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Comparison of cytotoxic and microbicidal function of bronchoalveolar and peritoneal macrophages

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Summary. Studies were carried out with mice to explore *in vitro* the effector function(s) of macrophages from two different anatomical compartments (peritoneal cavity and lungs). The cytotoxic capacity of macrophages was measured by determining their cytostatic and cytocidal effects on EL-4 tumour target cells, and the microbicidal capacity of macrophages was measured by determining their ability to kill or inhibit the intracellular protozoan, *Toxoplasma gondii*. Neither peritoneal macrophages (PM) nor bronchoalveolar macrophages (BAM) from normal mice were ever microbicidal or cytotoxic. Intravenous treatment with *Corynebacterium parvum* greatly enhanced (activated) both effector functions of PM but did not activate BAM. Chronic infection with Toxoplasma activated PM throughout the period of observation (> 140 days), but the presence of activated BAM was transient and appeared to coincide with the occurrence of an inflammatory response in the lungs.

Abbreviations: PM, peritoneal macrophages; BAM, bronchoalveolar macrophages; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's medium; FCS, foetal calf serum; CI, cytostatic index; and c.p.m. counts per minute.

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INTRODUCTION

Murine peritoneal macrophages (PM) are commonly employed in *in vitro* studies of macrophage effector function. The microbicidal function of PM (Mackaness, 1969; Remington, Krahenbuhl & Mendenhall, 1972) and their cytotoxic capacity for tumour cells (Evans & Alexander, 1970; Hibbs, Lambert & Remington, 1972; Olivotto & Bomford, 1974; Remington, Krahenbuhl & Hibbs, 1975) have been demonstrated in a variety of experimental systems. However, both of these effector functions do not necessarily co-exist in the same population of PM (Wing, Gardner, Ryning & Remington, 1977).

In a previous study from our laboratory (Ryning & Remington, 1977), mice chronically infected with *Toxoplasma gondii* were employed to compare the microbicidal capacity of PM with that of macrophages from a different anatomical compartment (bronchoalveolar macrophages [BAM]). The present report extends this study to include the cytotoxic as well as microbicidal function of PM and BAM and examines these effector functions in normal, Toxoplasma infected, and *Corynebacterium parvum* treated mice.

MATERIALS AND METHODS

Mice

Female Swiss Webster mice (Simonsen Laboratories, Gilroy, Calif.) weighed 20–24 g at the start of each experiment.

Toxoplasma infection

The relatively avirulent C56 strain of *T. gondii* was employed to establish chronic infection in mice by procedures described previously (Gentry & Remington, 1971). Briefly, Toxoplasma tachyzoites were obtained from the peritoneal fluid of mice infected 7 days previously with brain tissue containing cysts of the C56 strain of Toxoplasma. Mice were injected intraperitoneally with 1×10^5 tachyzoites and, beginning 3 days later, were treated for 21 days with sulfadiazine in their drinking water.

C. parvum *treatment*

Mice were injected intravenously with 1400 μg of killed *C. parvum* (Lot No. CA 380; Wellcome Laboratories, Research Triangle Park, N.C.).

Macrophages

PM were obtained from normal, infected, or treated mice as described previously (Ruskin & Remington, 1968). BAM were harvested using the procedures described by Hunninghake & Fauci (1976). Briefly, the lungs were removed from normal, infected, or treated mice and were pooled and minced in heparinized (10 u/ml) Hanks's balanced-salt solution (HBSS). The tissue fragments were pressed through a stainless steel screen (80 mesh) and the screen was washed with 10 ml of HBSS. The cell suspension was passed through a syringe containing two layers of sterile gauze to remove gross tissue fragments. Mononuclear cells were obtained from this cell suspension by Ficoll–Hypaque density centrifugation as described previously (Böyum, 1968). PM and BAM were washed twice with warm (37°) HBSS, resuspended in Dulbecco's medium (Grand Island Biological Co. [GIBCO], Berkeley, Calif.) containing 10% foetal calf serum (DMEM-10% FCS) and antibiotics (100 u/ml penicillin; 100 μg/ml streptomycin). The cells were counted in a hemacytometer, viability was determined by trypan blue exclusion, and cells were diluted to the appropriate concentration.

Microbicidal capacity of macrophage assay

To determine the microbicidal capacity of PM or BAM for Toxoplasma, monolayers were prepared by seeding a 0·05 ml drop of the appropriate cell suspension (5×10^6 cells/ml) into the centre of each chamber of two-chambered tissue culture slides (Lab–Tek Products, Naperville, Ill). After 1 hr of incubation to allow for cell adherence, each chamber was flooded with 1·0 ml warm DMEM-10% FCS and re-incubated. After an additional 2 hr, non-adherent cells were removed by washing, and the monolayers were challenged with a 1·0 ml suspension of RH strain Toxoplasma (5×10^5 tachyzoites/ml in the case of PM; 5×10^6 tachyzoites/ml in the case of BAM) as described previously (Ryning & Remington, 1977). After 60 min, extracellular organisms were removed by washing, sample slides were fixed (0·4% aminoacridine hydrochloride in 50% ethanol), and the remaining monolayers were replenished with 2·0 ml medium and fixed 18 hr later. After the monolayers were stained with Giemsa stain, the percent infected cells and number of intracellular organisms were determined.

Cytostatic and cytocidal capacity of macrophages

The cytostatic and cytocidal capacity of PM or BAM was determined by measuring their ability to inhibit ^{3}H-TdR uptake by tumour cells (cytostasis) or to effect the release of ^{51}Cr from pre-labelled tumour target cells (cytocidal capacity). Monolayers were prepared by seeding 0·1 ml of the appropriate cell concentration (1×10^6 for cytostasis; 4, 8, or 16×10^5 for cytocidal assays) into the wells of microtitre plates (96-well, flat-bottom; Falcon Plastics Inc., Los Angeles, Calif.). After 3 hr of incubation at 37°, non-adherent cells were removed by washing with warm (37°) HBSS, and adherent cells were replenished with 0·1 ml DMEM-10% FCS and challenged with 0·1 ml containing 10^4 EL-4 lymphoma cells (unlabelled in the case of the cytostasis assay; pre-labelled with ^{51}Cr for measuring cytocidal effects).

^{3}H-TdR uptake

Eighteen hours after incubation of PM or BAM monolayers with 10^4 EL-4 cells, 1·0 μCi ^{3}H-TdR (6 Ci/mM; Schwartz-Mann, Orangeburg, N.Y.) in 0·01 ml was added to each well. After an additional 6 hr of incubation, the cultures were processed with a MASH II automated sample harvester (Microbiological Associates, Bethesda, Md) and ^{3}H-TdR uptake was quantitated in a liquid scintillation counter as previously described (Gardner & Remington, 1978). Inhibition of ^{3}H-TdR by PM or BAM from *C. parvum*

treated or Toxoplasma infected mice was quantified by calculating the cytostatic index (*CI*) as follows:

$$CI = \frac{(N-E) \times 100}{N}$$

where $N = {}^3$H-TdR uptake (c.p.m.) by PM or BAM from control (normal) mice and E = c.p.m. in the presence of PM or BAM from treated or infected (experimental) mice.

^{51}Cr release

The assay for *in vitro* release of ^{51}Cr from target cells was adapted from the methods of Cerottini & Brunner (1971). Briefly, 5×10^6 EL-4 target cells were centrifuged at 150 ***g*** and resuspended in 1·0 ml tris phosphate buffered saline, pH 7·4, containing 5% FCS. One hundred microcuries of ^{51}Cr sodium chromate (specific activity 100–300 Ci/mM; New England Nuclear, Gardena, Calif.) were added, and the cell suspension was incubated for 30 min at 37° with gentle shaking every 5 min. The cells were then washed five times, centrifuged at 140 ***g***, and resuspended in 10 ml DMEM-10% FCS, and their concentration was adjusted to 1×10^5 viable (trypan blue exclusion) cells/ml DMEM-10% FCS. At the time of challenge of PM or BAM, 0·1 ml of pre-labelled EL-4 (1×10^4) cells was added to the appropriate wells. Release of ^{51}Cr was measured at 24 hr, and percentage release was measured according to the following formula:

$$\% \text{ release} = \frac{E-S}{R-S} \times 100$$

where E = experimental amount of ^{51}Cr released from cultures of EL-4 target cells cultured in the presence of macrophages (PM or BAM) from normal or Toxoplasma infected mice, $S = {}^{51}$Cr released spontaneously from cultures of EL-4 cells alone, and R = amount of ^{51}Cr released from EL-4 cells suspended for 6 hr in distilled water.

Preparations of lung tissue for histological examination

The lungs of normal mice, mice treated with *C. parvum*, and mice chronically infected with *T. gondii* were inflated with buffered formalin, and the tracheas were tied with the lungs fully distended. The lungs were immersed in buffered formalin for 24 hr before sectioning and staining with either Giemsa stain or hematoxylin and eosin.

RESULTS

In vitro microbicidal and cytostatic activity of BAM and PM at various time intervals after *C. parvum* administration

Neither BAM nor PM from normal mice were microbicidal for Toxoplasma (Table 1); intracellular growth of the organism was unrestricted. PM from *C. parvum* treated mice were markedly microbicidal for Toxoplasma (Table 1). At time zero, 68% ± 3% of PM from treated mice contained intracellular Toxoplasma, whereas 18 hr later there was not only a substantial reduction in the number of infected cells with no significant decrease in cell density of the monolayer but there was also a marked inhibition of multiplication of Toxoplasma. Many of these Toxoplasma were undergoing obvious degeneration as shown by loss of cellular definition and staining characteristics. In contrast to the microbicidal capacity of PM from *C. parvum* treated mice, BAM from the same animals did not

Table 1. *In vitro* microbicidal activity of bronchoalveolar and peritoneal macrophages from mice treated with *Corynebacterium parvum*

Group†	Macrophages	Microbicidal effect* 0 hr	12 hr	18 hr
Normal	bronchoalveolar	1·0 (27 ± 4)	1·4 (20 ± 2)	4·5 ± 0·3 (21 ± 4)
C. parvum treated	bronchoalveolar	1·0 (24 ± 3)	1·2 (24 ± 3)	4·3 ± 0·2 (26 ± 2)
Normal	peritoneal	1·0 (54 ± 2)	1·3 (56 ± 4)	5·5 ± 0·3 (60 ± 3)
C. parvum treated	peritoneal	1·0 (68 ± 3)	1·1 (26 ± 4)	1·2 ± 0·1 (22 ± 3)

* Mean number of Toxoplasma per vacuole of triplicate macrophage monolayers fixed 0, 12, and 18 hr after infection. Numbers in parentheses = % infected cells ± SEM.

† Treated mice injected intravenously with 1400 μg *C. parvum* 7 days previously.

inhibit the multiplication of Toxoplasma. Toxoplasma multiplied in cytoplasmic vacuoles of BAM to an extent which did not differ significantly from the number of Toxoplasma per vacuole in normal BAM. The initial infection rates of BAM from normal and treated mice were comparable, and there was no reduction in the percentage of infected cells at 18 hr of incubation in either group (Table 1).

When the cytostatic effects of these different macrophage populations were examined, PM from treated mice markedly inhibited ^{3}H-TdR uptake by EL-4 tumour target cells when tested 7, 10, and 14 days after *C. parvum* treatment as shown by cytostatic indices of 96, 99, and 96, respectively (Table 2). In contrast, BAM from the same treated mice were not cytostatic at a comparable mononuclear cell to tumour cell ratio of 100:1. Examination of the microbicidal capacity of macrophages from the peritoneal cavity and lungs in parallel yielded results similar to those seen above. PM from treated mice were capable of killing or inhibiting the multiplication of Toxoplasma 7 and 14 days after *C. parvum* administration. No evidence of microbicidal activity of BAM was observed at either 7 or 14 days after *C. parvum* treatment (Table 2).

The importance of the macrophage to tumour cell ratio in demonstrating cytostatic macrophages has been well documented (Krahenbuhl & Lambert, 1975). Our inability to demonstrate a cytostatic effect for BAM from treated mice at a ratio of 100 macrophages per tumour cell may have reflected an insufficient macrophage to tumour cell ratio. As shown by the results in Table 3, a ten-fold increase in the number of mononuclear cells per tumour cell did not result in cytostasis (Table 3).

Table 2. Effects of time elapsed since intravenous treatment with *Corynebacterium parvum* on *in vitro* cytostatic and microbicidal activity of bronchoalveolar and peritoneal macrophages

Group	Macrophages	Cytostatic index*	Microbicidal effect
Normal	bronchoalveolar	—	3·6±0·1†
C. parvum treated —7 days	bronchoalveolar	0	5·2±0·3
C. parvum treated —10 days	bronchoalveolar	3	ND‡
C. parvum treated —14 days	bronchoalveolar	1	4·2±0·2
Normal	peritoneal	—	5·6±0·3
C. parvum treated —7 days	peritoneal	96	1·4±0·2
C. parvum treated —10 days	peritoneal	99	ND
C. parvum treated —14 days	peritoneal	96	2·2±0·1

* Effector to target cell ratio = 100 mononuclear cells to one tumour cell.
† Mean number ± SEM of Toxoplasma per vacuole of triplicate monolayers fixed 18 hr after challenge.
‡ ND = not done.

Table 3. Effects of mononuclear cell to tumour target cell ratio on *in vitro* cytotoxicity of bronchoalveolar and peritoneal macrophages from mice 7 and 14 days after intravenous treatment with *Corynebacterium parvum*

Macrophages	Mononuclear cell: tumour target cell ratio	Day after *C. parvum* treatment 7	14
Bronchoalveolar	100:1	0*	2
	500:1	2	1
	1000:1	1	3
Peritoneal	100:1	99	98

* Cytostatic index.

In vitro cytostatic and microbicidal activity of BAM and PM from mice chronically infected with Toxoplasma

At all time periods tested, PM from Toxoplasma infected mice were both cytostatic for EL-4 target cells and microbicidal for Toxoplasma (Table 4). In contrast, BAM with cytostatic and microbicidal activity made a transient appearance in the lungs. Thirty days after Toxoplasma infection, BAM supported multiplication of Toxoplasma and failed to prevent the uptake of ^{3}H-TdR by EL-4 tumour target cells. In contrast, after infection with Toxoplasma for 60 and 70 days, BAM were cytostatic and were capable of inhibiting the multiplication of Toxoplasma (Table 4). By 140 days of chronic Toxoplasma infection, BAM were neither cytostatic nor microbicidal.

Cytocidal effects of BAM and PM from mice chronically infected with Toxoplasma

Cytocidal macrophages from the lungs of infected mice were detected only at 45 days after infection and only at a very high mononuclear cell to tumour cell ratio of 160:1. Parallel studies of the microbicidal capacity of BAM showed that this effect on function was also present only at 45 days of infection. In contrast, PM from infected mice were cytocidal at all periods tested at a mononuclear cell to tumour target cell ratio of 40:1. The need for the high ratio of mononuclear cells to tumour cells before cytocidal BAM were detected probably reflects the large number of non-adherent cells present in the lung at this time as demonstrated in previous experiments (Ryning & Remington, 1977). Despite the use of cell numbers that were comparable with those used at 45 days of Toxoplasma infection, BAM at 26 and 102 days were not cytocidal compared to PM (Table 5).

Effect of chronic Toxoplasma infection and *C. parvum* administration on lung architecture related to time after infection or *C. parvum* treatment

The time-course appearance of cytotoxic and microbicidal BAM from Toxoplasma infected mice coincided with a marked mononuclear cell infiltrate in the lungs. There were a number of small nodules grossly visible on the pleural surface of the lung. On histological examination, these nodules were composed of mononuclear cells with dark-staining nuclei occasionally intermingled with larger mononuclear cells with vacuolated cytoplasm. These mononuclear cell aggregates were observed throughout the lung parenchyma, around bronchi, and around blood vessels. In addition, there was an interstitial infiltrate of mononuclear cells and many more intra-alveolar cells were present than in uninfected mice.

No evidence of gross or histological alteration of lung architecture was noted in mice that received *C. parvum* except for modest hyperplasia of lymphoid tissue around bronchi.

Table 4. Effects of time elapsed since initiation of chronic Toxoplasma infection on *in vitro* cytostatic and microbicidal activity of bronchoalveolar and peritoneal macrophages

Macrophages	Days after infection	Cytostatic index*	Microbicidal effect†	
			Infected	Control‡
Bronchoalveolar	30	−5	3·4±0·2	3·7±0·2
	60	98	1·1±0·2	3·6±0·3
	70	96	1·3±0·3	4·1±0·2
	140	2	3·6±0·1	4·4±0·3
Peritoneal	30	99	1·1±0·2	5·6±0·3
	60	98	1·1±0·1	5·4±0·2
	70	96	1·2±0·3	5·2±0·2
	140	94	1·1±0·1	4·8±0·1

* Effector to target cell ratio = 100 mononuclear cells to one tumour cell.

† Mean number ± SEM of Toxoplasma per vacuole of triplicate macrophage monolayers fixed 0, 12, and 18 hr after infection.

‡ Age-matched controls.

Table 5. Effects of time elapsed since initiation of chronic Toxoplasma infection on *in vitro* cytotoxic and microbicidal activity of bronchoalveolar and peritoneal macrophages

Group	Time since infection (days)	Macrophages	Cytocidal effect % 40:1*	80:1	160:1	Microbicidal effect
Normal		bronchoalveolar	+1·2†	−2·0	−2·2	4·2±0·3‡
Normal		peritoneal	+1·7	+1·0	ND§	6·8±0·3
Infected	26	bronchoalveolar	−1·0	−0·4	−2·2	4·8±0·2
Infected	26	peritoneal	+31·9	+29·2	ND	1·2±0·2
Normal		bronchoalveolar	ND	ND	−2·5	4·8±0·4
Normal		peritoneal	−3·1	−2·4	ND	6·2±0·4
Infected	45	bronchoalveolar	−0·6	+4·2	+25·4	1·5±0·1
Infected	45	peritoneal	+25·7	+25·9	ND	1·0±0·1
Normal		bronchoalveolar	ND	ND	−8·7	6·4±0·3
Normal		peritoneal	−10·7	−11·1	−7·9	7·3±0·3
Infected	102	bronchoalveolar	−7·0	−4·9	−7·8	6·2±0·2
Infected	102	peritoneal	+20·6	+27·4	+27·8	1·8±0·3

* Effector cell to tumour target cell ratio.
† % specific ^{51}Cr release.
‡ Mean number ± SEM of Toxoplasma per vacuole of triplicate macrophage monolayers fixed 18 hr after infection.
§ ND = not done.

DISCUSSION

This study has shown a dissociation in the effects of intravenous *C. parvum* administration on macrophages from different body compartments. Intravenous administration of *C. parvum* resulted in a population of activated PM which was both cytotoxic for tumour target cells and microbicidal for *T. gondii*. In contrast, lung macrophages from the same mice were neither cytotoxic for tumour cells nor microbicidal for Toxoplasma.

The effects of *C. parvum* administration on lung macrophages differed markedly from the effect of chronic Toxoplasma infection on these macrophages. In contrast to the inability of *C. parvum* to activate lung macrophages, infection of mice with *T. gondii* resulted in the transient appearance of lung macrophages with both cytotoxic and microbicidal activity.

The results of the present report differ from those of Olivotto & Bomford (1974), who demonstrated apparent *in vitro* inhibition of tumour cell growth and DNA synthesis by lung macrophages from mice injected intravenously with *C. parvum*. In their study, maximum *in vitro* cytotoxicity for RI leukemia cells occurred at 14 days after intravenous injection of *C. parvum* and coincided with the appearance of a histiocytic infiltrate in the lungs. In the present study, the only histological abnormality which we observed following *C. parvum* treatment consisted of peribronchial hyperplasia. The diversity of findings between our study and those of Olivotto & Bomford could be due to factors such as mouse strain, tumour target cell, or lot of *C. parvum* employed.

Pertinent to the inability of *C. parvum* to activate macrophages in all body compartments were the findings of Swartzberg, Krahenbuhl & Remington (1975) who found that mice treated with *C. parvum* intravenously were not protected from an intravenous challenge with a virulent strain of *T. gondii*, despite the presence of PM that were highly activated to kill this strain of *T. gondii*. As shown in the present report, the inability of *C. parvum* treatment to activate BAM to kill *T. gondii* might establish the lung as a sanctuary for uninhibited multiplication of Toxoplasma and possibly explain the *in vivo* susceptibility of *C. parvum* treated mice to Toxoplasma.

Unlike the inability of *C. parvum* treatment to protect mice against a lethal challenge inoculum of virulent Toxoplasma strain, chronic infection of mice with this parasite affords protection when the mice are challenged with an identical inoculum (Swartzberg *et al.*, 1975). As Toxoplasma infection of the mouse in-

volves virtually every organ (Ito, Tsunoda & Suzuki, 1967), host defenses must be operative in all body compartments. Recent *in vitro* studies suggest that the activated macrophage with microbicidal activity might be an important factor in host resistance to Toxoplasma (Remington *et al.*, 1972). In the present study, we have shown that macrophages with microbicidal as well as cytotoxic activity are present in the lung and peritoneal cavity, thus expanding the results of our previous study (Ryning & Remington, 1977). However, in contrast to the peritoneal cavity where activated macrophages persist indefinitely during chronic Toxoplasma infection, there is a transient appearance of activated macrophages in the lungs. Their transient appearance in the lung, in contrast to the peritoneal cavity, may reflect inherent differences in the process of macrophage activation in the cell populations of these compartments or may be due to differences in the local macrophage populations themselves. Furthermore, there may be a persistent stimulus for activation (Toxoplasma antigen, T lymphocytes, or both) in the peritoneal cavity in contrast to the lungs.

In conclusion, the mouse has provided a useful model to examine the effects of immunostimulants and chronic intracellular parasite infections on lung macrophages. The demonstration that the effect of an immunostimulant is not operative in all body compartments may have important implications for the design of cancer treatment protocols in which immunostimulants might be expected to provide a significant contribution. The fact that chronic infection with Toxoplasma results in the transient appearance of macrophages with cytotoxic and microbicidal activity might provide a model for studying the role of different host and antigenic components in creating the stimulus necessary to activate macrophages in the lungs.

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