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Effects of *Corynebacterium parvum* treatment and *Toxoplasma gondii* infection on macrophage-mediated cytostasis of tumour target cells

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Summary. Injection of mice with *Corynebacterium parvum* or living or killed *Toxoplasma gondii* was studied to determine the efficacy of these treatments in activating peritoneal macrophages to inhibit the uptake of [^{3}H]TdR (cytostasis) by tumour target cells *in vitro*. In the presence of activated macrophages from mice treated i.p. with a wide dose range of either *C. parvum* or living *Toxoplasma*, cytostasis was usually greater than 99 per cent. This population of activated macrophages was transient in *C. parvum*-treated mice, but persists, probably for life, in *Toxoplasma*-infected mice. Whereas the i.p. route of administration of *C. parvum* was more efficient in activating macrophages than the i.v. route, the s.c. route appeared to be relatively ineffective. Treatment with killed *Toxoplasma* by any route was also relatively ineffective in activating macrophages. In contrast *Toxoplasma* infection resulted in highly activated peritoneal macrophages, regardless of the route of administration. Depending upon the route of initial treatment, the route of readministration of *C. parvum* had either no appreciable effect or resulted in a marked alteration in the cytostatic capacity of peritoneal macrophages.

INTRODUCTION

Treatment with killed *C. parvum* has been shown to induce anti-tumour activity in experimental animals (Woodruff & Boak, 1966; Halpern, Biozzi, Stiffel & Mouton, 1966) and man (Israel & Edelstein, 1974). One of the effector mechanisms of this anti-tumour effect may be activated macrophages, which, *in vitro*, have been shown to be nonspecifically cytotoxic to tumour target cells. Depending on the methods employed, these cytotoxic properties can be expressed as a killing effect (cytocidal) (Lohmann-Matthes, Schipper & Fischer, 1972) or as a marked inhibition of incorporation of radio-labelled thymidine into the DNA of tumour target cells (cytostasis) (Olivotto & Bomford, 1974; Ghaffar, Cullen, Dunbar & Woodruff, 1974; Scott, 1974a,b; Krahenbuhl & Remington, 1974). The present studies were designed to determine the duration of effect of the cytostatic capacity of activated macrophages and whether the degree of inhibition varies with the dose and route of administration of *C. parvum*. We have previously shown that *T. gondii* infection in mice also results in a population of activated peritoneal macrophages cytostatic to tumour target cells (Krahenbuhl & Remington, 1974; Remington, Krahenbuhl & Hibbs, 1975; Krahenbuhl & Lambert, 1975). Macrophages from *T. gondii*-infected mice were compared with those from mice inoculated with *C. parvum* to determine the efficacy of these treatments in producing macrophages capable of inhibiting tritium-labelled thymidine ([^{3}H]TdR) uptake by tumour target cells.

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MATERIALS AND METHODS

Mice

Outbred mice of the Swiss Webster strain were obtained from Simonsen Laboratories, Incorporated, Gilroy, California. All mice were females and weighed 20–25 g at the beginning of each experiment.

C. parvum *treatment*

A suspension of killed *Corynebacterium parvum* (Wellcome Research Laboratories, Beckenham, Kent, batch PX 425, 7 mg/ml dry weight) was employed. Dilutions of the stock suspension were prepared in isotonic saline. Depending upon the experiment, 0·2 ml of *C. parvum* was injected i.p., i.v. or s.c. in the left rear flank. In our experience and that of others (Scott, 1974a) i.v. administration of 0·2 ml stock *C. parvum* suspension (1400 μg) was occasionally lethal to the mice. Therefore in most experiments 700 μg was administered i.v.

T. gondii *infection*

Chronic *Toxoplasma* infection was established with either of two strains of *T. gondii*—the C-56 strain (intermediate virulence) and the C-37 strain (low virulence). Such chronically-infected mice were protected against a 1000 LD_{100} dose of the virulent RH strain. Living RH strain organisms were also employed as a booster in some studies and in the preparation of large numbers of pure *Toxoplasma* trophozoites for use as a killed antigen. Mice were infected i.p. with 1×10^5 *T. gondii* trophozoites of the C-56 strain and, to establish a chronic infection, were treated with sulphadiazine as previously described (Krahenbuhl & Remington, 1974). When the less virulent C-37 strain was employed; the methods of preparation were as above except treatment with sulphadiazine was not employed. In studies in which a booster dose of living *Toxoplasma* was employed, 1×10^5 trophozoites of the RH strain were administered by the appropriate route.

To prepare killed antigen, RH strain *Toxoplasma* trophozoites were harvested from the peritoneal cavities of mice infected 3 days earlier, separated from host cells by filtration (Remington, Bloomfield, Russell & Robinson, 1970), and killed with formalin (Krahenbuhl, Ruskin & Remington, 1972). Following lyophilization, a suspension of killed *Toxoplasma* was prepared in saline to consist of 7 mg (dry weight)/ml.

Macrophages

Mouse peritoneal cells (PC) were harvested from the unstimulated peritoneal cavity of normal or experimental mice. The PC were washed, counted, and resuspended in medium 199 (M199; Grand Island Biological Corporation, Berkeley, California) containing 20 per cent foetal calf serum (FCS) and antibiotics. 4×10^6 PC from the appropriate groups were seeded into each well of Linbro tissue culture plates (16 mm Fb-16-24-TC, Linbro Chemical Company, Los Angeles, California) and allowed to adhere for 2 h, at which time nonadherent cells were removed by repeated washing with saline. Of the remaining cells in the monolayer, 95–99 per cent were mononuclear cells capable of phagocytizing heat-killed *Candida albicans*.

Target cell challenge

L-929 (L cells), obtained from Dr Jorgen Fogh, Sloan-Kettering Institute, Rye, New York, were maintained by bi-weekly passage in M199-10 per cent FCS. Following trypsinization, suspensions of L cells were prepared in M199-20 per cent FCS at a concentration of 1×10^5 cells/ml. One millilitre of this suspension was added to freshly prepared macrophage monolayers.

Measurement of macrophage-effected cytostasis

Cytostasis was studied by measuring the ability of target cells to incorporate [^{3}H]TdR (specific activity 6 Ci/mM, Schwarz Mann, Orangeburg, New York) in the presence of macrophages during 6-h pulse intervals with 5 μCi [^{3}H]TdR (0–6 h and/or 18–24 h). At the end of each pulse interval, extracellular [^{3}H]TdR was removed by washing and the amount of isotope incorporated by adherent target cells was measured using methods previously described (Krahenbuhl & Remington, 1974).

Cytostatic index

The cytostatic index (CI) was calculated after the formula of Ghaffar *et al.* (1974):

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

where N = mean c.p.m. in cultures containing macrophages from control mice (normal) and E = mean c.p.m. in cultures containing macrophages from *C. parvum*- or *Toxoplasma*-treated mice (experimental). The *P* values were calculated from the Student's *t*-test, and unless otherwise qualified

Table 1. [^{3}H]TdR uptake by target cells cultured in the presence of macrophages from mice treated with *C. parvum* or infected with *Toxoplasma*

Day after treatment	Source of macrophages			
	Target cells only	Normal mice	*C. parvum*-treated mice*	*Toxoplasma*-infected mice†
3	120,435±27,496‡	87,383±3723	118±7 (99·86)§	93±11 (99·89)
6	529,188±5449	248,830±31,493	142±18 (99·94)	318±99 (99·87)
9	275,804±59,637	180,981±33,332	193±41 (99·89)	1901±687 (98·95)
15	120,026±34,822	68,820±22,105	309±138 (99·54)	280±190 (99·59)
20	241,233±2483	54,189±6903	2026±387 (96·26)	1100±488 (97·97)
30	153,539±32,289	88,672±21,384	1089±122 (98·77)	730±156 (99·18)
50	62,175±14,849	18,967±3762	1097±324 (94·21)	1288±262 (93·20)
80	140,904±35,848	57,407±13,174	56,550±5320 (1·50)	332±110 (99·42)
105	120,212±4595	28,354±4669	25,235±2130 (11·00)	188±30 (99·33)

* Injected i.p. on day 0 with 1400 μg *C. parvum*.
† Infected i.p. on day 0 with 10^5 C-56 strain *Toxoplasma*.
‡ Mean c.p.m.±s.d. of quadruplicate cultures pulsed for 6 h beginning 18 h after challenge.
§ Figures in parentheses = cytostatic index.

represent a comparison between the effects of experimental and normal macrophages.

RESULTS

Persistence of activated macrophages in mice treated with *C. parvum* or infected with *Toxoplasma*

To study the duration of time during which activated macrophages persist in the peritoneal cavity, groups of forty mice were treated i.p. with *C. parvum* or infected with *T. gondii*. At varying intervals thereafter, PC were harvested from four or five mice, macrophage monolayers were prepared and challenged with L cells. As controls, target cells were cultured alone or with normal macrophages. A 6-hr pulse of [^{3}H]TdR was applied 18 hr after challenge. As early as day 3 (Table 1), [^{3}H]TdR uptake by target cells was almost totally inhibited in the presence of macrophages from both *C. parvum*-treated and *T. gondii* infected mice (CI = 99·9 and 99·8, respectively). Macrophages from *C. parvum*-stimulated mice were markedly cytostatic for the target cells until the eightieth day after challenge. When the level of [^{3}H]TdR uptake by tumour cells cultured with normal macrophages or macrophages from *C. parvum*-treated mice was compared 80 days after treatment, there was no significant difference (CI = 1·5, $P > 0{\cdot}8$). On day 105 there was again no significant cytostasis observed with macrophages from the *C. parvum*-treated group (CI = 11, $P < 0{\cdot}5$). In contrast, when tested in parallel on days 80 and 105 macrophages from mice chronically infected with *T. gondii* did not appear to lose their cytostatic capability (CI = 99·4, $P < 0{\cdot}001$; CI = 99·3, $P < 0{\cdot}001$ respectively).

In two similarly designed experiments, peritoneal macrophages from *C. parvum*-treated mice lost their cytostatic capacity at 60 and 115 days respectively. Mice infected i.p. with living *Toxoplasma* consistently possessed macrophages which were highly cytostatic at all the times tested (as long as 150 days in this series of experiments and greater than 360 days in others [Swartzberg, Krahenbuhl, Remington, in preparation]).

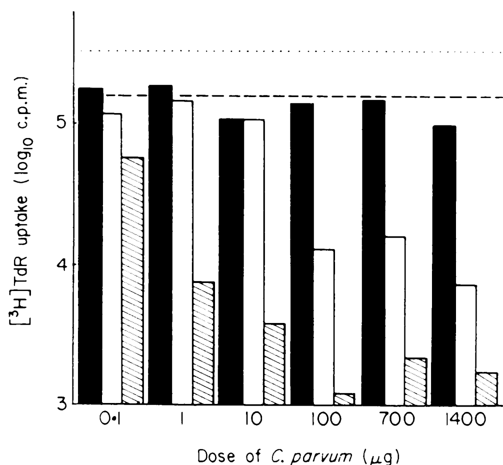

Figure 1. Cytostatic capacity of peritoneal macrophages from mice treated by different routes (solid column, s.c., open column, i.v. and hatched column, i.p.) with different doses (0·1, 1, 10, 100, 700, 1400 μg) *C. parvum*. Macrophage monolayers were prepared 7 days after treatment, challenged with target cells and pulsed with [^{3}H]TdR for 6 h beginning 18 h after challenge. The values shown represent the mean c.p.m. (triplicate cultures) of [^{3}H]TdR uptake. (......) [^{3}H]TdR uptake by target cells cultured alone. (– – –) [^{3}H]TdR uptake by target cells cultured with normal macrophages.

Effects of dose and route of administration of *C. parvum* or *T. gondii*

Experiments were performed to determine the effects of dose and route of administration of *C. parvum* on stimulation of a population of cytostatic peritoneal macrophages. Groups of five mice each were injected s.c., i.v. or i.p. with each of the following doses of *C. parvum*; 0·1, 1, 10, 100, 700 or 1400 μg. Untreated mice served as normal controls. Seven days later cultures of peritoneal macrophages were prepared from each group, challenged with target cells and pulsed at 18–24 h. Two such experiments yielded similar results, an example of which is shown in Fig. 1. Compared to the effects of normal macrophages, none of the doses of *C. parvum* administered s.c. stimulated a population of peritoneal macrophages, which significantly altered (i.e. $P<0{\cdot}05$) [^{3}H]TdR uptake by target cells. In these groups of mice (s.c.) CI ranged from 33 to −16. Intravenous treatment with *C. parvum* resulted in macrophages which were significantly cytostatic ($P<0{\cdot}01$) but only at the dose of 100 μg or greater (CI = 92·1 (100 μg), 90 (700 μg), 95·5 (1400 μg)). At the doses of 0·1, 1 and 10 μg i.v. treatment did not result in a population of peritoneal macrophages which significantly altered [^{3}H]TdR uptake by the target cells (CI = 24, 25, 29 respectively). Intraperitoneal administration of as little as 0·1 μg of *C. parvum* stimulated a population of peritoneal macrophages which significantly inhibited [^{3}H]TdR uptake by target cells ($P<0{\cdot}01$, CI = 64). Markedly increased cytostasis (CI > 95 was effected by macrophages from mice treated with 1 μg or greater (CI = 95·3 (1 μg), 97·6 (10 μg), 99·3 (100 μg), 98·6 (700 μg) and 98·9 (1400 μg)).

Experiments were performed to measure the cytostatic capabilities of peritoneal macrophages from groups of mice treated with equivalent amounts by dry weight of either *C. parvum* or formalin-killed *Toxoplasma*. The effect of varying both dose and route of administration were investigated. To compare the effects of dose, groups of mice were inoculated i.p. with the following doses of these preparations: 1400, 350, 87, and 22 μg. Seven days

Table 2. The effects of dose and route of administration of *C. parvum* or killed *Toxoplasma* antigen on activation of macrophages as measured by inhibition of [^{3}H]TdR uptake by target cells

Source of macrophages*	Route and dose of treatment: i.p. (μg) 22	i.p. (μg) 87	i.p. (μg) 350	i.p. (μg) 1400	i.v. (μg) 700	s.c. (μg) 1400
C. parvum	807 ± 337† (99·49)‡	709 ± 286 (99·55)	900 ± 120 (99·42)	363 ± 158 (99·77)	47,783 ± 18,102 (69·70)	237,758 ± 67,631 (−50·75)
Killed *Toxoplasma*	209,172 ± 38,716 (−32·63)	26,703 ± 2579 (83·07)	81,469 ± 13,303 (48·34)	229,564 ± 79,508 (−45·56)	61,192 ± 4792 (61·20)	294,990 ± 54,875 (−87·04)

* Groups of mice treated 7 days prior to harvest of macrophages.

† Mean c.p.m. ± s.d. of quadruplicate cultures pulsed for 6 h beginning 18 h after challenge. Control groups: target cells only = 265,176 ± 33,867, normal macrophages = 157,715 ± 16,715.

‡ Cytostatic index.

after treatment, macrophages were cultured from each of these groups, challenged with target cells, and their cytostatic capabilities measured (Table 2). Whereas the two intermediate i.p. doses of killed *T. gondii* (87 and 350 μg) caused a slight but significant inhibition of [^{3}H]TdR uptake by target cells (CI = 83 and 48 respectively, $P<0{\cdot}001$), all of the i.p. doses of *C. parvum* employed resulted in macrophages which were almost totally cytostatic (CI > 99·4). Studies of effects of route of administration (Table 2) revealed that the i.v. route of administration of 700 μg *C. parvum* or killed *T. gondii* resulted in a population of slightly but significantly activated macrophages (CI = 70 and 61 respectively, $P<0{\cdot}001$). Significant enhancement of [^{3}H]TdR uptake was seen in the presence of macrophages from mice treated s.c. with 1400 μg or i.p. with 22 μg killed *Toxoplasma* (CI = −87, $P<0{\cdot}01$; −33, $P<0{\cdot}05$, respectively).

To compare the effects of route of administration of C. *parvum* or chronic *T. gondii* infection groups

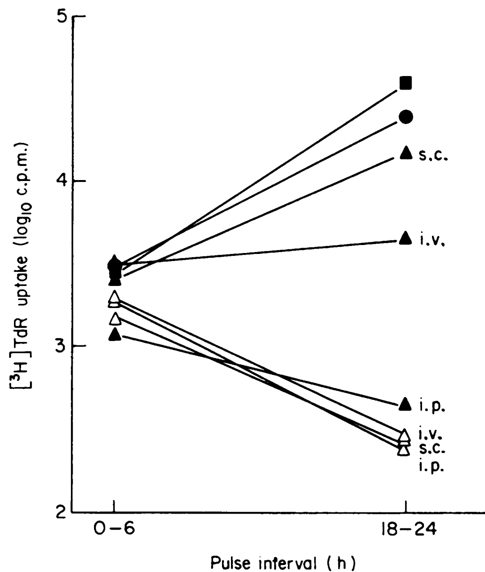

Figure 2. Cytostatic capacity of peritoneal macrophages from mice treated with *C. parvum* or infected with *Toxoplasma* by varying routes. Macrophage monolayers were prepared and challenged with target cells (0 h). The uptake of [^{3}H]TdR was determined during two 6-h pulse intervals. (■) Target cells cultured alone. (●) Target cells cultured with normal macrophages. (▲) Target cells cultured with macrophages from *C. parvum*-treated mice (7 days after treatment). (△) Target cells cultured with macrophages from *Toxoplasma*-infected mice (1 month after infection). Routes of treatment or infection are shown in figure.

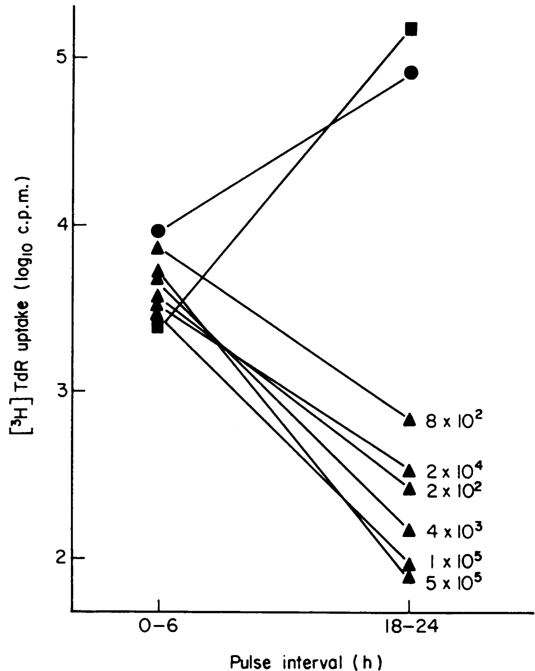

Figure 3. Cytostatic capacity of activated peritoneal macrophages from mice infected with varying numbers of *Toxoplasma*. Macrophage monolayers were prepared from groups of mice one month after infection with varying numbers of *Toxoplasma* organisms. The monolayers were challenged with target cells (0 h) and [^{3}H]TdR uptake was determined during two 6 h-pulse intervals. (■) Target cells cultured alone. (●) Target cells cultured with normal macrophages. (▲) Target cells cultured with macrophages from different groups of *Toxoplasma*-infected mice (infecting dose shown in figure).

of mice were injected i.p., i.v. or s.c. with either *C. parvum* 1 week, or living *T. gondii*, 4 weeks prior to preparation of macrophage monolayers and challenged with target cells. The data in Fig. 2 show [^{3}H]TdR uptake by target cells during two pulse intervals; 0–6 h and 18–24 h. The route of infection with *T. gondii* did not appear to be an important variable; s.c., i.v. or i.p. infection stimulated a population of activated macrophages which almost totally inhibited [^{3}H]TdR uptake by the target cells (CI = 98·9, 98·9 98·7, respectively). Comparison of [^{3}H]TdR uptake between these three groups revealed no significant difference in effectiveness ($P<0{\cdot}8$). In contrast, only the i.p. route of administration of *C. parvum* resulted in a population of macrophages with an equivalent degree of cytostasis (CI = 98). When compared with each of the routes of *T. gondii* infection this value was not significantly different.

Macrophages harvested from mice treated with *C. parvum* i.v. were cytostatic (CI = 81), but were significantly less cytostatic than macrophages from mice treated i.p. with *C. parvum* ($P < 0{\cdot}01$). Treatment with *C. parvum* s.c. resulted in a population of peritoneal macrophages which were only slightly cytostatic (CI = 40) but differed significantly from normal macrophages ($P < 0{\cdot}05$).

To determine the effects of infection with different doses of *Toxoplasma* groups of mice were infected i.p. with doses of the avirulent C-37 strain of *T. gondii* ranging from 200 to 500,000 trophozoites. This strain, rather than the more virulent C-56 strain, was chosen because treatment with sulphadiazine is not necessary to establish chronic infection, thus allowing an accurate evaluation of the effects of different doses of *T. gondii*. Thirty days after infection, cultures of macrophages were prepared from each group and challenged with target cells. The data shown in Fig. 3 respresent [^{3}H]TdR uptake by the target cells during two pulse intervals; 0–6 h and 18–24 h. Even the lower infecting doses of *T. gondii* stimulated a population of activated macrophages capable of almost totally inhibiting [^{3}H]TdR uptake by target cells (CI > 99, $P < 0{\cdot}001$). Similar results were obtained 60 and 90 days after infection (data now shown in figure).

The effects of route of readministration of *C. parvum* or *Toxoplasma*

Groups of twenty mice each were treated with *C. parvum* or infected with *T. gondii* by the i.v., i.p. or s.c. route. One week later, subgroups of five mice each received a second dose of the homologous agent administered by each of the three routes. Five mice in each group did not receive a second inoculation. Seven days later, cultures of macrophages from each subgroup were prepared and challenged with target cells. [^{3}H]TdR was added during a single 6-h pulse interval beginning 18 h after challenge. The results, shown in Table 3 reveal that infection of mice with *T. gondii* by any of the three routes was so efficient in stimulating a population of macrophages highly cytostatic to the target cells that readministration of *T. gondii* by another route statistically altered the cytostatic effect but resulted in little apparent change in cytostatic effect. In contrast certain routes of readministration of *C. parvum* appeared to markedly alter the effects of the initial treatment. Whereas mice which received only the initial i.v. dose of *C. parvum* possessed cytostatic macrophages (CI = 89, $P < 0{\cdot}001$), reinjection i.p. or s.c. significantly increased the cytostatic capacity of their peritoneal macrophages (CI = 98,

Table 3. The effects of route of readministration of *C. parvum* or *Toxoplasma* on the activation of peritoneal macrophages as measured by the inhibition of [^{3}H]TdR uptake by target cells

Source of macrophages	Route of initial treatment	Route of readministration*							
		None	*P*†	i.v.	*P*‡	i.p.	*P*‡	s.c.	*P*‡
C. parvum	i.v.	4607±74§ (89·15)	(<0·001)	—		727±209 (98·29)	(<0·001)	204±61 (99·52)	(<0·001)
C parvum	i.p.	103±39 (99·76)	(<0·001)	1673±159 (96·06)	(<0·001)	194±57 (99·54)	(<0·05)	227±19 (99·47)	(<0·01)
C. parvum	s.c.	86,489±5759 (−103·69)	(<0·001)	695±213 (98·36)	(<0·001)	304±127 (99·28)	(<0·001)	59,491±3169 (−40·08)	(<0·001)
Toxoplasma	i.v.	345±91 (99·19)	(<0·001)	348±148 (99·18)	(>0·8)	501±82 (98·82)	(<0·05)	546±51 (98·71)	(<0·01)
Toxoplasma	i.p.	315±39 (99·26)	(<0·001)	428±75 (99·00)	(<0·05)	92±6 (99·78)	(<0·001)	241±38 (99·43)	(<0·05)
Toxoplasma	s.c.	607±90 (98·57)	(<0·001)	438±30 (98·97)	(<0·05)	220±49 (99·48)	(<0·001)	411±99 (99·03)	(<0·05)

* Homologous preparations of either *C. parvum* or *Toxoplasma* were readministered i.v., i.p., or s.c. 1 week after the initial treatment. Macrophages were harvested 1 week later.

† Comparison with [^{3}H]TdR uptake in presence of normal macrophages.

‡ Comparison of effects of readministration with effects of initial treatment.

§ Mean c.p.m.±s.d. of quadruplicate cultures pulsed for 6 h beginning 18 h after culture. Cytostatic index in parenthesis. Control groups: target cells only = 97,478±10,419, normal macrophages = 42,469±3286.

$P<0.001$ and CI = 99·5, $P<0.001$, respectively). Mice treated only by the i.p. route had highly cytostatic macrophages (CI = 99·8) and readministration of *C. parvum* i.p. or s.c. did not appreciably alter the cytostatic effect of these macrophages although the changes were significant ($P<0.05$, $P<0.01$ respectively). However, in mice initially treated i.p., (CI = 99.8) a second injection of *C. parvum* i.v. resulted in a population of activated macrophages which were obviously less cytostatic (CI = 96) for target cells than those of unboosted mice. This shift in the cytostatic capacity of these activated macrophages represents a sixteen-fold increase ($P<0.001$) in [^{3}H]TdR uptake by the target cells. Also of special interest were the effects of booster treatment in mice which initially received *C. parvum* by the s.c. route. Treatment by the s.c. route alone yielded a population of macrophages which, compared to normal macrophages, significantly enhanced [^{3}H]TdR uptake by the target cells (CI = −104, $P<0.001$). A s.c. boost in this group did little to increase the cytostatic capacity of the macrophages; compared with normal macrophages significant enhancement was still observed (CI = −40, $P<0.001$). A second injection i.v. or i.p. markedly increased the cytostatic capacity of macrophages from these mice treated initially s.c. (CI = 98 and 99·3 respectively).

DISCUSSION

Recent studies have shown that i.v. (Olivotto & Bomford, 1974; Scott, 1974a) or i.p. (Ghaffar *et al.*, 1974; Scott, 1974b) administration of killed *C. parvum* to mice stimulates a population of activated macrophages cytotoxic to tumour target cells *in vitro*. Measurement of this cytotoxicity as a cytostatic effect (inhibition of DNA synthesis in target cells) allows quantitative comparison of the levels of activation in different populations of activated macrophages (Krahenbuhl & Remington, 1974; Krahenbuhl & Lambert, 1975). Such observations, made in the present report, provide *in vitro* evidence which partially define the potential strength and shortcomings of *C. parvum* treatment as a means of activating macrophages in the murine model.

Persistence of activated macrophages following a single administration of *C. parvum* was partially studied by Olivotto & Bomford (1974) who reported a reduction in the cytostatic effect of macrophages harvested 20 days after i.v. treatment. There was a total loss of cytostatic effect by 60 days (Bomford & Christie, 1975). Ghaffar *et al.* (1974) reported no obvious changes in the effects of peritoneal macrophages harvested at 4 and 7 days after i.p. treatment. In the present report the long-term persistence of cytostatic macrophages was studied following a single i.p. treatment with *C. parvum*. Cytostatic macrophages were present as early as 3 days and persisted for a prolonged, but finite period (2–3 months), after which they performed essentially as normal macrophages.

Comparison of the effectiveness of route of administration of *C. parvum* revealed that i.p. injection consistently yielded populations of activated peritoneal macrophages which had a greater cytostatic ability than those from mice injected i.v. The i.p. route of administration was also less dose-dependent than the i.v. Whereas 0·1 μg of *C. parvum* i.p. resulted in significantly cytostatic macrophages (CI = 64, $P<0.01$) the lowest i.v. dose to result in cytostatic macrophages was 100 μg (CI = 92, $P<0.01$). Regardless of dose, s.c. injection of *C. parvum* yielded populations of activated macrophages which were usually not even slightly cytostatic and often enhanced DNA synthesis by target cells.

Studies have been performed on the effects of multiple treatments with *C. parvum* on established tumours. In a model employing a s.c. transplanted mammary carcinoma it was shown that seven weekly treatments i.p. were more effective in retarding tumour growth than a single i.p. injection or four i.v. treatments (Fisher, Wolmark, Saffer & Fisher, 1975). In these studies multiple s.c. treatments failed to inhibit tumour growth; however, a pronounced effect was noted when multiple s.c. treatments followed an initial treatment administered i.p. In the present study, if the initial injection was given i.v. and the second injection i.p. or s.c. (i.v.- i.p., i.v.- s.c., respectively), macrophage cytostatic capacity was increased. i.p.- i.p. or i.p.- s.c. did not markedly alter the high level of cytostatis observed in macrophages from unboosted (i.p. only) animals. It was apparent that the initial i.p. treatment alone was so effective in activating macrophages to inhibit DNA synthesis of target cells that any increase in cytostatic capacity as a result of second treatment with *C. parvum* was difficult to measure. However, i.p.- i.v. treatment reduced the cytostatic capacity of the macrophages. The effect of s.c. treatment, ineffective

alone in activating peritoneal macrophages, was enhanced when the treatment was s.c.- i.v. or s.c.- i.p. but not by s.c.- s.c. These *in vitro* studies may provide a rationale for employing certain multiple treatment combinations, since the route by which *C. parvum* is readministered may have a marked effect on the cytostatic ability of peritoneal macrophages.

Whether these shifts in relative levels of cytostatic ability are due to qualitative changes in the macrophages themselves or to changes in the make-up of the peritoneal cell population (for example, the role of the 'macrophage disappearance' phenomenon, as described by Nelson and Boyden [1963]) is not known. Of interest is the observation of Wolmark & Fisher (1974) who observed that a second s.c. injection of *C. parvum* appeared to augment the stimulation of bone marrow macrophage colony production observed after an initial s.c. treatment, suggesting that changes in the macrophage population do occur following primary, as well as secondary, treatment with *C. parvum*.

The route of administration of *Toxoplasma* infection did not appear to be important—almost total inhibition of DNA synthesis was observed employing macrophages from mice infected with 2×10^2 to 5×10^5 parasites or injected i.v., i.p. or s.c. Because of the almost total cytostasis effected by macrophages from *Toxoplasma*-infected mice, rechallenge did not cause appreciable differences. Compared with *C. parvum* treatment, activated macrophages persisted in the peritoneal cavities of *Toxoplasma* infected mice for more prolonged periods (probably for life), due apparently to the lifetime persistence of antigen in the form of living organisms in virtually all of the tissues. The continuous interaction of this antigen with sensitized lymphocytes probably maintains a continuous population of activated macrophages (Krahenbuhl & Remington, 1971).

The presence of lymphocytes sensitized to *C. parvum* antigens is suggested by the work of Scott (1974b) who demonstrated delayed hypersensitivity (DH) to *C. parvum* in the footpads of mice treated s.c. with *C. parvum*, but not if treatment was given i.v. However, Christie & Bomford (1975), rather than testing for DH, employed an *in vitro* assay for a cell-mediated immune response to *C. parvum* and showed that normal macrophages become cytostatic for tumour cells when cultured in the presence of *C. parvum* and lymphocytes from mice treated i.v. with *C. parvum*. In addition, i.v. readministration of *C. parvum* 60 or 130 days after an i.v. presensitization dose resulted in the accelerated appearance of cytostatic peritoneal macrophages, again suggesting that lymphocytes sensitized to *C. parvum* do persist. The disappearance of cytostatic macrophages following a single treatment as in the study of Bomford and Christie (1975) and the present report is perhaps due to the eventual degradation and disappearance of *C. parvum* from the tissues. This may be analogous to the rapid waning of the nonspecific microbicidal (Mackaness, 1962; 1969) and cytostatic effects (Krahenbuhl & Remington, 1974) of activated macrophages which coincides with the elimination of the infecting organism from mice sublethally infected with BCG or *Listeria monocytogenes*.

Many of the comparative shortcoming of *C. parvum* treatment demonstrated *in vitro* in the present report may underlie certain disadvantages to its use *in vivo* in the immunotherapy of cancer. For example, recent studies (Scott, 1974b; Likhite & Halpern, 1974) have shown that direct intratumour injection of *C. parvum* is most advantageous in treating local tumours, often leading to complete regression. However, such local treatment had little effect on systemically administered tumour cells (Scott, 1974a). Although the contribution of activated macrophages in the *in vivo* resistance to tumours seen in *C. parvum* treated mice is unknown, in our study even repeated s.c. (local) treatment with *C. parvum* failed to markedly enhance the cytostatic capacity of peritoneal macrophages. This inability of local treatment to activate macrophages at distant sites may underlie these *in vivo* observations. In contrast, systemic administration of a single dose of *C. parvum* (i.p. or i.v.) markedly stimulates the reticuloendothelial system (Halpern, Prevot, Biozzi, Stiffel, Mouton, Morand, Bouthillier & Decreusefond, 1964; Adlam & Scott, 1972) and depending upon the tumour model, may inhibit established local or i.v. administered tumours as evidenced by slower rates of growth and prolonged survival (Scott, 1974a; Fisher *et al.*, 1975; Woodruff, Inchley & Dunbar, 1972; Likhite & Halpern, 1973; Milas & Mujagic, 1972). Systemic administration may also prolong the survival of mice challenged i.v. with tumour cells (Scott, 1974a; Milas, Hunter & Withers, 1974).

Finally, the results of the present report and others from our laboratory (Krahenbuhl & Remington, 1974; Remington, *et al.*, 1975) clearly demonstrate that chronic infection of mice with Toxoplasma is

a potent means of stimulating a population of activated macrophages which are cytotoxic to tumour cells *in vitro*. Neither the dose or route of infection, nor the strain of *Toxoplasma* employed, appear to be important variables; macrophages activated in this manner persist in the host, probably for life. As in the *in vivo* murine models, which employ such immunotherapeutic agents as BCG or *C. parvum*, *Toxoplasma* infection induces a marked resistance to the development of autochthonous or transplanted tumours (Hibbs, Lambert & Remington, 1971). The greatest disadvantage that might be associated with potential *Toxoplasma* immunotherapy lies in the use of a living infectious agent in a host who is immunologically compromised from either his disease (cancer) or treatment (radio- or chemotherapy). Such a problem has been recognized in patients being treated with living BCG (Bast, Zbar, Boros & Rapp, 1974; McKhann, Hendrickson, Spitler, Bonnarsson, Banerjee & Nelson, 1975). However, approximately 50 per cent of the adult population in the United States (and an even greater percentage in many other parts of the world) are chronically infected with *Toxoplasma*. Whether toxoplasmosis in man offers any protection against the development of cancer is unknown, but such individuals elicit strong cell mediated immunity to *Toxoplasma* antigen for as long as 20 years after infection (Krahenbuhl, Gaines & Remington, 1972). Although in uninfected mice, killed *Toxoplasma* antigen was relatively ineffective in activating peritoneal macrophages, preliminary studies from our laboratory suggest that in *Toxoplasma*-infected mice, resistance to established local or systemic murine tumours can be markedly enhanced by administration of killed Toxoplasma antigen.

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