Immunology 1978 **35** 685

Immunomodulation by *Corynebacterium parvum*

I. VARIABLE EFFECTS ON ANTI-SHEEP ERYTHROCYTE ANTIBODY RESPONSES

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Received 30 *September* 1977; *accepted for publication* 6 *March* 1978

Summary. *Corynebacterium parvum* injected i.p. 1–16 days prior to i.p. antigen inoculation virtually abolished both IgM and IgG primary responses to 1×10^8 SRBC. The suppression was significantly marked at antigen doses ranging from 1×10^6–1×10^9 SRBC but not at 5×10^9 SRBC. As little as 56μg *C. parvum* caused a marked suppression of the response to 1×10^8 SRBC. In secondary responses *C. parvum* given either one day before priming with 1×10^8 SRBC or one day before secondary challenge caused a dramatic suppression of both IgM and IgG PFC responses. In contrast with i.p. injected *C. parvum*, i.v. injection of the vaccine enhanced immune responses to i.p. or i.v. injected SRBC. Similarly *C. parvum* injected i.p. prior to i.v. immunization resulted in an augmented anti-SRBC response. An enhancement of anti-SRBC response was also noted when *C. parvum* was injected i.p. on the day of i.p. immunization. The suppressed responses in *C. parvum* injected animals could be explained partly by the reduced splenic localization of the antigen.

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0019-2805/78/1100-0685$02.00

INTRODUCTION

Corynebacterium parvum is a powerful stimulant of the reticuloendothelial system (Neveu, Branallec & Biozzi, 1964; O'Neill, Henderson & White, 1973; Warr & Sljivic, 1974a) and an effective immunopotentiator; it augments humoral immune responses (Biozzi, Stiffel, Mouton, Bouthillier & Decreusefond, 1968; Howard, Scott & Christie, 1973; O'Neill *et al.*, 1973; Warr & Sljivic, 1974b; Greenberg & Dimitrov, 1976) and exerts an anti-tumour effect in animals (Scott, 1974a; Woodruff, Dunbar & Ghaffar, 1973) and man (Reed, Gutterman, Mavligit & Hersh, 1975; Fisher, Rubin, Sartiano, Eunis & Wolmark, 1976). In contrast, the organism has been shown to suppress delayed hypersensitivity responses (Allwood & Asherson, 1972; Scott, 1974b). We now report that under certain conditions *C. parvum* also inhibitshumoral responses against sheep erythrocytes.

MATERIALS AND METHODS

Animals

Male 8–10 week-old $(C_{57}Bl\times DBA/_2)F_1$ (BDF_1) and C3H mice were used in all studies.

Antigen

Sheep erythrocytes (SRBC) were used as antigen.

Sheep blood was obtained from Cordis Laboratories, Miami, Florida, as 25% blood in Alsever's solution. SRBC were washed and suspended in saline at the appropriate concentration (usually 5×10^8 cells/ml) and the required number of cells were injected i.p. in 0·2 ml volume.

C. parvum

Formalin-killed suspension of *C. parvum* CN6134 containing 7 mg/ml dry weight of organisms was kindly given by Dr J. Whisnant of Burroughs Wellcome, Research Triangle, North Carolina. In most experiments 0·2 ml (1·4 mg) of this suspension was injected i.p. one day before the antigen. When varying doses of *C. parvum* were to be tested, the original suspension was diluted in saline so that the required dose could be administered in 0·2 ml volume.

Antibody plaque forming cell (PFC) assay

Spleens were removed 4 days after (unless otherwise stated) the inoculation of the antigen, and single cell suspensions were prepared by a method described earlier (Ghaffar & James, 1973). The humoral immune responses against SRBC were determined, for individual mice, by the method of Cunningham & Szenberg (1968) as modified by Dresser & Greaves (1973).

Indirect PFC were developed by rabbit anti-mouse IgG serum obtained from Miles Laboratories and IgG plaques were enumerated according to Dresser & Greaves (1973).

Splenic localization of SRBC

Mice were injected with 1×10^8 [^{51}Cr]-labelled SRBC by the i.p. or i.v. route and the amounts of radioactivity present in the spleen was measured 18h after injection, a time which was found to be optimal. The method for labelling SRBS with ^{51}Cr has been described elsewhere (Warr & Sljivic, 1974a).

Presentation of results

Nucleated spleen cell counts have been expressed as the geometric mean for the group. The PFC results have been expressed as $\log_{10}$ mean ±1 standard error for groups of mice. The splenic uptake of SRBC is represented by the mean counts per min. The significance of differences between means was determined by the standard two-tail t test.

RESULTS

Effect of *C. parvum* on the primary anti-SRBC responses

It is apparent from data summarized in Table 1 that 1·4 mg *C. parvum* injected one day before the antigen invariably suppressed both IgM and IgG responses. From the kinetics of the immune response summarized in Fig. 1 it is apparent that *C. parvum* injected i.p. one day before i.p. immunization did not merely alter the onset of the immune response: the supression of the response was consistent when tested between 3 and 8 days after the antigen administration.

The magnitude of the inhibition of anti-SRBC responses in these mice was dependent on the dose of *C. parvum* administered (Fig. 2). Thus, the immunosuppressive effect of the organism decreased with the decreasing amounts of *C. parvum* administered 1 day before the antigen. It is noticeable, however, that none of the doses caused an enhancement of the anti-SRBC response, and as little as 56 μg dry weight of *C. parvum* caused a significant decrease of both IgM and IgG PFC (Fig. 2).

The depression of anti-SRBC responses (both IgM and IgG) was apparent in mice receiving as high an antigen dose as 1×10^9 SRBC but not 5×10^9 SRBC (Table 2). In fact, *C. parvum* slightly augmented the immune response to the latter antigen dose, although only the increase in IgM PFC per spleen in *C. parvum* treated animals was found statistically significant. It is noticeable that control mice receiving this antigen dose had a significantly lower IgM and IgG responses compared with mice receiving 1×10^9 SRBC.

Data summarized in Table 3 indicate that *C. parvum* not only suppressed anti-SRBC response when given one day before the antigen, but it also suppressed IgM and IgG responses when injected up to 16 days before the antigen. In contrast, there was a significant augmentation of the immune response when *C. parvum* was injected immediately prior to the antigen. There was no significant effect on the anti-SRBC response when *C. parvum* was injected one day after the antigen.

C. parvum injected i.p. before the antigen also suppressed, in most instances, anti-SRBC responses in C3H mice (Table 4). On one occasion however, *C. parvum* treatment significantly increased both IgM and IgG responses in this strain of mice. It is

Table 1. Suppression of anti-SRBC responses by *C. parvum* in BDF_1 mice

Experiment no	Spleen index†	IgM PFC per spleen‡			IgG PFC per spleen‡		
		Normal	*C. parvum* treated	*P* value	Normal	*C. parvum* treated	*P*§ value
1	1·33	163,815 (4)	12,223 (4)	<0·001	N.T.	N.T.	
2	1·26	79,974 (5)	22,932 (5)	<0·01	N.T.	N.T.	
3	1·34	46,662 (4)	1846 (5)	<0.005	42,734 (4)	1667 (3)	<0.005
4	1.83	52,692 (6)	1188 (6)	<0.001	14,619 (6)	364 (6)	<0·001
5	1·29	44,269 (5)	879 (5)	<0.001	20,269 (5)	263 (5)	<0·001
6	1·29	104,116 (5)	2004 (5)	<0·001	63,181 (5)	134 (5)	<0·001
7	1·36	71,283 (5)	6503 (5)	<0·001	10,305 (5)	214 (5)	<0·005
8	2·09	72,729 (4)	12,475 (5)	<0·02	29,711 (4)	2885 (5)	<0.02

* 1·4 mg *C. parvum* injected i.p. one day before i.p. immunization with 1×10^8 SRBC, PFC responses measured four days thereafter.

† $\frac{\text{No of cells in } C.\ parvum \text{ treated spleen}}{\text{No of cells in the normal spleen}}$

‡ Geometric mean of PFC in individual spleens. Numbers in parentheses indicate the number of mice in each group. N.T.= not tested.

§ Comparison of normal and *C. parvum*-treated groups by the 2-tail Student's *t* test.

Table 2. Effect of *C. parvum* on the primary anti-SRBC responses in BDF_1 mice.* Influence of the antigen dose

Antigen dose	*C. parvum* treatment	No of mice/ group	Nucleated cells/ spleen $\times10^{-6}$	PFC/10^6†		PFC per spleen†	
				IgM	IgG	IgM	IgG
1×10^6	No	5	149	1·708±0·225 (51)	none detected	3·876±0·0220 (7508)	none detected
	Yes	5	253	0·230±0·108¶ (1·7)		2·637±0·109¶ (433)	
1×10^9	No	4	159	2·933±0·094 (858)	2·688±0·115 (487)	5·136±0·099 (136,634)	4·890±0·123 (77,585)
	Yes	4	192	2·270±0·213‡ (186)	1·820±0·187‡ (66)	4·554±0·203 (35,783)	4·105±0·161§ (12,730)
5×10^9	No	4	198	2·286±0·183 (193)	2·248±0·224 (177)	4·583±0·165 (38,312)	4·352±0·177 (22,505)
	Yes	4	276	2·597±0·046 (395)	2·352±0·087 (225)	5·019±0·059‡ (104,504)	4·796±0·102 (62,362)

* 1·4 mg *C. parvum* injected i.p. one day before antigen and tested four days thereafter.

† Log_{10} mean ± standard error; figures in parentheses are geometric means.

‡ *P* 0·05–0·025; § P 0·01—0·005; ¶ P <0·001.

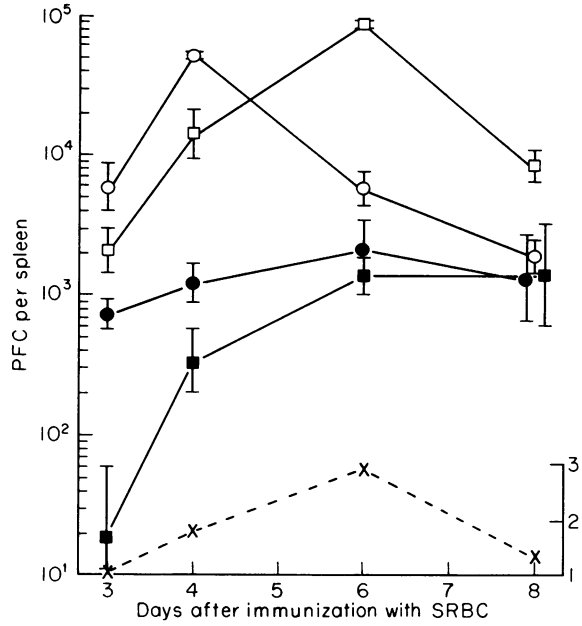

Figure 1. Supression of anti-SRBC responses by *C. parvum* in BDF_1 mice. 1·4 mg *C. parvum* or saline inoculated i.p. on day −1: 1×10^8 SRBC injected i.p. on day 0 and PFC responses tested on different days thereafter. (○), IgM in control mice; (●) IgM in *C. parvum*-treated mice; (□), IgG in control mice; (■), IgG in *C. parvum*-treated mice.

(×) $\frac{\text{Mean spleen weight in } C.\ parvum\text{-treated mice}}{\text{Mean spleen weight in control mice}}$

Vertical bars represent one s.d. of the mean

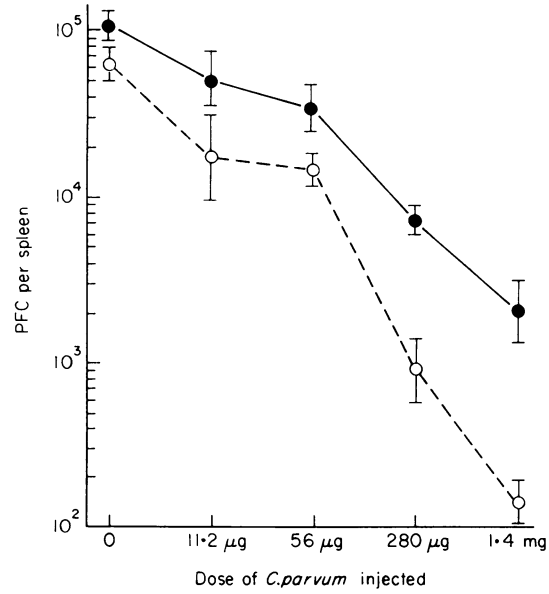

Figure 2. Suppression of anti-SRBC responses of BDF_1 mice by *C. parvum*. Effect of varying the dose of *C. parvum* injected i.p. on day −1, 1×10^8 SRBC injected i.p. on day 0 and tested for PFC responses on day 4. Vertical bars represent 1 s.d. of the mean (●), IgM; (○), IgG.

Table 3. Modification anti-SRBC* responses in BDF_1 mice: effect of timing of *C. parvum*† injection

Treatment (day)	No of mice tested	PFC per 10^6 nucleated cells‡		PFC per spleen‡	
		IgM	IgG	IgM	IgG
None	14	2·717±0·054 (521)	2·374±0·075 (236)	4·827±0·067 (67,209)	4·486±0·102 (30,592)
C. parvum					
(−16) §	5	1·225±0·138 (17)	1·158±0·166 (14)	3·745±0·148 (5,560)	3·679±0·188 (4,778)
(−7) §	4	0·949±0·191 (9)	0·491±0·232 (3)	3·488±0·211 (3,078)	3·059±0·275 (1,146)
(−1) §	6	0·763±0·321 (6)	0·230±0·109 (2)	3·075±0·137 (1,188)	2·561±0·259 (364)
(−0) ¶	5	3·067±0·143 (1167)	2·930±0·0699 (852)	5·220±0·201 (165,768)	5·083±0·093 (121,059)
(+1) **	5	2·788±0·147 (614)	1·934±0·579 (86)	4·827±0·179 (67,138)	3·977±0·578 (9,495)

* 1×10^8 SRBC i.p.
† 1·4 mg *C. parvum* i.p.
‡ $\log_{10}$ mean ± 1 s. d.; figures in parentheses are geometric means
§ Significant suppression
¶ Significant augmentation
** No significant change in the PFC responses of *C. parvum*-treated animals.

Table 4. Effect of *C. parvum* on anti-SRBC* primary responses in C3H mice

Experiment no	Spleen† index	IgM PFC per spleen‡			IgG PFC per spleen‡		
		Normal	*C. parvum* treated	*P*§ value	Normal	*C. parvum* treated	*P*§ value
1	1·95	32,995	11,703	N.S.	1978	548	N.S.
2	1·78	18,262	61,830 ¶	<0·01	1786	14,378 ¶	<0·05
3	1·29	109,358	51,522	<0·01	39,816	20,338	N.S.
4	2·06	33,135	9147	<0·05	N.D.	N.D.	—
5	1·15	74,389	12,094	<0·02	47,387	4378	<0·05

* 1·4 mg *C. parvum* injected i.p. one day before i.p. immunization with 1×10^8 SRBC, PFC responses measured four days thereafter.
† No. of cells in *C. parvum* treated spleen / No. of cells in the normal spleen
‡ Geometric mean of PFC in individual spleens. Numbers in parentheses indicate the number of mice in each group
§ Comparison of normal and *C. parvum*-treated groups by the 2-tail Student's *t* test.
¶ Significantly enhanced. *P* values greater than 0·5 were considered not significant

noticeable that the magnitude of suppression in C3H mice is smaller than that observed in BDF_1 mice.

Effect of *C. parvum* on the secondary anti-SRBC responses

C. parvum not only suppressed the primary anti-SRBC responses but it also inhibited the priming effect of 1×10^8 SRBC. Thus, mice which received *C. parvum* one day before 1×10^8 SRBC and rechallenged 16 days later with the same dose of the antigen showed a significantly reduced IgM and IgG anti-SRBC responses (Table 5). Furthermore, animals which received 1·4 mg *C. parvum* one day before the secondary challenge also showed significantly depressed immune responses. As anticipated, *C. parvum* administered both before priming and before

secondary challenge also effectively suppressed the secondary IgM and IgG responses. Thus *C. parvum* not only inhibits the maturation of the primary immune responses but also prevents the development of immunological memory, as well as abolishing the pre-existing memory.

Effect of route of *C. parvum* and antigen inoculation

The suppression of the primary anti-SRBC responses was critically dependent on the route of *C. parvum* injection as well as the route of antigen administration. Thus, while i.p. injection of *C. parvum* one day before immunization suppressed the anti-SRBC response, i.v. inoculation of the vaccine amplified this response (Fig. 3). Furthermore, neither i.p. nor i.v. injected *C. parvum* suppressed the immune response to i.v. injected SRBC. On the contrary, the response to i.v. SRBC was augmented by pretreatment with *C. parvum* either i.v. or i.p. (Fig. 3).

Effect of *C. parvum* on the splenic localization of SRBC

The uptake of the antigen injected by the i.p. or the i.v. route was studied in normal and *C. parvum*-treated animals. The results summarized in Fig. 4 indicate that i.p. injected *C. parvum* caused a significant diminution in the splenic localization of SRBC injected i.p. In contrast, i.p. injection of *C. parvum* caused an elevated uptake of i.v. injected SRBC. Similarly i.v. injection with *C. parvum* resulted in an increased uptake of both i.p. and i.v. injected SRBC. These results correspond very closely with the effect of route of *C. parvum* injection on the response of mice to SRBC described in the preceding section.

DISCUSSION

The results described in this report clearly demonstrate that *C. parvum* can exert variable effects on the humoral immune responses to SRBC. The effect is critically dependent on the route of administration of *C. parvum* and SRBC, the timing of *C. parvum* treatment in relation to the antigen and partly on the strain of animals tested, but is not so dependent on the dose of *C. parvum* or the dose of the antigen.

The suppression noted in our studies does not merely reflect an alteration in the development or kinetics of the response but is evident throughout the course of the response. This is in contrast with observations reported by Knight and Lucken

Table 5. Effect of *C. parvum* on the secondary anti-SRBC response in BDF_1 mice*

Expt no.	*C. parvum* treatment	No of mice/ group	Nucleated cells per spleen $\times 10^{-6}$	PFC/10^6 nucleated cells‡		PFC per spleen	
				IgM	IgG	IgM	IgG
1	None	6	89·5	1·477±0·053 (30)	2·634±0·100 (431)	3·431±0·100 (2,698)	4·585±0·080 (38,466)
	Before priming	3	156·7	0·146±0·221 (1·4)	1·140±0·080 (13·8)	2·332±0·202 (215)	3·333±0·186 (2,155)
	Before 2° challenge	5	100·8	0·322±0·123 (2·1)	1·336±0·078 (21·7)	2·336±0·102 (217)	3·341±0·063 (2,191)
	Before 1° and 2° challenge	4	176·0	0·139±0·122 (1·3)	0·919±0·127 (8·3)	2·362±0·176 (230)	3·164±0·175 (1,460)
2	None	5	194	2·079±0·124 (120)	3·144±0·122 (1,393)	4·369±0·131 (23,383)	5·432±0·128 (270,628)
	Before priming	5	403	0·519±0·076 (3·3)	1·544±0·134 (35)	3·118±0·071 (1,312)	4·156±0·116 (14,317)
	Before 2° challenge	5	295	0·699±0·203 (5·0)	1·968±0·172 (93)	3·169±0·189 (1,475)	4·441±0·175 (27,607)

* Mice injected i.p. with 0·2 ml (1·4) mg *C. parvum* one day before priming, one day before secondary challenge or on both occasions. 1×10^8 SRBC were administered i.p. for both priming and secondary challenge sixteen days apart.

‡ Log_e mean ± 1 s.d.; figures in parentheses show the geometric mean of the PFC response. Responses in all mice treated with *C. parvum* by all regimens are significantly lower than controls ($P < 0{\cdot}005$).

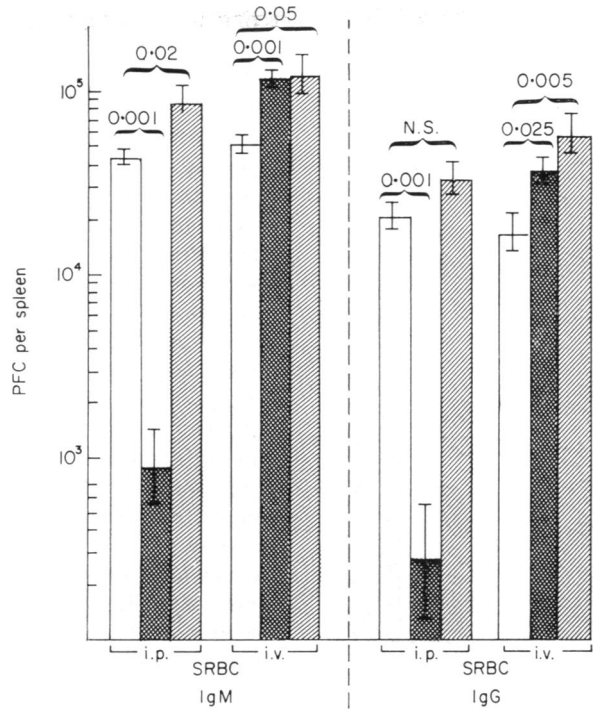

Figure 3. Effect of route of *C. parvum* and antigen inoculation on the immune response to SRBC in BDF_1 mice. Numbers above the bars indicate *P* values. *P* values greater than of 0·05 were considered not significant (N.S.). Open column, control; hatched column, *C. parvum* i.v.; crosshatched column *C. parvum* i.p.

(1975) who found that *C. parvum* only retarded the development of the response to tetanus toxoid.

The impairment of humoral anti-SRBC immunity by *C. parvum* was not restricted to animals receiving low doses of the antigen, as reported by others (Warr & Sljivic, 1974b). In the present studies, *C. parvum* suppressed immune responses of animals receiving high concentrations of the antigen, 1×10^9 SRBC. Only the animals receiving the supra-optimal dose of the antigen (5×10^9) were found to be refractory. It is to be noted that at this antigen dose, immune responses of control mice were significantly lower than the response of animals receiving a lower antigen dose (1×10^9). This phenomenon may be attributed to the stimulation of suppressor cells by the very high antigen dose (Whisler & Stobo, 1976). The accelerated clearance of the antigen in *C. parvum* treated animals may reduce the threshold of the antigen necessary for the stimulation of suppressor cells and thus result in the increased immune response to the very high antigen dose observed in our studies.

The suppressive effect of *C. parvum* was dependent on the timing of its injection in relation to the antigen. Thus, when given 1–16 days before antigen, *C. parvum* suppressed the immune response whereas when injected on the same day with antigen it augmented the response.

It has been reported that while higher doses of *C. parvum* may abrogate anti-tumour immunity, smaller doses tend to augment tumour resistance (Woodruff, Ghaffar & Whitehead, 1976). In studies reported here, this does not seem to be the case with the humoral anti-SRBC response, for as little as 56 μg *C. parvum* significantly suppressed both IgM and IgG PFC. It is, however, possible that small doses of *C. parvum* might augment cell mediated immune responses which are operative in the rejection of tumour transplants.

The suppressive effect of *C. parvum* was not restricted to BDF_1 mice. It also suppressed the response of C3H animals. It is however, interesting that the magnitude of suppression in G3H mice was smaller than that in BDF_1 mice. In fact, in one

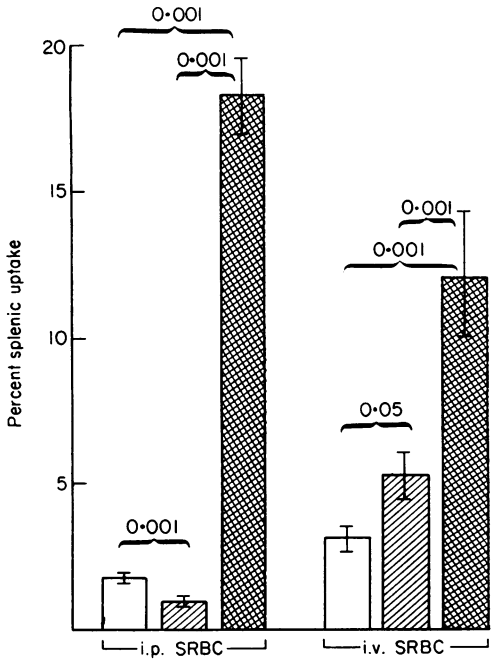

Figure 4. Effect of *C. parvum* on the splenic uptake of [^{51}Cr]-SRBC in BDF_1 mice. Numbers above the bars indicate *P* values. Open column, control; hatched column *C. parvum* i.p.; cross hatched column, *C. parvum* i.v.

experiment it augmented the response. It is therefore possible that the effect of *C. parvum* might be strain dependent.

C. parvum not only abrogated the primary responses but it also prevented the development of memory when given before the primary antigen, and decreased the expression of existing memory when injected before the secondary challenge.

Ths severe depression of anti-SRBC responses is in contrast with most of the reported findings on the effect of *C. parvum* on humoral immune responses (Biozzi *et al.*, 1968; Howard *et al.*, 1973; O'Neill *et al.*, 1973; Warr & Sljivic, 1974b; Greenberg & Dimitrov, 1976). A number of factors can be listed to explain this diversity of results. Most of the studies reporting augmented immune responses in *C. parvum*-treated animals have been carried out in CBA mice. It is interesting to note that i.p. injection of *C. parvum* in C3H mice produced less severe depression of anti-SRBC response and in one instance it caused an augmentation. It is also interesting to note that other biological effects of *C. parvum* may be dependent on the strain of mice tested (Otu, Russell & White, 1977; unpublished data).

Another factor common to the majority of studies reporting an augmentation of humoral immune responses is that they employed either a combination of i.p. and i.v. routes for *C. parvum* and SRBC administration or have used i.v. routes for both *C. parvum* and the antigen. It is not surprising that, in all cases, an augmentation of the immune response was observed. This is indeed confirmed by out studies reported here.

C. parvum might cause immunosuppression by rapid elimination of the antigen or altered localization in lymphoid organs. Impaired splenic uptake of i.p. injected [^{51}Cr]-SRBC in animals injected i.p. with *C. parvum* may explain partly the depressed response of these animals. The effects of route *C. parvum* and SRBC injection on the immune response paralleled the localization of the antigen in the spleen. The inverse correlation between the antigen dose injected i.p. and the magnitude of the suppression by i.p. injected *C. parvum* also indicates that the rate of antigen elimination might be a contributory factor in the suppressive effect of *C. parvum.* Among other alternative mechanisms is the generation of suppressor cells and inhibition of antigen-induced lymphoid cell proliferation by the activated macrophages. Macrophages activated by *C. parvum* (Olivotto & Bomford, 1974; Ghaffar, Cullen & Woodruff, 1975; Otu, Russell, & White, 1977), and by other agents (Keller, 1973, 1976) have been reported to cause inhibition of DNA synthesis in malignant as well as normal cells. *C. parvum*-activated macrophages have also been shown to inhibit DNA synthesis in mitogen stimulated lymphoid cells (Scott, 1972). Studies are currently in progress to test if the above mechanisms are operative in immunosuppression by *C. parvum.*

ACKNOWLEDGMENTS

The authors are grateful to Miss Sheila Long for excellent technical assistance. This work was supported by USPHS Contract NO1-CM-57041 from the National Cancer Institute, the Division of Cancer Treatment.

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