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Cell Mediated Cytotoxicity in Dogs Following Systemic or Local BCG Treatment alone or in Combination with Allogeneic Tumour Cell Lines*

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Abstract—*Normal dogs were treated with intravenous BCG* (0.1 mg/kg *body wt*) *on a doubling interval schedule previously found to be clinically effective. A rise in non-specific NK cytotoxicity against* ^{51}Cr *labelled canine tumour target cells was observed. Intrapleural injections were also effective. To determine the adjuvant efficacy of BCG either intravenously or in Freund's adjuvant, alloimmunisation of dogs with tumour cell lines with or without BCG was performed. Dogs in all groups showed strong non-specific peripheral blood cytotoxic responses to alloimmunisation in most cases. Spleen cells were usually less cytotoxic. Specific adjuvant effects of BCG were not pronounced. Lung macrophage activation was observed after intravenous BCG treatment.*

INTRODUCTION

THE EXPRESSION of neoantigens on malignant cells (see [1] for review) provides a basis for immunotherapy [2]. Spontaneous neoplasms in man and animals may however fail to express specific tumour antigens. Cytotoxicity can be detected in both tumour bearers and clinically normal controls [1]. Naturally arising canine tumours may express antigens in autochthonous tests for blastogenesis [3], cytotoxicity or indirect leucocyte migration inhibition tests [4]. Tests performed with allogeneic cell lines showed no evidence for type-specific antigens in direct leucocyte migration inhibition [5] or cytotoxicity tests [6] but revealed NK activity, resembling the situation in man. Effective BCG immunotherapy in the dog has been reported for osteosarcoma [7, 8] mammary carcinoma [9] and allogeneic transmissible venereal tumour [10].

Natural killer (NK) cell activity acting against non-specific target cells shows variation with age, and, to a lesser degree, with time, and can be stimulated by a variety of agents [11] including poly I-C which shows a rapid response typical of interferon mediation. The NK cell is thought to be bone marrow derived and, in the mouse, controlled by genetic factors [12] in contrast to the human situation [13, 14]. It is thymus independent [1] and does not express fully either T or B lymphocyte markers although receptors for the Fc portion of immunoglobulin and low affinity spontaneous erythrocyte rosette formation resembles activated T lymphocytes [1, 15]. The apparent non-specificity of NK cells may include a selective component as shown by competitive inhibition studies [16]. This component was protease sensitive and could be restored by cytophilic antibody present in human serum [13, 14]. Foetal calf serum determinants acquired in culture [17] are probably not the target for NK activity [18, 19].

The effect of BCG on NK activity of healthy canine donors [6], either *per se* or in combination with alloimmunisation, is now reported.

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Abbreviations used: BCG = Bacillus Calmette Guerin strain of Mycobacterium bovis, FCS = Foetal Calf Serum, CFA = Complete Freund's Adjuvant, HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, SEC = Splenic Effector Cells, LMP = Lung Macrophage, NK = Natural Killer Cell.

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MATERIALS AND METHODS

Animals

Outbred adult healthy dogs were housed in conventional kennels. The BCG immunotherapy protocol used clinically was repeated for studies in healthy recipients. BCG for percutaneous use ($5–25 \times 10^7$ viable organisms/3 mg, Glaxo, Greenford, Middx, U.K.) was resuspended in normal saline and injected intravenously (0.1 mg moist BCG/kg body wt.).

Dogs receiving repeated doses were premedicated with 1 mg chlorpheniramine ('Piriton', Allen and Hanburys, London) to reduce the risk of anaphylactic reactions. Intrapleural inoculation was carried out under local anaesthesia and BCG injected into the pleural cavity. Subcutaneous or perivascular deposition of BCG organisms was carefully avoided. Timings of repeated inoculations are given in Results.

Adjuvant activity of BCG on cytotoxic responses to alloimmunisation was also evaluated. Dogs were immunised with allogeneic malignant cell lines (see below) by the following protocols:
(i) cells alone in 2 ml Hanks' BSS intravenously, (ii) cells admixed with BCG organisms in 2 ml Hanks' BSS intravenously and (iii) cells emulsified in Freund's incomplete adjuvant (1 ml, Difco lab, Detroit, U.S.A.) supplemented with BCG (=CFA) and injected intramuscularly into alternating hind legs. Alloimmunisation was repeated after 1 and 3 weeks from the commencement of treatment. Peripheral blood was collected by jugular venepuncture before and throughout the immunisation programme. One week after the final immunisation, dogs were killed with pentobarbitone sodium ("Euthatal", May and Baker, Essex, U.K.) and spleen and lungs were immediately removed aseptically.

Allogeneic cell lines

Canine tumour cell lines were established from surgical specimens excised from spontaneous cases of neoplasia referred by veterinarians to the Veterinary School, Cambridge. The derivation and characterisation of these lines has been previously reported [6, 20–23]. The cell line melanoma RVC347 [24] was provided by Prof. W. Plowright, Royal Veterinary College, London. Cell lines were maintained in medium RPMI 1640 plus 10% FCS and antibiotics. Cell monolayers were detached with antibiotic/trypsin/EDTA solution for subculture or use as antigen or target cells. For immunisation, single cell suspensions were washed three times in Hanks' BSS and inocula of 5×10^7 or 1×10^8 cells were injected intravenously (with or without BCG) or emulsified in CFA containing BCG and injected intramuscularly.

Effector cell isolation

Details of the ^{51}Cr release assay employed have been previously published [6]. Briefly, gelatin sedimented buffy-coat cells were layered onto Ficoll (Pharmacia, Uppsala, Sweden)-Hypaque (Winthrop, U.S.A.) gradients and lymphocytes isolated from the interface fraction after centrifugation. Where specified in Results, whole blood was preincubated with carbonyl iron (20 mg/ml, GAF Ltd., London, U.K.) at 37°C in a shaking waterbath for 30 min before sedimentation and isopycnic centrifugation in order to deplete phagocyte cells. All effector cell preparations were washed and resuspended in RPMI 1640 plus 10% FCS, antibiotics and 10 mM Hepes buffer and incubated overnight (unless otherwise stated) in 25 cm^2 plastic culture flasks (Falcon, Calif., U.S.A.). Non-adherent mononuclear cells were then decanted off and tested as effector cells after counting and resuspending in fresh medium. Effector cell purity of >90% mononuclear cells in Giemsa-stained cytocentrifuge preparations was regarded as satisfactory.

Spleen effector cells (SEC) were prepared from diced spleen tissue subjected to 0.25% trypsin (Difco, Detroit, U.S.A.) on a spinner for 1 hr at 20°C. Mononuclear cells were then purified by Ficoll–Hypaque centrifugation as above. Lung macrophage populations (LMP) were prepared by tracheal lavage with Hanks' BSS accompanied by gentle massage of the lung tissue. The lung cells obtained were purified on Ficoll–Hypaque gradients and mononuclear cells collected from the interface. Macrophages were purified from this heterogeneous population by adherence to 75 cm^2 plastic culture flasks (Falcon, Oxnard, Calif. U.S.A.) for 1 hr, washing and removal of contaminating fibroblasts by trypsinisation. Adherent macrophages were cultured overnight in fresh medium and harvested the following day with EDTA solution.

Cytotoxicity assay

The ^{51}Cr release assay was performed in round bottomed 96-well microtest plates

(Nunc, Denmark) using a 200 μl inoculation volume of medium RPMI 1640 plus 10% FCS, antibiotics and 10 mM HEPES buffer. Wells contained 5×10^3 ^{51}Cr labelled allogeneic tumour target cells and an effector: target cell ratio range of 20:1 to 160:1 or 10:1 to 80:1. The results for 8 hr % ^{51}Cr release with background spontaneous release values (usually <20%) are shown. Details of the cytotoxicity test and analysis of log transformed means of triplicate % release values by analysis of the variance and multiple range testing at the $P<0.05$ level are given in [6]. 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%. Only data significant at $P<0.05$ is presented as positive cytotoxicity.

RESULTS

Effects of BCG alone

To examine the effect of BCG inoculation *per se*, 6 dogs were treated with intravenous BCG (dogs 1–3) or intrapleural BCG (dogs 4–6). Cell-mediated cytotoxicity was measured throughout the inoculation programme (BCG injections on weeks 1, 2, 4, 8, 16 and 32). Pretreated tests revealed no cytotoxic activity in 5/6 days but NK activity against all target cells was present in dog 4. This activity was absent on re-testing and later rose again in the final test in which all BCG treated dogs had become significantly cytotoxic for the most sensitive target, osteosarcoma H72-1503 (Table 1). Splenic effector cells were also cytotoxic for osteosarcoma target cells in all dogs tested at the end of the BCG treatment programme. The apparent selectivity of BCG

Table 1. Cytotoxic responses following BCG inoculation in healthy dogs

Route of Inoculation:		Intravenous				Intrathoracic		
Cytotoxicity against Target Cells:		A	B	C		A	B	C
No. of BCG Injections	Dog No.				Dog No.			
0	1	—	—	—	4	25.2	15.0	14.0
	2	—	—	—	5	—	—	—
	3	—	—	—	6	—	—	—
1	1		—	—	4	ND	ND	ND
	2	ND	ND	ND	5	—	—	—
	3	—	—	—	6	—	—	—
2	1	26.7	21.8	13.4	4	—	—	—
	2	—	—	—	5	ND	ND	ND
	3	—	—	—	6	—	—	—
3	1	15.2	—	—	4	—	—	—
	2	—	ND	—	5	—	ND	—
	3	—	ND	—	6	—	ND	—
5–6	1	—	31.6*	—	4	—	23.8	2.4
	2	—	18.9	—	5	—	19.8	—
	3	—	29.8	—	6	—	11.8	—

Target Cells: A = Melanomas VI, H71-1843 or RVC-347.
B = Osteosarcoma H72-1503.
C = Mammary Carcinoma H73-2295.

BCG inoculations were carried out with Glaxo percutaneous BCG at 0.1 mg/kg body wt either intravenously or into the pleural space intrathoracically (three dogs in each group). Specific cytotoxicity was calculated from the 8 hr ^{51}Cr release at an 80:1 effector: target cell ratio except for * where a 40:1 ratio was used. ND = not tested. All positive cytotoxicities were significant at $P<0.05$.

induced cytotoxicity for osteosarcoma target cells was also observed in other experiments (data not shown).

Effect of BCG on responses to alloimmunisation

To test whether the potential effects of BCG immunotherapy could be attributed to an adjuvant effect in addition to the non-specific effects described above, comparison of cytotoxicities following alloimmunisation of dogs with (i) i.v. allogeneic cells, (ii) i.v. allogeneic cells plus BCG and (iii) i.m. allogeneic cells plus CFA containing BCG was performed. Four groups of those dogs were immunised with four allogeneic cell lines respectively. Cell-mediated cytotoxicity of peripheral blood lymphocytes was assayed against the immunising cell line and unrelating control target cells.

Pretreatment tests demonstrated NK activity against osteosarcoma cells in 5/11 dogs and three donors lysed other target cells (Table 2). Post-immunisation, 8/9 dogs were cytotoxic for osteosarcoma target cells (2/3 given cells alone, 3/3 given cells + BCG, 3/3 given cells + CFA) 9/12 dogs lysed other target cells (3/4 given cells alone, 3/4 given cells + BCG, 3/4 given cells + CFA). One dog in group 3 treated with intravenous osteosarcoma cells alone remained negative throughout. However all other dogs showed the induction or elevation of cytotoxicity in response to alloimmunisation.

Specificity of cytotoxicity

The specificity of the reactivity was determined using the specific immunising cell and control cells as targets through the course of immunisation. Unexpectedly, immunisation produced a rise in cytotoxic activity against both the specific and control target cells (Table 2, Fig. 1). In most cases, the non-specific cytotoxicity was pronounced with

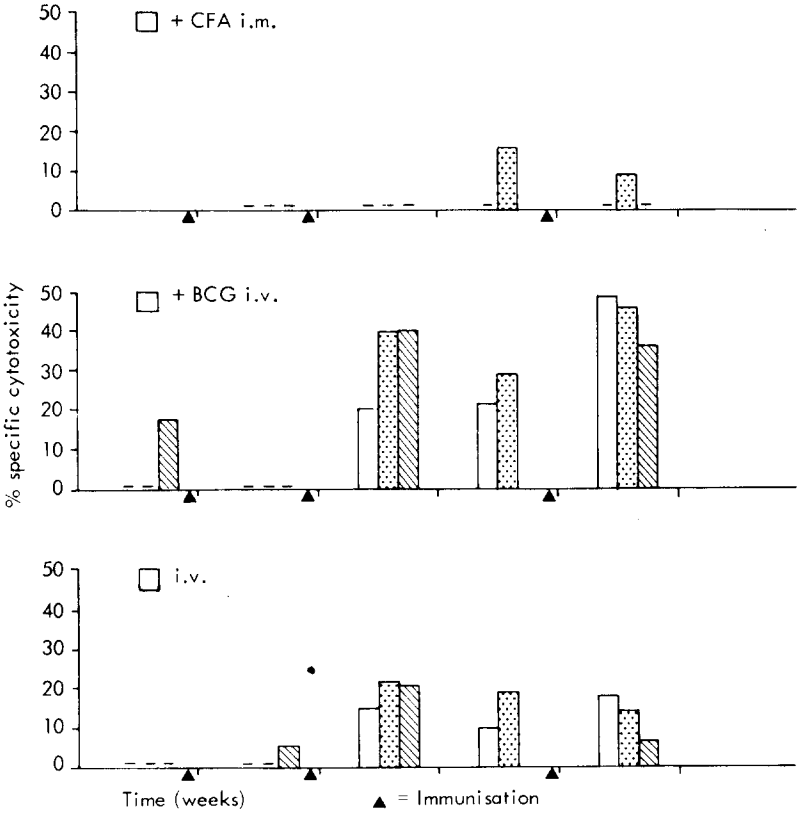

Fig. 1. Time course of cytotoxic response to alloimmunisation. Three dogs were immunised with VI melanoma cells either alone (i.v.), with BCG i.v. or in CFA i.m. (see text). Peripheral blood mononuclear cell cytotoxicity was repeatedly assayed using three target cells. □ *= melanoma (specific) target cell.* ⊡ *= osteosarcoma target cell control.* ▨ *= mammary carcinoma target cell control.* – – – *= no significant cytotoxicity.*

Table 2. Cytotoxicity following immunisation with allogeneic cell lines

Immunisation protocol		Cells i.v.			Cells + BCG i.v.			Cells + CFA i.m. (with BCG)		
% Specific cytotoxicity at 80:1 ratio against target cells		A	B	C	A	B	C	A	B	C
Group I	Effector cells									
Immunisation with	Pretest PBL	—	—	—	—	—	—	10.2	16.3	8.5
mammary carcinoma	Post immunisation:									
	PBL	18.2	ND	29.1	48.8	ND	64.8	44.2	ND	41.0
	SEC	—	—	—	—	—	—	ND	ND	ND
Group II										
Immunisation with	Pretest PBL	—	—	—	—	—	17.2	ND	ND	ND
melanoma A1	Post immunisation:									
	PBL	18.4	14.3	6.7^{4hr}	48.1	45.7	35.9^{4hr}	—	8.8	—
	SEC	—	16.2	2.6^{4hr}	6.3	15.5	2.5^{4hr}	12.4	25.8	7.6^{4hr}
	LMP	—	—	6.2	37.5	16.7	28.3	6.7	—	9.2
Group III										
Immunisation with	Pretest PBL	—	—	ND	—	—	ND	9.8	13.8	ND
osteosarcoma B	Post immunisation:									
	PBL	—	—	—	—	8.4	—	8.0	15.8	4.0
	SEC	—	—	—	—	—	—	—	—	—
	LMP	—	—	3.6	6.4	12.1	6.2	—	6.9	2.6
Group IV										
Immunisation with	Pretest PBL	—	11.8	ND	—	8.1	ND	—	11.8	ND
melanoma A2	Post immunisation:									
	PBL	5.3	30.9	8.1	3.5	11.1	—	10.9	35.8	17.8
	SEC	—	—	—	—	—	—	—	—	—

Target cells: A = Melanoma line VI(A1) (Groups I–III) or melanoma line H71-1843 (A2) Group IV).
B = Osteosarcoma line H72-1503.
C = Mammary Carcinoma line H73-2295.

Effector cells were prepared from peripheral blood prior to immunisation (Pretest PBL) or after immunisation from peripheral blood (PBL), spleen (SEC) or lung macrophages (LMP) (Groups III and IV only). Immunisation was carried out by the intravenous inoculation of a single cell suspension of tissue culture cells alone (cells i.v.) or after administration with viable BCG (cells plus BCG i.v.) or by the intramuscular inoculation of tissue culture cells emulsified in Freund's adjuvant containing BCG (cells + CFA i.m.). ND = not tested; 4hr = 4 hr release value. All positive cytotoxicities were positive at $P<0.05$.

osteosarcoma target cells, as previously observed. Sequential testing nevertheless indicated a rising specific component in the cytotoxic reactivity in some cases (Fig. 1). Since pretreatment testing of one group was performed in the same experiment as post-immunisation testing of the previous group, variations in test sensitivity for technical reasons were not responsible for the rise in cytotoxicity observed.

In order to further examine the specificity of these reactions, competitive inhibition of lysis by the addition of unlabelled target cells was examined. Titrations of effector: target cell ratios against cytotoxicity (% ^{51}Cr release) under various conditions are given in Fig. 2. Donors were immunised against mammary carcinoma target cells (group 1, Table 2) and all developed strong cytotoxic activity for both specific and unrelated target cells, as did the control given BCG alone. Addition of excess (5:1 ratio) unlabelled mammary carcinoma inhibitor cells depressed cytotoxicity against both specific and control target cells. Non-specific competitive inhibition was regularly demonstrable in other experiments (data not shown). The possibility of non-specific cytotoxicity being an artefact resulting from the overnight inoculation of effector cells before testing was eliminated as effector cells prepared immediately prior to testing were similarly active (Fig. 2).

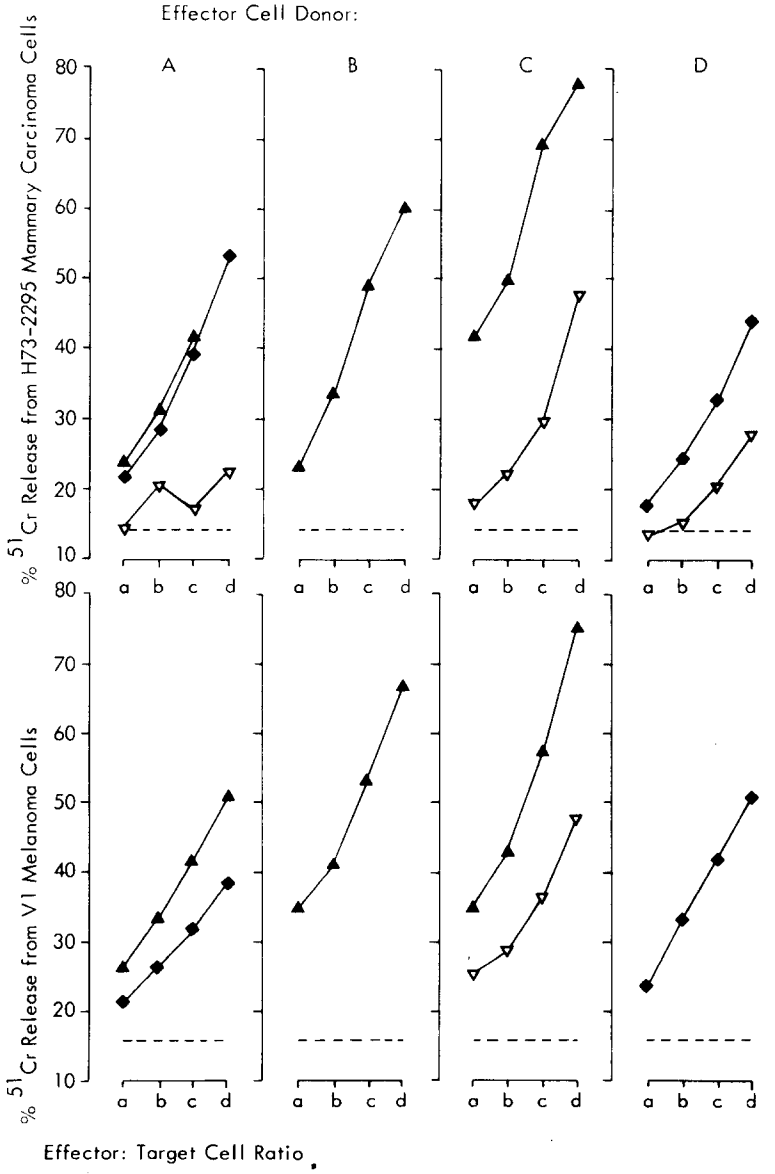

Fig. 2. Competitive inhibition of cytotoxicity and effect of overnight incubation of effector cells. Effector cell donors were immunised with H73-2295 cells i.v. (A), H73-2295 cells + BCG i.v. (B), H73-2295 cells + CFA i.m. (C) or BCG only i.v. (D). Peripheral blood lymphocytes were prepared on Ficoll–Hypaque and tested immediately (◆) or after overnight incubation (▲). The addition of unlabelled competitive inhibitor cells (▽) significantly reduced cytotoxicity. – – – = spontaneous release in medium alone. Eight hour release values shown. a = 20:1, b = 40:1, c = 80:1, d = 160:1. Effector: target cell ratio.

Distribution of effector cells

In an experiment designed to compare the cytotoxicity activity of peripheral blood mononuclear cells with or without carbonyl iron depletion of mononuclear phagocytes, no depression of lytic activity was observed and in some cases activity was increased (Fig. 3). The cytotoxic activity of spleen cells was also tested and found again to be non-specific in nature with all target cells lysed. Comparison of splenic vs peripheral blood lytic activities showed no constant relationship (Fig. 3) although peripheral blood cells were usually most cytotoxic. Further comparisons were made using peripheral blood, spleen and lung effector cells. In this experiment, dogs were primed with melanoma cells plus CFA intramuscularly following which peripheral cells were cytotoxic only for osteosarcoma target cells. After two subcutaneous challenges with melanoma cells in Hanks' BSS, strong peripheral blood cytotoxic activity developed against both immunising and unrelated control target cell (Fig. 4). Spleen non-adherent mononuclear cells and lung macrophages tested the following day exhibited lower levels of cytotoxicity (Fig. 4). Other comparisons between peripheral blood, spleen and lung effector cell activities after three alloimmunisation protocols are presented in Table 2. Again specificity of lysis was not observed and each effector cell population showed independent expression of cytotoxic activity.

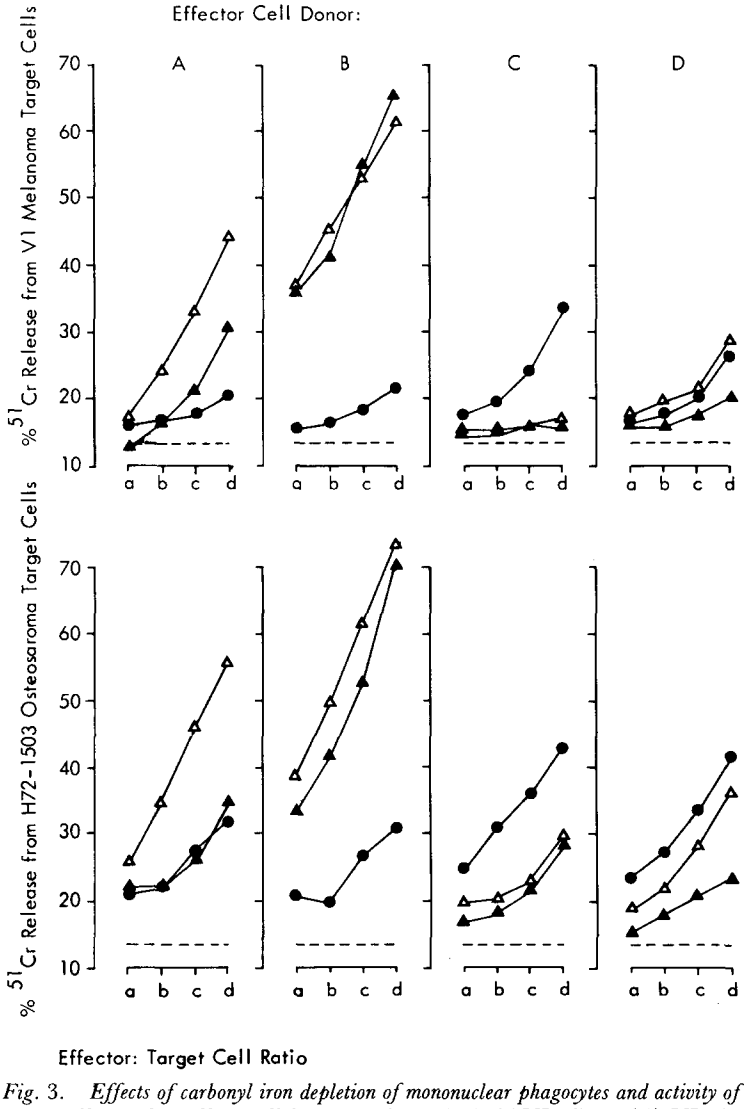

Fig. 3. Effects of carbonyl iron depletion of mononuclear phagocytes and activity of splenic effector cells. Effector cell donors were immunised with VI *cells i.v. (A),* VI *cells + BCG i.v. (B) or* VI *cells + CFA i.m. (C), Donor D was a healthy control. Peripheral blood cells were prepared with (△) or without (▲) carbonyl iron depletion of phagocytic cells. Spleen cells = ●, — — — = spontaneous release in medium alone, a = 20:1, b = 40:1, c = 80:1, d = 160:1. Effector: target cell ratio.*

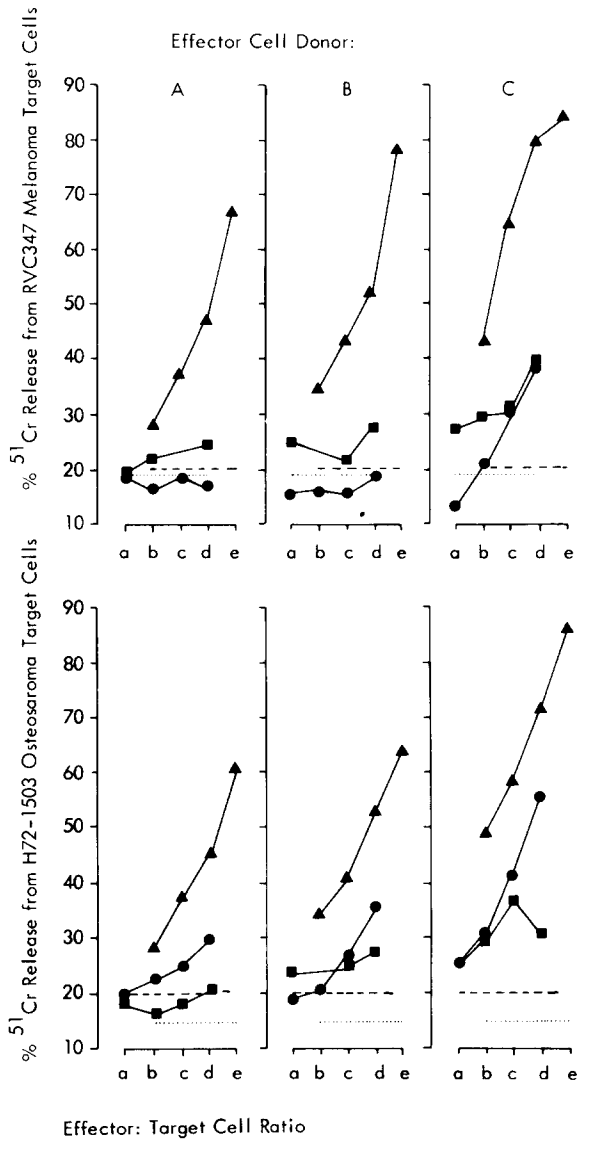

Fig. 4. Comparison of cytotoxic activity of peripheral blood, spleen and lung effector cells. Effector cell donors (A, B, C) were immunised with RVC 347 melanoma cells in CFA followed by two subcutaneous RVC 347 challenges. Peripheral blood lymphocytes (▲) showed strong cytotoxicity against both target cells (– – – –) spontaneous release in medium alone. Spleen non-adherent mononuclear cells (●) and lung macrophages (■) tested the following days were less cytotoxic (. . . .) = spontaneous release. a = 10:1, b = 20:1, c = 40:1, d = 80:1, e = 160:1. Effector: target cell ratio.

DISCUSSION

Many investigations of BCG immunotherapy have failed to include controls for the effects of BCG alone. In rodent models, BCG has been shown to be a potent activator of macrophages which may be selectively active against neoplastic cells [25]. Furthermore, cytotoxic lymphocyte resembling NK cells may appear [2, 26]. NK cell activity has been previously identified in the dog [6] in both tumour bearer and healthy control groups. The data presented here indicate that NK activity in the dog can be induced or augmented by treatment with BCG alone and was preferentially expressed against certain cell lines, e.g., osteosarcoma H72-1503. Stimulation of cytotoxicity was non-specific and normal contact-inhibited canine kidney cells were also susceptible to lysis (unpublished data). To be effective *in vivo*, the immunostimulation detected *in vitro* as non-specific cytotoxicity would require interaction(s) with as yet undefined tumour specific factors to result in the antitumour effect observed in clinical trials [7, 10].

Mechanisms for the induction of NK activity are unknown but may be mediated by soluble factors [11, 27, 28]. In this study, depletion of phagocytes failed to remove NK activity from peripheral blood effector cells whereas lung macrophage populations showed cytotoxicity in BCG treated dogs, confirming earlier findings [29]. Several mechanisms may therefore be operative in BCG immunotherapy including intermediate mediators such as interferon [11].

Adjuvant effects of BCG given intravenously or in CFA intramuscularly were not consistently identifiable over and above the effect of alloantigens alone. Further studies are required to determine whether or not different doses of BCG can result in varying responses as seen in mice [30, 31]. Route and timing of antigen restimulation also require optimisation.

The non-selective nature of the cytotoxic reactions in alloimmunised dogs suggested that a large bystander effect was the result of either shared antigens on the target cells tested or polyclonal lymphocyte activation in response to antigenic stimulation. "Anomalous" non-specific cytotoxic reactions to allogeneic cells have been seen *in vitro* during early mixed lymphocyte reactions [32, 33]. Cell lines employed as target cells were free of Mycoplasma infection and hence anti-Mycoplasma reactivity could not be operative. Although cytophilic antibody against unknown determinants on target cell lines [13, 14] remains a theoretical possibility, this could not explain the non-specific cytotoxicity observed with spleen cells prepared by trypsinisation without subsequent exposure to dog serum.

Shared antigens or a non-requirement for specific antigen recognition could result in non-specificity. Competitive inhibition studies and preabsorption of effector cells on cell monolayers (to be submitted) point to these two possible mechanisms rather than the effector cell population being a composite of

specific effector cells against a wide range of antigens [13, 16]. Although certain alleles of the DLA histocompatibility complex show a high population frequency [34], it is unlikely that all the target cell lines used share one or more common specificities against which alloantigen stimulated cytotoxic clones would react. Efforts to identify DLA specificities on the cell lines employed have proved technically difficult (Betton and Vriesendorp, unpublished data).

The inability to provide autochthonous or partly or fully DLA compatible effector: target cell combinations may also contribute to the low degree of specificity in contrast to studies in the autochthonous situation [4]. The use of NK sensitive established target cell lines rather than primary cultures resistant to lysis [6] could however also explain these differences.

In conclusion, BCG *per se* was capable of stimulating NK activity in the dog. Alloimmunisation with tumour cell lines, with or without BCG, also generated a non-specific cytotoxic response in peripheral blood effector cells in most cases. Whether tumour bearing dogs are equally capable of such a response [6] and if the elevation of NK activity is of therapeutic value requires further investigation.

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