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TUMOUR GROWTH, PHAGOCYTIC ACTIVITY AND ANTIBODY RESPONSE IN *CORYNEBACTERIUM PARVUM*-TREATED MICE

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SUMMARY

Serum from both normal and T cell-deprived female adult CBA mice shows a background titre of antibody to *Corynebacterium parvum* of about 2–4 $\log_2$ units by a latex agglutination test. Intraperitoneal injection of *C. parvum* causes a marked rise in titre which reaches its peak after about a month, and a second injection at that time evokes a further response. Treatment with mercaptoethanol reduces the background titre, and also the titre 1–3 weeks after immunization by 1–2 log units. Subcutaneous injection of *C. parvum* on the other hand evokes little or no antibody response.

Both the antitumour effect of *C. parvum*, and its effect on clearance of colloidal carbon from the blood stream, can occur in the presence of high levels of antibody directed against the organism. Theoretical and possible therapeutic implications of these findings are discussed.

INTRODUCTION

Injection of a killed vaccine of some strains of *Corynebacterium parvum* has been shown to stimulate reticuloendothelial (RE) activity in mice (Halpern *et al.*, 1964), and to inhibit the growth of isogeneically transplanted tumours (Woodruff & Boak, 1966; Woodruff & Dunbar, 1973; Woodruff & Inchley, 1971; Woodruff, Inchley & Dunbar, 1972).

Several of these organisms have been reported to be immunogenic (Turpin *et al.*, 1959), and it seemed possible that there might be a relationship between the antibody response to the organism and its effect on phagocytic activity and tumour growth. Some data bearing on this question are available, but they are too fragmentary to point to any conclusions. It is known, for example, that the antitumour effect of *C. parvum* depends very much on the route of injection (Woodruff & Inchley, 1971; Woodruff *et al.*, 1972) but is much the

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same in T cell-deprived as in normal mice (Woodruff, Dunbar & Ghaffar, 1973); the effect of these particular variables on the antibody response to the organism, however, does not seem to have been studied.

It seemed of interest therefore to make a comparative study of the antibody response to *C. parvum* and the effect of the organism on phagocytic activity and tumour growth under a variety of experimental conditions, the main variables being the route of injection of *C. parvum* (intraperitoneal or subcutaneous) and the immunological status of the animals (intact or T cell-deprived, and whether or not pre-immunized with *C. parvum*).

MATERIALS AND METHODS

The experimental protocol is shown in Table 1.

C. parvum

Two strains of *C. parvum* have been used. One, designated CN 6134 Batch WEZ 174,* was supplied in the form of a formol-killed suspension by the Wellcome Foundation. The other, designated NCTC 10390, was obtained originally from the National Collection of Type Cultures, Colindale, and maintained in the Department of Bacteriology. Formol-killed suspensions were prepared for experimental use.

The dose, expressed as dry weight of organisms, was either 1·4 or 0·7 mg. In either case this was suspended in 0·2 ml of saline.

Animals and tumour

The mice were adult (18–22 g) female CBA/H. The tumour was a fibrosarcoma which had been induced in this strain with methylcholanthrene and was in its fifteenth transplant generation. During the experiments transplantation was performed by s.c. inoculation of 10^4 viable cells, prepared with pronase as described previously (Woodruff & Boak, 1966). As in a previous experiment (Woodruff *et al.*, 1973) we have used the sum of tumour diameters in each mouse, measured every 2 days up to some fixed time, as the index of tumour growth. The 95 per cent confidence limits for the group mean sums were calculated as $\bar{x} \pm ts/\sqrt{n}$, where $\bar{x}$, s and n denote respectively the group mean sum, its standard deviation, and the number of observations. The value of Student's function t for a 2-tailed test based on n-1 degrees of freedom and a probability (P) of 0·05 has been taken from published tables. Where it seemed appropriate group means were compared by a standard Student's t-test.

T cell-deprived mice were prepared as described by Woodruff *et al.* (1973).

Titration of antibody to C. parvum

Anti-*C. parvum* antibodies were assayed by a modification of the latex agglutination test of Florman & Scoma (1960).

The bacterial and latex (Difco; 0·81 μm) suspensions were first adjusted to give optical densities (540 nm) of 38 and 192 respectively. From these stock suspensions a mixture containing 5% bacteria and 0·5% latex (v/v) was prepared in glycine buffer, pH 8·2, containing 0·1% BSA (GBSA). The buffer was added last after giving the bacteria a few

* In previous papers (Woodruff & Inchley, 1971; Woodruff *et al.*, 1972; Woodruff & Dunbar, 1973) this organism was designated either EZ 174 or WEZ 174.

TABLE 1. Experimental protocol

	Mice		Procedure			*C. parvum*		
	Intact or T cell-deprived	Number	Day 0*	Day 28	Day 31	Strain	Dose (mg)	Parameters observed
1	Intact	12		Tumour s.c.‡				
2	Intact	12		Tumour s.c.	*C. parvum* i.p.	CN 6134	1·4	
3	Intact	6		Tumour s.c.	*C. parvum* s.c.	CN 6134	1·4	Tumour growth and
4	Intact	12	*C. parvum* i.p.†	Tumour s.c.		CN 6134	1·4	antibody response
5	Intact	17	*C. parvum* i.p.	Tumour s.c.	*C. parvum* i.p.	CN 6134	1·4	to *C. parvum*
6	Intact	6	*C. parvum* i.p.	Tumour s.c.	*C. parvum* s.c.	CN 6134	1·4	(Group 1 for
7	Intact	6		Tumour s.c.	*C. parvum* i.p.	CN 6134	0·7	background only)
8	Intact	6		Tumour s.c.	*C. parvum* i.p.	NCTC 10390	0·7	
9	Intact	6	*C. parvum* i.p.			CN 6134	1·4	
10	Intact	6	*C. parvum* s.c.			CN 6134	1·4	
11	Intact	6	*C. parvum* i.p.		*C. parvum* i.p.	CN 6134	1·4	Antibody response
12	T cell-deprived	6	*C. parvum* i.p.			CN 6134	1·4	to *C. parvum*
13	Intact	16						
14	T cell-deprived	6						
15	Intact	12		*C. parvum* i.p.		CN 6134	1·4	
16	T cell-deprived	4		*C. parvum* i.p.		CN 6134	1·4	
17	Intact	5		*C. parvum* s.c.		CN 6134	1·4	
18	Intact	6	*C. parvum* i.p.			CN 6134	1·4	Phagocytic index
19	Intact	7	*C. parvum* i.p.	*C. parvum* i.p.		CN 6134	1·4	on day 32
20	Intact	5	*C. parvum* i.p.	*C. parvum* s.c.		CN 6134	1·4	
21	Intact	5		*C. parvum* i.p.		CN 6134	0·7	
22	Intact	5		*C. parvum* i.p.		NCTC 10390	0·7	

* In this table day 0 is the day on which the whole experiment was begun.
† i.p. = intraperitoneal injection.
‡ s.c. = subcutaneous injection.

minutes to coat the latex particles. The sera were diluted (25-μl volumes) in GBSA using 'Cooke' microtitre equipment. One volume of GBSA was added to each well followed by one volume of the bacteria-latex suspension. The plates were incubated at 37°C for 2 hr, spun at 500 ***g*** for 20 min and read directly for agglutination over a diffuse light source. The ability of the pellet to stream when the plate was upright was also read. The titres were checked after overnight incubation at 4°C. The results are expressed as the $\log_2$ reciprocal of the antiserum end point dilution.

Carbon clearance test

The phagocytic index, based on the rate of clearance of intravenously injected colloidal carbon, was determined as in previous experiments by the method of Biozzi *et al.* (1954). The optical density of serum samples obtained 1, 3, 6, 9 and 12 min after i.v. injection of the carbon was measured with a spectrophotometer and for each mouse a linear regression line showing the regression of log (optical density) on time was fitted by the method of least squares. The slope of this line, multiplied by -1, represents the phagocytic index (k). Group mean values and 95% confidence limits were calculated as already described, and where it seemed appropriate group means were compared by a Student's t-test.

RESULTS

A general summary of the results in qualitative terms is shown in Table 2. More detailed information is given below, in Figs 1 and 2, and in Tables 3 and 4.

Antibody response

Intact mice. There was quite a high background of antibody to *C. parvum* in non-immunized intact mice, and a marked rise in titre after a single i.p. injection of standard *C. parvum*, which reached its peak after about a month (Fig. 1). The subsequent decline in titre was slightly accelerated in animals inoculated with tumour at about the time the peak had been reached. In mice which had been inoculated with tumour 3 days before *C. parvum* injection

TABLE 2. Summary of results

C. parvum		Mice		*C. parvum* antibody response	Effect on phagocytic index	Tumour inhibition
Dose	Route of injection*	Pre-immunized or not	Intact or T cell-deprived			
			Intact	+	+	+
1·4 mg	i.p.	No	T cell-deprived	+	+	+‡
1·4 mg	i.p.	Yes	Intact	+	+	+
1·4 mg	s.c.	No	Intact	−†	−	−
1·4 mg	s.c.	Yes	Intact	−	−	−
0·7 mg	i.p.	No	Intact	+	+	+

* i.p. = intraperitoneal; s.c. = subcutaneous.

† − = either no effect, or so slight that it is not significant at $P = 0{\cdot}05$ level in present experiments.

‡ From Woodruff, Dunbar & Ghaffar (1973).

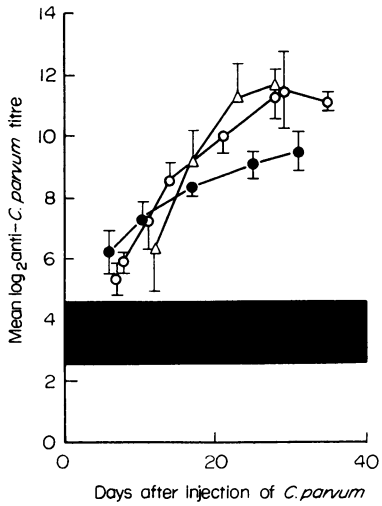

FIG. 1. Antibody response to a single injection of *C. parvum* (1·4 mg dry weight organisms) in intact and T cell-deprived female CBA mice. (○) Intact mice; no tumour; *C. parvum* i.p. (●) Intact mice; inoculated with tumour 3 days prior to i.p. injection of *C. parvum*. (△) T cell-deprived mice; no tumour; *C. parvum* i.p. The vertical bars represent 95 per cent confidence limits. The mean titre following s.c. injection of *C. parvum* did not rise above the background level represented by the solid area.

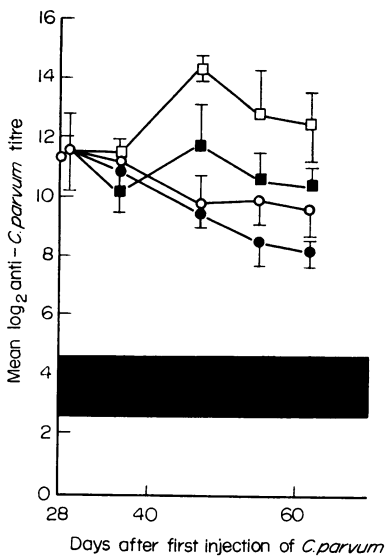

FIG. 2. Primary and secondary antibody response to *C. parvum* in intact CBA female mice. (○) *C. parvum* i.p. day 0; no tumour. (□) *C. parvum* i.p. days 0 and 31; no tumour. (●) *C. parvum* i.p. day 0; tumour inoculation Day 28. (■) *C. parvum* i.p. days 0 and 31. Tumour inoculation day 28. Each dose of *C. parvum* was 1·4 mg dry weight of the organism. The vertical bars represent 95 per cent confidence limits. Background level is represented by the solid area.

TABLE 3. Effect of *C. parvum* injection on tumour growth in CBA female mice

Group number	Treatment	Number of mice	Σ d, i.e. Sum of tumour diameters (mm) in each mouse measured every 2 days up to 24 days after tumour inoculation	
			Observed values	Mean with 95% confidence
1	Controls no treatment	12	66, 85, 94, 107, 86, 50, 76, 78, 31, 89, 78, 83	76·9 ± 12·8
2	*C. parvum* CN 6134, 1·4 mg i.p. 3 days after tumour inoculation	12	54, 50, 41, 41, 30, 25, 53, 53, 59, 58, 17, 45	43·8 ± 8·6
3	*C. parvum* CN 6134, 1·4 mg s.c. 3 days after tumour inoculation	6	78, 80, 82, 71, 81, 69	76·8 ± 5·8
4	*C. parvum* CN 6134, 1·4 mg i.p. 28 days before tumour inoculation	12	62, 46, 58, 55, 52, 75, 70, 58, 80, 70, 75, 72	64·4 ± 6·8
5	*C. parvum* CN 6134, 1·4 mg i.p. 28 days before and 3 days after tumour inoculation	17	60, 49, 58, 43, 29, 49, 41, 27, 68, 22, 27, 57, 43, 53, 67, 59, 50	47·2 ± 7·3
6	*C. parvum* CN 6134, 1·4 mg i.p. 28 days before and s.c. 3 days after tumour inoculation	6	77, 84, 72, 66, 70, 75	74·0 ± 6·5
7	*C. parvum* CN 6134, 0·7 mg i.p. 3 days after tumour inoculation	6	46, 41, 47, 55, 28, 50	44·5 ± 9·8
8	*C. parvum* NCTC 10390, 0·7 mg i.p. 3 days after tumour inoculation	6	47, 34, 56, 49, 51, 51	48·0 ± 7·9

Comparison of group mean

Groups compared	*t*	*P*
1, 2	4·712	< 0·001
1, 4	0·6451	> 0·1, N.S.
1, 3, 6	Clearly no significant difference	
2, 5, 7, 8, 9	Clearly no significant difference	

TABLE 4. Effect of *C. parvum* injection on the phagocytic index in CBA female mice

Group number	Mice		*C. parvum*		Carbon clearance (k values) on Day 0*	
	Intact or T cell-deprived	Number in group	Strain	Dose, route and day of injection†	Observed values	Mean with 95% confidence limits
13	Intact	16		Uninjected controls	2·6, 5·8, 1·7, 2·7, 1·8, 2·8, 1·6, 2·1 2·0, 1·9, 2·1, 2·0, 2·0, 1·7, 1·7, 2·9	2·34±0·54
14	T cell-deprived	6		Uninjected controls	2·6, 2·0, 1·4, 3·7, 2·1, 2·0	2·30±0·82
15	Intact	12	CN 6134	1·4 mg i.p., day −4	13·1, 6·6, 13·7, 11·8, 10·9, 7·9, 7·9 12·6, 5·8, 5·7, 9·7, 11·6	9·78±1·84
16	T cell-deprived	4	CN 6134	1·4 mg i.p., day −4	6·0, 5·4, 11·5, 6·4	7·33±4·48
17	Intact	5	CN 6134	1·4 mg s.c., day −4	6·3, 4·0, 2·8, 3·3, 2·1	3·66±2·03
18	Intact	6	CN 6134	1·4 mg i.p., day −32	2·1, 1·2, 4·8, 3·4, 1·8, 2·0,	2·70±1·3
19	Intact	7	CN 6134	1·4 mg i.p. days −32 and −4	6·1, 3·1, 8·5, 6·1, 7·0, 7·2, 9·4	6·77±1·84
20	Intact	5	CN 6134	1·4 mg i.p., day −32 and 1·4 mg s.c., day −4	2·9, 2·1, 2·3, 2·7, 1·2	2·24±0·83
21	Intact	5	CN 6134	0·7 mg i.p., day −4	7·6, 4·7, 10·5, 8·4, 5·2	7·28±2·96
22	Intact	5	NCTC 10390	0·7 mg i.p., day −4	12·0, 8·6, 11·9, 8·3, 8·9	9·94±2·29

* Day 0 is the day on which the k values were determined.
† i.p. = intraperitoneal injection; s.c. = subcutaneous injection.

there was still a marked antibody response, but the peak titre was somewhat less than in mice not bearing tumour. Treatment with 0·2 M 2-mercaptoethanol for 30 min at 37°C reduced the titre of the background antibody, and also of the titre 1–3 weeks after immunization, by 1–2 $\log_2$ units. A second injection of *C. parvum* a month after the first in mice not bearing tumours evoked a definite secondary response (Fig. 2).

Subcutaneous injection of *C. parvum* evoked little or no primary or secondary antibody response. A few animals showed a slight increase in titre but the mean value did not rise significantly above the control level.

T cell-deprived mice. The background level, and also the antibody response to a single i.p. injection of *C. parvum* (Fig. 1), were similar to those observed in normal mice.

Tumour growth

The results are set out in Table 3.

As expected, a single i.p. injection of *C. parvum* 3 days after tumour inoculation markedly inhibited tumour growth. A similar degree of inhibition occurred in mice injected i.p. with *C. parvum* 28 days before and 3 days after tumour inoculation, as judged by the mean tumour diameter, although the variance was considerably greater; a single i.p. injection 28 days prior to tumour inoculation, on the other hand, was significantly less effective.

A subcutaneous injection of *C. parvum*, whether or not it was preceded by an i.p. injection 28 days previously, did not significantly influence tumour growth.

Tumour growth in T cell-deprived animals was not studied in these experiments since, as mentioned in the Introduction, it has already been reported (Woodruff *et al.*, 1973) that intraperitoneal injection of *C. parvum* inhibits tumour growth in these animals to about the same extent as in normal mice.

Phagocytic activity

The results of the carbon clearance tests are shown in Table 4. It will be seen that an i.p. injection of *C. parvum* markedly stimulated phagocytic activity, as judged by a carbon clearance test performed 4 days later, in intact mice which had received an immunizing injection of *C. parvum* 4 weeks previously, and also in non-immunized T cell-deprived mice, though not to quite the same extent as in intact, non-immunized mice.

The mean k values in mice which were given *C. parvum* by s.c. injection, whether or not this was preceded by an i.p. injection 28 days previously, did not differ significantly from the value in the untreated controls. (Comparing groups 13 and 17, $t = 2{\cdot}28$, $n = 19$, $P > 0{\cdot}1$).

DISCUSSION

It seems clear from the findings that both the antitumour effect of *C. parvum*, and its effect on macrophage activity as judged by the carbon clearance test, can occur in the presence of high levels of antibody directed against the organism. It is difficult to assess why this should be so. The number of organisms injected was large (about 5×10^{10}) and this may have obscured any depressive effect due to the presence of antibody, but we have no direct evidence of this. The failure of subcutaneous injection to produce a significant response in respect of any of the parameters studied, might appear to suggest, on the other hand, that the RE stimulatory and tumour inhibitory effects actually depend on the presence of a

primed state towards the organism. This hypothesis is in line with the observation of Schinitsky (1973) that BCG is effective as an immunotherapeutic agent in patients with cancer only so long as the host can mount a strong cell-mediated response to purified protein derivative of the organism (PPD) but, while it is consistent with the present findings, the evidence is clearly inconclusive.

The relatively high level of antibody to *C. parvum* in normal untreated animals is perhaps not surprising in view of the ubiquitous nature of the anaerobic Corynebacteria and their high degree of serological cross-reactivity (Johnson & Cummins, 1972). It is noteworthy that this background titre can readily be decreased by absorption of the sera with *C. parvum*.

The antibody response in T cell-deprived mice suggests that *C. parvum* behaves as a thymus-independent antigen, but this does not seem to be true in an absolute sense since in experiments now in progress (McBride, Dunbar & Woodruff, unpublished observations) nude mice have so far not shown any antibody response to an i.p. injection of 1·4 mg of *C. parvum*. It must, however, be stressed that the dose of *C. parvum* was not well tolerated by the nude mice; no firm conclusion can therefore be drawn until further observations have been made with smaller doses.

However, even if *C. parvum* does regularly fail to evoke an antibody response in nude mice, it is not altogether surprising that it evokes a response, and especially a 19S response, in T cell-deprived mice of the kind we have used. These animals do not produce significant amounts of antibody in response to sheep erythrocytes (injected without *C. parvum* or other adjuvant) and, as reported previously (Woodruff *et al.*, 1973), do not reject skin allografts, but they are by no means entirely devoid of T cells. Moreover, *C. parvum* can function both as an antigen and as an adjuvant, and in its latter capacity can amplify not only the 19S, but also the 7S response, to sheep erythrocytes in standard thymectomized, irradiated and reconstituted mice (Howard, Scott & Christie, 1973). Whether this is due to expansion of the small T-cell population in these mice or, as Howard *et al.* (1973) have suggested, to some kind of T-cell by-pass mechanism, remains to be seen.

The slight reduction of both the primary and secondary antibody response to *C. parvum* in tumour-bearing animals is of interest in relation to the work of James, Ghaffar and Milne (1974), who found that prior or simultaneous inoculation of viable sarcoma cells completely suppressed the antibody response to alum BSA, but often augmented the response to sheep erythrocytes and Type III pneumococcal polysaccharide.

In discussing the immunogenicity of *C. parvum* we have so far been concerned only with its capacity to stimulate the production of antibody capable of agglutinating a suspension of whole organisms. The extent to which various components of the organism stimulate production of non-agglutinating antibody is however being investigated.

Although the theoretical significance of the results reported in this paper is difficult to assess the finding that the antitumour effect of *C. parvum* does not appear to be blunted by the presence of antibody may be of therapeutic importance, because a high level of background antibody is typically present also in humans (McBride, Tuach, Jones, Dawes & Weir, unpublished). It raises the question, moreover, of the possible therapeutic effect of repeated *C. parvum* injection. It might be hazardous to give successive intravenous injections to a patient, and, if the mouse model is a satisfactory guide, a single subcutaneous or intramuscular injection seems unlikely to be of value; the effect of a single intravenous injection followed by repeated subcutaneous or intramuscular injections would seem, however, to merit investigation.

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