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Antiproliferative effects of bacillus Calmette-Guérin and interferon α2b on human bladder cancer cells in vitro

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Abstract Direct inhibitory effects of bacillus Calmette-Guérin (BCG) and interferon α2b (IFNα2b) on six human bladder carcinoma cell lines, UCRU-BL-13, UCRU-BL-17, UCRU-BL-28, 5637, T24 and J82, were studied using an in vitro proliferation assay. Effects on proliferation following exposure to BCG or IFNα2b were analysed by [^{3}H]thymidine incorporation over 7 days. BCG had an antiproliferative effect on all bladder lines, while sensitivity to IFNα2b varied greatly, being as remarkably low as 1 U/ml for some lines. The antiproliferative effect was greatest when cells were exposed continuously to either agent, but was still evident with a limited exposure. When clinical concentrations were simulated in vitro, BCG+IFNα2b was more effective than BCG alone and as effective as a double BCG concentration. We conclude that, in addition to their immunomodulatory effects, BCG and IFNα2b directly inhibit the proliferation of human bladder cancer cells, and often at extremely low concentrations.

Key words Bladder neoplasms · Carcinoma transitional cell · BCG vaccine · Interferon · Immunotherapy

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Introduction

Bacillus Calmette-Guérin (BCG) immunotherapy has been successfully used for the treatment and prophylaxis of transitional cell carcinoma (TCC) of the bladder since 1976 [1]. The mechanism of action is not fully understood but seems to involve both direct effects and indirect immune effects. Despite the serious side-effects that occur in 5% of patients, it is an established agent for bladder cancer [2].

Since an intact thymus-dependent immune response was shown to be necessary for BCG-mediated antitumour activity [3], much in vitro research on BCG has focussed on its effect on immune cells [4]. Although it has been reported that BCG is not cytotoxic toward TCC cell lines [4], internalised and degraded BCG has been identified in urothelial cells both in vitro and in vivo [5]. When BCG is infused directly into the bladder cavity, attachment of viable BCG to tumour cells is an essential first step in the BCG-mediated antitumour process [6]. In T24 cells, attachment and ingestion of BCG are partly mediated by the α5β1 integrin receptor and are enhanced by fibronectin [7]. BCG is internalised and processed by tumour cells and BCG antigens are then presented to T lymphocytes [8].

The use of intravesical interferon α2b (IFNα2b) for the treatment of low-grade superficial bladder cancer was first reported in 1988 [9]. IFNα2b is an effective treatment for some patients and is associated with minimal local and systemic toxicity. IFNα2b has antiproliferative activity against human bladder carcinoma cells in vitro [10] and has antiproliferative, surface-membrane, antiviral and immunomodulatory activities in vivo [11]. When IFNs bind to their receptors various signal-transduction pathways are activated, which then activate different sets of genes [12, 13]. The action of IFNα2b in cell growth is thought to be based on IFN-induced alterations in the level of gene expression. However, variant cell lines exist in which IFN activate only some of the genes they activate in the wild-type line. There are more than 30 IFN-inducible proteins that have levels that increase to different degrees after IFN

treatment. The functions of many IFN-induced proteins are unknown, and many relevant genes, and the proteins they encode, are not yet identified.

As BCG is more effective than most chemotherapeutic agents in the prophylaxis and treatment of superficial bladder tumours [2], it may be beneficial to combine a reduced dose of BCG with an antiproliferative agent such as IFNα2b, which has minimal side-effects [11]. Our previous studies showed that BCG has a direct effect on bladder tumour cells in the absence of any immune cells [14]. This study suggests that this may be due to direct antiproliferative activities of BCG and IFNα2b.

Materials and methods

Target cells

The UCRU-BL-13 [15] and UCRU-BL-17 [16] cell lines were established from xenografted human primary bladder carcinomas and UCRU-BL-28 [17] was established from a relapsed, cisplatin-resistant human TCC. The 5637, T24 and J82 cell lines were obtained from the American Type Culture Collection (ATCC, Bethesda, Md., USA). Normal growth medium was RPMI-1640 (Gibco Laboratories, N.Y., USA) with 10% fetal bovine serum (FBS) (P. A. Biologicals Co, Sydney, Australia), 25 mM HEPES [Commonwealth Serum Laboratories (CSL) Victoria, Australia] and 4 mM glutamine (CSL). All cultures tested negative for *Mycoplasma* (Gen-probe Inc., Calif., USA).

BCG and cytokines

BCG, living organisms of an attenuated strain of *Mycobacterium tuberculosis* (Pasteur strain, $7 \times 10^6 - 15 \times 10^6$ colony-forming units/mg), was obtained from CSL. Human recombinant IFNα2b was obtained from Schering Corporation, N.J., USA.

Proliferation assay

The proliferation assay was adapted from that described by Hawkyard et al. [18]. The cells were plated at $(0.2-1) \times 10^4$ cells/ml in 96-well flat-bottomed tissue-culture plates (Corning, N. Y., USA) for 48 h at 37 °C. The medium was aspirated and replaced with medium containing BCG (0–2260 mg/ml) and/or IFNα2b ($0-1 \times 10^7$ U/ml). The simulated clinical concentrations of BCG and BCG/IFNα2b were those calculated to be equivalent to intravesical therapeutic doses of 60 mg and 120 mg BCG, or 60 mg BCG in combination with 10×10^6, 30×10^6, 60×10^6 or 100×10^6 international units (IU) of IFNα2b. In some experiments BCG and IFNα2b were added to selected wells for 2, 4 or 8 h only, then replaced with normal growth medium to compare the proliferation with that of cells continuously exposed for 7 days. Each treatment was added to quadruplicate wells for the six cell lines. Fresh BCG- or IFNα2b-supplemented medium was added to the plates every 2–3 days. Replicate wells were pulsed with 0.5 μCi/well [^{3}H]thymidine (specific activity 5.0 Ci/mmol, Amersham, Ill., USA) for 18 h then harvested, each day for 7 consecutive days. The plates were then washed twice with phosphate-buffered saline (PBS) and cells detached with trypsin/ethylenediaminetetraacetic acid [trypsin/EDTA; 0.5% trypsin, 5.3 mM Na_4EDTA (10 ×); MultiCel, Cytosystems, Australia]. The cells were harvested with an Inotech automated cell harvester and counted in Ready Safe scintillation fluid (Beckman Instruments Inc., Calif., USA) on a Tri-Carb 1500 liquid scintillation analyser (Packard, Groningen, The Netherlands).

Percentage inhibition was calculated as follows:

$$\text{Inhibition } (\%) = [1 - \left(\frac{^3\text{H test}}{^3\text{H control}}\right)] \times 100$$

where ^{3}H radioactivity was measured as dpm.

Cell counts

To determine their effect on cell number, BCG or IFNα2b was added to cells that had been plated for 24 h. Fresh BCG- or IFNα2b-supplemented medium was added on day 4. The cells were counted on days 3, 5 and 7. Cells were washed twice with PBS, detached with trypsin/EDTA and counted by 0.04% trypan blue (Flow Laboratories, Irvine, Scotland) exclusion using a Neubauer haemocytometer.

Statistical methods

The results were expressed as the mean ± SD and the data were analysed using the Mann-Whitney *U*-test. Each figure shows a representative experiment with four replicate wells per treatment/mean. All experiments were repeated at least once for each of the six cell lines,

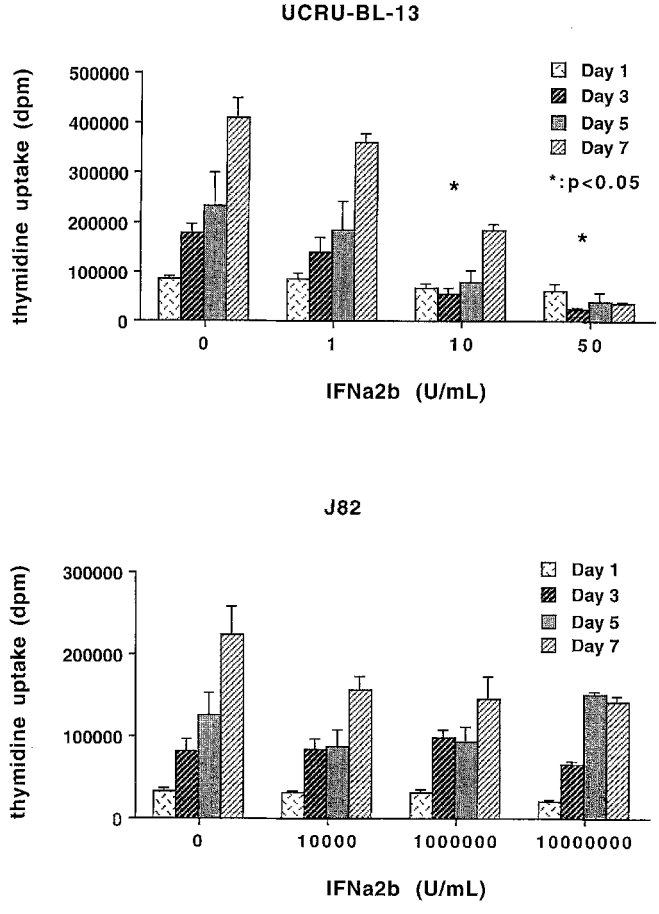


Fig. 1 Effect of a 7-day continuous exposure to interferon α2b (*IFNα2b*) on proliferation of UCRU-BL-13 and J82 cell lines. This figure shows the most sensitive and most resistant cell lines to IFNα2b of the six cell lines tested. The log-transformed correlation coefficients for increasing IFNα2b concentration with UCRU-BL-13 cells were statistically significant ($r^2 = 0.75$, $P < 0.05$ on day 7). The results show a representative experiment with four replicate wells per mean ± SD. * Significant inhibition ($P < 0.05$) of proliferation over 7 days for IFNα2b-treated cells versus untreated cells

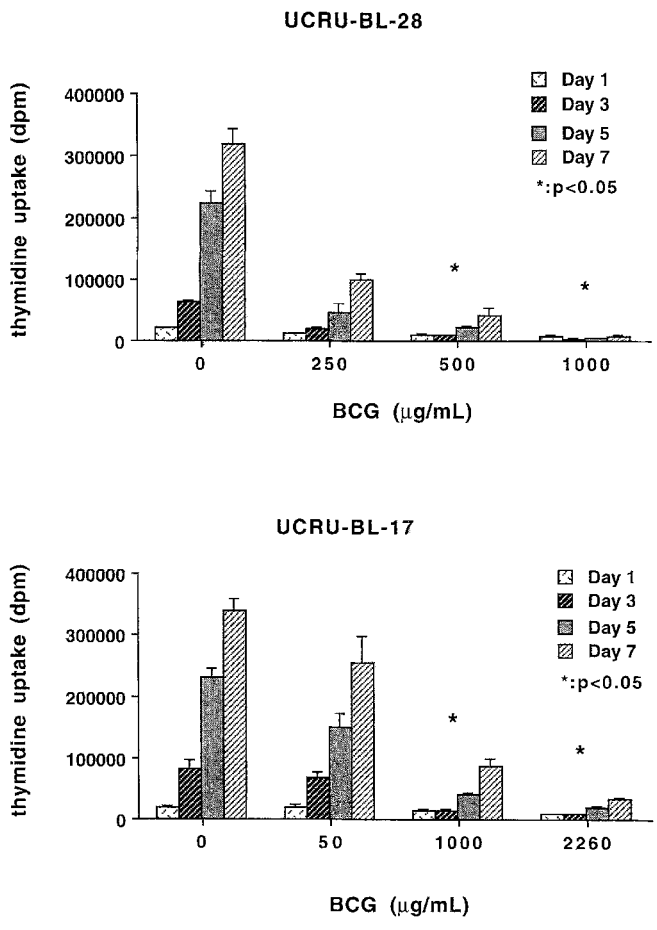


Fig. 2 Effect of a 7-day continuous exposure to bacillus Calmette-Guérin (*BCG*) on the proliferation of UCRU-BL-28 and UCRU-BL-17 cells. This figure shows the most sensitive and most resistant cell lines to BCG of the six cell lines tested. The correlation coefficients for increasing BCG concentration were statistically significant for both lines at day 7 (for UCRU-BL-28 $r^2 = 0.598$, $P = 0.024$; for UCRU-BL-17 $r^2 = 0.691$, $P = 0.011$). The results show a representative experiment with four replicate wells per mean ± SD. * Significant inhibition ($P < 0.05$) of proliferation over 7 days for BCG-treated cells versus untreated cells

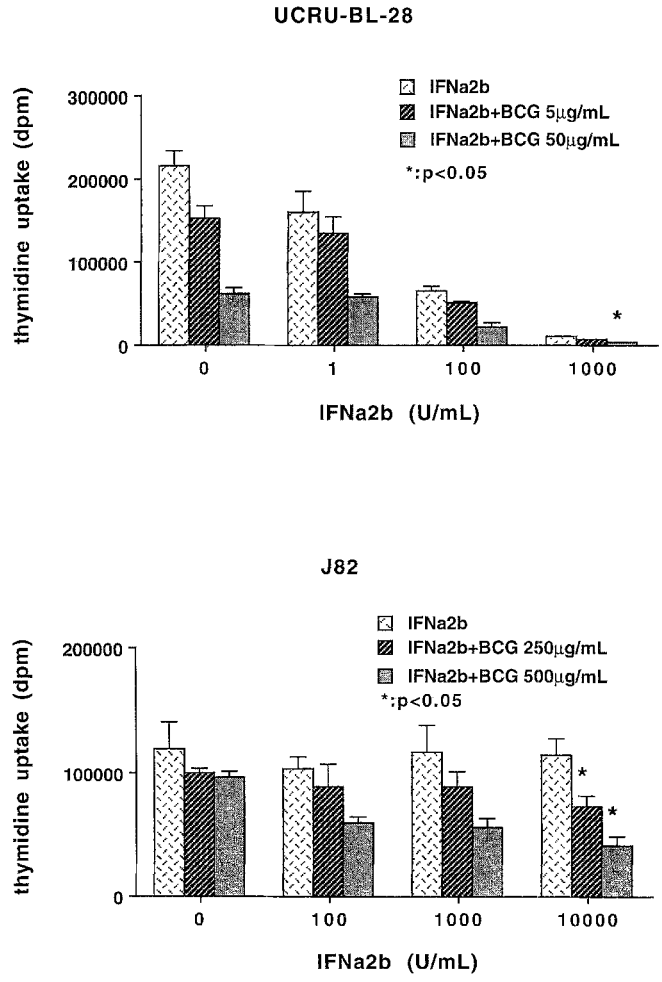


Fig. 3 Effect of a 7-day continuous exposure to IFNα2b ± BCG on UCRU-BL-28 and J82 cells. This figure compares BCG-sensitive, IFNα2b-sensitive and BCG-sensitive, IFNα2b-resistant cell lines + IFNα2b ± BCG out of six cell lines tested. The log-transformed correlation coefficients were significant ($P < 0.05$) for the UCRU-BL-28 cell line with IFNα2b alone ($r^2 = 0.941$) and in combination with BCG 5 μg/ml ($r^2 = 0.96$) and BCG 50 μg/ml ($r^2 = 0.966$). The results show a representative experiment with four replicate wells per mean ± SD. * Significant inhibition ($P < 0.05$) of proliferation over 7 days of cells grown in the presence of BCG + IFNα2b compared with IFNα2b alone

except those involving high-dose IFNα2b, which were only done for the IFNα2b-resistant 5637, T24 and J82 lines. Regression analyses were performed for data that concerned a dose/response effect. For logarithmic increases in BCG or IFNα2b dose, the raw data were log-transformed before regression analyses were performed.

Results

Effect of IFNα2b

The influence of IFNα2b on the proliferation of six tumour cell lines was investigated. UCRU-BL-13 cells were extremely sensitive to IFNα2b, being 12% inhibited by as little as 1 U/ml IFNα2b, and significantly inhibited ($P < 0.05$) by 10 U/ml (55% inhibited) and 50 U/ml (91% inhibited) on day 7 (Fig. 1). The other UCRU lines were very sensitive to IFNα2b, while the 5637 and T24 lines were more resistant (data not shown). The J82 line demonstrated extreme resistance to high-dose IFNα2b (inhibition on day 7 was 31% for 1×10^4 U/ml, 35% for 1×10^6 U/ml and 37% for 1×10^7 U/ml) (Fig. 1).

Effect of BCG

The influence of BCG on the proliferation of six tumour cell lines was investigated. The antiproliferative responses to BCG occurred over a much narrower dose range than the responses to IFNα2b (Fig. 2). When BCG-treated UCRU-BL-28 cells were compared with controls over 7 days, proliferation decreased with increasing BCG dosage and was reflected by decreasing P values (250 μg/ml, $P = 0.087$; 500 μg/ml, $P = 0.028$; 1000 μg/ml, $P = 0.001$). Although

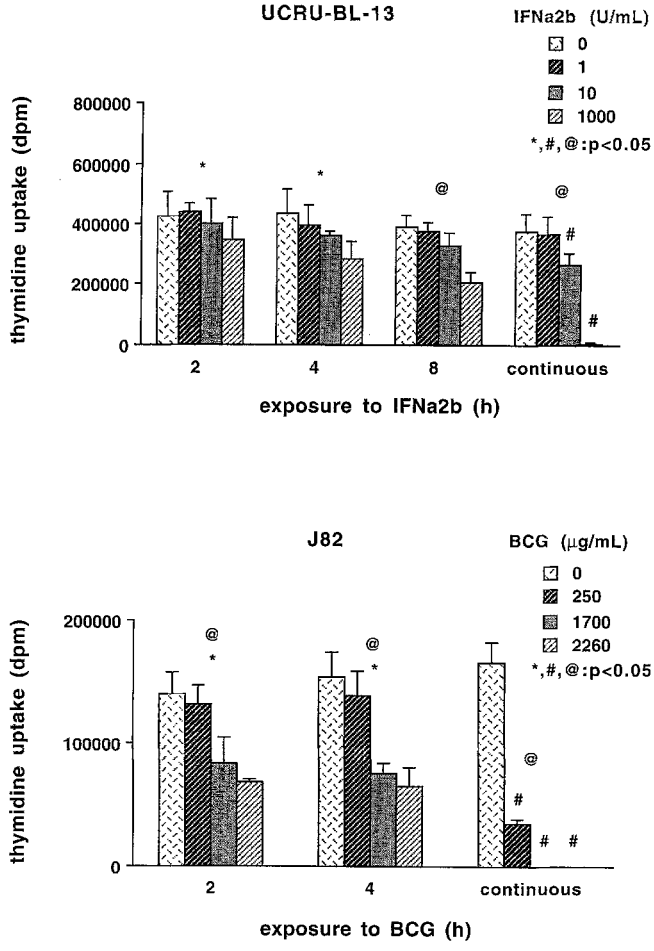


Fig. 4 Comparison of a 2-h, 4-h, 8-h and continuous exposure to IFNα2b of UCRU-BL-13 cells and a 2-h, 4-h and continuous exposure to BCG of J82 cells on day 7. This figure shows cell lines sensitive to IFNα2b and BCG. A regression analysis of the log-transformed data showed a more significant dose response with increased exposure to IFNα2b of UCRU-BL-13 and to BCG of J82. The results show a representative experiment with four replicate wells per mean ± SD. # Significant inhibition ($P < 0.05$) of proliferation of cells due to increasing dose; * significant difference ($P < 0.05$) between continuous exposure and short exposures; @ significant correlation coefficient ($P < 0.05$) for dose response for a given time of exposure (all statistical comparisons on day 7)

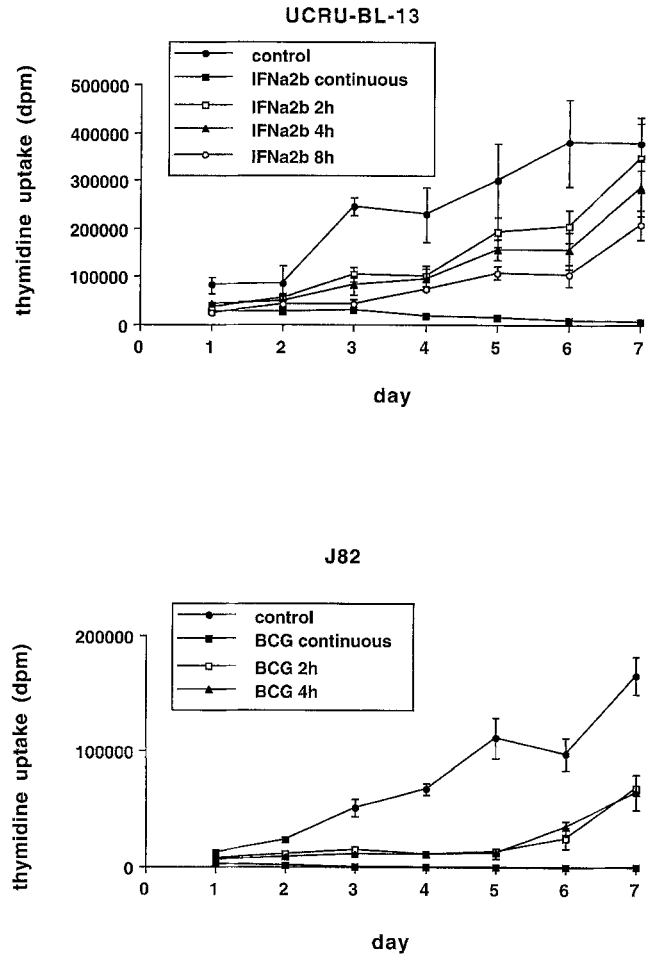


Fig. 5 Thymidine uptake measured daily for 7 days after a 2-h, 4-h, 8-h and continuous exposure of UCRU-BL-13 cells to IFNα2b and after a 2-h, 4-h and continuous exposure of J82 cells to BCG. This figure shows cell lines sensitive to BCG and IFNα2b. The results show a representative experiment with four replicate wells per mean ± SD

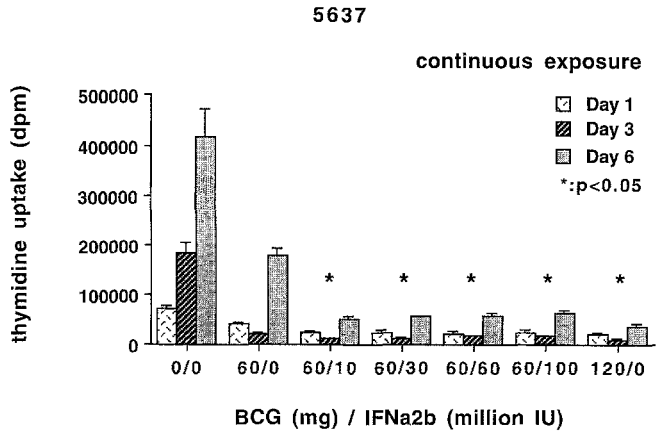
decreased proliferation also occurred with increasing BCG dosage for UCRU-BL-17 cells (50 µg/ml, $P = 0.655$; 1000 µg/ml, $P = 0.025$; 2260 µg/ml, $P = 0.004$) (Fig. 2), some proliferation occurred in the presence of 2260 µg/ml BCG, a simulated clinical concentration of 120 mg. Although J82 cells were extremely resistant to IFNα2b (Fig. 1), they had similar sensitivity to BCG as UCRU-BL-28, and the results of the other lines were between those of UCRU-BL-28 and UCRU-BL-17 (data not shown).

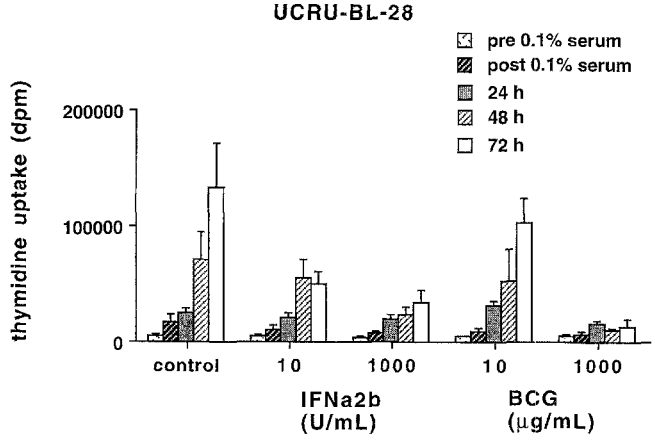
Combination of BCG and IFNα2b

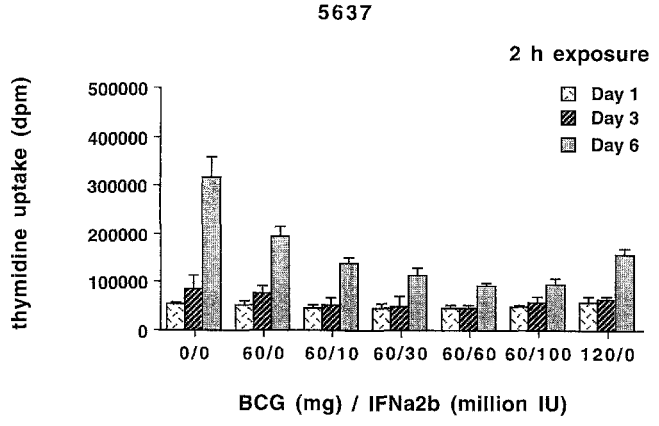
The effect of a combination of BCG and IFNα2b on the proliferation of six tumour cell lines was investigated. The proliferation of UCRU-BL-28 cells was inhibited by low doses of both BCG and IFNα2b (Fig. 3). On day 7 UCRU-BL-28 cells were significantly inhibited ($P < 0.05$) by 1000 U/ml IFNα2b alone (95% inhibition), and in combination with 5 µg/ml and 50 µg/ml BCG (97% and 98% inhibition respectively). When the three groups were compared, there was a significant difference ($P < 0.05$) between IFNα2b and IFNα2b + BCG 50 µg/ml only. The addition of BCG to IFNα2b-resistant J82 cells (Fig. 3) caused a significant difference ($P < 0.05$) in proliferation between the three groups IFNα2b, IFNα2b + BCG 250 µg/ml and IFNα2b + BCG 500 µg/ml.

Short exposures of cells to BCG or IFNα2b

Clinically BCG or IFNα2b is administered for short periods only, so we compared short exposures with continuous exposure of six tumour cell lines to BCG or IFNα2b. When cells were sensitive to BCG or IFNα2b, an antiproliferative effect could be seen as long as 7 days after a 2-, 4- or 8-h exposure to these agents (Fig. 4). On day 7, UCRU-BL-13







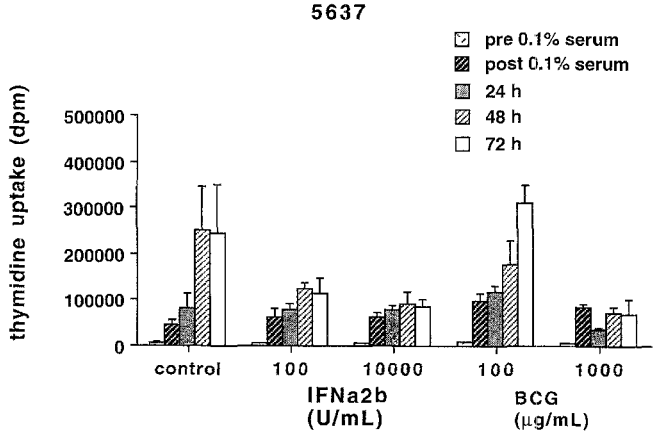


Fig. 6 Effects of simulated clinical concentrations of BCG ± IFNα2b applied continuously or for 2 h only to 5637 cells. This figure shows cells that have intermediate sensitivity to high-dose IFNα2b. The results show a representative experiment with four replicate wells per mean ± SD. * Significant inhibition ($P < 0.05$) of proliferation over 6 days of 5637 cells grown continuously in the presence of 60 mg BCG + IFNα2b or 120 mg BCG alone compared with untreated controls

Fig. 7 Effects of BCG and IFNα2b on the re-establishment of proliferation of UCRU-BL-28 and 5637 cells, which were growth-arrested in limiting serum (0.1% fetal bovine serum) for 72 h. This figure shows cells with high and intermediate sensitivity to BCG and IFNα2b out of six cell lines tested. The results show a representative experiment with four replicate wells per mean ± SD. The decreases in proliferation shown were not statistically significant

cells showed significantly decreased proliferation ($P < 0.05$) in response to increased time of exposure to IFNα2b and increased IFNα2b concentration (Fig. 4). Likewise J82 cells significantly decreased proliferation ($P < 0.05$) in response to increased exposure to BCG and increased BCG concentration (Fig. 4). UCRU-BL-13 cells exposed to IFNα2b for short periods returned to a proliferative state more quickly than J82 cells exposed to BCG for a short period (Fig. 5).

Simulated clinical doses of BCG and BCG + IFNα2b

In order to reproduce doses used in the clinical setting, we exposed 5637 cells, which had intermediate sensitivity to BCG and IFNα2b, to simulated clinical concentrations of BCG and BCG+IFNα2b either continuously for 6 days, or for 2 h only on day 1, followed by normal growth medium (Fig. 6). The simulated clinical concentrations we used were equivalent to the concentration of reconstituted doses of 60 mg and 120 mg BCG alone, and 60 mg BCG in combination with 10×10^6, 30×10^6, 60×10^6 or 100×10^6 IU IFNα2b. When compared with controls, both 60 mg and 120 mg BCG caused a significant antiproliferative effect ($P < 0.05$), but a dose of 60 mg BCG was not significantly different from 120 mg BCG or from 60 mg BCG combined with any dose of IFNα2b. However, when compared with controls, a combination of 60 mg BCG and IFNα2b was as effective as 120 mg BCG alone ($P < 0.05$). Antiproliferative effects on 5637 cells were also evident 6 days after a 2-h exposure to BCG or a combination of BCG and IFNα2b, but these were not significant (Fig. 6). Similar results were obtained with the T24 and J82 cell lines (data not shown).

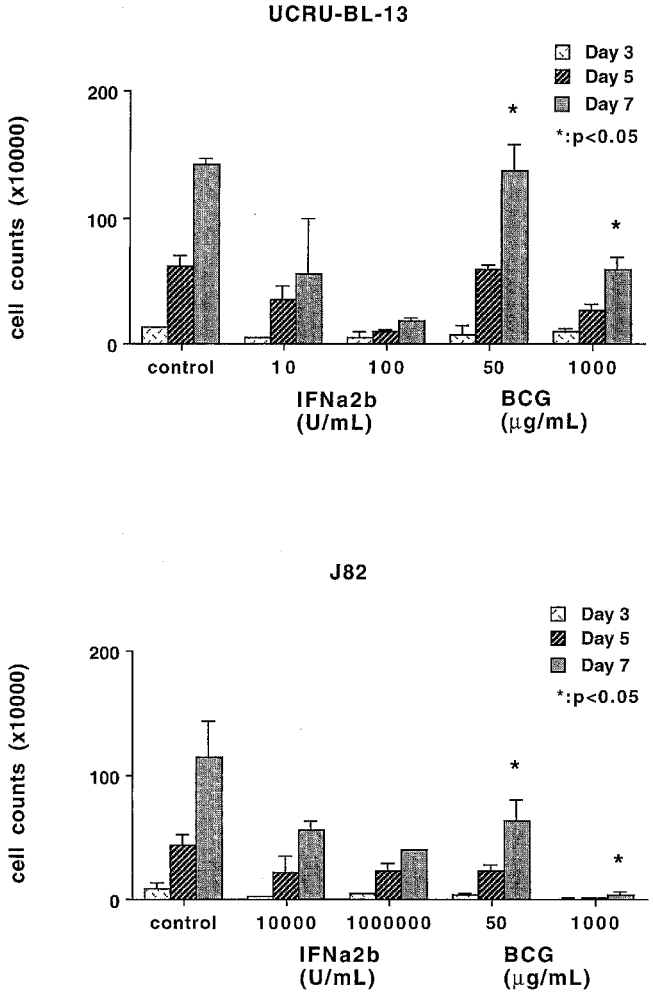


Fig. 8 Effects of BCG and IFNα2b on UCRU-BL-13 and J82 cell number. This figure shows the most sensitive and the most resistant cell lines to IFNα2b of the six cell lines tested. The results show a representative experiment with three replicate wells per mean ± SD. * Significant correlation coefficients ($P < 0.05$) for a regression analysis of cell number data and proliferation data on day 7

Re-establishment of proliferation after incubation in limiting serum

We investigated whether the presence of BCG or IFNα2b in normal growth medium would deter viable, non-proliferating cells from re-establishing proliferation. After the six cell lines were growth-arrested in limiting serum (0.1%–1% FBS) for 3–5 days, normal growth medium (10% FBS) was re-introduced with or without BCG or IFNα2b (Fig. 7). The limiting concentration of serum needed to stop proliferation was 1% for UCRU-BL-13, 5637 and T24 and 0.1% for UCRU-BL-17, UCRU-BL-28 and J82. Growth-arrested bladder cells returned to normal growth medium with BCG or IFNα2b showed decreased proliferation compared to controls, but this was not statistically significant.

Cell counts

Counts of viable cells were made to compare with the results obtained using the proliferation assay. A decrease in cell counts occurred with similar BCG or IFNα2b concentrations to those producing a decrease in proliferation (Fig. 8). When a regression analysis of cell counts versus proliferation at day 7 was done for the six cell lines, the correlation coefficients were significant ($P < 0.05$) for UCRU-BL-13 and J82 cells exposed to BCG.

Discussion

We have observed a cell line, UCRU-BL-13, which is exquisitely sensitive to IFNα2b (Fig. 1). Such sensitivities to IFN have previously only been demonstrated with IFNγ [19]. The six bladder tumour lines we studied showed remarkably different responses to IFNα2b, ranging from UCRU-BL-13 cells which stopped proliferating in response to the very low dose of 50 U/ml, to J82 cells which proliferated in the presence of 1×10^7 U/ml. In vitro growth inhibition of five bladder lines has been observed at final IFN concentrations of 50–1000 U/ml [10], while doses of up to 1000×10^6 IU have been used clinically [9].

The antiproliferative effects of IFN are thought to relate to target cell receptor number or binding affinity [19–21]. However an IFNα2c-resistant cell line had more IFN receptors per cell than two IFNα2c-sensitive lines, while the IFN affinity was similar for all three lines [20]. Likewise the more IFNγ-sensitive of two lines tested had a lower density of receptors, while the affinity of the receptor for its ligand was similar for both lines [19]. Differences in receptor numbers may not reflect response to IFNγ, or whether cells respond with HLA-DR expression and/or proliferation inhibition [21]. However, results obtained with different cell lines and IFNs may not predict the outcome of receptor studies using IFNα2b with our cell lines.

The antiproliferative effect of IFNγ has been studied, as this cytokine is present in the urine of patients after intravesical BCG therapy [22]. The onset and magnitude of inhibition of proliferation by IFNγ varied between three human TCC lines, RT4 (grade I), RT112 (grade II) and MGHU1 (grade III), and high-grade tumour cells have been reported to be the least sensitive to IFNγ [18]. Our IFNα2b-sensitive TCC cell lines were grade II (UCRU-BL-13, UCRU-BL-28) and grade III (UCRU-BL-17) [15–17], while our IFNα2b-resistant lines were grade II (5637, ATCC), grade III (T24 [23]) and grade III–IV (J82 [24]). Therefore the response of bladder tumour cells to IFNα2b is probably not linked to tumour grade. Our most BCG-resistant line represented grade III (UCRU-BL-17) and most sensitive lines represented grade II (UCRU-BL-28) and grade III–IV (J82), indicating that sensitivity to BCG is also independent of tumour grade.

The cell doubling time also appeared to be unrelated to sensitivity to either BCG or IFNα2b. IFNα2b-resistant,

BCG-sensitive J82 cells doubled in 30 h [24], compared with IFNα2b-sensitive, BCG-sensitive UCRU-BL-28 cells (62.4 h) [17], and T24 cells which showed intermediate sensitivity to both agents (19 h) [23]. Non-cycling tumour cells are more sensitive targets for IFN, indicating that intracellular events, together with extracellular IFN concentration, determine the magnitude of the antiproliferative response [25].

Naturally produced IFNα is stable after 7 days in tissue-culture medium at 37 °C. Natural IFNα and four recombinant subtypes have equivalent antiproliferative effects and inhibitory effects on colony formation [10]. Thus the response of our cell lines to IFNα2b is probably independent of the assay length or IFNα type.

We have found that BCG exerts a direct, antiproliferative effect on bladder tumour cells in the absence of immune mechanisms (Fig. 2) [14]. Similar antiproliferative effects of both heat-killed and viable BCG on a panel of human bladder transitional-cell carcinoma lines have been described [26]. These authors postulate that, if the tumour system does not respond to BCG therapy by secreting cytokines and expressing ICAM-1, then it may not be susceptible to cellular effector mechanisms employed by the immune system [27]. Biochemical pathways activated after BCG attachment to tumour cells have not been explored; nor have detrimental effects caused by intracellular accumulation of BCG. Urothelial cells and tumour cells internalise and present BCG antigens to CD4 cells, via the MHC class II pathway [28]. Non-immunological mechanisms include a direct toxic effect and injury to bladder tumour cells by products of inflammatory events [8].

We observed additive antiproliferative effects of BCG and IFNα2b (Fig. 3), which may indicate similar sites of action. A combination of BCG and IFNα2b at simulated clinical concentrations had a similar antiproliferative effect to a double dose of BCG alone (Fig. 6). This has important clinical possibilities, as one of the drawbacks of BCG therapy is the toxic side-effects [2]. Previous data support the clinical efficacy of combining IFN with cytotoxic agents such as doxorubicin, as IFN reduces the proliferation rate rather than causing tumour cell death [29].

In our susceptible lines we measured decreased proliferation 7 days after a 2-, 4- or 8-h exposure to BCG or IFNα2b (Fig. 4). This agrees with previous findings, which showed that, although the maximal effect of BCG was observed after the most prolonged exposure, exposure to BCG for as little as 1 h significantly decreased the proliferative capabilities of susceptible lines [26]. This may be clinically relevant, as the bladder cavity receives only short exposures to BCG or IFNα2b, typically 2 h/week for 6 weeks [1]. For BCG-susceptible J82 cells (Fig. 5), the antiproliferative effect after a short exposure to BCG was longer lasting than the antiproliferative effect after short exposures of IFNα2b-sensitive UCRU-BL-13 cells (Fig. 5) to IFNα2b. It remains to be determined whether this results from cell-line differences or whether BCG has a more persistant antiproliferative effect on tumour cells. Others found no significant inhibition of colony formation when RT112 bladder carcinoma cells were exposed to IFNα for 1 h then washed, and greater inhibition occurred when cells were refed every 4 days with IFNα-containing media than if they were exposed for 4 days only [10]. We have also observed that the presence of BCG and IFNα2b stops susceptible quiescent cells undergoing maximum proliferation when favourable conditions for tumour growth arise (Fig. 7), which is an important consideration for a tumour with a high recurrence rate.

Our results indicate that BCG and IFNα2b have a direct, antiproliferative effect on bladder tumour cells, which augments their immune effects, and that it may be clinically beneficial to combine an antiproliferative agent with a cytotoxic agent to reduce toxic side-effects. Understanding the reasons for different sensitivities of different tumour lines to therapeutic agents may help to determine which agent or combination would give maximum benefit to a particular patient.

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