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Original Paper

Antitumour Immunity of Bacillus Calmette–Guerin and Interferon Alpha in Murine Bladder Cancer

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Intravesical Bacillus Calmette–Guerin (BCG) immunotherapy is currently the optimal choice for aggressive superficial bladder cancer, with a 70% response rate. This study investigated whether the antitumour response elicited by BCG could be improved by the addition of recombinant interferon alpha (IFNα) in the subcutaneous murine MB49 bladder tumour model. The combination of BCG and IFNα had superior and earlier antitumour activity than BCG alone for MB49 cells in culture. A total of 14/15 BCG plus interferon-treated mice and 8/16 BCG-treated mice became tumour free after treatment. BCG or the combination treatment significantly raised the T-helper 1 (Th1) cytokine IFNγ levels compared with levels in all other groups. Whilst BCG therapy alone increased $CD4^+$ and $CD8^+$ populations in spleens, the combination of BCG and IFNα also increased $\alpha\beta^+$ T cells significantly. Our results suggest that the combination of BCG and IFNα may represent a more efficacious therapeutic than BCG alone for superficial bladder cancer.

Key words: Bacillus Calmette–Guerin, interferon alpha, bladder cancer, immunotherapy
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INTRODUCTION

INTRAVESICAL Bacillus Calmette–Guerin (BCG) has been shown to eliminate tumours and prevent recurrences of transitional cell carcinomas (TCC) of the bladder with routine complete response rates of 60–70% [1, 2]. Whilst BCG is the most successful example of immunotherapy to date, the mechanism by which it inhibits tumour growth remains unclear. Furthermore, two major problems exist: a significant proportion of patients do not respond to BCG therapy and side-effects are common [3]. These limit its use in clinical practice.

Other than BCG, interferon alpha-2b (IFNα2b) has also been used as an intravesical agent for superficial bladder cancer, with some success. However, its response rate of 40% has been overshadowed by that of BCG [4, 5]. Nevertheless, a significant proportion of BCG non-responders has been cured with IFNα2b [6]. In contrast to BCG, intravesical IFNα therapy has few and usually mild side-effects [4, 5].

Intravesical instillation of BCG causes a non-specific activation of the local cellular immune response. This response is T-lymphocyte dependent [7–9] and is modulated by T-helper 1 (Th1) and T-helper 2 (Th2)-like cytokines, primarily interleukin-2 (IL-2), interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα), IL-6, IL-8, IL-10 and IL-12 [10]. BCG also causes upregulation of major histocompatibility complex (MHC) class II proteins [11, 12] and intercellular adhesion molecule-1 (ICAM-1) [10]. Unlike BCG, the antitumour effects of IFNα2b are believed to be primarily direct. IFNα can inhibit tumour proliferation and induce differentiation. Interestingly, it enhances the expression of tumour-associated antigens and MHC molecules [13, 14]. It can also expand the activity of natural killer (NK) cells [15].

These studies suggest that BCG and IFNα2b may act through different mechanisms and their combined use could be synergistic. Our previous studies on their direct antiproliferative and cytolytic activity, and the modulatory effect on cytokine production by human bladder carcinoma cell lines [16, 17] raise the possibility that combined use of these intravesical agents may be beneficial for clinical practice. Therefore, we investigated the antitumour effects of BCG or/and IFNα on MB49, a murine bladder cancer cell line, *in vitro* and in the subcutaneous murine bladder carcinoma

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model *in vivo*. We also examined and compared the phenotype of splenocytes from mice receiving the different treatments as well as their cytokine expression profile in the spleen.

MATERIALS AND METHODS

Animals and tumour cells

C57BL/6J female mice (approximately 6 weeks old) were obtained from the Laboratory Animals Centre of the National University of Singapore and maintained at the Animal Holding Unit of the university. Murine bladder cancer cell line MB49 was kindly provided by Timothy L. Ratliff (University of Iowa, U.S.A.). The cells were routinely grown in RPMI-1640 supplemented with 5% fetal bovine serum (FBS), 5 mM sodium pyruvate, 4 mM glutamine and a 2% solution of antibiotics (penicillin G 5000 U/ml and streptomycin sulphate 5000 μg/ml) (all from Sigma, St Louis, Missouri, U.S.A.), incubated at 37°C in the presence of 5% CO_2.

BCG and IFNα

BCG, living organisms of an attenuated strain of *Mycobacterium bovis* (Connaught strain, 13.78×10^8 colony-forming units/vial, containing 81 mg freeze-dried BCG powder), was obtained from Connaught (Willowdale, Canada). Murine recombinant IFNα was purchased from Gibco BRL (Gaithersburg, Maryland, U.S.A.).

Cytotoxicity assay

The assay detected the cytolytic activity of BCG and/or IFNα by the release of radioactivity from the target cells as described previously [16]. Briefly, MB49 cells were labelled while in the exponential growth phase with 2 μCi of [^{14}C]-thymidine for 24 h at 37°C. The labelled cells were plated in 96-well flat-bottomed tissue culture plates (1×10^4 cells/well). They were incubated with or without BCG (500 μg/ml) or/and IFNα (1000 U/ml) at 37°C for 72 h. The supernatants were harvested and the radioactivity measured with a liquid scintillation counter. Spontaneous release was measured in target cells cultured in medium alone and maximum release was measured from cells which had been lysed in 1% sodium dodecyl sulphate (SDS). This experiment was repeated three times in quadruplicates.

The percentage specific release was calculated as follows:

$$\text{Specific release (\%)} = \frac{\text{c.p.m. test release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. maximal release} - \text{c.p.m. spontaneous release}} \times 100$$

Tumour transplant and treatment schedule

A single cell suspension containing 5×10^5 MB49 cells in 0.1 ml phosphate-buffered saline (PBS, pH 7.2) was injected subcutaneously into the right thigh. After 24 h, the mice were randomised to different treatment groups, each to be treated intralesionally with PBS, IFNα (1000 U), BCG (500 μg = 8.5×10^6 colony-forming units) or combined BCG (500 μg) and IFNα (1000 U) in 0.1 ml injections. In another experiment, mice were injected with subcutaneous tumours for 1 week before treatment. Only those with a uniform tumour size of approximately 0.12 cm^3 were selected and randomised into four groups for the intralesional treatments. Treatment was given once a week for 5 weeks. Tumour size was measured with callipers and a ruler at various time points after injection up to day 27. The calculation for tumour volume was performed by the formula: length×width×height.

For rechallenge experiments, mice were rested for 4 weeks after the last intralesional treatment before they were injected subcutaneously with 5×10^5 MB49 cells on their left thighs. Controls included age-matched naïve mice, as well as non-tumour-bearing mice that had similarly received BCG or BCG and IFNα treatments for 5 weeks and rested for 4 weeks.

Spleen cell harvesting and RNA extraction

24 h after the last treatment, the mice were sacrificed and the spleens aseptically removed. Single cell suspensions from the spleen were harvested. Total RNA was extracted from 1×10^7 spleen cells using 1 ml Trizol reagent (Gibco BRL) according to the manufacturer's instructions.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Five micrograms of total RNA was reverse transcribed with 0.2 μM oligo-dT [16], 1 μM deoxynucleotide triphosphates (dNTP), 50 U M-MLV-RT (Promega, Madison, Wisconsin, U.S.A.) and 30 U RNasin (Angewandte Gentechnolgie Systeme, Heidelberg, Germany). The reaction was performed at 37°C for 1 h.

PCR was carried out in an automatic DNA thermal cycler (Hybaid Omni-Gene, Middlesex, U.K.). For the amplification of cDNA, 0.2 mM dNTP, 0.6 U *Taq* polymerase (Finnzymes OY, Espoo, Finland) and 0.4 μM sense and antisense primers were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control and a curve of cDNA concentration was plotted against the amplified GAPDH values. A concentration within the linear portion of the graph was used for all subsequent reactions and all other cytokine values were normalised to the corresponding GAPDH values for comparisons between samples. Relative mRNA levels were determined using the BioProfile software of a photoimager (Vilber Lourmat, Marne La Vallee, France).

The sequences of the primers for the PCR were as follows: GAPDH: antisense CACCCTGTTGCTGTAG; sense GTCGGTGTGAACGGAT; IL-6: antisense CTCCTTCTGTGACTCC; sense GCCTTCCCTACTTCAC; IL-10: antisense CTCTTCACCTGCTCCA; sense ACTGCTATGCTGCCTGC; GM-CSF: antisense GAAATCCGCATAGGTGG; sense GGCCATCAAAGAAGC; IFNγ: antisense CCGCTTCCTGAGGCTG; sense ACTGCCACGGCACAGTC; TNFα: antisense CAGATTGACCTCAGCGC; sense GAACTTCGGGGTGATCG; IL-2: antisense GAGTCAAATCCAGAACATGCCGCAG; sense TGATGGACCTACAGGAGCTCCTGAG; IL-4: antisense CATGGTGGCTCAGTACTA; sense GTCTCTCGTCACTGAGGGG.

Flow cytometry

Single cell suspensions were labelled with anti-CD4 and anti-CD8 antibodies that were conjugated to phycoerythrin (PE; Pharmingen, San Diego, California, U.S.A.). αβ T cells were labelled with antibodies conjugated to biotin (Pharmingen), followed by streptavidin conjugated to fluorescein isothiocynate (FITC; Boehringer Mannheim, Indianapolis, Indiana, U.S.A.). Isotype control antibodies were also included (Pharmingen). The labelled cells were analysed on a dual laser flow cytometer (Coulter Epics Elite, Miami, Florida, U.S.A.).

Statistical methods

The data were analysed using the Mann–Whitney test for the *in vitro* cytotoxicity and cytokine mRNA expressions. The Mann–Whitney test was also used for comparing tumour growth among the different treatment groups at individual time points and the two-way ANOVA test was used to compare tumour growth between treatment groups over time. P values of < 0.05 are deemed significant. All statistical analyses were performed with the GraphPad Prism 2.0 software (California, U.S.A.).

RESULTS

Cytolytic effect of BCG or/and IFNα on MB49 cells

The effect of BCG and IFNα on MB49 cells was examined by treating them with 500 μg/ml BCG and 1000 U/ml IFNα for 3 days, after initial experiments showed these to be the optimal doses (data not shown). BCG and IFNα alone exerted a low but significantly different cytolytic effect on MB49 cells (6.2% and 2.5%, respectively, $P < 0.0001$). A combination of BCG and IFNα had an additive cytolytic effect of 9% on MB49 cells and this was significantly higher than BCG treatment alone ($P = 0.0043$).

Intralesional therapy on tumour growth

The effects of BCG and IFNα, either alone or in combination, on locally growing MB49 bladder carcinoma are presented in Figure 1(a). Significant tumour growth inhibition was observed in the BCG-treated group compared with the PBS control group at day 25 ($P = 0.002$) and day 27 ($P = 0.005$). Combination treatment with BCG and IFNα resulted in a much earlier inhibition at day 14 ($P = 0.018$), day 18 ($P = 0.003$), day 25 ($P = 0.003$) and day 27 ($P = 0.003$) compared with the PBS control group, achieving a much more pronounced inhibitory effect on tumour growth than BCG alone. Surprisingly, treatment with IFNα showed enhanced tumour growth when compared with the PBS control group. Although there was no significant increase in tumour size between the IFNα-treated group and the PBS control group at any single time point examined, except at day 21 ($P = 0.018$), the overall increase in tumour growth of the IFNα-treated group compared with the control group over time was significant, as measured by the two-way ANOVA test ($P = 0.0004$). Similarly, both the BCG ($P = 0.023$) and the combination ($P < 0.0001$) treatment showed a significant inhibition of tumour growth over time compared with the PBS controls. In fact, the overall inhibition of growth by the combination treatment was significantly higher than that effected by BCG alone ($P < 0.0001$). When the numbers in two separate experiments were combined, 14/15 combination-treated mice had complete tumour regression compared with 8/16 BCG-treated mice (Chi square test, $P < 0.001$).

To control for the variability in the number of tumour cells injected, the experiment was repeated by selecting animals with an initial uniform tumour size and randomly dividing these animals into the four treatment groups as described in Materials and Methods. The tumour growth pattern was similar to the previous experiment (Figure 1b). Both the BCG ($P = 0.012$) and the combination ($P < 0.0001$) treatments showed a significant reduction in tumour size compared with the PBS controls over time. The combination treatment was also significantly better than the BCG treatment over time ($P = 0.019$). However, none of the mice had complete tumour regression.

Cytokine expression in splenocytes

We compared cytokine expression in the splenocytes of tumour-bearing mice with those from normal mice to see if tumour growth affected endogenous cytokine production. Both tumour-bearing mice and control (no tumour) mice expressed mRNAs for IL-2, IL-6, IL-10, TNFα, GM-CSF and IFNγ. Except for IFNγ, there was no significant difference in the expression of the mRNAs of these cytokines among all the groups. After treatment with BCG, expression of IFNγ mRNA in these mice was significantly higher than the PBS-treated tumour-bearing group ($P = 0.001$) and the IFNα-treated group ($P = 0.009$) (Figure 2). Tumour-bearing mice treated with BCG and IFNα also expressed more IFNγ mRNA compared with the PBS-treated group ($P = 0.001$) and the IFNα-treated group ($P = 0.09$). However, mice treated with BCG alone expressed slightly more IFNγ mRNA than those treated with BCG and IFNα. BCG treatment could also induce a higher IFNγ mRNA expression in normal non-tumour-bearing mice, but these levels were not as high as those induced in the tumour-bearing mice (Figure 2a). The combination treatment failed to induce higher IFNγ mRNA levels compared with the other treatment groups in the non-tumour-bearing mice.

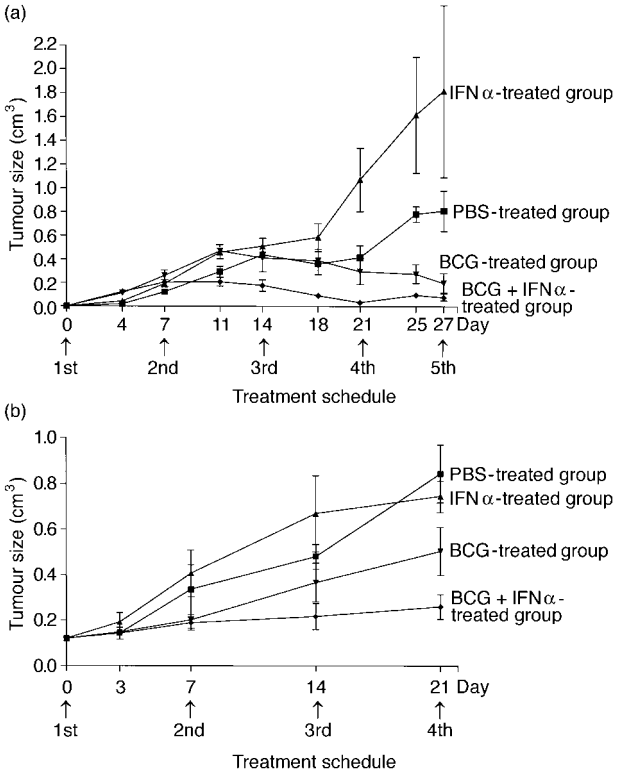

Figure 1. Effect of the various treatments on tumour progression. (a) Mice were treated 24 h after tumour cell injection. Arrows on the *x*-axis represent when the first to the fifth intralesional treatments were given. Data represent the ± standard error of the mean (SEM) of seven, six, five and five mice in the phosphate-buffered saline (PBS)-, Bacillus Calmette–Guerin (BCG)-, interferon alpha (IFN-α)- and the combination-treated groups, respectively. (b) Mice were injected with tumour cells 1 week before treatment commenced. Data represent the ± SEM of seven mice in the PBS-, IFNα- and combination-treated groups and eight mice in the BCG-treated group.

Phenotypic characterisations of splenocytes

Flow cytometric analysis of splenocytes from mice with phenotype-specific monoclonal antibodies showed that the $CD4^+$, $CD8^+$ and $\alpha\beta^+$ populations in tumour-bearing, PBS-treated mice were decreased compared with the non-tumour-bearing controls (Table 1). After treatment with

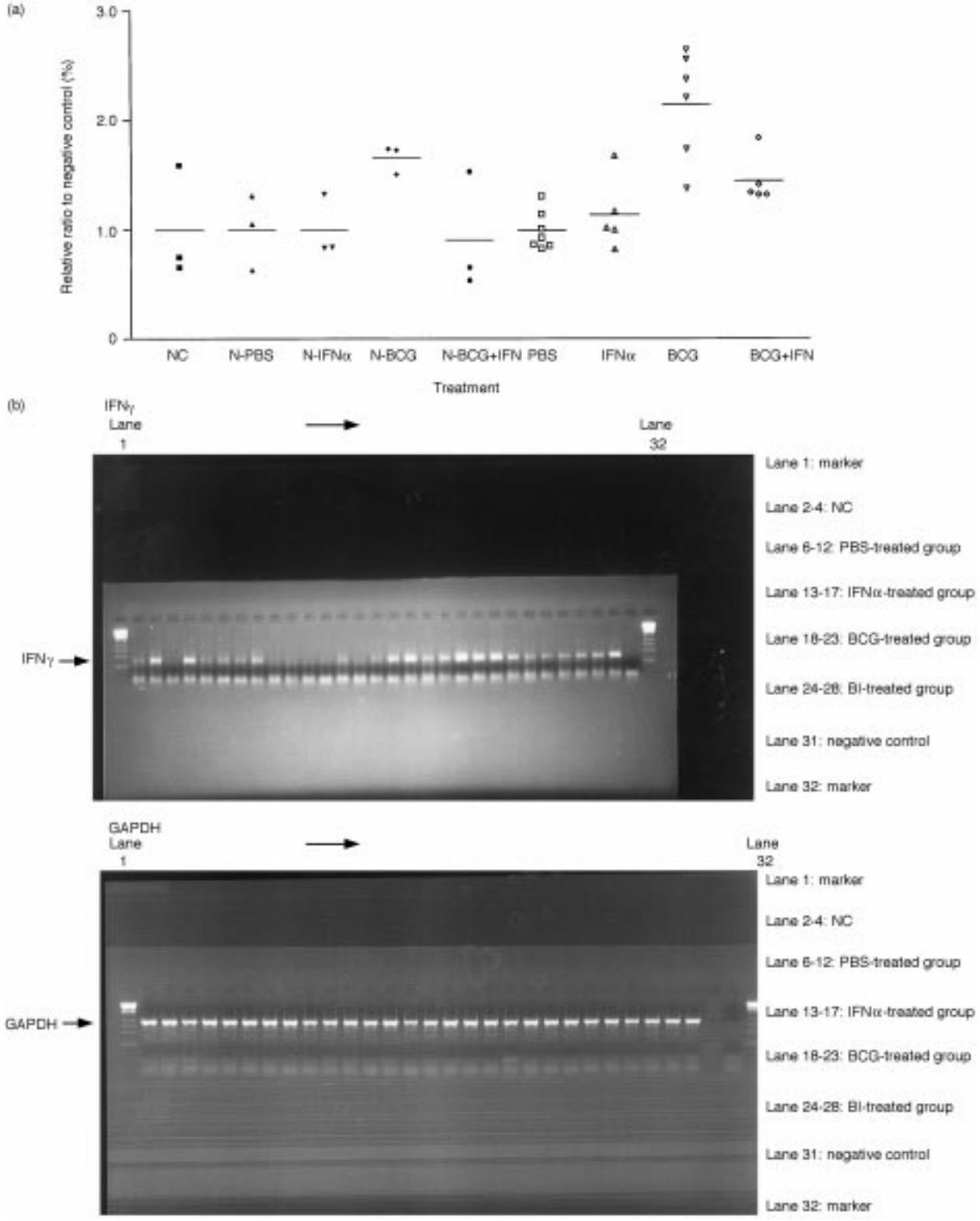

Figure 2. (a) The relative mRNA levels for interferon gamma (IFNγ) in the splenocytes of mice. Each symbol represents one mouse belonging to the non-tumour-bearing control (NC), phosphate-buffered saline (N-PBS)-, Bacillus Calmette–Guerin (N-BCG)-, interferon alpha (N-IFNα)-, BCG plus IFNα (N-BCG+IFNα)-treated groups, and tumour-bearing PBS (PBS)-, IFNα (IFNα)-, BCG (BCG)- and BCG plus IFNα BCG-IFNα-treated groups. The mean mRNA level for the NC mice was arbitrarily set at 1. (b) A representative set of gels showed the IFNγ mRNA level in the splenocytes of the different groups of mice.

BCG or the combination of BCG and IFNα, the $CD4^+$ and $CD8^+$ populations returned to slightly higher levels than the non-tumour-bearing control group and these levels were much higher than the PBS-treated tumour-bearing group. The combination of BCG and IFNα also significantly increased the $\alpha\beta^+$ percentage ($P = 0.036$).

In order to ascertain if these changes effected by the various treatments were particular to tumour-bearing mice, we examined the effects various treatments had on normal non-tumour-bearing mice. The $CD4^+$ population was higher in all the treated groups compared with the PBS-treated controls. Therefore, tumour-bearing mice had suppressed T cell subset percentages. BCG and the combination treatment in all mice raised the percentage of $CD4^+$ T cells.

Secondary challenge of BCG- and combination-treated mice with tumour

In two separate experiments, 5/6 and 9/9, of the combination-treated group remained tumour free 4 weeks after the termination of intralesional treatment while only 5/8 and 3/8 from the BCG-treated group were tumour free. To see if mice that had eliminated the original tumour could be protected from a subsequent tumour challenge, the nine combination-treated mice and three BCG-treated mice that were tumour free in the second experiment were given a secondary challenge of tumour cells. Up to 3 weeks after the rechallenge, none of the above-mentioned mice had any tumour (Figure 3). In contrast, the age-matched naïve control mice, including non-tumour-bearing mice previously treated with BCG or BCG and IFNα, had tumours. However, tumour size in all control groups did not consistently increase by the second week. By the third week, the naïve control mice had completely eliminated their tumours, whilst the non-tumour-bearing mice previously treated with BCG or BCG and IFNα had smaller tumours (data not shown).

Table 1. *Phenotypic characterisations of T cell subsets in the spleen*

Treatment	CD4%	CD8%	αβ%
PBS	12.7 ± 1.3	5.8 ± 0.3	24.0 ± 0.2
BCG	17.5 ± 0.2	10.5 ± 1.1	24.7 ± 8.7
IFNα	14.5 ± 1.2	7.2 ± 0.3	39.9 ± 5.4
BCG + IFNα	17.8 ± 0.6	8.9 ± 0.7	45.7 ± 7.0
NC	16.1 ± 1.0	9.3 ± 0.3	35.0 ± 1.3
N-PBS	16.7 ± 0.7	11.2 ± 0.1	30.7 ± 0.4
N-BCG	20.3 ± 0.8	9.5 ± 0.3	34.8 ± 3.5
N-IFNα	20.7 ± 0.4	9.7 ± 0.5	32.7 ± 1.6
N-BCG + IFNα	21.3 ± 1.9	9.6 ± 3.1	36.0 ± 3.3

PBS, phosphate-buffered saline; BCG, Bacillus Calmette–Guerin; IFNα, interferon alpha; NC, non-tumour-bearing control. The percentages of T cell subsets among total splenocytes at day 27 from the PBS-, BCG-, IFNα and BCG + IFNα-treated mice, as well as non-tumour-bearing mice (NC) similarly treated (N-PBS, N-BCG, N-IFNα, N-BCG + IFNα), are expressed as ± standard error of the mean (SEM) where $n = 3$ for each group.

DISCUSSION

Several clinical investigations (reviewed in [18]) have raised the possibility of treating superficial bladder carcinoma with a combination of BCG and IFNα that may result in milder side-effects and a higher response rate. Previous studies [16, 17] have demonstrated that both BCG alone and this combination have additive direct cytolytic and anti-proliferative effects on human bladder cancer cell lines. Thus, we compared the antitumour effects of this combination with BCG or IFNα alone, and examined the various immune parameters for correlation to antitumour immunity in the MB49 murine bladder cancer model.

The direct cytolytic effect of BCG or/and IFNα on MB49 *in vitro* was low and the combined use had small (approximately 4% more), but significant, additive effects. *In vivo*,

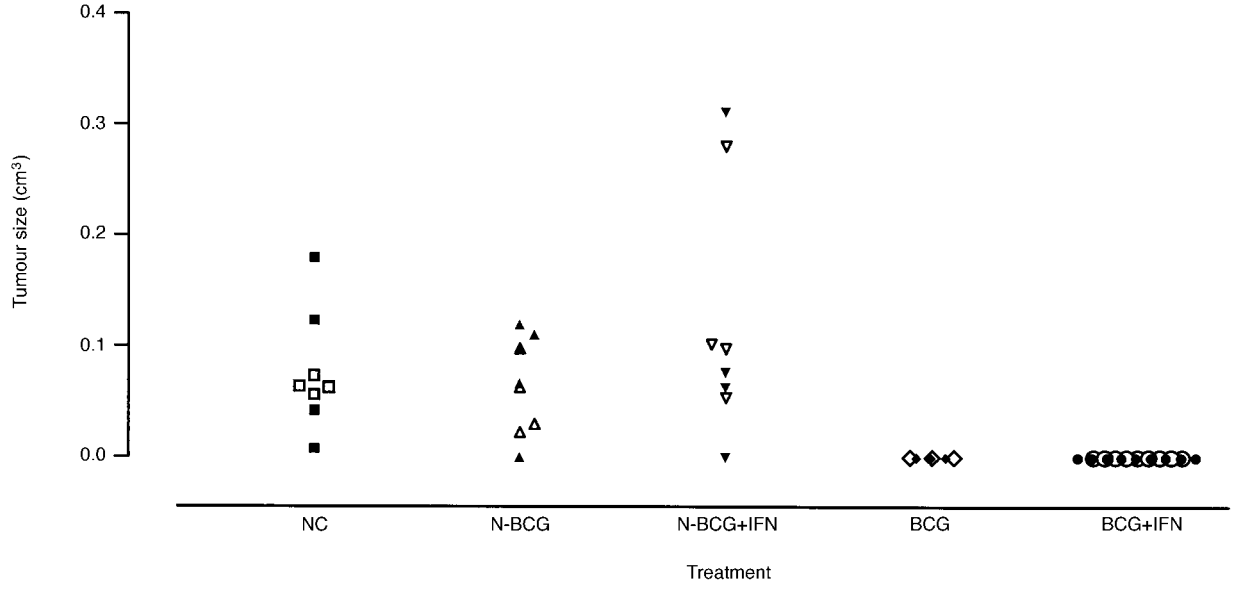

Figure 3. The tumour size 1 week (open symbols) and 2 weeks (solid symbols) after secondary challenge of MB49 cells. The groups included age-matched naïve mice (NC, $n = 4$), non-tumour-bearing Bacillus Calmette–Guerin (BCG)-treated mice (N-BCG, $n = 5$), non-tumour-bearing BCG and interferon alpha (IFNα)-treated mice (N-BCG + IFNα, $n = 4$), BCG-treated mice (BCG, $n = 3$) and combination-treated mice (BCG + IFNα, $n = 9$).

BCG exhibited antitumour effects. The combination of BCG and IFNα showed much earlier and superior tumour reduction than BCG alone in this study. These results are in contrast with the findings of another study [19] where the combined use of BCG and IFNα showed no significant reduction in tumour volume compared with the single agent alone and BCG did not reduce tumour volume significantly compared with controls. These differences may be due to their use of a different strain of BCG and a tumour model involving C3H/HeN mice and MBT-2 tumour cells, which are more resistant to BCG than MB49 cells [20]. The enhanced tumour growth seen in IFNα-treated mice in our study could be due to the newly reported immunosuppressive effects of IFNα [21–23]. IFNα can downmodulate MHC class II and ICAM-1 expression on antigen-presenting cells [21, 22]. It has also been shown to interfere with the synthesis of various pro-inflammatory cytokines such as IL-1 and IL-8 [21].

It is believed that BCG immunotherapy for bladder cancer involves a local non-specific immunological reaction requiring the presence of CD4 and CD8 T cell subsets [9]. Our results showed a decrease in splenic $CD4^+$ and $CD8^+$ populations in tumour-bearing mice. The significant increase in $CD4^+$ and $CD8^+$ populations in the spleen after local BCG treatment suggests that BCG had a systemic effect. This is not surprising since a transient peripheral immune activation has also been reported in bladder cancer patients treated with intravesical BCG [24]. Interestingly, the combination treatment resulted in an increase not only in the $CD4^+$, $CD8^+$ T cells but also in the $\alpha\beta^+$ populations. The increase in CD4 and CD8 subsets in the spleen may be associated with tumour reduction.

Although there was an increase in CD8 T cells and IFNγ expression in mice where tumours regressed, we could not detect any MB49-specific immunity in the bulk splenocytes, although they exhibited a low non-specific cytotoxicity against YAC cells, which are NK sensitive targets (data not shown). This is consistent with the results of Ratliff and colleagues [9] who demonstrated the lack of cytolytic T cell activity in the spleen as well as the draining lymph nodes in the intravesical BCG MB49 murine model. However, it is possible that the cytotoxic T cell precursor frequency was too low to be detected in unseparated cultures despite *in vitro* expansion in our experiment.

Cytokines have been implicated to play an important role in the cellular immune response responsible for tumour ablation in BCG immunotherapy [25]. In the murine MB49 model, McAveney and associates [26] found that BCG treatment resulted in IFNγ and TNFα, but not IL-4 mRNA expression in the local bladder wall after intravesical BCG treatment. These findings suggest a Th1 response to BCG. The Th1 pattern is typically characterised by the production of IFNγ by both $CD4^+$ and $CD8^+$ T cells [27]. It has been shown that live BCG induced an early production of Th1 cytokines followed by Th2 cytokines later [28].

Our data on cytokine expression demonstrate that the growth of the murine TCC MB49 in syngeneic mice induces a complex cytokine response in spleen cells and this is modulated by BCG and IFNα treatments. The results obtained upon BCG and combination treatment of MB49 tumours are consistent with the strong induction of a Th1 cytokine IFNγ, which correlates to inhibition of tumour growth.

Survivors treated with BCG or BCG and IFNα were protected against recurrent tumour incidence upon rechallenge with MB49 tumour. Surprisingly, the tumours evident in naïve control mice 1 week after the challenge completely regressed by the third week. Through two repeated experiments, we consistently found that implanted tumours grew slowly and regressed spontaneously within 2–3 weeks in older (16 weeks) mice compared with the 6-week-old mice. The more mature immune system of the older mice most probably restrained the tumour. This raises doubts about the suitability of using this model for the study of long-term protection in bladder cancer. However, the protection we observed with the BCG- or combination-treated mice is still valid and could be due to a combination of age-related effects and the priming and expansion of immune cells capable of eradicating the tumours. We are currently examining the effects of age on tumour incidence as well as the possible presence of a tumour-specific protective response in long-term rechallenge experiments to answer this question.

The results presented in this study demonstrate for the first time the superior effects on murine bladder tumour reduction by a combination treatment of BCG and IFNα intralesionally. More animals were completely cured of tumours when treated with BCG and IFNα than with BCG alone. Tumour growth inhibition correlated with an increase in CD4 and CD8 T cells in the spleen and increased IFNγ production. It is possible that the combination of BCG and IFNα exerted a higher antiproliferative and cytolytic effect on the tumour cells than all other groups, thus limiting the size of the tumour to facilitate its effective destruction by immune cells. This is evident from the kinetics of earlier tumour reduction in the combination group. Thus, the combination of BCG and IFNα intravesical therapy could be superior to BCG alone in the treatment of superficial bladder cancer in clinical practice. This possibility is currently being investigated in a clinical trial at our centre.

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