,h}$	61.2(±3.0)	53.7(±4.4)
$t_{6\,h}$	5.5(±0.8)	7.9(±2.0)

[1] 1 × 10^6 B16 radiolabelled cells given i.v. at t_0.

In the third group of experiments, the effects of TA on endotoxin-pretreated mice were examined. All mice received endotoxin 3 days prior to retention experiments at t-72 h, B16 cells were injected at t-0 and mice then received either TA or its vehicle at t-5 min. The results in Table I show that the vehicle did not modify lung retention patterns when compared with mice receiving endotoxin only. However, TA markedly inhibited the increased clearance evident in endotoxin-treated mice. Six hours after melanoma cells were injected, a 92% endotoxin-induced acceleration of clearance was converted to an almost 3-fold decrease. Similarly, by t-24 h, previously increased clearance was replaced by a nearly 4-fold decrease.

In order to determine whether the increased lung retention induced by TA was mediated by direct effects of the agent on melanoma cells rather than by host-related factors, radiolabelled B16 melanoma cells were pre-treated *in vitro* with TA as described and injected into otherwise untreated tumor-bearing hosts. The results in Table II show that lung retention patterns were not different in animals receiving TA-treated or vehicle-treated cells for at least 6 h after tumor cells were injected.

DISCUSSION

In order to develop into metastases, cancer cells liberated from the primary tumor must be arrested elsewhere in the body. The initial arrest of tumor cells will determine the dose delivered to a particular organ but it is the degree of retention of these cells which determines the numbers remaining to develop into covert or overt lesions. Previous studies with rodents (Fidler, 1970; Weiss *et al.*, 1979; Glaves, 1980) have shown that shortly after tail-vein injection of cancer cells, the majority of these become arrested in the lungs. However, this arrest is transient and over the next 24 h as many as 99% of cells initially arrested may be re-released from the pulmonary vasculature. Following release or clearance from the lungs, cancer cells may be arrested in other organs from which the majority are released yet again, and the release process, in common with circulatory trauma, impairs their metastatic capacity, thereby contributing to metastatic inefficiency (Weiss, 1980, 1981). The majority of cells leaving the lungs are probably dead (Weiss, 1980; Glaves, 1980) and the chance of extra-pulmonary tumor formation is therefore low. Thus, release of arrested cancer cells from the vascular endothelium may in itself represent a host defense reaction.

Previous work (Glaves, 1980) has shown that stimulation of reticuloendothelial system (RES) activity by administration of endotoxin markedly enhances the release or clearance of arrested tumor cells from the lungs of tumor-bearing mice. Bacterial endotoxins are well known to affect a number of homeostatic mechanisms, including activation of the coagulation and complement systems, enhancement of fibrinolysis and stimulation of the phagocytic and secretory activities of cellular components of the RES such as macrophages and polymorphs (Bradley, 1979; Westphal, 1975). Glucocorticoid steroids also exert a wide variety of pharmacologic effects, most of which are antagonistic towards the endotoxin-mediated modification already mentioned (Fauci, 1979; Latour and Leger, 1975). It was therefore of interest to determine whether triamcinolone acetonide could also inhibit the endotoxin-induced acceleration of cancer cell clearance from the lungs.

Before discussing the present experiments in detail, it must be emphasized that the data in Table I show that, compared with the overall controls, the vehicles used for administration of endotoxin and TA may have their own effects on retention of radiolabelled melanoma cells, in accordance with previous observations (Weiss and Glaves, 1978). This is especially true in the case of the experiments where TA is administered by the intraperitoneal route suspended in 0.5 ml 20% ethanol in PBS, and both these animals and the vehicle controls must suffer from transient alcohol intoxication. Our discussion will therefore be largely limited to comparisons of results from experimental groups with their own vehicle controls.

Endotoxin treatment alone led to a marked acceleration in clearance of melanoma cells from the lungs. This confirms the results of a previous study which also showed that the final incidence of pulmonary nodules after injection of non-radiolabelled cells was correspondingly decreased after endotoxin treatment (Glaves, 1980). However, triamcinolone acetonide alone, after accommodation for the effects of its vehicle, had the opposite effect to endotoxin and increased lung retention (Table I). These results are consistent with those of Fidler and Lieber (1972) who also found that pulmonary tumor incidence was significantly increased in steroid-treated mice. In animals treated with both agents, it was found that the stimulatory effects of endotoxin on clearance processes were markedly inhibited by TA treatment even though retention rates were not completely restored to the levels of untreated controls.

Among the actions of endotoxin likely to be relevant to the re-release of arrested cancer cells is the enhanced secretion of proteolytic enzymes from cells of the stimulated RES including macrophages, polymorphs, etc., and also the vascular endothelium (Davies and Bonney, 1979; Goldstein, 1976) since lysomal activation is known to facilitate cell detachment *in vitro* (Weiss, 1965, 1977). If these lysosomal enzymes are the principal factors involved, then antagonism of cancer cell release by TA would be expected because glucocorticoids are classical lysosomal stabilizing agents (Weissman, 1969) and, in addition, a similar glucocorticoid has been shown to inhibit lysosome-mediated cell detachment *in vitro* (Weiss, 1965).

Also included in the array of proteolytic enzymes released by the RES upon bacterial endotoxin stimulation are those involved in fibrinolysis, especially plasminogen activator (Gordon *et al.*, 1974; Cohn, 1978). Although the initial arrest of cancer cells at the vascular endothelium does not involve blood-clotting mechanisms (Glaves and Weiss, 1978), the temporary deposition of fibrin around arrested cancer cells (Chew *et al.*, 1976; Warren, 1973) ap-

pears to stabilize this process. Therefore, an endotoxin-mediated increase in the local release of fibrinolysins is expected to enhance the rate of secondary release of tumor cells. Glucocorticoids, which are known to inhibit plasminogen activation in both phagocytes (Vassalli *et al.*, 1976) and vascular endothelium (Shepro *et al.*, 1980) are thus expected to at least partially negate the endotoxin-mediated increase in release. It is possible that the RES may also participate in clearance directly, since macrophages (Keller, 1976) and polymorphs (Takasugi *et al.*, 1975) exhibit contact-mediated cytotoxicity for malignant cells. Also, macrophage cytolysis is potentiated by *in vivo* (Ruco and Meltzer, 1978) and *in vitro* (Sone and Fidler, 1980) treatment with bacterial endotoxins. Conversely, glucocorticoids have been shown to suppress such cytotoxicity *in vitro* (Gallily and Eliahu, 1976) and macrophages from stressed mice exhibit depressed cytotoxic potential (Schultz *et al.*, 1979) which the authors attributed to elevated levels of circulating glucocorticoids.

A contribution of enzyme activity associated with tumor cells themselves to their own release is unlikely in the present system, because the results in Table II show that pre-treatment of melanoma cells with TA *in vitro,* prior to injection into otherwise untreated mice, has no influence on retention, at least over the initial 6-h period when treatment of the host with TA leads to increased lung retention (Table I). However, *in vitro,* endotoxin facilitates the detachment of L929 fibroblasts and Ehrlich ascites carcinoma cells from protein-coated glass (Neiders and Weiss, 1973) and hydrocortisone inhibits this effect (Weiss, 1978).

Many endotoxin-induced interactions are mediated by fibrinolysins and lysosomal enzymes. Therefore, although the interactions of both agents used in the present studies are complex, as discussed, we have emphasized the role of these enzymes as the final mechanisms in the endotoxin-enhanced clearance of arrested cancer cells from the pulmonary vasculature. This non-exclusive hypothesis is supported by the triamcinolone acetonide-inhibition of the effects of endotoxin reported here; the reduced incidence of metastasis reported in fibrinolysin-treated animals by some investigators as critically reviewed by Weiss (1981) and by the enhanced cell detachment produced by lysosomal activation *in vitro* and the inhibition of these effects by hydrocortisone (Weiss, 1965, 1980; Neiders and Weiss, 1973).

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