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Kinetics of BCG Mediated Tumour Cell Destruction in the Rat*

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Abstract—*The rate of destruction of syngeneically transplanted rat ascites tumour cells in BCG treated rats has been determined by monitoring urinary excretion of* ^{125}I *from* ^{125}I*UdR-labelled cells. Intraperitoneal injection of BCG* 2 days *before or together with labelled cells prevented their growth but did not accelerate loss of label within a* 6-day *period. However, rats pre-treated* 4–10 days *previously with BCG showed increased* ^{125}I *excretion immediately after cell injection. Other rats receiving heat-killed or irradiated cells similarly showed accelerated loss of* ^{125}I, *but this was not consistently apparent until* 24 hr *after cell injection. These studies indicate that there is a delay of several* days *after BCG administration into the tumour environment before tumour cell destruction occurs, probably reflecting the time for local generation of non-specific host responses.*

INTRODUCTION

BCG INJECTED locally into the site of solid or ascitic tumours may suppress their development and current evidence indicates that both non-specific and immunologically specific host responses are involved in this destruction of malignant cells [1, 2]. Using the peritoneal cavity as a model site, local non-specific host responses generated by BCG have been evaluated *in vitro* by assessment of the cytotoxicity of peritoneal exudate cells for cultured tumour cells and *in vivo* by adoptive transfer of tumour resistance (reviewed in [2–4]). These approaches have demonstrated considerable discrepancies in the responses to BCG-stimulated peritoneal exudates since they are generally highly cytotoxic *in vitro* for cultured tumour cells whereas little or no *in vivo* tumour suppressive capacity is revealed when these cells are injected together with tumour cells [2]. The objective of the present study was to determine more directly the rate of generation by BCG of local host responses capable of *in vivo* destruction of malignant cells. For this, the kinetics of destruction of cells of an ascitic rat hepatoma (D23As) have been assessed in normal rats and in animals in which tumour development is controlled by local, i.p., injection of BCG. The time of commencement of cell death and the rate of destruction have been monitored by following urinary excretion of ^{125}I from ^{125}IUdR-labelled cells.

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MATERIALS AND METHODS

Tumour

Hepatoma D23 was originally induced in an adult male rat of the Department's inbred WAB/Not strain by oral administration of 4-dimethyl-aminoazobenzene and maintained by s.c. implantation in syngeneic rats of the same sex [5]. Subsequently it was converted to an ascitic form (D23As) and carried by weekly i.p. passage of 10^7 cells [6]. Cells were routinely harvested by peritoneal lavage with Hank's solution containing 5 U/ml of heparin. This tumour is immunogenic, rats immunized by repeated i.p. injection of radiation-attenuated D23As cells rejecting i.p. challenge with up to 5×10^5 cells, although inocula of 10^3 cells will develop in untreated control rats.

Cell labelling

One to four days after i.p. injection of 10^7 D23As cells, rats received three i.p. injections of a sterile aqueous solution of 5-[^{125}I]iodo-2'-deoxyuridine (^{125}IUdR) (Radiochemical Centre, Amersham, Buckinghamshire,

England) in three divided doses at 2–24 hr intervals to a total dose of 4–18 μCi/rat. Cells were harvested by peritoneal lavage with heparinized Hank's solution 6–48 hr after the final ^{125}IUdR injection. Alternatively, cells were labelled *in vitro* by incubation of freshly harvested cells at 37°C in tissue culture medium 199 at a concentration of 4×10^6 D23As cells/4 μCi ^{125}IUdR/ml for 2–4 hr. In both cases, labelled cells were washed 4 times in Hank's solution before *in vivo* injection. There were no apparent differences in subsequent *in vivo* behaviour between cells labelled in these two ways. Levels of labelling varied from 10^4 to 10^6 counts/min 10^7 D23As cells and all radioactivity counts were carried out in an LKB Wallac 80000 gamma counter.

Bacillus Calmette Guérin (BCG)

Freeze-dried BCG vaccine (percutaneous) was supplied by Glaxo Research Laboratories, Greenford, Middlesex, England. On reconstitution in water this vaccine contains 10 mg moist weight of organisms per ml.

Experimental protocol

To assess the rate of *in vivo* destruction of killed D23As cells, rats received labelled D23As cells (5×10^6 to 4×10^7) heated in a boiling water bath for 5 min, or exposed to 15,000 rad ^{60}Co γ-irradiation. The rate of destruction in BCG treated rats was assessed in animals receiving 0.5 mg moist weight of BCG injected i.p. at the time of labelled cell injection, or 2–10 days previously. In one test, rats received BCG both 10 days before and at the time of tumour cell injection. Animals were subsequently housed in groups of 2–4 in all-glass metabolism cages ("Metabowls", Jencons, Hemel Hempstead, Herts., England), allowing separate collection of urine and faeces, with drinking water containing 0.1% sodium iodide. Urine was collected daily and the amount of ^{125}I excreted expressed as a percentage of injected activity with reference to a retained aliquot of labelled cells. After 4–6 days animals were returned to conventional cages and killed when moribund with ascitic tumour growth.

RESULTS

Figure 1 shows the rate of urinary excretion of ^{125}I from untreated rats injected i.p. with free ^{125}IUdR, viable ^{125}IUdR-labelled cells or labelled cells killed by heating or γ-irradiation. ^{125}I was excreted rapidly from free ^{125}IUdR (Fig. 1a), 75% of injected activity being recovered in the urine 24 hr after injection. In contrast, only 15–20% of label was excreted in the first 24 hr from rats receiving viable ^{125}IUdR-labelled cells (5×10^6 to 4×10^7 cells/rat) and this rate of excretion continued over the 4-day observation period (Figs. 1a–f). All of the rats

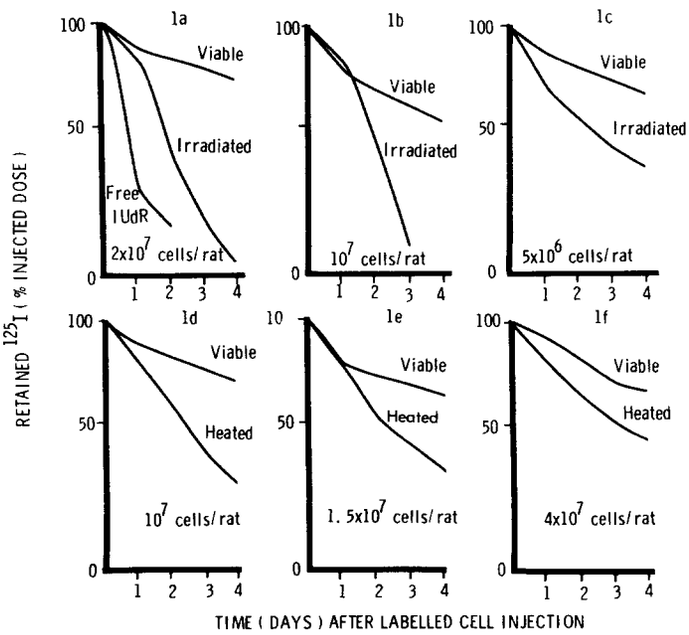


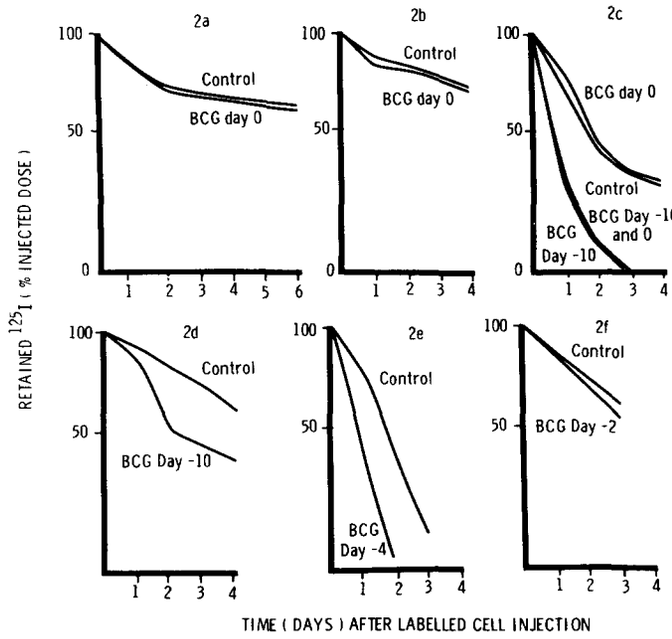
Fig. 1. Urinary excretion of ^{125}I from untreated rats injected i.p. with free ^{125}IUdR, viable ^{125}IUdR-labelled D23As cells, and with heat and radiation killed labelled cells. 2–4 rats/group.

receiving viable labelled cells subsequently developed ascitic tumour growth after 10–20 days. Rats receiving killed cells showed accelerated loss of label (Figs. 1a–f) although in some cases (Figs. 1a, b, e) this was not apparent until the second day after cell inoculation, the excretion of ^{125}I over the first 24 hr parallelling that from animals receiving viable cells.

Figure 2 illustrates urinary excretion of ^{125}I from labelled cells injected into normal and BCG treated rats receiving 10^5 labelled cells, Treatment with BCG both 10 days before and at the time of labelled cell injection did not increase ^{125}I excretion over and above that seen only with treatment 10 days before (Fig. 2c).

DISCUSSION

The kinetics of *in vivo* destruction of tumour cells injected i.p. can be monitored by prelabelling cells with $^{125}IUdR$ and measuring the *in toto* animal radioactivity or its urinary



Fig, 2. *Urinary excretion of* ^{125}I *from normal, and BCG treated rats injected i.p. with* 10^5 *viable* $^{125}IUdR$*-labelled D23As cells. BCG dose* 0.5 mg *moist weight of organisms injected i.p.* 2–4 *rats/group.*

this being the maximum suppressible by BCG treatment. Injection of BCG together with tumour cells had no effect on the rate of cell destruction with a 4–6 day period (Figs. 2a–c). None of the BCG treated rats developed ascites growths, in contrast to the untreated animals, all of which developed tumours. These findings indicate that active cell destruction does not take place within 4–6 days after local application of BCG and further tests were therefore carried out with rats pretreated with the vaccine. Rats treated with BCG 2 days before labelled cell injection showed ^{125}I excretion only marginally different from untreated animals (Fig. 2f). Treatment 4 or 10 days before tumour cell injection, however, markedly increased the rate of excretion of label (Figs. 2c–e). excretion. The label is released from cells on their death, its metabolites undergoing rapid urinary excretion with minimal re-utilization, so that the rate of loss of radioactivity is proportional to the rate of cell death [7–11]. ^{125}I-UdR may itself be toxic for cells, and this technique is not therefore suitable for determining the absolute rate of cell death *in vivo* but only for comparing cell destruction in normal animals with that in animals receiving anti-tumour therapy, such as BCG. The growth rate of labelled ascites cells in the present work was comparable with that from unlabelled cells suggesting that the toxic effects are relatively small in this system.

In the present studies, accelerated loss of label was seen from animals receiving cells pre-killed by heat or γ-irradiation. This, and

the rapid excretion of label from free ^{125}I-UdR indicates that re-utilization is small in this animal system and that the rate of destruction and catabolism of cells may be followed by this technique. When tumour growth was suppressed by regional application of BCG, however, there was no accelerated tumour cell destruction within 4–6 days after simultaneous injection of labelled cells and BCG. In these experiments, the rate of excretion of ^{125}I from labelled cells in control animals varied from experiment to experiment, but rats treated with BCG at the same time as labelled cell injection consistantly showed identical excretion patterns as rats receiving no treatment in three separate tests (Figs. 2a–c). Animals pre-treated 4–10 days previously, were, however, capable of immediate destruction of tumour cells. This was most clearly shown in animals pre-treated 10 days beforehand where there was accelerated loss of label in three groups of animals compared with rats having no BCG treatment, or treated on the day of challenge (Figs. 2c–d).

These observations indicate that there is a delay after regional BCG injection before host responses become adequate to effectively destroy malignant cells. These are probably local, rather than systemic responses, since BCG injected at other anatomical sites is unable to suppress i.p. tumour development with the model system used here [12]. These responses may be non-specific or immunologically specifically directed against tumour associated antigens. The weight of evidence indicates, however, that non-specific responses predominate in this form of regional immunotherapy since tumour suppression is obtained in immunosuppressed syngeneic animals and in congenitally athymic nude mice [13–15]. In contrast, treatment of animals with agents designed to impair phagocytic cell function abrogates tumour suppressive properties of regionally applied BCG for solid, s.c. tumour growths [16–18] and ascitic tumours such as that used in the present study [19]. These agents, e.g., silica and carrageenan, deplete host phagocytic cells, and it was originally proposed that locally activated host macrophages destroyed malignant cells in model systems such as described here. In support of this, peritoneal exudate macrophages from BCG treated animals are cytotoxic for tumour cells at least in *in vitro* assays, although their *in vivo* effects on adoptive transfer are less clear cut [20–22]. It is possible that in the present system i.p. injected BCG produces macrophages cytostatic for malignant cells and that cytotoxicity does not develop for several days. This would account for the failure to detect cell destruction within the observation periods when BCG and tumour cells were given together. More recent observations, however, indicate that these agents designed to deplete host phagocytic cells, may influence other host responses [4] including the generation of normal killer (NK) cells [23], probably via their depletion of macrophages and the precise nature of non-specific host responses destroying malignant cells at the site of regionally applied BCG remains unresolved. Certainly NK cells as well as activated macrophages can be recovered from the peritoneal cavity following local injection of BCG or *C. parvum* [24–27] and, perhaps significant in the present context, NK activity has been reported to peak 3–4 days after a single i.p. injection [24, 25, 27] whereas cytotoxic phagocytic cells do not reach maximal limits for 14–25 days, and experimentally they are most frequently harvested after repeated i.p. injection of BCG [28, 29].

In summary, these studies demonstrate that there is a delay of probably 4–6 days after regional application of BCG before malignant cell destruction is evident. This probably reflects the time needed for the generation of local non-specific host responses. The techniques developed here may be a useful adjunct to *in vitro* assays for determining the time of appearance of effective *in vivo* host cell destruction of malignant cells in animals undergoing immunotherapy.

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REFERENCES

1. R. W. Baldwin and M. V. Pimm, BCG in tumor immunotherapy. *Advanc. Cancer Res.* **28,** 91 (1978).
2. R. W. Baldwin and V. S. Byers, Immunoregulation by bacterial organisms and their role in the immunotherapy of cancer. *Semin. Immunopathol.* **2,** 79 (1979).

3. W. DEN OTTER, H. F. J. DULLENS, H. VAN LOVEREN and E. PELS, Anti-tumour effects of macrophages injected into animals: a review. In *The Macrophage and Cancer.* (Edited by K. James, B. McBride and A. Stuart) p. 119. Edinburgh University (1977).
4. R. KELLER, Mononuclear phagocytes and antitumour resistance: a discussion. In *The Macrophage and Cancer.* (Edited by K. James, B. McBride and A. Stuart) p. 31. Edinburgh University (1977).
5. R. W. BALDWIN and C. R. BARKER, Tumour specific antigenicity of aminoazo-dye induced rat hepatomas. *Int. J. Cancer* **2,** 355 (1967).
6. R. A. ROBINS, Serum antibody response to an ascitic variant of rat hepatoma D23. *Brit. J. Cancer* **32,** 21 (1975).
7. S. L. COMMERFORD, Biological stability of 5-iodo-2′-deoxyuridine labelled with ^{125}-iodine after its incorporation into deoxyribonucleic acid of the mouse. *Nature (Lond.)* **206,** 949 (1965).
8. K. G. HOFER, W. PRENSKY and W. L. HUGHES, Death and metastatic distribution of tumour cells in mice monitored with ^{125}I-iododeoxyuridine. *J. nat. Cancer Inst.* **43,** 763 (1969).
9. D. D. PORTEUS and T. R. MUNRO, The kinetics of the killing of mouse tumour cells *in vivo* by immune responses. *Int. J. Cancer* **10,** 112 (1972).
10. T. E. SADLER and P. ALEXANDER, Trapping and destruction of bloodborne syngeneic leukaemia cells in lung, liver and spleen of normal and leukaemic rats. *Brit. J. Cancer* **33,** 512 (1976).
11. S. ORBACH-ARBOUYS, J. LHERITIER, M. ALLOUCHE and P. POUILLART, Intense tumour-cell destruction by syngeneic mice: role of macrophages, complement activation and tumour-cell factors. *Brit. J. Cancer* **36,** 743 (1977).
12. M. V. PIMM and R. W. BALDWIN, BCG therapy of pleural and peritoneal growth of transplanted rat tumours. *Int. J. Cancer* **15,** 260 (1975).
13. M. MOORE, N. LAWRENCE and N. W. NISBET, Tumour inhibition mediated by BCG in immunosuppressed rats. *Int. J. Cancer* **15,** 897 (1975).
14. M. V. PIMM and R. W. BALDWIN, BCG immunotherapy of rat tumours in athymic nude mice. *Nature (Lond.)* **254,** 77 (1975).
15. M. V. PIMM and R. W. BALDWIN, Influence of whole body irradiation on BCG contact suppression of a rat sarcoma and tumour-specific immunity. *Brit. J. Cancer* **34,** 199 (1976).
16. D. L. CHASSOUX and J.-C. SALOMON, Therapeutic effect of intratumoral injection of BCG and other substances in rats and mice. *Int. J. Cancer* **16,** 515 (1975).
17. D. G. HOPPER, M. V. PIMM and R. W. BALDWIN, Silica abrogation of mycobacterial adjuvant contact suppression of tumour growth in rats and athymic mice. *Cancer Immunol. Immunother.* **1,** 143 (1976).
18. M. MOORE and N. W. NISBET, Abrogation of BCG-contact induced tumour inhibition by silica: implications for the mechanism of action. *Develop. biol. Stand.* **38,** 233 (1978).
19. R. KELLER, Abrogation of antitumour effects of *Corynebacterium parvum* and BCG by anti-macrophage agents. *J. nat. Cancer Inst.* **59,** 1751 (1977).
20. R. KELLER, Promotion of tumor growth *in vivo* by anti-macrophage agents. *J. nat. Cancer Inst.* **57,** 1355 (1976).
21. I. B. PARR, E. WHEELER and P. ALEXANDER, Selective mobilisation of specifically cytotoxic T-lymphocytes at sites of inflammation in relation to BCG-induced resistance to implants of syngeneic sarcoma in mice. *J. nat. Cancer Inst.* **59,** 1659 (1977).
22. L. J. PETERS, W. H. MCBRIDE, K. A. MASON, N. HUNTER, N. BAŠIĆ and L. MILAS, *In vivo* transfer of antitumor activity by peritoneal exudate cells from mice treated with *Corynebacterium parvum*: reduced effect in irradiated recipients. *J. nat. Cancer Inst.* **59,** 881 (1977).
23. J. R. OEHLER and R. B. HERBERMAN, Natural cell-mediated cytotoxicity in rats. III. Effects of immunopharmacologic treatments on natural reactivity and on reactivity augmented by polyinosinic-polycytidylic acid. *Int. J. Cancer* **21,** 221 (1978).
24. J. R. OEHLER, L. R. LINDSAY, M. E. NUNN, H. T. HOLDEN and R. B. HERBERMAN, Natural cell-mediated cytotoxicity in rats. II. *In vivo* augmentation of NK-cell activity. *Int. J. Cancer* **21,** 210 (1978).

25. E. Ojo, O. Haller, A. Kimura and H. Wigzell, An analysis of conditions allowing *Corynebacterium parvum* to cause either augmentation or inhibition of natural killer cell activity against tumor cells in mice. *Int. J. Cancer* **21,** 444 (1978).
26. D. E. Tracey, S. A. Wolfe, J. M. Durdik and C. S. Henney, BCG-induced murine effector cells. I. Cytolytic activity in peritoneal exudates: an early response to BCG. *J. Immunol.* **119,** 1145 (1977).
27. S. E. Wolfe, D. E. Tracey and C. S. Henney, BCG-induced murine effector cells. II. Characterization of natural killer cells in peritoneal exudates. *J. Immunol.* **119,** 1152 (1977).
28. R. N. Germain, R. M. Williams and B. Benacerraf, Specific and non-specific antitumor immunity. II. Macrophage mediated non-specific effector activity induced by BCG and similar agents. *J. nat. Cancer Inst.* **54,** 709 (1975).
29. J. B. Hibbs, L. H. Lambert and J. S. Remington, Possible role of macrophage mediated nonspecific cytotoxicity in tumor resistance. *Nature New Biol.* **235,** 48 (1972).