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The Effects of *Corynebacterium parvum* in Dogs and a Study of its Distribution Following Intravenous Injection

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Abstract—*The pathological effects of* C. parvum *vaccine in the dog were found to be similar to, but less marked than, BCG, at the dose given. Studies in distribution using* ^{125}I C. parvum *showed this to be similar to that occurring in the mouse. Alveolar macrophages from normal dogs were found to be not cytotoxic to canine tumour cells and normal cells* in vitro; *alveolar macrophages from dogs receiving i.v.* C. parvum *produced a marked cytotoxic effect on similar cells. Treatment of dogs bearing mastocytoma by surgical excision followed by injection of* C. parvum *at the operation site did not prevent tumour recurrence in seven of nine cases.*

INTRODUCTION

IN PREVIOUS studies [1, 2] the effects of i.v. BCG in normal dogs and in dogs with spontaneous osteosarcoma or mammary tumours have been described. While the appearance of lung metastases appeared to be delayed in osteosarcoma and was definitely delayed in dogs subjected to mastectomy for mammary carcinoma there were some toxic side-effects, in particular marked granuloma formation in the liver.

It has been suggested [3] that there is an antimetastatic effect of *Corynebacterium parvum* which appears to be mediated through macrophages in concert with a subpopulation of T-lymphocytes. The work now described was designed to determine the pathological effects of *C. parvum* in the dog as compared to BCG, to study its distribution following i.v. administration when compared to other animals and to examine the possible cytotoxic effects of alveolar macrophages on tumour cells *in vitro*. Results in dogs bearing spontaneous recurrent mastocytoma treated by surgical excision followed by injections of *C. parvum* at the operation site are also given.

MATERIALS AND METHODS

Experimental dogs

Approximately 1–2-yr-old adult male and female mongrel dogs were used. These were clinically normal and vaccinated against distemper, canine viral hepatitis and leptospirosis. Weights varied from 7.5 kg to 14 kg with a mean of 11 kg.

C. parvum *vaccine (CPV)*

This was a formol-killed vaccine supplied by Wellcome Reagents Ltd., Beckenham, Kent, U.K. CN6, 134 Batch No. PX416 containing 7 mg dry weight per ml.

Injections of 0.5 ml (3.5 mg) CPV were made to 13 dogs by routes shown in Table 1; dilution of the vaccine with saline being made in some instances. In two dogs (7, 8) an i.m.

Table 1. *Route and number of injections of* 0.5 ml *of* C. parvum *vaccine into experimental dogs*

No. of dog	Route of administration	Dilution of vaccine	Repeat doses (weeks interval)
1	s.c.	No	3 × 1
2	s.c.	No	2 × 1
3	s.c.	× 4	1 × 1
4	i.p.	× 10	1 × 1
5	intrathoracic	No	3 × 1
6	i.v.	No	3 × 1
7*	i.v.	× 20	2 × 2
8*	i.v.	No	2 × 2
9	i.v.	No	1 × 2
10	i.v.	No	1 × 2
11	i.v.	No	1 × 2
12	i.m.	No	1 × 2
13	i.m.	× 4	1 × 2

*Dogs injected i.m. with 10 mg chlorpheniramine 30 min previously.

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injection of 10 mg of the antihistamine drug chlorpheniramine ('Piriton' Allen and Hanbury's Ltd., London, England) was made 30 min before the CPV injection.

Four additional dogs used for *in vitro* studies on alveolar macrophages received i.v. injections of 3.5 mg of CPV on two occasions with a 2-week interval.

Pathological studies

Rectal temperatures were measured with an Elab electrical thermometer for periods of up to 36 hr post injection. Haematological examination and estimations of serum alkaline phosphatase values were made on four occasions post injection. Intradermal tests were made before and after treatment with streptokinase, BCG (heat-killed) and PPD (human). The leucocyte migration inhibition test was performed in four dogs receiving i.v. CPV. In the dogs used for pathological studies euthanasia was carried out 2–3 weeks after the final injection of CPV and histological examinations made on many organs and tissues.

^{125}Iodine-*labelled* C. parvum (^{125}I-*CPV*)

The technique described by Scott and Milas [4] was used. In brief this involves the addition of 1 mCi of ^{125}I (Radiochemical Institute, Amersham, England) to 2.5 ml CPV. Following treatment with sodium bisulphite and sodium iodide the suspension is centrifuged and dialysed.

For 3 days before administration of the labelled vaccine, dogs were dosed orally with 1.5 g sodium iodide in 20 ml of water. Following the i.m. injection of 10 mg of chlorpheniramine as an antihistaminic 30 min earlier, doses of 3.5 mg of ^{125}I-CPV were administered to experimental dogs. The injected dogs were kept in metabolism cages and all urine collected. At the end of the period designated for study the dogs were given deep Halothane anaesthesia and exsanguinated. In one dog this was performed 1 hr after administration of ^{125}I-CPV and in other dogs at 24 hr and 11 days. One dog was injected with soluble ^{125}I and killed 24 hr later.

Gamma counting

All samples of homogenised tissues, blood and urine were made up to a volume of 50 ml with water in calibrated plastic pots. The samples were counted using a Nuclear Enterprise gamma ratemeter SR5 gamma counter with appropriate background counting and allowance for decay being made. In the case of kidney, spleen, liver and lung, five samples of each organ were counted, for thyroid and various lymph nodes whole organs were counted.

Fluorescein-labelled C. parvum (*FITC-CPV*)

The method of Scott and Milas [4] was employed using fluorescein isothiocyanate (Grand Island Biological Co., New York, U.S.A.) An i.v. injection of 3.5 mg FITC-CPV was given to one dog and 14.0 mg i.v. to a second dog. Euthanasia was as described for dogs given ^{125}I-CPV. On post-mortem examination samples of various organs were immediately frozen in liquid nitrogen and were later cut on a cryostat. Blood samples were taken in heparin and smears made of these and also of bone marrow. Sections and smears were examined by Dr. T. M. Scott using a Leitz fluorescent microscope.

Cytotoxicity of alveolar macrophages

Four dogs which had received i.v. two injections of 3.5 mg CPV with a 2-week interval between injections and 2 normal dogs were anaesthetised and exsanguinated.

Macrophages were harvested by lung lavage based on the technique of Myrvik [5] and following adhesion to plastic subsequently obtained in a purity of 95–99%. Each alveolar macrophage preparation was resuspended at a concentration of 4×10^6 cells/ml for use in a ^{51}Cr release assay. The details of allogeneic target cells used in this test have been discussed in previous papers [6–9] and are summarised in Table 2. The target cells were detached from monolayers by trypsin–EDTA solution and resuspended in 10 ml of RPMI 1640 medium plus 10% FCS and sodium ^{51}chromate (Code CJSIP, Amersham, England) added at a rate of 5.0 μCi/10^5 cells. ^{51}Cr labelling occurred during $1\frac{1}{2}$–2 hours incubation at 37°C and any unbound isotope was removed by 4–5 washes in fresh

Table 2. Details of allogeneic cells

Identification	Histological type	Age (yr)	Sex	Breed
VI	Melanoma	13	F	Poodle
RVC 347	Melanoma	7	M	Boxer
H72. 1503	Osteosarcoma	2	F	Alsatian
H73. 2295	Mammary carcinoma	14	F	Mongrel

medium. The labelled cells were resuspended at a concentration of 5×10^4 cells/ml in RPMI 1640 plus 20% FCS and 10 mM Hepes, 100 μl aliquots of the effector macrophage population were pipetted into a 96-well round-bottomed T.C. grade plate (MRC-96-TC Linbro, Conn., U.S.A.). Doubling dilutions of the effector cells from 80:1 to 10:1 ratios were made and triplicate samples for each ratio dispersed. To these were added 100 μl aliquots of the relevant target cells and the plates sealed with adhesive film. The plates were incubated at 37°C for either 4 or 8 hr on a rocking platform. After the set incubation period the plates were centrifuged at 600 ***g*** for 5 min to pellet all the cells. Half of the supernatant, 100 μl, of each well was pipetted into plastic tubes (LPS Luckham). After drying, the plates were cut into individual wells and paired wells and respective supernatants were counted for gamma activity in a Wallac Gamma counter. After correction for background activity the percentage ^{51}Cr release was calculated from:

Percentage ^{51}Cr release $= 2 \times S/SIC \times 100\%$

S = activity of 100 μl aliquot of supernatant

C = activity of well containing cells and residual 100 μl of supernatant

Release values were computed and their log transformed values showing uniform variance were employed in an analysis of variance [10] to obtain a residual standard error value. Comparison of replicate means was performed using Duncan's Multiple Range Test [11].

Clinical cases

In eight dogs with grade 1 or grade 2 mastocytoma, tumours recurred following surgical excision in all cases. In these eight dogs and one further dog with a mastocytoma not previously treated surgically the tumours were again excised and usually within 1–4 weeks post-operation the site was infiltrated with 0.5 ml *C. parvum* vaccine diluted to 2 ml with saline. In seven dogs a second similar injection was given 2 weeks after the first injection.

RESULTS

Toxicity

No dogs showed an anaphylactic-like response following i.v. injection of CPV whether animals had been injected with an antihistamine drug or not. Behaviour and food intake appeared to be normal. Rectal temperature in some dogs rose 0.7–1.0°C 4–6 hr post i.v. injection but had returned to normal by 8–24 hr. No rise in temperature occurred in the dog given soluble ^{125}I.

Intradermal tests and the leucocyte migration inhibition test were negative and alkaline phosphatase values remained within the normal range. Haematological values showed no significant change except in one dog injected s.c. and one dog injected i.m. which developed abscesses and in this instance a polymorphonuclear leucocytosis occurred.

Following s.c. injection of CPV a hyperaemia and exudation led to scab formation (dogs 1 and 2). Injection at another site 1 week later (dog 2) led to a greatly increased reaction and severe oedema. A similar effect resulted from a third injection at a different site in the same dog and was this time followed by abscessation. In dog 3 (Table 1) the s.c. injection of a 0.5 ml dose of vaccine diluted to 2 ml and repeated on two occasions at 1-week intervals produced no local reaction. A similar severe reaction followed i.m. injections of undiluted CPV (dog 12) but following similar dilution the vaccine was well-tolerated (dog 13).

Post-mortem and histological changes

Gross post-mortem changes were few and in the dogs injected s.c. with undiluted vaccine were confined to the local changes of necrosis and oedema. In the dog injected i.p. no gross changes were seen in the peritoneum or elsewhere. In the dog injected intrathoracically and in two dogs injected i.v. a few very small whitish foci were seen in the lungs. In dogs given ^{125}I-CPV and in which euthanasia was performed with Halothane and exsanguination, the spleen appeared grossly normal in size and the liver weight expressed as a percentage of the total weight of the dog was below mean values quoted for normal dogs. This may have been due to the exsanguination.

Histological changes were similar but considerably fewer and less marked than in dogs injected i.v. with BCG. In the one dog injected intrathoracically and in five dogs injected i.v. a few small granulomata in the lungs and very few granulomata in the liver were found.

Distribution of ^{125}I-CPV

The relative *in vivo* persistence of ^{125}I-CPV in various organs following i.v. administration

Table 3. *Relative* in vivo *persistence of active* C. parvum *in the dog following* i.v. *injection*

Organ	Percentage of total dose per g 1 hr	24 hr	11 days
Liver	0.258	0.073	0.007
Spleen	0.303	0.068	0.010
Lung	0.336	0.013	<0.001
PLN	0.015	0.007	0.002
Kidney	0.054	0.027	0.001

is shown in Table 3. The per gram values of radioactivity in the liver, lung and spleen at 1 hr were similar, but this activity declined at a more rapid rate in lung than in liver or spleen (Fig. 1). On an organ basis the highest levels of activity were found in the liver with approximately 48% being present at 1 hr compared with approximately 19% in the lungs and 4.5% in the spleen. Levels of activity in lymph nodes, kidneys and thyroid were low at all times.

Measurement of the radioactivity in the urine showed that the majority of ^{125}I released from the labelled CPV was excreted by the kidneys. In the dog killed 11 days after injection of ^{125}I-CPV, 95% of the total radioactivity was recovered in urine collected over this period. Organs from the control dog injected with the soluble ^{125}I contained very low doses of radioactivity, no organ exceeding values of 0.01% (percentage of total dose per g) except the thyroid at 0.017% and the blood at 0.011%/ml. By 24 hr 41% of the total dose had been excreted.

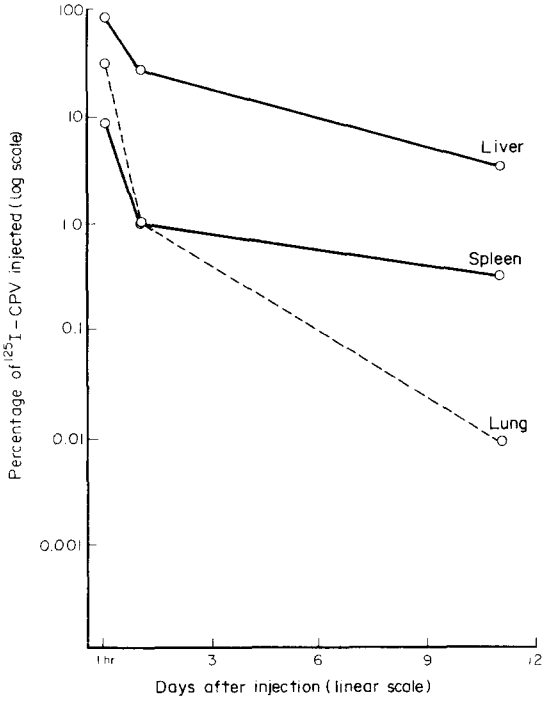

Fig. 1. *Amounts of* ^{125}I-*CPV in liver, lung and spleen at various times after i.v. administration.*

Distribution of FITC-CP

In the dog injected with 3.5 mg CPV i.v. it was possible to demonstrate the presence of intact FITC-CP in the liver and spleen. Preparations from all other organs appeared to be negative. In the dog injected with 14 mg CPV i.v. a similar result in liver and spleen was obtained and in addition occasional labelled cells were seen in the bone marrow and lymph nodes.

Alveolar macrophage cytotoxicity

The mean values of the percentage ^{51}Cr release from the allogeneic cells at the ratios examined are presented in Tables 4 and 5. These results indicate that the alveolar macrophages from three of the four CPV injected dogs exhibited some degree of significant target cell lysis.

Results of clinical cases

The results of treatment of mastocytoma in dogs by excision followed by injection of *C. parvum* at the operation site are given in Table 6. Tumour recurrence was rapid in seven cases and in only two cases was there no tumour recurrence at 1 and 2 yr respectively.

DISCUSSION

Provided that the vaccine was diluted before s.c. or i.m. injection the toxicity was found to be minimal and the pathological effects while similar to those seen following i.v. injection of BCG in dogs have been considerably less severe at the doses used. The studies in distribution showed that CPV given i.v. in the dog followed approximately the same pattern as in other mammalian species, notably the mouse [4] and it would appear likely that a similar distribution would occur in man. In the dog, CPV appeared to be cleared from organs more rapidly than in mice possibly indicating that the endothelial system is more efficient in the dog (Table 7).

Following i.v. administration of CPV in the mouse [4], guinea pig, rat [12] and rabbit, [13] there was hepatosplenomegaly. This did not occur to a detectable extent in the dog at the relatively small dosage used in the present study. Perhaps larger doses may produce this effect. McBride *et al.* [14] in mice have shown

Table 4. Specific cytotoxicity of alveolar macrophages from CPV injected dogs

	Target cell											
	VI						H73.2295					
Effector: target cell ratio	4 hr			8 hr			4 hr			8 hr		
	1	2	3	1	2	3	1	2	3	1	2	3
80:1	ND	14.23	8.90	ND	30.05	16.44	ND	10.51	10.48	ND	22.86	17.26
40:1	ND	12.28	9.52	ND	29.43	18.11	ND	10.04	9.40	ND	21.39	16.66
20:1	9.98	10.43	9.13	22.18	24.80	18.20	9.97	9.14	10.16	16.54	19.09	16.06
10.:1	10.49	10.07	9.77	22.37	26.68	17.56	10.12	9.89	9.99	18.45	18.69	15.86
Medium	8.96	8.96†	8.96	17.21	17.21	17.21	8.96	8.76	8.76	16.32	16.32†	16.32

*All values expressed as percentage ^{51}Cr release.
†Significant differences between ratios within the group $P<0.05$. Assessed by Duncan's multiple range test.

Table 5. Specific cytotoxicity of alveolar macrophages from CPV injected dogs

	Target cell											
	H72.1503						RVC 347					
Effector: target cell ratio	4 hr			8 hr			4 hr			8 hr		
	4	5	6	4	5	6	4	5	6	4	5	6
80:1	15.78	14.47	10.24	29.29	33.09	25.30	12.21	8.99	10.27	13.20	15.65	ND
40:1	12.66	14.35	9.67	29.27	32.92	25.63	14.66	8.30	9.87	11.13	12.90	ND
20:1	11.63	9.38	9.30	25.37	21.50	23.72	12.74	7.96	9.13	11.54	11.53	13.22
10:1	8.18	8.47	9.19	21.90	ND	24.16	11.67	6.98	9.91	12.42	12.35	12.97
Medium	8.49†	8.49†	8.49	25.27	25.27†	25.27	8.11	8.11	8.11	12.81	12.81	12.81

*All values expressed as percentage ^{51}Cr release.
†Significant differences between ratios within the group $P<0.05$. Assessed by Duncan's multiple range test.

Table 6. Results of treatment of mastocytoma in dogs by excision followed by injection of C. parvum *at the operation site*

Case and site	Grade of tumour	No. of injections	Time interval between injections (weeks)	Time of recurrence after last injection
(1) Labrador. Interdigital area—metastasis to popliteal node (91)	2	1	—	2 weeks
(2) Collie-cross. Eyelid (1380)	1	2*	2	2 weeks
(3) Spaniel (13 yr). Flank	1	1†	—	2 weeks—with v. rapid growth
(4) Staffordshire bull terrier. Thorax (785)	1	2	4	3 weeks
(5) Mongrel. Stifle (1454)	2	2	4	2 months
(6) Boxer. Hock (735)	1	2	4	2 months
(7) Labrador. Metatarsus (1819)	1	2	2	3 months
(8) Labrador. Lip (570)	2	2	4	No recurrence at 1 yr
(9) Boxer. Neck (71)	2	2	4	No recurrence at 2 yr

*Only 0.05 ml *C. parvum* used.
†Injected 3 months post-operation.

Table 7. Comparison of in vivo *distribution of* C. parvum *in mouse and dog*

		Percentage of total dose injected			
		1 hr	24 hr	11 days	15 days
Lung	Mouse*	4.0	0.2		0.01
	Dog	19.0	1.1	0.006	
Liver	Mouse	70.0	40.0		10.0
	Dog	48.0	15.0	2.0	
Spleen	Mouse	3.0	1.0		0.3
	Dog	4.6	0.8	0.2	

*Mouse values after Scott and Milas (1977)

that the *in vitro* anti-tumour activity of CPV correlated better with splenomegaly than with stimulation of phagocytic index.

The prognoses of grade 1 mastocytoma—survival 18 weeks—and grade 2 mastocytoma—survival 28 weeks—have been established by Bostock [15]. In only two of nine dogs treated by injection of *C. parvum* at the operation site was the expected recurrence considerably delayed. It is of interest that in these two cases there was marked local hyperaemia and some swelling at the injected site even though the vaccine was diluted. One of these cases had been previously operated upon twice for tumour recurrence.

If it can be demonstrated that i.v. *C. parvum* is as effective as BCG in delaying the onset of metastasis it would be an advantage to use because of its decreased toxicity. No information is yet available on the i.v. use of CPV in dogs with mammary carcinoma as it is for BCG but this information should be available in 3 yr time following the completion of a World Health Organization international trial which will compare large numbers of dogs with mastectomy alone with dogs having mastectomy followed by either i.v. BCG or i.v. *C. parvum*. It is hoped that the information in this present article will stimulate further interest and collaboration in this trial and provide the necessary background information.

It is of considerable interest that alveolar macrophages stimulated by CPV were found to be cytotoxic to canine malignant tumour cells *in vitro*. It has been previously demonstrated that macrophages obtained from BCG in injected dogs were also cytotoxic to tumour cells whereas those obtained from normal dogs showed no such cytotoxicity [16]. The present results with CPV stimulated macrophages parallel those of Olivotto and Bomford [17] in mice.

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