

ACTIVATED MACROPHAGES RELEASE A FACTOR WHICH
LYSES MALIGNANT CELLS BUT NOT NORMAL CELLS*

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When peritoneal exudate macrophages are activated by a variety of stimuli they acquire the capacity to inhibit the growth of tumor cells. Such activated macrophages can be obtained by the addition of agents such as endotoxin or double-stranded RNA to normal unsensitized macrophages in vitro (1). They can also be obtained from animals carrying chronic infections (2), by the addition of specific antigen to macrophages obtained from suitably sensitized mice (3) or by the addition of lymphokines to normal peritoneal exudate cells (4). The toxic effects of such activated macrophages show no immunological specificity but appear to show selectivity for transformed cells, although the mode of target cell damage detected by Hibbs (5) (lysis, stasis, or detachment) was unclear. This apparent selectivity for transformed cells may not be absolute in that lysis of normal erythrocytes by macrophages has also been described (6). Embryonic cells may also be damaged by activated macrophages but only after prolonged tissue culture and their susceptibility seems to be associated with spontaneous in vitro transformation (7). However in other studies (8) the lysis of apparently untransformed embryo cells was detected. The apparent selectivity for transformed cells described by Hibbs has also been noted in other test systems (4). In the studies of Alexander and Evans (1) and in those of Hibbs and his colleagues (2) the toxicity of macrophages for transformed target cells appeared to be strictly localized to the region of the macrophages and it was concluded that close cellular apposition is a prerequisite for target cell damage. Both groups were unable to detect toxic activity in supernatant cell-free media. We therefore designed the following experiments to examine the cytotoxic effects of endotoxin-activated rat macrophages on a variety of normal and malignant rat cells and to evaluate the possible role of supernatant macrophage products in any such cytotoxicity.

Materials and Methods

Target Cells. Adherent peritoneal exudate (PE) cells from both Hooded and August rats were tested for cytotoxic activity on the following target cells. ASBP₁ is a benzpyrene-induced sarcoma syngeneic in August rats, HSN, a similar sarcoma syngeneic in Hooded rats, NAK and 2NAK were both cells obtained from normal August adult kidneys and were both of epithelial morphology, and NAD₁ a cell line derived from the diaphragm of a normal adult August rat. All the normal cells used in this study showed contact inhibition, (and were pre-labeled with ¹²⁵I-iodo-

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deoxyuridine [^{125}I UdR] when far from confluent) and all the cells were tested within the first 10 passages in vitro. The target cells were obtained from the normal and malignant tissues by dissociation with 0.1% trypsin plus 0.1% collagenase, and were maintained in culture in RPM1 1640 plus HEPES and 10% fetal bovine serum (FBS) in disposable plastic flasks.

Cytotoxicity Assay. Stock cultures of subconfluent target cells were prelabeled with ^{125}I UdR (ca. 100 mCi/mg, Radiochemical Centre, Amersham, England) at 1 $\mu\text{Ci}/\text{ml}$ in complete medium overnight at 37°C. They were then washed and removed from the flask by exposure to 0.1% trypsin for 5 min. The single cell suspensions obtained were washed, the concentration adjusted, and then they were employed as targets by addition to the wells of microplates in 0.1-ml aliquots in complete medium.

PE cells from normal inbred rats of either August or Hooded strains were washed thrice and seeded into the wells of microplates (Falcon 3040; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at a concentration of $5 \times 10^6/\text{ml}$ in 0.1-ml vol. After incubation at 37°C for 2 h the wells were vigorously washed with two changes of medium and then fed with RPM1 1640 containing 20 mM HEPES, antibiotics, and 10% FBS. *Salmonella typhosa* lipopolysaccharide B (Difco Laboratories, Detroit, Mich.) was added at 25 $\mu\text{g}/\text{ml}$ to the wells. This dose was found to be optimal in pilot experiments. The plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. Prelabeled target cells were then added in 0.1-ml vol containing 5×10^3 cells to the wells already containing activated PE cells in 0.1 ml medium. The plates were then incubated and at various time intervals triplicate wells were aspirated, the contents spun at 300 g for 10 min and the supernates subsequently counted in a Packard Autogamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The lysis of target cells was expressed as the percentage of ^{125}I released from the cells, each point representing the mean of triplicate observations. Treatment of the cells with 10% sodium dodecyl sulphate for 10 min indicated that the total releasable ^{125}I was always greater than 95%. It is unlikely that the presence of macrophages significantly affected the detection of released ^{125}I since Evans (9) has shown that in a similar system negligible ^{125}I was associated with macrophages after a 24 h test period. Morphological examination of the target cells indicated that the release of ^{125}I was associated with their overt destruction. Quantitative cell counts performed on the target cells gave similar results to those obtained using ^{125}I release.

Results and Discussion

Endotoxin-treated adherent PE cells from normal adult August rats produced significant lysis of ASBP₁ and HSN cells but had no effect on NAK and NAD cells (see Fig. 1). The lytic effect was usually detectable at 18 h and rose progressively thereafter. Hooded PE cells, endotoxin treated, similarly lysed the ASBP₁ tumor cells but had no lytic effect on 2NAK cells. Macrophages from both Hooded and August rats, without treatment with endotoxin, produced lytic effects on the malignant cells (but not the normal cells). (A typical result is shown in Table I.) This was substantially less than that produced after endotoxin treatment and this result resembles those of Meltzer and his colleagues (10). Alexander and Evans (1) demonstrated that their activated PE cell monolayers contained 99% macrophages. We tested the cytotoxicity of our PE cell monolayers on ASBP₁ sarcoma cells before and after extensive washing and although the number of cells other than macrophages judged by morphological criteria was reduced by the washing to less than 0.1%, the cytotoxic activity of the monolayers was retained, indicating that the activated effector cells are macrophages.

Having demonstrated selective (but not immunologically specific) lysis by endotoxin-treated macrophages we went on to examine the effects of supernatant cell-free media from such cultures on the various target cells. In this system the target cells were prelabeled as before but after washing were seeded into

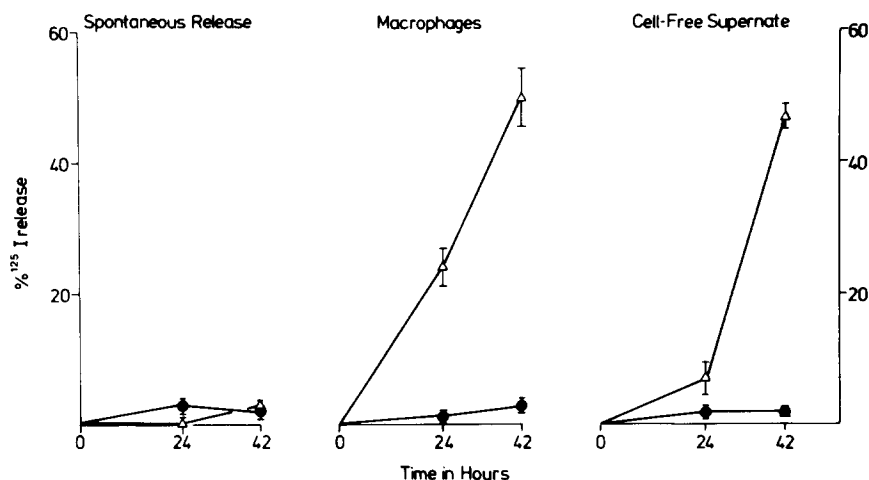


FIG. 1. Release of ^{125}I from ASBP₁ sarcoma and normal August kidney (NAK₁) cells after addition to control media, endotoxin-activated syngeneic macrophages, or a 24 h supernate from such macrophages. The results show the mean percentage of ^{125}I released from the cells. The spontaneous release of ^{125}I from the target cells was extremely low under these assay conditions. (●), NAK₁ cells; and (Δ), ASBP₁ cells.

TABLE I
Lytic Activity of Unstimulated and Endotoxin-Treated Macrophages on Normal and Malignant Syngeneic Rat Target Cells

Effector cells tested	^{125}I released from the cells after 42 h	
	NAK ₁ (normal)	ASBP ₁ (sarcoma)
Nil (i.e., spontaneous release)	3.8 ± 0.4	4.6 ± 0.5
Untreated macrophages	4.9 ± 0.4	22.3 ± 1.1
Macrophages + 25 μg/ml endotoxin	4.6 ± 0.3	54.2 ± 2.3

Falcon no. 3040 microplates at 5×10^3 /well in 0.1 ml of complete medium directly into wells already containing 0.1 ml of the supernatant medium freshly taken from endotoxin-activated 24-h adherent PE cell cultures. These media were taken from the cultures of macrophages in the wells of Falcon no. 3040 plates as described above. These supernates were centrifuged at 300 g for 10 min before use and were cell free. Control media consisted of 24-h-aged medium only, with or without endotoxin. The plates were then incubated and the media aspirated at different time points, spun, and counted as before.

The 24-h supernatant medium collected from endotoxin-treated August PE cells, cultured in Falcon no. 3040 microplate wells, produced significant lysis of ASBP₁ and HSN cells but had no effect on NAD or NAK cells. The control media with and without endotoxin had no lytic effects on any target cell tested. Supernates from Hooded PE cells after endotoxin treatment also produced lysis of ASBP₁ cells but not 2NAK. The absence of toxic activity in the control supernates indicates that the presence of residual or degraded endotoxin cannot be incriminated in the toxic effects. The degree and time-course of the lysis produced by supernates suggests that the lytic activity of the activated macro-

TABLE II
Some Properties of the Selective Cytolytic Supernate from Endotoxin-Activated August Rat Macrophages Produced in the Absence of Serum

Treatment of Supernate	Test Conditions	¹²⁵ I released from ASBP ₁ sarcoma cells at 42 h
		%
Fresh untreated	3% FBS	48.0 ± 2.3
Fresh untreated	10% FBS	46.0 ± 2.1
Fresh untreated	33.3% FBS	8.5 ± 0.5
Fresh untreated	10% FBS	47.5 ± 1.1
Heated 56°C for 30 min	" "	1.5 ± 0.5
Stored 7 days at -20°C	" "	13.0 ± 1.0
Stored 24 h at 4°C	" "	33.0 ± 2.2
Diluted serially in serum-free medium		
1:2	" "	45.1 ± 1.5
1:8	" "	23.5 ± 1.2
1:32	" "	0

phages may be wholly attributable to the supernatant lytic material. The lytic factor was also found to be active when added after attachment of the target tumor cells to the plastic substratum.

When the supernate from adult August macrophages was produced in the absence of added FBS, its lytic activity was increased, a finding which suggests that serum may have some inhibitory effect on the lytic factor. The test phase of the assay does, however, necessitate the presence of calf serum since the target cells will not survive without it. A batch of lytic supernate was therefore produced in the absence of FBS and tested on prelabeled ASBP₁ cells in medium containing either 3, 10, or 33.3% FBS. The lysis obtained after 42 h incubation is shown in Table II. As these results indicate, the lytic activity of the supernate was detectable in 3 and 10% serum but was greatly reduced when the serum concentration was raised to 33.3%. Attempts to store supernatant media from endotoxin-treated macrophages indicated that the lytic activity is labile. Endotoxin by itself had no lytic effect on the malignant target cells. However, it could be argued that the endotoxin is degraded by exposure to macrophages and that a lytic endotoxin fraction is responsible for the effects seen. Brailowsky et al. (11) have indeed claimed that bacterial glycolipids of short carbohydrate chain length show some toxic effects on transformed cells. However, they showed that the effect of these glycolipids was growth inhibitory, had no lytic activity, and had no effect on the plating efficiency of the cells. We are currently examining modes of activation other than bacterial endotoxin.

The failure to detect a toxic supernatant factor by Alexander and Evans (1) and by Hibbs and his colleagues (2), among others, can be accounted for by the lability of the lytic factor, its inhibition by serum, and by the many variables involved in the assay materials and techniques employed.

Many factors have been detected in the supernatant medium from macrophages and can be arbitrarily classified into three different categories: (a) Factors which are lytic; (b) those which inhibit cell proliferation; and (c) those which inhibit the incorporation of labeled DNA precursors, i.e. free thymidine (12). In the first category, that with the most relevance to the present study, the lysis of mammalian cells, bacteria, and erythrocytes have been documented.

Bast and his colleagues (13) have shown that immunologically activated macrophages release a heat-labile factor which kills *Listeria monocytogenes*. Melsom and his co-workers (6) have described a labile factor released from macrophages which will lyse syngeneic and allogeneic erythrocytes. However, when the supernatant factor from our activated August rat macrophages was tested for its ability to lyse syngeneic and allogeneic erythrocytes we were unable to duplicate these observations. An immunologically specific macrophage cytotoxin has been described by McIvor and Weiser (14). The tumor-directed lytic factor detected in our assay, having no such immunological specificity, is clearly unrelated to this specific cytotoxin. However its activity does resemble the factor described by Meltzer and Bartlett (15), which is released from spleen cells specifically stimulated with purified protein derivative of tuberculin.

The biological basis of the apparent tumor selectivity of the macrophage product is unclear. There would seem to be two alternative explanations. The macrophage factor may be in some way an inhibitor of cell growth, unable to distinguish between normal and malignant cells and the subsequent apparently selective lysis of the malignant cells may merely be a reflection of their intrinsic inability to withstand prolonged cytostasis. This sort of explanation would deny any selective recognition process occurring at the surface of the malignant cells. Selective recognition of characteristic features of the malignant cell surface by a macrophage product (say, an enzyme) would represent the major alternative hypothesis.

Summary

When rat macrophages are activated by exposure to bacterial endotoxin they acquire the capacity to lyse sarcoma cells in tissue culture. Although showing no immunological specificity this lytic effect was only detectable on malignant cells; normal cells were unaffected. This tumor-selective lytic effect is mediated by a labile supernatant product.

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