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Enhanced Fc receptor expression by a sub-population of murine intra-tumour macrophages following intravenous *Corynebacterium parvum* therapy

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Summary Intravenous injection of *Corynebacterium parvum* (*C. parvum*) 4 days after s.c. inoculation of 5×10^5 cells derived from the immunogenic fibrosarcoma FSA/R induced tumour growth inhibition over a period of 21 days in syngeneic C3H/Buf mice. This was not accompanied by a change in the proportions of host cells within the tumour, but the activation state of tumour-infiltrating macrophages was increased following *C. parvum* therapy. Two macrophage subpopulations were identified in FSA/R tumours after fractionation by unit gravity velocity sedimentation. After i.v. *C. parvum* therapy the tumour-infiltrating macrophage subpopulation which sedimented between 1 and $6\,\mathrm{mm\,h^{-1}}$ was consistently activated as determined by measurement of Fc receptor avidity. Other intra-tumour macrophages were generally unaffected by *C. parvum* treatment. We have previously shown that the host cell fraction sedimenting between 1 and $6\,\mathrm{mm\,h^{-1}}$ is enriched with monocytes and the data presented in this paper suggest that these cells may enter the tumour in a pre-activated state following intravenous *C. parvum* therapy.

Many experimental (Kerbel & Pross, 1976; Moore & Moore, 1977) and human (Wood & Gollahon, 1977; Wood *et al.*, 1978) tumours exhibit a significant level of macrophage (mφ) infiltration and this is believed to be a manifestation of the host response (Alexander, 1976) involved in the restraint of primary tumour growth and metastatic dissemination.

Although the spontaneous regression of experimental virus-induced murine tumours has been demonstrated to be associated with alterations in the activation status of tumour-infiltrating mφ (Russell & McIntosh, 1977) no such correlation has before been demonstrated in either chemically-induced tumours or those of spontaneous origin. However, mφ isolated from non-immunogenic tumours have been demonstrated to stimulate tumour cell growth rate *in vitro* whilst those isolated from immunogenic tumours were cytostatic (Mantovani, 1978). In human cancer patients a correlation has been found between the number of mφ present within primary breast tumours and melanomas and extent of subsequent tumour dissemination (Gauci & Alexander, 1975).

These data indicate that mφ associated with tumours may exert anti-tumour functions under the appropriate circumstances, but generally even those tumours which contain very high levels of Macrophages, isolated from such progressively growing chemically-induced rat (Moore & Moore, 1980) and mouse tumours (Moore & McBride, 1980), exhibit functions which suggest that these cells are at an arrested state of differentiation such that they are not fully activated. These tumours do not generally regress spontaneously and in the present study we have used immunotherapy-induced inhibition of tumour growth as a model to investigate the role that mφ may play in this process.

It has been shown previously that inhibition of tumour growth following systemic administration of Cornyebacterium parvum (*C. parvum*) is not associated with an increase in the numbers of tumour-infiltrating mφ (Thomson *et al.*, 1979). We confirm this but also demonstrate that *C. parvum* induced-tumour growth inhibition is associated with an enhancement of tumour mφ activation state. The particular subset of mφ that is activated maximally with regard to Fc receptor function is that with sedimentation characteristics of the recently-emigrated monocyte and this suggests that *C. parvum* therapy causes peripheral pre-activation of monocytes before they enter the tumour.

Materials and methods

Mice

Inbred C3H/Buf/Kam strain mice aged between 6–10 weeks were used in all experiments. These mice were taken from a colony established from breeding pairs obtained from the laboratory where the FSA/R tumour was induced.

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Tumour

The tumour used (FSA/R) was a highly immunogenic fibrosarcoma originally induced in C3H/Buf/Kam mice by methylcholanthrene (Suit & Kastelan, 1970). It was maintained by serial passage in syngeneic mice and the tumour was used between passage 13 and 19.

C. parvum *treatment*

Tumours were induced in groups of 10 mice by s.c. inoculation of 5×10^5 *in vivo* derived tumour cells. Four days later 0.25 mg of Corynebacterium parvum (*C. parvum,* Burroughs Wellcome Ltd., Beckenham, Kent, England), suspended in 0.25 ml PBS was injected in the tail veins of the test group. Control mice received no treatment.

Tumour growth was monitored by taking the average of opposing diameters of tumours measured with skin calipers. Both groups of mice were monitored for up to 22 days of tumour growth and groups were killed by cervical dislocation at various intervals for tumour excision and analysis of intra-tumour host cells.

Cell fractionation

Following excision non-necrotic tumour tissue was pooled within groups and single cell suspensions prepared by enzymatic disaggregation with a mixture of Dispase/DNase as previously described (Moore & McBride, 1980) for 30 min at room temperature. After washing and resuspension 10^8 cells were subjected to unit gravity velocity sedimentation (Miller & Phillips, 1969) for 3 h and fractions were collected which contained cells sedimenting over the range of 1 to 14 mm h^{-1}.

The composition of cell suspensions before and after fractionation was determined by differential counting of Jenner–Giemsa stained cytospin films prepared from cells incubated for 1 h at 37 °C with 1.1 μm diam. polystyrene latex in medium containing 20% foetal calf serum (FCS).

Preparation of Macrophage monolayers

1) *From non-fractionated tumour cell suspensions*:

Cells were suspended at 2×10^6 ml^{-1} in MEM containing 20% FCS and 0.05% Dispase to prevent adherence of tumour cells and the suspension pre-warmed at 37 °C. One-half millilitre of the cell suspension was then dispensed in pre-warmed Costor tissue culture plates (Arnold R. Horwell Ltd., London, England) and the plates incubated for 10 min before washing off the non-adherent cells ×3 with Hanks solution and finally adding 1 ml of Hanks solution before EA rosetting. These mφ were denoted as rapidly adherent.

2) *From fractionated tumour cell suspensions*:

Cells isolated after velocity sedimentation were suspended at 5×10^5 ml^{-1} in MEM containing 20% FCS and 0.05% Dispase. This cell suspension was treated as above but the mφ were allowed to adhere for 30 min at 37 °C to allow relatively less adherent mφ and monocytes to attach. Both procedures gave monolayers made up of more than 85% of phagocytic mononuclear cells as determined by 1.1 μm diam. polystyrene latex uptake (Moore & McBride, 1980).

Measurement of Fc receptor avidity

Fc receptor (FcR) avidity was measured as previously described (Moore & McBride, 1980). Briefly, aliquots of bovine erythrocytes were sensitized with doubling dilutions of hyperimmune rabbit IgG. These EA suspensions were then sedimented by centrifugation onto mφ monolayers and after an incubation for 30 min at room temperature excess EA were washed off and the number of cells forming rosettes (EA RFC) was enumerated microscopically.

For each mφ population the EA_{50} value, as a measure of FcR avidity, was calculated as follows:

$EA_{50} = 1000 \div$ Concentration of sensitising antibody, in μg ml^{-1} required to induce 50% of total EA RFC to form rosettes.

In addition to the standard technique where 8 batches of EA sensitised with antibody diluted over the range of 1/8 to 1/1024 a modified technique was used to measure FcR avidity of monolayers prepared from fractions isolated after velocity sedimentation. This was performed by preparing two mφ monolayers and measuring the number of cells forming rosettes with EA sensitised with antibody diluted at 1/16 (EA_{16}) or 1/256 (EA_{256}). The results were expressed for each cell monolayer by use of the following formula:

$$\frac{\%\text{ of cells forming rosettes with } EA_{256}}{\%\text{ of cells forming rosettes with } EA_{16}} \times 100.$$

Results

I.v. inoculation of 0.25 mg of *C. parvum* 4 days after a s.c. inoculation of 5×10^5 FSA cells caused inhibition of tumour growth (Figure 1). Typically *C. parvum* treatment had little influence on the early

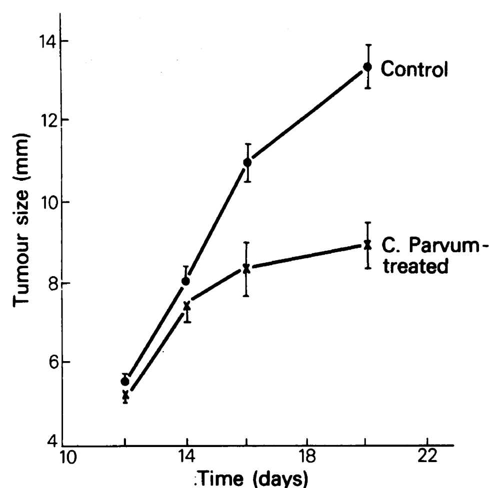

Figure 1 Effect of i.v. *C. parvum* treatment on growth rate of FSA/R tumours. 5×10^5 tumour cells were injected s.c. to groups of 8 mice at Day 0 and 0.25 mg of *C. parvum* was injected at Day 4 to the test group. Tumour size expressed as the mean of opposing diameters. Error bars ± s.e. of the mean of 8 tumours in each group.

stages of tumour growth with its effects becoming obvious 10 days after *C. parvum* was given.

After enzymatic disaggregation of tumour tissue the host cell content of the resultant cell suspension was determined by differential counting (Table I). It was not possible to detect a difference in cellular composition between tumour cell preparations derived from control and *C. parvum*-treated animals during the period of tumour growth inhibition following Day 14 after tumour inoculation.

F_c receptor avidity of unfractionated tumour mφ

The activation state of rapidly adherent non-fractionated mφ within individual tumours was measured between 13 and 22 days after tumour inoculation. A consistent increase in EA_{50} values was detected for those mφ isolated from tumours which were undergoing *C. parvum* induced growth inhibition (Table II). The mean increase in EA_{50} was 67% (range 25%–144%) and was highly significant ($P < 0.001$) by Student's *t*-test.

Velocity sedimentation fractionation of tumour mφ

When cell suspensions from either *C. parvum* treated or control tumours were subjected to velocity sedimentation for 3 h, no difference could be detected between them with respect to the elution profiles of total cells or EA rosette forming cells. In both situations 2 major cell peaks were consistently found (Figure 2b). The smaller peak sedimenting at 1 to 5 mm h^{-1} contained mainly host cells, whilst a larger more rapidly sedimenting peak (>6 mm h^{-1}) contained tumour cells and large mφ. Although this second peak contained only 20% mφ, in absolute numbers 50% of the total mφ in the tumours sedimented in excess of 6 mm h^{-1}. Rapidly sedimenting large, vacuolated mφ were found distributed throughout the lower regions of the gradient with sedimentation velocities of up to 14 mm h^{-1} (Table III).

After isolation of mφ from each fraction of the gradient, their FcR activation level was determined by evaluating the ratio of the number of cells forming EA rosettes with ORBC sensitized by IgG diluted 1/16 to the number of cells forming rosettes with ORBC sensitized by a 1/256 dilution of IgG. This method was used because it could be carried out on a limited number of mφ, and therefore was ideal for assaying small fractions isolated from the sedimentation procedure. Fractionation of control tumour suspensions from untreated animals demonstrated that the more highly activated mφ sedimented at 6 to 10 mm h^{-1}. Within the tumour cell suspension prepared from *C. parvum* treated animals a shift in the sedimentation profile of the activated mφ population was observed when

Table I Differential count of cell suspensions prepared from tumours excised from control or *C. parvum* treated mice

	Tumour cells	*Macrophages/ monocytes*	*Lymphocytes*	*Polymorphonuclear neutrophils*
Control	80 ± 4.4[a]	12.5 ± 2.8	5.0 ± 3.4	1.0 ± 1.1
C. parvum treated	83 ± 3.7	10.5 ± 2.7	4.5 ± 2.0	2.0 ± 1.5

Each group is the mean of differential counts on 10 different tumour cell preparations.
[a] ± s.d.
Differences between each group were not significant in a Student's *t*-test.

Table II EA_{50} of unfractionated tumour-infiltrating macrophages

*Days after tumour transplant**	EA_{50} *control*	EA_{50} C. parvum *treated***	*% Increase*
13	305	380	25
14	322	448	39
16	241	588	144
17	333	650	95
17	292	500	71
17	294	500	70
17	277	385	39
17	185	286	55
21	223	428	92
21	294	422	44
22	339	550	62
Mean (±s.e.)	282 (±15)	467 (±31)***	67 (±10)

*5×10^5 *in vivo* derived tumour cells inoculated s.c. on Day 0.
**0.25 mg *C. parvum* injected i.v. on Day 4.
***$P<0.001$ when the means of the EA_{50} values are compared by Student's *t*-test.

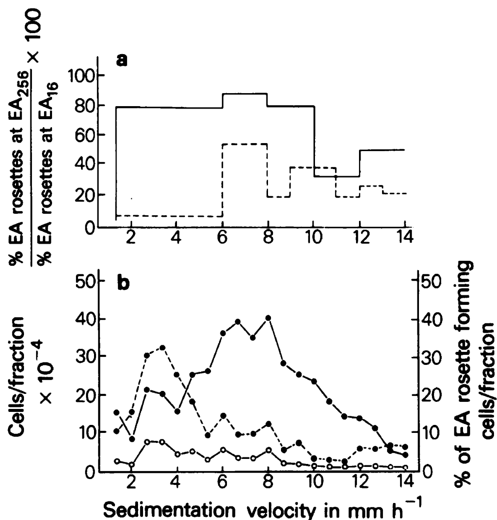

Figure 2 Velocity sedimentation fractionation of tumour infiltrating macrophages. a. Fc receptor avidity: (———) *C. parvum*; (-----) Control. b. (■-----■) % of EA rosette forming cells per fraction; (■———■) total cells per fraction $\times 10^{-4}$; (□———□) total EA rosette forming cells per fraction.

tumours were tested during the period of growth inhibition. This was largely restricted to those mφ present within the major host cell fraction containing cells sedimenting at 1 to 5.5 mm h^{-1}. In the experiment shown in Figure 2a, a less marked enhancement of FcR avidity was also found in the mφ sedimenting between 6 and 10 mm h^{-1} but this increase was not a consistent finding in all *C. parvum*-treated tumours. Macrophages sedimenting in excess of 11 mm h^{-1} were unaffected by *C. parvum* therapy.

Discussion

The host cell content of immunogenic tumours undergoing *C. parvum*-induced growth inhibition does not differ significantly from progressor tumours growing in untreated syngeneic mice. *C. parvum*-induced tumour growth inhibition in this syngeneic system is however, T-cell dependent (McBride *et al.*, 1980) and thus one might expect some qualitative, if not a quantitative change, in those intra-tumour host cells representative of the cellular immune system. Changes in the activation state of tumour infiltrating mφ have been shown to be intimately associated with regression or progression of MSV tumours (Russell *et al.*, 1977) and in the present study we have demonstrated changes in this cell population to be associated with inhibition of growth of a chemically induced tumour following i.v. immunotherapy with *C. parvum*. Measurement of the activation state of unfractionated mφ isolated from regressor or progressor tumours indicated that when means of the two groups were compared a 67% increase in FcR avidity was found.

We have previously demonstrated that tumour infiltrating mononuclear phagocytes are

Table III Differential count on Day 14 fractionated control and *C. parvum* treated tumours

	Tumour cells		*Macrophages*		*Lymphocytes*		*Polymorphonuclear neutrophils*	
	%	(*cells* × 10^4)	%	(*cells* × 10^4)	%	(*cells* × 10^4)	%	(*cells* × 10^4)
				CONTROL				
Unfractionated	82		14		3		1	
I 1–5.5 mm h^{-1}	23	(131)	48	(274)	23	(131)	6	(34)
II 5.5–8.5 mm h^{-1}	78	(640)	21	(172)	1	(8.2)	0	(0)
III 8.5–11.5 mm h^{-1}	95	(979)	5	(56)		—		—
IV 11.5–14 mm h^{-1}	97	(412)	3	(22)		—		—
				C. parvum TREATED				
I 1–5.5 mm h^{-1}	33	(249)	40	(302)	26	(196)	1	(8)
II 5.5–8.5 mm h^{-1}	82	(1013)	17	(209)	1	(12)	1.5	(6)
III 8.5–11.5 mm h^{-1}	95	(1064)	5	(51)		—		—
IV 11.5–14 mm h^{-1}	97	(425)	3	(24)		—		—
Unfractionated	82		13		4		1	

heterogeneous with respect to FcR avidity (Moore & McBride, 1980). In that study we demonstrated the presence of two populations of mφ exhibiting different activation levels of FcR and in the present study we determined which mφ subpopulation was the target for *C. parvum* induced activation to gain some insight into how *C. parvum* was influencing mφ differentiation within the tumour micro-environment.

When tumours from *C. parvum*-treated animals were subjected to fractionation by unit gravity velocity sedimentation no difference could be detected in the distribution of EA rosette forming cells when rosettes were formed using ORBC sensitised with high concentrations of antibody. However, when the mφ were rosetted with ORBC sensitised with low levels of antibody, only the activated mφ formed rosettes. This technique demonstrated that the smaller sized population of phagocytic cells within the tumour which sedimented at 1 to 5 mm h^{-1} exhibited the greatest increase in activation state in response to *C. parvum* treatment. An eight-fold increase in FcR avidity was detected within this population in contrast to a mean 67% increase in the rapidly adherent non-fractionated mφ preparations. This was caused by the presence within monolayers derived from non-fractionated cells of large, rapidly sedimenting mφ which comprise ~50% of the total within the tumour. These mφ are unaffected by *C. parvum* therapy and consequently their presence results in an apparent lowering of FcR avidity when heterogeneous monolayers are examined. Although cell monolayers analysed in these experiments were routinely >85% phagocytic it has to be considered that a minor non-phagocytic cell population expressing FcR that increase in avidity after *C. parvum* treatment may be responsible for the observed changes. Response to *C. parvum* treatment in this model is T-lymphocyte dependent (McBride *et al.*, 1980) and tumour infiltrating T-lymphocytes may be activated simultaneously with cells of the mononuclear phagocyte series. Activated T-lymphocytes (Yoshida & Anderson, 1972) and B-lymphocytes (Basten *et al.*, 1972) express FcR which would form rosettes under the conditions used, i.e. with ORBC sensitised with high concentrations of antibody if they did contaminate the mφ monolayers. It is unlikely however, that the presence of these contaminating lymphocytes would contribute to the FcR avidity changes observed in the monolayers isolated from the slower sedimenting lymphocyte enriched fractions. Although activation of T-lymphocytes does increase FcR expression, this level is still 10 times less than that of proteose peptone-elicited peritoneal mφ (Anderson & Grey, 1974). Earlier studies with the FSA/R tumour (Moore & McBride, 1980) indicated that the FcR avidity of the slower sedimenting tumour infiltrating mφ in control animals was at least equal to that of proteose peptone-elicited mφ. In the present study we are measuring increases in excess of this level of FcR expression which eliminates the possibility of activated T-lymphocyte interference in the assay.

Results of our earlier study indicated that the intra-tumour phagocytic mononuclear cells sedimenting at the same velocity as those activated by *C. parvum* therapy, contain mφ which are less differentiated than the larger more rapidly

sedimenting cells and probably represents cells which have recently entered the tumour from the circulating monocyte pool. Systemic administration of *C. parvum* induces a generalised stimulation of the reticuloendothelial system (Baum & Breese, 1976) and monocytes may therefore be activated before extravasation to the solid tumour. We have previously demonstrated that full activation of mφ is not achieved within the microenvironment of FSA/R tumours when growing progressively (Moore & McBride, 1980). The activation pathway of the intra-tumour mφ in *C. parvum* treated mice may circumvent this problem by activating the mφ at a site distant to the tumour. If intra-tumour mφ differentiation is normally inhibited by the high concentration of suppressor factors at the tumour site (Spitalny & North, 1977) then *C. parvum* induced mφ activation may be occurring where these factors are at an ineffective concentration. Thus activated monocytes/macrophages may enter the tumour where they are able to exert anti-tumour mechanisms before being inactivated.

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