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Effect of *Corynebacterium parvum* on Cellular and Humoral Antitumoral Immune Effector Mechanisms*

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Abstract—*In an attempt to characterize the mechanism of action of* Corynebacterium parvum (C. parvum) *an analysis was performed of the changes in specific antitumoral immune effector mechanisms in C_3H mice showing increased protection against challenge with high inocula of the allogeneic L*1210 *leukemia after treatment with this immunoadjuvant.* C. parvum*-treated spleens displayed an increase in DNA synthesis, total cell number and in the percentage of macrophages; the percent of theta bearing lymphocytes was concomitantly decreased whereas no modification in the percent of EA-rosette forming cells was observed. Although the efficiency of target cell lysis by immune splenocytes was unchanged, the raise in spleen cellularity of* C. parvum*-treated mice resulted in a marked increase in this organ's capacity to express cell-mediated cytotoxicity. Treatment with this adjuvant produced also a sizable increase in the spleen capacity to mediate antibody-dependent cellular cytotoxicity whereas arming and potentiating serum activities and the levels of complement-dependent cytolytic antibody were not modified.*

INTRODUCTION

THE POTENTIAL of *Corynebacterium parvum* (*C. parvum*) in the immunotherapy of experimental tumors has been demonstrated in various studies [1–8] and this agent has also proven of value in the treatment of human neoplastic conditions [9]. The exact mechanisms through which *C. parvum* can induce tumor growth retardation are still not completely clarified. The powerful stimulation of the reticuloendothelial system by *C. parvum* has been repeatedly confirmed [10–12] and evidence has been presented supporting the contention that in the antitumoral effect of this compound a primary role is played by increases in non specific resistance through an activation and multiplication of macrophages [7, 13–15]. However, the possibility that this agent also induces a stimulation of specific immune responses could not be ruled out especially since a detailed analysis of the changes in humoral and cellular antitumoral immune effector mechanisms induced by *C. parvum* is still lacking.

In this study, an experimental system in which treatment with *C. parvum* conferred increased protection against challenge with high inocula of an allogeneic tumor has been employed for evaluating the effects of this agent on cell-mediated cytotoxicity (CMC) and the capacity to mediate antibody-dependent cellular cytotoxicity (ADCC) by spleen cells as well as on complement-dependent, arming and potentiating serum activities.

MATERIAL AND METHODS

Animals and tumor

Female C_3H mice (Charles River, Italy; 22 ± 2 g of body weight) were used throughout. The mice were injected intraperitoneally on

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day 0 with 15×10^6 cells of the allogeneic L1210 leukemia which had been maintained by weekly i.p. passages in compatible DBA/2 hosts. Leukemia cell viability was assessed microscopically by dye exclusion and only preparations showing over 95% viability were used for transplantation.

Drug

C. parvum (Lot No. PX 374) was obtained from Wellcome Research Laboratories, Beckenham, England, and a dose of 0·7 mg was injected intravenously at different times before tumor allografting.

Sera

Blood was collected from the retroorbital plexus of 4–6 mice per group on various days after tumor transplantation; the sera (henceforth referred to as S6, S7, etc. according to the day of collection) were heat inactivated (56°C for 30 min) and stored at −20°C until used.

Spleen cell populations

An anti-theta C_3H serum prepared in AKR/J mice [16] was used to determine the percentage of T cells on preparations from individual spleens resuspended after twice washing in RPMI 1640 medium (Biocult, Scotland) supplemented with 10% fetal calf serum (FCS). Specifically, 2×10^5 spleen cells in 50 μl RPMI-FCS were incubated for 45 min at 37°C with 50 μl of 1:8 anti-theta serum and 50 μl of 1:4 diluted guinea pig serum previously absorbed with agarose [17] as a source of complement. The number of viable cells was counted by Trypan blue exclusion on 5 replicates per test; in the conditions described the anti-theta serum killed > 95% of C_3H thymocytes and < 5% of bone marrow cells. Identification of leukemic cells in spleen cell suspensions was routinely performed using morphologic criteria after preliminary experiments in which the same suspensions were also treated with cytotoxic C_3H anti-L1210 serum had confirmed the reliability of this approach.

The percentage of EA-rosette forming cells (EA-RFC) was determined as described by Kedar *et al.* [18] employing sheep erythrocytes (E) sensitized with rabbit anti-E serum. At least 200 cells per sample were scored with 5 replicates per test; those elements having 3 or more erythrocytes bound at their periphery were considered EA-RFC.

The percentage of spleen macrophages was evaluated by a modification of the procedure of Evans [19]. Spleen cells (2×10^6/ml RPMI-FCS) were incubated in a hemocytometer for 60 min at 37°C in a humidified 95% air–5% CO_2 atmosphere in the presence of carbon particles [20]; the hemocytometer was then immersed in Hank's and the floating coverslips lifted off, thus removing non-ingested carbon and non-adhering cells. The number of adherent cells which had ingested carbon particles was then determined microscopically. Depletion of macrophages from spleen cell preparations was performed by the carbonyl iron method [21].

DNA synthesis in vivo

Mice were injected i.p. with 50 μCi of thymidine-methyl-^{3}H(^{3}H-TdR; The Radiochemical Center; specific activity 18·4Ci/mmol) 1 hr before sacrifice. After twice washing, 10^7 spleen cells in 1 ml PBS were layered on 0·8 μ filters (Sartorius GMBH) in a Millipore multimanifold sample collector. Filters were then submitted to 3 washings with 5 ml PBS and 1 with 5% cold trichloroacetic acid, dried overnight and radioactivity then determined in a liquid scintillation counter. At least 3 replicates were counted per spleen with 5 animals per group per time.

Cell-mediated cytotoxicity

A modification of the ^{51}Cr release assay described by Brunner *et al.* [22] was used and experimental conditions were as previously described [23, 24]; 3 replicates per individual spleen were used with 5 spleens per experimental group. Specific cytotoxicity was calculated by subtracting the percent isotope release in the presence of normal splenocytes which averaged 12·5 ± 1·5% in different experiments.

Antibody-dependent cellular cytotoxicity

In this study a separate analysis was performed of *C. parvum* effects on serum potentiating and arming activity and on the capacity of the spleen cells to mediate ADCC. The effects of *C. parvum* treatments on ADCC-effector cells were investigated by assessing the cytotoxic capacity of control and test splenocytes in the presence of reference C_3H anti-L1210 ADCC-positive sera. To tubes containing labelled target cells (5×10^4 in 1 ml RPMI-FCS), 0·1 ml of 1:10 diluted normal or reference serum were added followed by 5×10^6 splenocytes in 0·2 ml RPMI-FCS from control or *C. parvum*-treated mice. Results presented are means of 3 replicates per individual spleen employing 5 animals per experimental group. Reference ADCC-positive sera (S15, S29, etc.) were obtained on various days after the i.p. transplantation of 5×10^6 L1210 cells into C_3H mice

and their arming and potentiating activities characterized as described below. An index expressing the total spleen capacity to mediate ADCC was calculated from the formula:

(% specific cytotoxicity × No. × 10^6 of spleen cells)/100, where per cent specific cytotoxicity in this case is the difference between the isotope released by spleen cells in the presence of ADDC-positive and normal C_3H serum.

For the evaluation of serum arming activity, 0·1 ml of the test serum was mixed with 5×10^4 L1210 cells in 1 ml RPMI-FCS and splenocytes from normal C_3H donors added at an attacker to target cell (A:T) ratio of 100:1. For serum potentiating activity, immune splenocytes were employed, i.e. cells harvested from C_3H mice 7 days after the i.p. inoculation of 5×10^6 L1210 cells, a challenge which is resisted by 100% of the C_3H hosts. Since the same serum may result in a potentiation or in a blocking of CMC depending on the A:T ratio employed in the assay [25], for evaluating serum potentiating activity a range of A:T ratios (20:1 to 100:1) was used.

In the absence of lymphoid cells all sera employed in these studies did not modify the isotope release from target cells as compared to normal C_3H serum, thus excluding the presence of feeder or toxic effects.

Complement-dependent cytolytic antibody

Conditions of testing were as described in reference 24. The mean percentage of isotope release in the presence of normal serum and complement was subtracted and the highest serum dilution still giving at least 20% specific cytotoxicity taken as cytotoxic antibody titer.

Statistical analysis

Statistical significance of *in vitro* data was assessed by analysis of variance, multiple tests, and *in vivo* results were analyzed by Fisher's exact test.

RESULTS

Effect of C. parvum *on allograft rejection*

When an inoculum of 15×10^6 cells of the allogeneic L1210 leukemia was given i.p. to C_3H mice, from 50 to 65% of the recipients succumbed to progressive tumor growth with a mean survival time of 9·2 ± 2·0 days (Table 1). If the C_3H recipients were pretreated with single i.v. injections of *C. parvum*, either increased or decreased protection against this leukemia challenge could be observed depending on the day of immunoadjuvant injection.

Table 1. *Effect of* C. parvum *on tumor allograft rejection*

C. parvum	Day of treatment	MST*	D/T†
−	—	9·2 ± 2·0	12/20
+	−2	10·4 ± 2·5	16/20
+	−4	—	2/20§
+	−5	10·5 ± 1·3	4/20‡
+	−9	10·4 ± 3·1	20/20§

*Mean survival time in days ± S.E.
†Dead with leukemia over total number of mice injected.
‡$P < 0·05$.
§$P < 0·01$.

In fact when *C. parvum* was given 4 or 5 days before transplantation, from 90 to 100% of the hosts were capable of rejecting the tumor whereas when the treatment preceded leukemia inoculation by 9 days an enhancement of tumor takes was observed since 100% of the animals died of leukemia versus 60% of the controls. Administration of *C. parvum* 2 days before tumor transplantation either had no effect or was followed by a slightly increased incidence of progressive tumor takes.

In view of the fact that the highest antitumoral resistance in these conditions was seen after injecting *C. parvum* 4 days before leukemia allografting, this schedule of treatment was selected for subsequent analysis of the immunological modifications accompanying protective treatments with *C. parvum.*

Effect on spleen cell populations

Injection of *C. parvum* induced marked changes in the spleen cellularity of both normal and tumor-transplanted mice (Fig. 1). In the former group the number of mononuclear spleen cells was significantly increased already 3 days after treatment with a peak reached on day 5 at values which were approximately

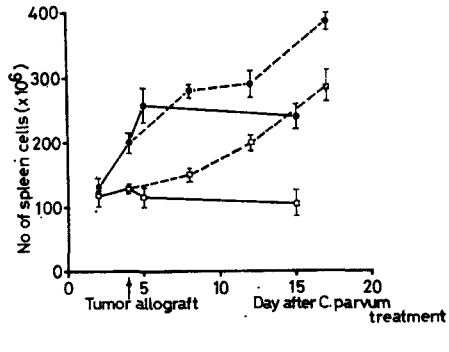

Fig. 1. *Spleen cell numbers in control* (□) *and* C. parvum-*treated* (●) *mice. Continuous and dotted line represent normal and tumor allografted mice respectively.*

130% higher than controls, and no significant decreases in spleen cellularity were observed up to day 15 when observation was discontinued. In tumor-allografted control mice, a progressive increase in spleen cell number was observed from day 4 after leukemia inoculation and on day 15 the values found were still 140% above control level; a similar pattern was seen in those mice which had been pretreated with *C. parvum*, the difference between control and stimulated animals averaging approximately 100 million cells throughout the period of observation. Values presented in Fig. 1 have been subtracted of the leukemia cells present in the organ, which never exceeded a value of 5% in these conditions.

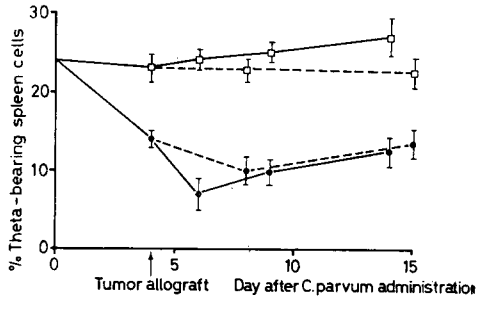

Fig. 2. Percent theta-bearing cells in control (□) and C. parvum*-treated (●) spleens. Continuous and dotted line represent normal and tumor allograft mice respectively.*

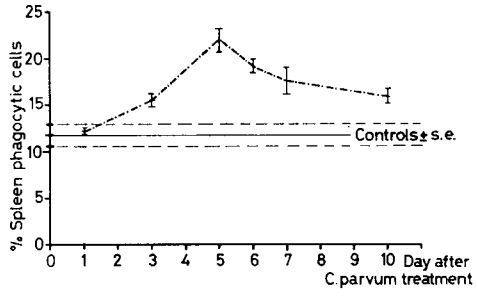

Fig. 3. Percent macrophages in non-tumor allografted mice, treated with C. parvum.

The percentage of theta-bearing spleen cells was also evaluated in these animals (Fig. 2). It was observed that treatment with *C. parvum* was followed in both normal and tumor-allografted mice by a consistent and marked decrease in the percentage of T cells which reached its nadir (7%) 6 days after adjuvant injection. Although a tendency to an increase in this value was present at later times, on day 15 after *C. parvum* the percent of theta-positive cells in the spleen was still about one half (12·5%) of control values. As can be seen in Fig. 2, the changes observed in normal mice were practically indistinguishable from those seen in allografted hosts. Taking into account the increase in organ cellularity present in *C. parvum*-treated mice, the total number of theta-bearing splenocytes was not however significantly changed in respect to normal values.

In normal, untreated mice the percentage of spleen macrophages was found to be $11{\cdot}8 \pm 1{\cdot}8\%$; treatment with *C. parvum* resulted in an increase in the percentage of phagocytic-adherent cells which was detectable already 3 days after drug injection (Fig. 3), reached a peak on day 5 ($22 \pm 1{\cdot}4\%$) and slowly declined thereafter. Results obtained in tumor-transplanted, adjuvant-pretreated mice were qualitatively and quantitatively similar. Considering the increase in organ cellularity following *C. parvum* injection, the total number of phagocytic elements in treated spleens was greater than is apparent from Fig. 3. Indeed, on day 5 the number of macrophages in adjuvant-treated organs was calculated to be $31{\cdot}5 \pm 3{\cdot}3 \times 10^6$ as compared to $12{\cdot}4 \pm 2{\cdot}1 \times 10^6$ of the controls.

Table 2. EA-rosette forming cells in the spleen of control and C. parvum*-treated mice*

Day after tumor allograft*	Experimental group	% EA-RFC
0	Control	$42 \pm 1{\cdot}2$
	C. parvum	$43 \pm 1{\cdot}3$
3	Control	$49 \pm 1{\cdot}5$
	C. parvum	$48 \pm 1{\cdot}9$
7	Control	$50 \pm 1{\cdot}1$
	C. parvum	$52 \pm 2{\cdot}1$

**C. parvum* was injected 4 days before challenge with allogeneic tumor cells.

As shown in Table 2, no differences were observed in the percentage of EA-RFCs between control and *C. parvum*-treated animals, whether tumor allografted or not. Therefore, taking as average a value of 100% for the increase in spleen cellularity given by the *C. parvum* treatment employed, the total number of EA-RFCs in this organ was in effect approximately doubled during the 5–15 day period after agent injection.

In order to evaluate the role played by cell proliferation in the observed rise in spleen cell numbers in adjuvant-treated mice, *in vivo* DNA synthesis was investigated in these animals. Figure 4 shows that a slight (22%) though significant ($P < 0{\cdot}05$) increase in ^{3}H-TdR uptake was present already one day after *C. parvum*; the maximum was found on day 2 with levels which were approximately 90% higher than baseline values, thereafter a tendency

towards a gradual decline in ^{3}H-TdR uptake was recognizable.

Effect on immune effector mechanisms

The first immune parameter examined in these mice showing increased protection against allogeneic tumor transplants after *C. parvum*, was spleen CMC. Since it had been reported [7, 14, 15] that cellular cytotoxicity could be non specifically stimulated by *C. parvum* also in non-tumor transplanted animals, a group of these animals was also included in the study. Splenocytes harvested 4 days after treatment with *C. parvum* of normal C_3H mice did not show any significant cytotoxic capacity towards L1210 cells, the values observed being indistinguishable from background values (Table 3). Similar results were obtained with cells collected up to 12 days after *C. parvum* administration.

Spleen anti-L1210 CMC was than evaluated

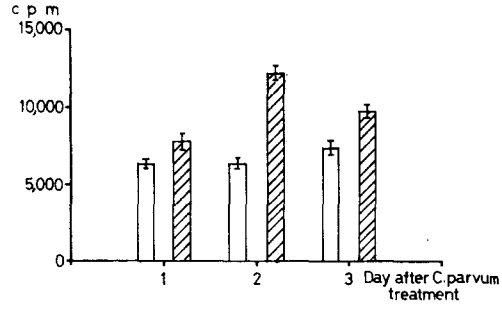

Fig. 4. ^{3}H-TdR incorporation in control (white column) and C. parvum*-treated (hatched column) spleens.*

in tumor allografted mice pretreated or not with *C. parvum*; no significant differences in cytotoxic capacity between the 2 groups of animals could be observed (Table 3) at any of the times examined over the day 5–30 range investigated and employing A:T ratios of 100:1 to 500:1. When splenocytes from both groups were tested against the EL_4 leukemia employed as a specificity control, ^{51}Cr release did not rise above background values. Since the total number of spleen mononuclear cells was markedly increased in *C. parvum*-treated hosts, the total spleen CMC capacity was in effect significantly higher in these animals showing an increased tumor resistance after *C. parvum* treatment. Prior removal of phagocytic cells from these splenocyte suspensions did not reduce their cytotoxic activity (Table 4); it may be noted on the contrary that, in some experiments, a significantly increased cytotoxic capacity could be seen in phagocyte-deprived cellular preparations.

The effects of *C. parvum* on the cellular and

Table 3. Spleen CMC in control and C. parvum*-treated mice*

Day after tumor allograft	Experimental group	A:T Ratio	% Specific cytotoxicity (± S.E.)
0	Control	100:1	—
		500:1	—
	C. parvum	100:1	—
		500:1	—
5	Control	100:1	4·3 ± 1·2
		500:1	5·9 ± 0·9
	C. *parvum**	100:1	2·0 ± 0·6
		500:1	3·9 ± 1·1
7	Control	100:1	9·9 ± 0·5
		500:1	18·3 ± 2·1
	*C. parvum**	100:1	7·3 ± 1·4
		500:1	18·6 ± 0·8
13	Control	100:1	10·4 ± 2·5
		500:1	27·9 ± 3·0
	*C. parvum**	100:1	14·2 ± 3·1
		500:1	31·9 ± 2·8
15	Control	100:1	14·4 ± 1·8
	*C. parvum**	100:1	10·3 ± 2·5
30	Control	100:1	13·6 ± 0·8
	*C. parvum**	100:1	10·9 ± 0·9

**C. parvum* was injected 4 days before challenge with allogeneic tumor cells.

humoral arms of ADCC were then examined. When the capacity of spleen cells from adjuvant-injected, non-tumor-allografted hosts to mediate ADCC in the presence of a reference arming serum (S29) was investigated, significantly higher cytotoxicity values were observed than seen with untreated controls (Table 5). Moreover, significant cytotoxicity was detected also when splenocytes from *C. parvum*-treated mice were tested in the presence of a late serum (S64) unable in the conditions employed to render cytotoxic normal C_3H spleen cells.

Table 4. Effect of removal of phagocytic cells on spleen cell-mediated cytotoxicity

Experimental group*		Treatment with carbonyl iron	% Specific cytotoxicity
Exp. 1	Control	−	14·4 ± 0·9
	Control	+	34·4 ± 3·0†
	C. parvum	−	10·3 ± 0·4
	C. parvum	+	12·7 ± 0·7
Exp. 2	Control	−	13·6 ± 1·1
	Control	+	13·4 ± 1·8
	C. parvum	−	10·9 ± 1·5
	C. parvum	+	14·8 ± 1·3

**C. parvum* was injected 4 days before tumor allograft. Spleen cells were collected 15 days (exp. 1) or 18 days (exp. 2) after tumor transplantation.

†$P < 0{\cdot}01$ versus untreated control spleen cell preparations.

Table 5. *Effect of* C. parvum *on spleen effector cells involved in antibody-dependent cellular cytotoxicity*

Splenocytes	Arming serum	% Specific cytotoxicity	No. cells/ spleen ($\times 10^6$)	Spleen ADCC activity index
Control*	S29	4·6 ± 0·4†	128·6 ± 3·8	5·9
*C. parvum**	S29	9·2 ± 0·4†‡	198·6 ± 13·1	18·3
Control*§	S29	4·2 ± 0·4†	103·0 ± 10·1	4·3
*C. parvum**§	S29	8·3 ± 0·3†‡	160·5 ± 19·3	13·4
Control*	S64	1·2 ± 0·8	114·8 ± 19·5	2·4
*C. parvum**	S64	4·8 ± 0·6†‡	258·0 ± 25·6	12·4
Control¶	S15	10·2 ± 0·9†	130·5 ± 15·2	13·2
C. parvum¶	S15	12·3 ± 1·4†	205·2 ± 18·8	25·2
Control	S15	11·7 ± 1·2†	94·6 ± 6·9	11·1
Sheep erythrocytes‖	S15	4·4 ± 0·9†	178·4 ± 22·7	7·8

*Spleen cells were collected from normal mice 6 days after treatment with *C. parvum*.
†$P < 0{\cdot}05$ versus samples containing normal C_3H serum.
‡$P < 0{\cdot}05$ versus control splenocytes.
§Phagocytic cells were removed by carbonyl iron.
¶Spleen cells were collected 6 days after tumor allograft, i.e., 10 days after *C. parvum*.
‖4×10^8 sheep erythrocytes were injected i.p. 5 days before testing.

Results presented in Table 5 were obtained using spleen cells collected 6 days after *C. parvum* injection, but no qualitative differences were seen when cells harvested from 5 to 10 days after adjuvant injection were tested or other arming sera employed. Considering the increase in spleen cell numbers present in *C. parvum*-treated mice, the total capacity of this organ to mediate ADCC was increased to a greater extent than it would appear from the *in vitro* data. The spleen ADCC index was in fact more than tripled in respect to control on day 6 and for days 5, 8 and 10 the calculated values were 17, 19 and 13 respectively. Table 5 also shows that increases in spleen cell numbers of a degree comparable to that given by *C. parvum* could be seen after the i.p. injection of 4×10^8 sheep erythrocytes; however, the spleen ADCC index computed for this group of animals was not significantly different from normal control levels. From the same table it can also be observed that previous removal of phagocytic cells did not modify the ADCC-mediating capacity of spleen cell preparations from control and *C. parvum*-treated mice.

At variance with non-tumor-transplanted mice, in leukemia allografted mice pretreated with *C. parvum* the capacity of splenocytes to mediate ADCC in the presence of a potentiating serum was not significantly higher than found in the appropriate controls (Table 5). Again, however, the total spleen ADCC indexes were markedly higher taking into consideration the raised cell number present in this organ.

Finally, the effects of *C. parvum* treatment on the serum cytolytic, arming and potentiating activities were investigated. As shown in Table 6, where the results of 2 representative experiments are pooled, no significant differences were observed between adjuvant-treated and untreated mice in the levels of complement-dependent cytolytic antibody in sera taken from 6 to 13 days after tumor transplantation. In these experimental conditions a significant serum arming activity was first detected in

Table 6. *Anti-L*1210 *complement-dependent cytolytic antibody levels in control and* C. parvum*-treated mice*

Day after tumor allograft	Experimental group	Antibody titer
6	Control	1:2
	*C. parvum**	1:2
7	Control	1:4
	*C. parvum**	1:4
11	Control	1:32
	*C. parvum**	1:32
13	Control	1:128
	*C. parvum**	1:128

**C. parvum* injected 4 days before tumor transplantation.

control mice 8 days after tumor inoculation, increasing progressively thereafter until day 13 when observation was discontinued (Fig. 5); no differences in the kinetics of the response nor in the levels of this serum activity were observed in adjuvant-injected hosts. Results presented in Fig. 5 were obtained employing final serum dilutions of 1:15, but no differences between control and *C. parvum* sera were detected with lower (1:7) or higher (up to

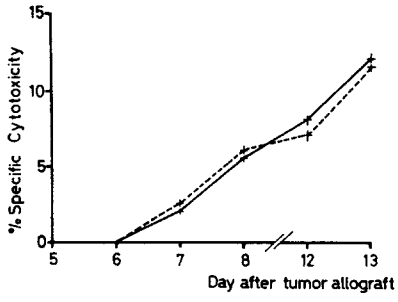

Fig. 5. *Serum arming activity in control (continuous line) and* C. parvum*-treated (dotted line), tumor-allografted.*

1:60) serum dilutions. Potentiating activity was also evaluated in the same sera, employing for these tests A:T ratios ranging from 20:1 to 100:1; representative results employing S8 are given in Table 7. It can be seen that when a low A:T ratio was used, serum from adjuvant-treated and untreated donors potentiated CMC to a similar extent, showing 5–6% increases in specific cytotoxicity. At a higher (100:1) A:T ratio neither *C. parvum* nor control sera significantly modified the cytotoxic capacity displayed by immune splenocytes. Results qualitatively similar to those just described were obtained employing sera taken 7, 9 and 11 days after tumor transplantation.

DISCUSSION

The results obtained in this study confirm previous observations that treatment with *C. parvum* can increase host's antitumor resistance. As described by others also in various syngeneic tumor models, in this system the conditions employed for adjuvant treatment were critical, since either no effect, increased protection or enhanced tumor takes could be seen depending on the relative timing between *C. parvum* injection and tumor transplantation. In this regard *C. parvum* does not therefore appear to be different from other immunomodulators such as BCG or Levamisole, agents for which a careful choice of treatment conditions must also be made in order to obtain an antitumoral effect [24, 26–29].

Table 7. *Serum potentiating activity in control and* C. parvum*-treated mice*

	% Specific cytotoxicity (± S.E.)	
Serum*	20:1†	100:1†
Normal	1·1 ± 0·9	58·9 ± 1·1
Control	6·7 ± 1‡	55·8 ± 1·4
C. parvum§	7·1 ± 1·2*	56·3 ± 1·3

*Serum was collected 8 days after tumor transplantation.
†A:T ratio.
‡$P < 0{\cdot}05$ versus normal serum.
§*C. parvum* was administered 4 days before tumor allograft.

The main purpose of this study was to investigate the modifications in immune parameters following a protective treatment with *C. parvum* in the effort to obtain a better knowledge of the mechanism(s) of action of this adjuvant. An increase in the number and functional capacity of macrophages has been proposed as playing the major role in the antitumoral activity of systemically injected *C. parvum* [6, 7, 11, 13], a well known reticulo-endothelial stimulant [10, 22]. In addition, peritoneal macrophages from normal animals treated with *C. parvum* have been reported to be cytostatic against a variety of tumor cells *in vitro* [7, 14, 15] as seen also with other stimulants such as BCG or endotoxin [30–32]. In support of the view that non specific mechanisms underly the antitumor effect of *C. parvum*, were also the facts that a depression of T-cell function can be seen in animals given this adjuvant systemically [33] and that the antitumor effect of i.v. *C. parvum* could still be observed in T-cell deprived mice [34]. The finding, however, of a direct relationship between tumor immunogenicity and the protective effect of *C. parvum* and *C. granulosum* [3, 35] suggested that the antineoplastic activity of these agents involves other mechanisms in addition to non-specific stimulation of macrophages. More directly, the development of a systemic antitumor immunity has been reported to occur after injections of *C. parvum* [4, 8, 36]. The possibility that a stimulation of specific immune mechanisms plays a role also in the antitumoral effect of i.v. *C. parvum*, at least in allogeneic conditions, appears to be supported by the results presented here.

Considering first CMC, although the cytocydal efficiency of splenocytes from adjuvant-treated mice was not significantly higher than that of control cells, because of the increase in spleen cellularity the total capacity of this organ to attack tumor cells was in effect substantially raised and could thus have significantly contributed to the increased tumor resistance displayed by *C. parvum* injected animals.

In allogeneic tumor systems, both T and non-T cells appear to mediate CMC [37]; in view of the fact that splenocyte preparations from adjuvant-treated mice displayed the same *in vitro* cytocydal capacity of control cells in spite of a lower percentage of T cells, non-T elements would thus seem to contribute to

cytotoxicity in a larger measure than in controls. This observation is in keeping with previous evidence that B-cell responses are potentiated by *C. parvum* [38], whereas spleen T-cell responses such as PHA and mixed lymphocyte reactivity or GVH-inducing capacity [33] are depressed after i.v. injections of this agent. The lower percentage of T cells observable in lymphoid organs of *C. parvum*-treated mice could provide an explanation of this depressed reactivity, for which an interaction requiring cell contact between activated macrophages and T lymphocytes has also been proposed [39] as an additional mechanism. The latter mechanism could also explain our observations of a higher CMC in macrophage-depleted splenocyte preparations.

The increase observed in spleen ADCC-mediating capacity can also be regarded as an important factor in the higher antitumoral resistance given by *C. parvum* in our conditions, as this effector mechanism can indeed play a crucial role in tumor resistance [40, 41]. A similar increase in ADCC-mediating activity has been reported by Allison [42] following treatment with Freund's complete adjuvant. Consensus exists that T cells are not involved in mediating ADCC [43] but the precise nature of the relevant population(s) is still unclear [43, 44]. Our data showing a higher number of EA-RFCs in the spleens of *C. parvum*-treated mice displaying an increased ADCC effector activity, cannot resolve this point since this type of rosette can be formed by B and other types of cells, such as macrophages or granulocytes [18].

The possible role of macrophages in mediating the increased tumor resistance found in our conditions cannot be assessed with certainty. We could not confirm that the administration of *C. parvum* to normal animals is followed by the appearance of unspecific tumorocydal activity in the spleen [7, 15]. As previously mentioned, the removal of phagocytes from splenocyte preparations from allografted, adjuvant-treated mice did not impair their CMC and ADCC capacities. These results would apparently seem to argue against the possibility that macrophages acted as effector cells in these conditions. A definite conclusion on this point cannot, however, be reached; not only the short term cytotoxicity assay employed might not have provided the best conditions for revealing a cytocydal macrophage activity, but results obtained in the spleen may not be representative of the conditions operative in the peritoneal cavity where tumor rejection took place.

Whether on the other hand, the marked and prolonged rise in macrophage numbers found in the spleen after *C. parvum* plays a role in the inductive if not effector phases of antitumoral response remains also to be resolved.

Increased lymphocyte trapping has been reported to play a major role in the higher spleen cellularity given by *C. parvum* [45] and activated macrophages considered the principal mediators in this process [46]. Whether this occurred also in our conditions was not tested; if so it may be reasoned that non-T cells possibly constituted the majority of the lymphoid cells trapped in the spleen. From our data it is clear however that this immunoadjuvant also induced a marked degree of cell multiplication in this organ, whose kinetics paralleled the increase in cellularity. In this connection, the fact that the total number of T cells in the spleen did not change and that only a dilution occurred, may be taken as an indication that non-T elements and macrophages were the populations mostly undergoing multiplication.

Evidence has been presented that *C. parvum* can stimulate the antibody response to various types of antigens in normal and tumor-bearing animals [12, 47]. In our conditions, however, an increase in the humoral response to this tumor was not detectable and such a mechanism did not appear to significantly contribute to the higher capacity to reject an allogeneic leukemia induced by this adjuvant.

In conclusion, our results indicate that the enhanced resistance towards allogeneic neoplasms caused by *C. parvum* is accompanied by an increase in the total spleen CMC activity and ADCC-mediating capacity. Both these findings can be explained by an increase in the total number of non-T cells observable in these animals, which is, at least partially, the consequence of a higher cell multiplication occurring in the organ. These results thus indicate that after systemic injections of this adjuvant an augmentation of specific immune responses occurs and not only an activation of non specific mechanisms as proposed by others [7]. Whether these conclusions also hold true for syngeneic tumor systems is currently under investigation.

For its possible therapeutic implications, one point deserves comment, namely the possible interest in associating *C. parvum* treatments leading to a potentiation of ADCC-mediating cells with passive immunotherapy with ADCC-positive sera. Preliminary data have in fact given clear indications of therapeutic synergism employing this combined approach.

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