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Alveolar Macrophage Cytotoxicity in Dogs Following Intravenous BCG*

N. T. GORMAN

Division of Immunology, Department of Pathology, Addenbrooke's Hospital, Hills Road, Cambridge, England

Abstract—*Normal dogs were treated with intravenous BCG on a doubling interval schedule previously found to be clinically effective. Alveolar macrophages retrieved from the treated dogs exhibited marked non-specific cytotoxic effects on normal and neoplastic allogeneic cells. This was not observed with alveolar macrophages from normal unstimulated dogs. The relevance of these findings to the therapy of pulmonary metastases is discussed.*

INTRODUCTION

THE APPLICATION of BCG to the therapy of neoplasia has only become prominent over the past 2 decades and the reports on the efficacy of this mode of treatment have been both encouraging and conflicting [1–6]. The adoption of non-specific BCG therapy however to canine neoplasia has been limited. This is unfortunate as many spontaneous canine neoplasms closely resemble the situation in man [7]. Owen and Bostock [8] first reported that in canine osteosarcoma, following amputation of the affected limb, a course of i.v. BCG (percutaneous strength 3 mg) administered after 1 week, 2 weeks, 4 weeks and then every 8 weeks, significantly delayed the appearance of pulmonary metastases. These initial findings have been reaffirmed [9] and applied to certain defined forms of mammary carcinoma [10].

A diffuse macrophage infiltration in the lung was consistently found in these studies following the i.v. BCG treatment [8–11]. It was postulated that this infiltration may provide a pool of effector cells with the potential to destroy some of the metastatic tumour cells within the lung. This mechanism would therefore contribute to the delayed onset of pulmonary metastases, although a concurrent specific or non-specific lymphocytotoxicity may also be operative at a local and/or systemic level.

Macrophages form a large percentage of cells within certain rodent [12] and human tumours [13] *in vivo*. Indeed, in the rodent models this commitment to the growing tumour was apparently sufficient to induce an anergic state [14]. A clear difference between the number of macrophages in regressing Maloney sarcomas compared to progressing tumours has also been demonstrated [15]. *In vivo* suppression of the mononuclear phagocyte system with silica was found to abolish the normal rejection mechanism in rodent models [16, 17]. However, in contrast, an array of macrophage stimulants administered *in vivo* enhanced tumour rejection in some experimental models [16–21]. *In vitro*, stimulated or activated macrophages have been shown to exhibit cytostatic and cytolytic effects on tumour cells (see [22] for review). This, under certain experimental techniques has been demonstrated to be selective for neoplastic cells [23]. However, this concept is somewhat unresolved and may relate to rate of cell division of the target cells [24].

This and other evidence has contributed to the current interest in the role of the macrophage in tumour rejection mechanisms. The purpose of this paper is to describe the cytolytic ability of alveolar macrophages from normal and BCG stimulated dogs.

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Abbreviations used: CAM, Canine alveolar macrophages; BCG, Bacillus Calmette-Guerin (Glaxo Laboratories, Greenford, England); FCS, Foetal Calf Serum; EDTA, Ethylenediaminetetra-acetic acid; BSS, Basic salt solution; PBS, Phosphate buffered saline.

MATERIALS AND METHODS

Animals

Standard 30–35 lb mongrel dogs were used in this study. Ages of the animals varied from 12 to 24 months. BCG (3mg percutaneous strength) was administered (i.v.) to the trial dogs on the injection protocol as indicated in the Introduction for the therapy of dogs bearing spontaneous tumours.

Isolation of the effector cell population

Canine alveolar macrophages (CAM), with other contaminating cells, were isolated by lung lavage based on the technique of Myrvik [25]. Macrophages were purified from the resultant heterogeneous cell population by adherence to plastic tissue culture flasks for 1 hr. This produced a 95–99% pure monolayer of CAM. The adherent cells were characterised by morphology, electron microscopy, cytochemistry, surface receptors and phagocytic properties [11]. Once purified the CAM were incubated overnight in RPMI plus 10% FCS as an adherent monolayer in 75 cm^2 plastic tissue culture flasks (Nunc, Denmark). At the end of this period the CAM were removed by treatment with EDTA (0.02%), collected in 25 ml conical tipped universal centrifuge tubes and spun at 750 ***g*** for 5 min. The cells were washed in Hanks BSS and finally resuspended in RPMI plus 20% FCS. The cell concentration was adjusted to 4×10^6 cells/ml.

Preparation of target cells

Target cells used in the ^{51}Cr release assay were obtained from *in vitro* cultures of canine neoplastic and non-neoplastic cells. A summary of the target cells employed in this study is shown in Table 1 with the relevant references to their original description. The Madin-Darby normal kidney line (MDCK) was purchased from Gibco-Biocult, Scotland. A target cell suspension for the assay was obtained by subjecting the monolayer to antibiotic-trypsin-versene (ATV). Following centrifugation the resultant cell pellet was resuspended in 10 ml of RPMI + 10% FCS and a cell and viability count performed. The cells were labelled with sodium ^{51}Cr (Na_2 $^{51}CrO_4$, Radiochemical Centre, Amersham, Bucks, England), at a rate of 5.0 μCi/10^5 cells, agitating the cell suspension and isotope for 2 hr in a water bath at 37°C. The labelled cells were subsequently centrifuged at 750 ***g*** for 5 min, the radioactive supernatant decanted and the cells resuspended in Hanks' BSS plus 5% FCS. The cells were washed a further 5 times and after the final wash resuspended in RPMI + 20% FCS at a concentration of 5×10^4 cells/ml.

Macrophage supernatants

The cytolytic potential of the supernatants from macrophage cultures was also examined. To the target cells was added either 100 μl or either neat or 1/100 supernatant in place of the CAM. The ^{51}Cr release was then determined at 4 and 8 hr.

^{51}Cr release assay

The assay was performed using a microtechnique in a 96 round bottom well plate (Nunc, Denmark, T.C. grade), each well with a 200 μl capacity. In each experiment at least 3, preferably 4 or more CAM populations were examined. The effect of each macrophage population was examined at 4 ratios (80:1–10:1 effector:target cell ratio with doubling dilutions) with triplicate samples for each ratio, (100 μl target cells, 5×10^3, 100 μl CAM) incubated in a 5% CO_2/air atmosphere to stabilise the pH, sealed with self-adhesive non-porous mylar strips (35PS, Biocult Laboratories, Scotland) and incubated at 37°C on a rocking platform. All the preparation and pipetting was carried out under sterile conditions.

Duplicate sets of plates were prepared so that the ^{51}Cr release could be determined at 4 and 8 hr. The cells in each well were pelletted by centrifugation at 1000 ***g*** for 5 min, 100 μl of the supernatant only withdrawn from each well and transferred to corresponding LP2 tubes (Luckham, England). The remaining medium in the cells was allowed to dry out at room temperature prior to the individual wells being cut with an electrically heated wire and placed with the accompanying supernatant in trays.

The cells and supernatant samples were placed in a γ well counter (LKB Wallac 80,000 Gamma sample counter) in a sequential order for time and other variables. The samples were counted for 6000 counts of 400 seconds, whichever was the shorter.

Both percentage and specific ^{51}Cr release were calculated:

(A) Percentage ^{51}Cr release from:

$$\% \ ^{51}Cr \text{ release} = 2.0 \times \frac{S}{S+C} \times 100\%$$

$S = ^{51}Cr$ counts in the supernatant

$C = ^{51}Cr$ counts in the cell plus residual aliquot.

Table 1. Details of allogeneic cells used in the ^{51}Cr release assay

Identification	Histological type	Breed	Age (yr)	Sex	Site	Established	References
H71-1843	Melanoma	English setter	1	M	Oral I^y	10.12.71	[26–28]
H72-1503	Osteosarcoma	Alsatian	2	F	Lung II^y	20.8.72	[26, 27, 29]
H73-2295	Mammary carcinoma	X-bred	14	F	Lung II^y	3.12.73	[28–31]
RVC-347	Melanoma	Boxer	7	F	Oral I^y	22.5.61	[27, 30]
VI	Melanoma	Poodle	13	F	Oral I^y	6.11.71	[26–28]
MDCK	Normal kidney	Spaniel	—	M	Kidney	58	

I^y = primary tumour.
II^y = secondary tumour.

Table 6. Alveolar macrophage effector cells from dogs bearing spontaneous tumours. Percentage ^{51}Cr release at 4 hr

Target cell	H72-1503				H72-2295				H71-1843				H76-1369			
Identification	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Effector: target cell ratio																
80:1	37.4	N.D.	10.9	9.9	32.0				11.8		33.2	14.3			14.4	17.0
40:1	37.7	10.0	10.1	10.8	35.8	14.6			12.6	9.7	15.7	11.5			14.6	N.D.
20:1	40.1	11.7	10.3	9.6	29.4	13.9			12.4	10.8	12.3	10.2			16.4	17.7
10:1	40.8	12.7	10.0	10.0	35.4	13.1			13.4	10.4	12.1	10.3			N.D.	16.7
Medium	34.7	10.5	9.5	9.5	28.8	12.7			11.6	9.9	13.3	13.3			17.8	17.8

1 = H75.1886 Male 3 yr old. Osteosarcoma right femur II^y involvement lung.
2 = GBA. Quinn. Male 9 yr old. Osteosarcoma right femur II^y involvement lung.
3 = H76.1369 Male 3 yr. old. Melanoma oral II^y involvement lung, regional lymph nodes.
4 = BL I Female 13 yr old. Lipoma left thigh. No II^y.

Values for S and C were first corrected for incorporated counts of background activity. Statistical analysis was performed on log transformed data to equalise S.E.'s of high and low percentage ^{51}Cr release values in the groups. Significant results within a group were assessed by Duncan's multiple range test [32] with the S.E. derived from the residual mean square value in the analysis of variance. Differences at a level of $P<0.05$ were taken as an indication of positive cytolysis within a group.

(B) For comparison between experiments, values for % specific cytotoxicity are presented for 80:1 effector:target cell ratios at 8 hr from:

Specific cytotoxicity = [% release by test effectors − % spontaneous release ÷ (100% release − % spontaneous release) × 100].

RESULTS

The cytolytic potential of normal canine alveolar macrophages was found to be insignificant. However, *in vivo* BCG stimulated macrophages were highly cytolytic as determined by the ^{51}Cr release assay described.

This is demonstrated by the results presented in Figs 1, 2 and 3. In this example

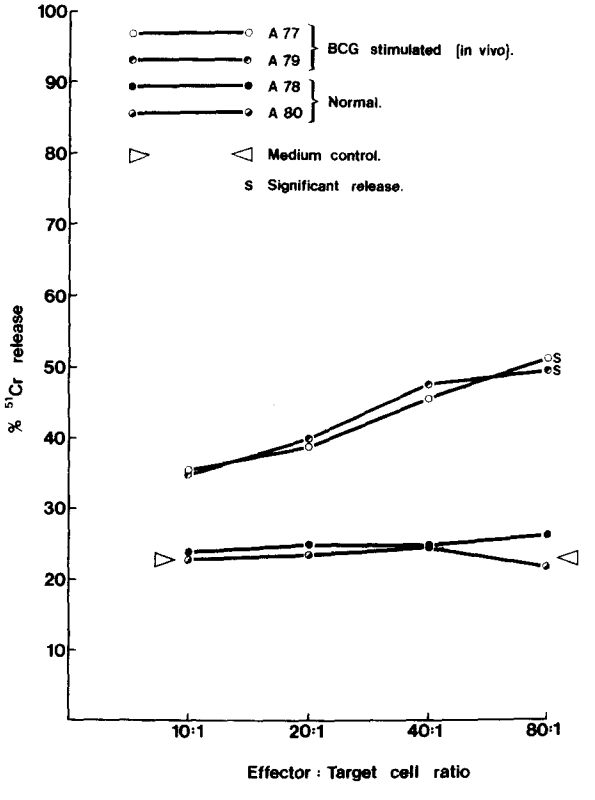

Fig. 1. Comparison of percentage ^{51}Cr release from the canine osteosarcoma cell line H72—1503 *with effector canine alveolar macrophages from two normal dogs* (A78, A80) *and two BCG stimulated dogs* (A77, A79), *at different effector: target cell ratios.*

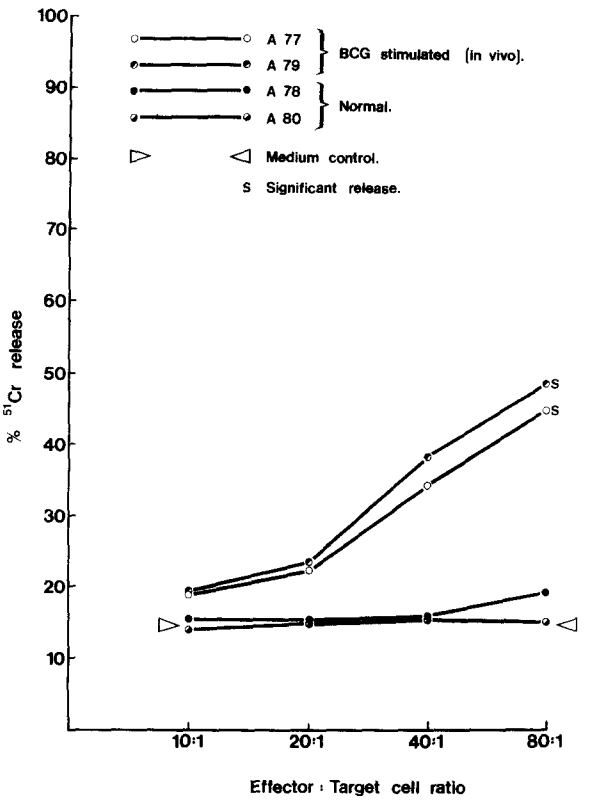

Fig. 2. Comparison of percentage ^{51}Cr release from the canine melanoma cell line RVC-347 *with effector canine alveolar macrophages from two normal dogs* (A78, A80) *and two BCG stimulated dogs* (A77, A79) *at different effector: target cell ratios.*

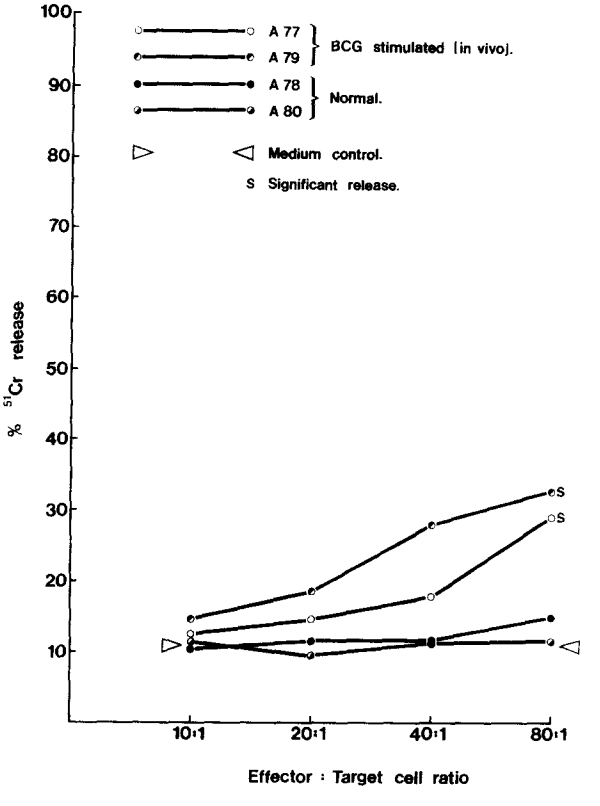

Fig. 3. Comparison of percentage ^{51}Cr release from the canine kidney cell line MDCK with effector canine alveolar macrophages from two normal dogs (A78, A80) *and two BCG stimulated dogs* (A77, A79) *at different effector: target cell ratios.*

dogs A77 and A79 received 2 i.v. BCG injections on the schedule outlined in the Introduction, and CAM collected 2 weeks after the final immunisation. Dogs A78 and A80 were controls and received i.v. placebo treatment (PBS 3 ml). As can be seen the macrophage effector cells exhibited a significant % ^{51}Cr release at 8 hr, whereas the control effector population produced no significant release. The specific cytotoxicity at both 4 and 8 hr is provided in Table 2.

This *in vivo* stimulation of the alveolar macrophages by i.v. BCG is further emphasised in Table 3. The alveolar macrophages from 8 dogs were tested:

(A) D, 1, 3, 5, 7 received 3 i.v. BCG injections on the standard schedule, and the CAM collected 25 days after the final injection.

(B) D, 2, 4, 6, 8 received 3 i.v. PBS injections and the macrophages collected at the same time interval as above. The results clearly indicate that the macrophage effector cells of the test group produced a highly significant ^{51}Cr release from all the target cells examined. This was not observed in the control group.

A further series of dogs was examined to assess the cumulative effect of multiple either i.v. (A10, A19) or intrapleural (A14, A20) BCG injections on the alveolar macrophage population. Table 4 indicates that there was no consistent significant cytolysis by the CAM of A10 and A14 where the final BCG injection had been administered 14 weeks prior to the experiment. A similar result was found in dogs when the final injection was 10.5 weeks prior to testing.

Table 2. Specific cytotoxicity of alveolar macrophages from normal and BCG stimulated dogs

Specific cytotoxicity. Effector: target ratio 80:1								
	4 hr				8 hr			
Target cell	A77	A78	A79	A80	A77	A78	A79	A80
H72-1503	22.0	2.7	24.0	4.4	31.0	0.2	28.7	0.0
RVC-347	23.9	0.9	24.8	0.0	35.5	5.0	39.5	0.8
MDCK	12.2	1.8	17.8	0.5	19.7	4.0	25.0	0.0

Table 3. Specific cytotoxicity of alveolar macrophages from normal and BCG stimulated dogs

Specific cytotoxicity 8 hr. Effector:target ratio 80:1								
Target cell	D1	D3	D5	D7	D2	D4	D6	D8
H73-2295	38.9	35.3	58.2	44.0	3.2	3.1	0.5	1.3
MDCK	28.5	12.1	40.1	27.0	0.9	1.4	0.0	0.8
H71-1843	40.9	13.9	58.5	29.0	1.7	2.9	1.4	0.0
H72-1503	50.6	24.3	58.3	27.8	3.1	2.0	0.7	2.5

Table 4. Specific cytotoxicity of alveolar macrophages from dogs which received multiple BCG injections

Specific cytotoxicity 8 hr. Effector:target ratio 80:1				
	Intravenous BCG		Intrapleural BCG	
Target cell	A10	A19	A14	A20
H71-1843	0.6	N.D.	0.3	N.D.
H73-2295	0.0	N.D.	1.3	N.D.
H72-1563	0.6	0	0.9	0
RVC-347	N.D.	0	N.D.	0

A10—received 6 i.v. BCG injections. Macrophages collected 14 weeks after last injection. A14—received 6 intrapleural BCG injections. Macrophages collected 14 weeks after last injection. A19—received 7 i.v. BCG injections. Macrophages collected 10 weeks after last injection. A20—received 7 intrapleural BCG injections. Macrophages collected 10 weeks after last injection.

The involvement of soluble products of macrophages as mediators of cytostatic and cytolytic reactions had been implicated in other reports. To investigate the possibility in this system, the activity of all macrophage supernatants was examined as described. An example of one is presented in Table 5. It can readily be appreciated that although the macrophage effector cell populations from dogs A77, A79 were highly active the respective supernatant failed to induce a comparable significant ^{51}Cr release at the dilutions examined.

Alveolar macrophages were isolated from dogs bearing spontaneous tumours and tested against the allogeneic cell lines available. The results are summarised in Table 6, cases H76-1369 and BL-1 were included in the same ^{51}Cr assay but the other examples were in different experiments. Neither significant non-specific nor specific ^{51}Cr release was observed in these tests. None of these dogs had received any intravenous BCG therapy.

with a protein moiety may be operative. If these criticisms were found to be justified false negative results may have been produced, although this was considered unlikely.

Hibbs [35] reviewed the current data and concluded that persistence of the antigen *in vivo* was a pre-requisite for the maintenance of non-specifically activated macrophages. Moreover, it was essential that this was present in the macrophage compartment under investigation. The results in this study were consistent with such a hypothesis where CAM which had not recently been stimulated with BCG were non-cytolytic. To clarify the situation would require careful examination to discern whether or not any increased cytolytic activity following each infection was due to a recent infiltration of CAM or as a result of restimulation of the effector cells already present. Betton, Gorman and Owen [36] demonstrated that in experimental alloimmunisation of dogs with i.v. BCG plus allogeneic cells induced an active population of CAM.

Table 5. Comparison of alveolar macrophage cytotoxicity with the cytolytic activity of macrophage culture supernatants

	% ^{51}Cr release at 4 hr								
	Effector: target ratio 80:1			S1			S2		
Target cell	H72-1503	RVC-347	MDCK	H72-1503	RVC-347	MDCK	H72-1503	RVC-347	MDCK
A77	29.5	32.0	17.1	10.5	13.1	7.5	10.4	12.0	6.4
A78	11.9	12.1	7.4	11.1	10.1	8.1	10.5	10.8	7.0
A79	31.3	33.3	22.4	11.4	11.6	8.4	11.2	11.0	9.2
A80	13.5	11.1	6.1	12.1	11.5	7.9	11.0	12.2	8.4
Medium	9.5	11.3	5.6	9.5	11.3	5.6	9.5	11.3	5.6

S1 = 100 μl of macrophage culture supernatant.
S2 = 100 μl of 1/100 macrophage culture supernatant.

DISCUSSION

The results indicated that normal canine alveolar macrophages failed to produce significant ^{51}Cr release in the experiments performed. It was clearly demonstrated, however, that following a course of i.v. BCG administration the macrophage population obtained *in vitro* exerted a significant effect on the allogeneic cells. This was a consistent finding and comparable to the results of other workers [33, 34]. It must be considered that whilst the ^{51}Cr techniques used in this study were suitable to detect cell lysis it may have underestimated target cell interaction by not accounting for cytostasis, this property would have to be independently examined. Furthermore, possible uptake of ^{51}Cr by the effector cells either as a free ion or combined

In contrast, i.m. cells plus Freund's adjuvant or i.v. cells alone failed to produce such a reaction. A consistent feature of this present study and that of Betton *et al.* [36] is the marked total lack of specificity against the allogeneic target cells including normal kidney cell line (MDCK). It should be considered that as this cell line has been in culture since 1958 during which time numerous spontaneous changes could have occurred to define it as normal may be incorrect. However, the findings of previous studies have indicated that the MDCK cell line has different functional and growth properties to the other cell lines of neoplastic origin used in this study ([26–28], L.N. Owen personal communication). It is therefore difficult to state whether or not these present results are consistent

with the hypothesis of either Hibbs [23] or Keller [24].

Macrophage mediated cell death *in vitro* is incompletely understood but the presence of soluble mediators has been implicated [37–40]. The evidence in this study could not demonstrate that such a mediator was operative. Furthermore in contrast to experiments with cytostatic assays [41] other unrelated culture supernatants failed to induce a significant ^{51}Cr release from the target cells.

The *in vivo* significance with respect to the control of pulmonary metastases is important. It was clear that the mononuclear phagocytes associated with the development of granulomas, produced after i.v. BCG, exhibited a marked non-specific cytolytic potential *in vitro* [36]. It would seem appropriate to suggest that the lack of pulmonary metastatic development observed clinically could be mediated by the pool of mononuclear phagocytes. This may operate either alone or in combination with enhanced N.K. cell activity following i.v. BCG in the dog previously reported [36].

This work supported the findings in experimental rodents where spontaneous or induced pulmonary metastases were restricted by i.v. BCG [42, 43]. In these experimental systems control of pulmonary metastases was similarly attributed to the granulomatous lesions in the lung tissue and thought comparable to the restriction of tumour growth when BCG is administered locally in skin grafts [44, 45].

This hypothesis on the control of pulmonary metastases can only be an extension of experimental work. However, the inability to obtain CAM from BCG treated tumour bearing dogs has obviated any direct evidence. In the clinical cases cited 3 had confirmed pulmonary metastases but this alone was insufficient to induce a pool of either specific or non-specific cytolytic macrophages *in vitro*.

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