Bacillus Calmette–Guerin (BCG) enhances monocyte- and lymphocyte-mediated bladder tumour cell killing

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Summary A cytotoxicity assay was used to study the action of bacillus Calmette–Guerin (BCG) and cytokines on four human bladder cancer cell lines. Monocytes and lymphocytes from peripheral blood were incubated with or without BCG or cytokines for 24 h, after which [3H]thymidine-labelled target cells were added and the 72 h percentage specific release determined. BCG had a direct cytotoxic effect against tumour cells and significantly enhanced monocyte/macrophage and enhanced lymphocyte cytotoxicity against one cell line (UCRU-BL-17). Supernatants (SNs) from BCG-activated monocytes/macrophages and lymphocytes increased the percentage specific release of [3H]thymidine from UCRU-BL-17 cells. Interferon alpha (IFN-α) and interleukin 2 (IL-2) were cytotoxic towards UCRU-BL-17. No synergy occurred between BCG and cytokines at the concentrations tested. The results suggest that BCG is superior to IFN-α, interferon gamma (IFN-γ) and IL-2 in enhancing cell-mediated cytotoxicity.

Keywords: bladder neoplasms; BCG vaccine; immunotherapy

Since the first report of the use of intravesical BCG for the treatment of recurrent superficial bladder tumours (Morales et al., 1976), clinical trials have confirmed that BCG is an effective immunomodulator which provides superior results to those from chemotherapy (Lamm, 1992). However, the mechanism of action of BCG remains unclear and therapy may cause significant side-effects (Lamm et al., 1992).

Interferons have immunomodulatory and antiproliferative effects and may stimulate phagocytosis by polymorphonuclear leucocytes and increase macrophage and natural killer (NK) cell activity (Torti and Lum, 1987). IFN-α has been used successfully clinically for the treatment of superficial bladder cancer, and patient responses are achieved with minimal local and systemic toxicity (Torti et al., 1988). IFN-γ (Prescott et al., 1990) and IL-2 (De Jong et al., 1990) are detected in the urine as part of the local immune response to BCG therapy.

In normal human urothelium T lymphocytes are present within the mucosal lining of the ureter and urinary bladder (El-Demiry et al., 1986). Most of these CD3⁺ T lymphocytes are CD8⁺ (suppressor/cytotoxic), although CD4⁺ (helper/inducer) cells are present. Monocytes/macrophages are found less frequently.

After repeated BCG instillations for superficial bladder cancer, mononuclear infiltrates are induced in the bladder wall of the patient. Infiltrating cells include T lymphocytes and smaller numbers of macrophages and B lymphocytes (De Boer et al., 1991a). Analysis of mucosal bladder leucocyte subpopulations shows that most cells are lymphocytes which are associated with macrophages and eosinophils (Peuchmaur et al., 1991). The major leucocyte subpopulation is the CD4⁺ T lymphocyte, while NK cells are uncommon and do not appear to make a major contribution to the anti-tumour activity of BCG (Ratliff et al., 1986).

Clinical studies showed a marked increase in the number of leucocytes, mostly granulocytes, in the urine 24 h after repeated BCG instillations (De Boer et al., 1991b). Monocytes/macrophages and lymphocytes, mostly CD4⁺ T cells, were also present. After treatment, the absolute numbers of all subpopulations increased, but the increase in monocytes/macrophages was most marked.

As monocytes/macrophages and lymphocytes appeared to be the major leucocyte subpopulations involved in the immune response to BCG, we developed a cytotoxicity assay in which monocytes and lymphocytes were pretreated with BCG and cytokines and then co-incubated with bladder tumour cells to determine the anti-tumour activity of immune cells.

Materials and methods

Target cells

The UCRU-BL-17 cell line was derived from a transitional cell carcinoma (Russell et al., 1988) and the 5637, T24 and J82 cell lines were obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). The cells were grown in RPMI-1640 (UCRU-BL-17, 5637, T24) or MEM (J82) (P.A. Biologicals, Sydney, Australia) with 10% foetal bovine serum (FBS), 25 mM Hepes, 4 mM glucose and 2% solution PS (penicillin G 5000 U ml⁻¹ streptomycin sulphate 5000 µg ml⁻¹) [all from Commonwealth Serum Laboratories (CSL), Victoria, Australia] and were mycoplasm free (GenProbe, CA, USA).

BCG and cytokines

BCG, living organisms of an attenuated strain of Mycobacterium tuberculosis [Pasteur strain, 7–15 × 10⁶ colony forming units (c.f.u.) mg⁻¹], was obtained from CSL. Human, recombinant cytokines included IFN-α2b (Schering Corporation, NJ, USA) and IL-2 and IFN-γ (both from Boehringer Mannheim, Mannheim, Germany).

Monocyte and lymphocyte isolation

Monocytes and lymphocytes were isolated from healthy volunteers’ peripheral blood, which was collected into EDTA Vacutainers (Becton Dickinson, NJ, USA). Mantoux tests identified donors who were positive for sensitivity to mycobacteria. Monocytes were isolated according to the Nycomed monocytes (Nycomed, Oslo, Norway) separation procedure. The monocyte-depleted Nycodenz pellet was resuspended in Hank’s balanced salt solution without calcium and magnesium (HBSS) (P.A. Biologicals) and lymphocytes were then isolated from it by the Ficoll-Paque (Pharmacia, Uppsala, Sweden) method. The washed Ficoll interface was plated in cell culture medium for 1 h at 37°C to remove adherent cells. Peripheral blood mononuclear cells (PBMCs) were isolated according to the Ficoll-Paque protocol.

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Flow cytometry

The purity of isolated monocytes and lymphocytes was estimated by flow cytometry (Profile 11, Coulter, FL, USA). Monoclonal antibodies (Mabs) were linked with fluorescein isothiocyanate (FITC) or phycocerythrin (PE). Monocytes and lymphocytes were stained for CD14 (Leu-M3-PE, monocytes), CD16 (Leu-11a-FITC, NK cells), CD2 (T11-FITC, T lymphocytes) and CD20 (B1-FITC, B lymphocytes). The negative controls were CD1 (T6-FITC, thymocytes) and Ms IgGl-PE. The cells were stained with MAbs for 30 min at 4°C. Mabs of the Leu series were obtained from Becton Dickinson (CA, USA) and T6, T11, B1 and Ms IgGl-PE were from Coulter. Within the monocyte population there were 78% CD14, 27% CD16, 2% CD20 and 15% CD2 positive cells, while the lymphocyte population contained 63% CD2-positive, 8% CD20-positive, 26% CD16-positive and 4% CD14-positive cells. To separate T lymphocytes and NK cells, lymphocytes isolated using our protocol (described above) were stained with CD16 and CD3 (Leu-4, T lymphocytes) for 30 min on ice. The lymphocytes were then sorted by flow cytometry (Epics V, Coulter) into a T lymphocyte (97% pure) and an NK cell (94% pure) group.

Cytotoxicity assay

The assay detected the cytolytic activity of monocytes and lymphocytes (effectors) by the release of a DNA radiolabel from the target cells. [3H]thymidine was selected for its low background release over long incubation periods as significant macrophage cytotoxicity cannot be measured before 15 h (De Weger et al., 1986). For this reason the standard 4 h chromium 51Cr-release assay was not chosen. Effectors were incubated in 96-well flat-bottomed tissue culture plates (Corning, NY, USA) for 24 h at 37°C. Monocytes were called monocytes/macrophages after plating in tissue culture conditions. BCG (5, 50, 250 or 500 µg ml⁻¹) and/or cytokines (10, 100 or 1000 U ml⁻¹) were then added to the plated effectors in triplicate wells for 24 h. The effects of BCG and IFN-α2b individually and in combination were calculated (Goldstein et al., 1989) to determine whether they were supra-additive. Target cells were plated at 3 × 10⁶ targets in 75 cm² tissue culture flasks (Corning) for 24 h then labelled while in the exponential growth phase with 60 µCi of [3H]thymidine (specific activity 5.0 Ci mmol⁻¹; Amersham, IL, USA) for 24 h at 37°C. The labelled cells were passaged with trypsin-EDTA (0.5% trypsin, 5.3 mM tetrasodium EDTA (10 ×); Gibco, NY, USA) then added to the effectors at an effector-target (E/T) ratio of 10:1 for 72 h at 37°C. This time frame was chosen as significant macrophage cytotoxicity cannot be measured before 15 h (De Weger et al., 1986) while BCG-induced killer activity in PBMCs has been shown to appear within 24 h and decrease after 72 h (Koga et al., 1991). The optimal E/T ratio for maximising percentage specific release of [3H]thymidine was predetermined (data not shown). There were 3000–9000 targets per well, depending on the target cell selected and the monocyte yield. In some experiments, effectors were incubated in 24 well flat-bottomed tissue culture plates (Corning), then BCG (250 or 500 µg ml⁻¹) was added to the plated effectors for 24, 48 or 72 h. The filtered SNs were added to labelled UCRU-BL-17 target cells plated in separate 96-well flat-bottomed tissue culture

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Figure 1 The percentage specific release of incorporated [3H]thymidine by bladder tumour cells UCRU-BL-17 (a), S637 (b), T24 (c) and J82 (d) increases after a 72 h incubation with BCG. A dose–response effect is seen for T24 cells (r² = 0.98, P = 0.0012) and J82 cells (r² = 0.912, P = 0.0113). Results are expressed as the mean ± the standard error (s.e.) of the number of experiments (n) (*P < 0.05).
plates. After 72 h the plates were centrifuged and the SNs counted in the Pico-Fluor 40 scintillation fluid (Packard, Groningen, The Netherlands) on a Tri-Carb 1500 liquid scintillation analyser (Packard). Spontaneous release was measured in target cells cultured in medium alone and maximum release in cells which had been lysed in 1% sodium dodecyl sulphate (SDS). The percentage specific release was calculated as:

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

where d.p.m. = disintegrations per minute.

Different bladder lines showed different mean percentage spontaneous (background) release of [\(^3\)H]thymidine as follows: UCRU-BL-17, 18.5 ± 1.4; 5637, 37.0 ± 2.5; T24, 9.2 ± 1.5; and J82, 19.6 ± 2.1.

**Cell counts**

To determine the effect of BCG on cell number, BCG was added to plated tumour cells for 72 h. Cells were washed twice with phosphate-buffered saline (PBS), detached with trypsin–EDTA and counted by 10% trypsin blue (Flow Laboratories, Irvine, UK) exclusion using a Neubauer haemocytometer. Cell number and cell viability were determined.

**Statistical methods**

The data were analysed using the Mann–Whitney U-test. Within each experiment (n) there were three replicate wells per treatment. The results were expressed as the mean of the percentage specific releases ± the standard error (s.e.). A regression analysis was performed for data which concerned a dose–response effect.

**Results**

**Effects of BCG alone**

The four target cell lines, UCRU-BL-17, 5637, T24 and J82, were incubated for 72 h at 37°C with 5, 50, 250 or 500 \(\mu\)g ml\(^{-1}\) BCG. BCG increased the percentage specific release of [\(^3\)H]thymidine from the targets and for the T24 (\(r^2 = 0.98, P = 0.0012\)) and J82 (\(r^2 = 0.912, P = 0.0113\)) cell lines in a dose-dependent manner (Figure 1) \(r^2 = 0.82, P = 0.0343\), for UCRU-BL-17, \(r^2 = 0.218, P = 0.5332\), for 5637. Decreasing significant P-values with increasing BCG were seen for the T24 cells (5–50 \(\mu\)g ml\(^{-1}\), \(P = 0.1747\); 5–250 \(\mu\)g ml\(^{-1}\), \(P = 0.0268\); and 5–500 \(\mu\)g ml\(^{-1}\), \(P = 0.0062\)).

To confirm that the release of [\(^3\)H]thymidine represented a cytotoxic effect, we selected two cell lines UCRU-BL-17, which was sensitive to effector-mediated killing, and J82, which showed a dose-dependent direct response to BCG, and exposed them to BCG for 72 h to compare the cytotoxicity assay method with cell counts (Figure 2). For both cell lines, counts significantly decreased (for UCRU-BL-17 \(P = 0.0374\) with BCG 250 \(\mu\)g ml\(^{-1}\) and \(P = 0.0163\) with BCG 500 \(\mu\)g ml\(^{-1}\), \(r^2 = 0.679, P = 0.1758\); for J82 \(P = 0.0051\) with BCG 250 \(\mu\)g ml\(^{-1}\) and \(P = 0.0039\) with BCG 500 \(\mu\)g ml\(^{-1}\), \(r^2 = 0.862, P = 0.0716\)) and percentage specific release increased (for UCRU-BL-17 \(P = 0.0209\) with both 250 and 500 \(\mu\)g ml\(^{-1}\) BCG, \(r^2 = 0.789, P = 0.1116\); for J82 \(P = 0.0209\) with 50, 250 and 500 \(\mu\)g ml\(^{-1}\) BCG, \(r^2 = 0.594, P = 0.229\)) with increasing BCG concentration. We found no increase in the percentage of dead cells with increasing BCG at the concentrations tested, indicating that lysis was complete (data not shown).

To determine whether a donor's previous exposure to BCG may make their immune cells more responsive to BCG in a cell culture system with UCRU-BL-17 target cells, effector cells were isolated from peripheral blood from healthy donors of different Mantoux status. We selected a group of six donors,
of whom two were Mantoux negative (−ve), two Mantoux positive (+ve) and two had had a recent BCG injection following a negative Mantoux test (BCG). We found no correlation between in vitro effector response to BCG and donor exposure to BCG (−ve vs +ve; \( P = 0.7488 \); −ve vs BCG, \( P = 0.7488 \); +ve vs BCG, \( P = 0.631 \)) (Figure 3).

Unstimulated lymphocytes had a significantly higher cytotoxic activity (19.6% ± 4.6%) than unstimulated monocytes/macrophages (6.8% ± 1.6%) (\( P = 0.0041 \)) against the cell line UCRU-BL-17, and both were significantly higher than the spontaneous release by target cells alone (monocytes vs control \( P = 0.0013 \); lymphocytes vs control \( P = 0.0001 \)) (Figure 4). This trend was observed for the 5637 and J82 cell lines but was not statistically significant (data not shown). As the UCRU-BL-17 cell line appeared to be more sensitive to effector-mediated killing, it was selected to test BCG- and cytokine-enhanced effector cytotoxicity. The cytotoxic activity of both monocytes/macrophages and lymphocytes against UCRU-BL-17 target cells was increased by pre-exposure to BCG (5, 50 or 250 μg ml\(^{-1}\)) (Figure 5). Monocyte/macrophage cytotoxicity was significantly enhanced by BCG in a dose-dependent fashion (from 7.8% ± 2.1% to 16.3% ± 2.4% with BCG 5 μg ml\(^{-1}\), \( P = 0.0093 \); to 25.8% ± 3.0% with 250 μg ml\(^{-1}\) and 25.9% ± 3.4% with 500 μg ml\(^{-1}\), \( P = 0.0001 \) for both, \( r^2 = 0.73, P = 0.1455 \)). Lymphocyte cytotoxicity increased at the higher concentrations (from 21.3% ± 4.4% to 30.6% ± 6.3% with 250 μg ml\(^{-1}\) BCG, \( P = 0.2517, r^2 = 0.869, P = 0.0676 \)). Results obtained from experiments with the 5637, T24 and J82 cell lines did not demonstrate a consistent trend (data not shown). When the filtered SNs of BCG-treated monocytes/macrophages or lymphocytes were added to UCRU-BL-17 target cells, the percentage specific release was significantly higher than that from targets incubated with SNs from untreated effectors (\( P = 0.0209 \) for each BCG concentration and incubation time for both effectors) (Figure 6). Similar significant differences were seen when using the J82 target line (data not shown).

To determine whether BCG pretreatment made target cells more susceptible to effector-mediated cytolysis, UCRU-BL-17 cells were pretreated for 48 h with BCG. Treated targets were no more susceptible than untreated targets to lysis by BCG- or cytokine-stimulated monocytes/macrophages or lymphocytes (data not shown).

Monocytes and lymphocytes were isolated from the peripheral blood of two pretherapy bladder cancer patients. The patients were male, 61 (patient 1) and 74 (patient 2) years old, and information on previous exposure to BCG was not available from their medical records. BCG-stimulated effector cells isolated from patient 2 but not from patient 1 (monocytes, \( P = 0.009 \); lymphocytes, \( P = 0.0947 \)) (Figure 7).

**图 4** Unstimulated lymphocytes caused a significantly higher 72 h percentage specific release by target cells UCRU-BL-17 than unstimulated monocytes (\( P = 0.0041 \)). The \([\text{H}]\text{thymidine}\) release in the presence of both effector types was significantly higher than the spontaneous release by the control, UCRU-BL-17 target cells alone (monocytes, \( P = 0.0013 \); lymphocytes, \( P = 0.0001 \)) (*\( P < 0.05 \)).

**图 5** UCRU-BL-17 cells were co-cultured with BCG-treated monocytes (a) or lymphocytes (b) for 72 h. Monocyte/macrophage cytotoxicity towards UCRU-BL-17 cells was significantly enhanced by BCG (\( P = 0.0093 \) with 5 μg ml\(^{-1}\) BCG; \( P = 0.0001 \) with 250 or 500 μg ml\(^{-1}\) BCG). Lymphocyte cytotoxicity increased from 21.3% ± 4.4% to 30.6% ± 6.3% with 250 μg ml\(^{-1}\) BCG (\( P = 0.2517 \) (*\( P < 0.05 \)).

**图 6** When filtered SNs from monocytes/macrophages (a) and lymphocytes (b) were added to UCRU-BL-17 target cells, the target cell lysis was significantly greater if the effectors had been incubated with BCG (\( P = 0.0209 \)). There was no difference between the incubation times (24, 48 or 72 h) or BCG doses (250 or 500 μg ml\(^{-1}\)) tested (*\( P < 0.05 \)).

**图 7** Effects of cytokines As the enhanced cytotoxicity in the presence of BCG appeared to be mediated by cytokine release, we tested the...
effects of specific cytokines on effector-mediated cytotoxicity. The cytokines IFN-γ, IFN-α and IL-2 at concentrations of 10, 100 or 1000 U ml⁻¹ were added to the tumour target cells alone (control) or with effectors. IFN-α2b and IL-2 caused an increase in percentage specific release by UCRU-BL-17 cells (for IFN-α2b: for an increase of 10 to 100 U ml⁻¹, \( P = 0.5715, 10 \text{ to } 1000 \text{ U ml}^{-1}, \ P = 0.5119, \ r^2 = 0.675, \ P = 0.1783; \) for IFN-γ: 10 to 100 U ml⁻¹, \( P = 1.0, 10 \text{ to } 1000 \text{ U ml}^{-1}, \ P = 0.8046, \ r^2 = 0.068, \ P = 0.7391; \) for IL-2: 10 to 100 U ml⁻¹, \( P = 0.8977, \ r^2 = 0.919, \ P = 0.1836, \) but this was not statistically significant (Table I). There was no observed increase for the other three cell lines (data not shown). IFN-α2b, IFN-γ and IL-2 did not significantly enhance monocyte/macrophage or lymphocyte killing of any of our cell lines at the doses tested (data not shown).

Combinations of BCG and cytokines

The effects of BCG combined with IFN-α2b, IFN-γ or IL-2 at the concentrations stated above were investigated using the four cell lines. In case the effects were due to timing, BCG and IFN-α2b were added together or sequentially, one 6 h after the other, in either order. We found neither evidence of supra-additivity nor any advantage in adding the biological response modifiers together as compared with separately in either order (data not shown).

T-cell/natural killer (NK) cell cytotoxicity

T cells and NK cells were compared with peripheral blood mononuclear cells (PBMCs) and lymphocytes in the above assay at 72 h and 144 h (Figure 8). The percentage specific release for all populations increased with time and BCG concentration. Addition of 250 μg ml⁻¹ BCG to effectors increased their 72 h percentage specific release as follows: control, 0% to 7.2%; PBMCs, 16.1% to 30.6%; lymphocytes, 11.7% to 25.4%; T cells, 4.4% to 6.3%; and T cells + 20% NK cells, 5.7% to 7.2%. These increases were not statistically significant (\( P = 0.1732 \)). At 144 h the percentage specific release in all effector populations strongly correlated with BCG concentration (control, \( r^2 = 0.727, \ P = 0.3499; \) PBMCs, \( r^2 = 1, \ P = 0.0066; \) lymphocytes, \( r^2 = 0.996, \ P = 0.0421; \) T cells, \( r^2 = 0.941, \ P = 0.1557; \) and T cells + 20% NK cells, \( r^2 = 0.998, \ P = 0.0275 \).

Discussion

BCG enhanced cytotoxic activity of monocytes and lymphocytes against bladder cancer cell lines in vitro and also had a small direct effect on these target cells. In four bladder tumour target lines, BCG mediated an increase in percentage specific release, accompanied by a decrease in cell numbers but not cell viability, suggesting an antiproliferative rather than a cytotoxic effect. BCG can bind in a dose-dependent manner to T24 cells (Mitzutani et al., 1991) and is internalised by both mouse and human bladder tumour cells (Becich et al., 1991). Although direct cytotoxicity of BCG or cytokines alone was not demonstrable against bladder cancer cell lines (Bohle et al., 1993), it could result from release of bacterial components during BCG degradation or from proliferation of intracellular bacteria.

Previous exposure of a donor to BCG failed to enhance in vitro BCG-mediated effector cytotoxicity, but effectors from two pretherapy bladder cancer patients showed different responses. An understanding of the basis of patient response variation may help to predict who would benefit from BCG immunotherapy.

Monocytes and lymphocytes have been identified in immune infiltrates in the bladder following BCG administration (De Boer et al., 1991a; Peuchmaur et al., 1991) and an intact thymus-dependent immune response is required for the anti-tumour activity of BCG (Ratliff et al., 1987; Ratliff, 1992). Flow cytometric analysis indicated that lymphocyte fractions used in our study contained T, B and NK cells and some polymorphonuclear (PMN) cells, while the monocyte population was more homogeneous (78% CD14⁺). PBMCs and unstimulated lymphocytes were more cytotoxic than sorted T cells or T cells + 20% NK cells, which showed a slower response, requiring over 144 h. Each effector group was more cytotoxic at 144 h than at 72 h. Others have shown that PBMCs incubated with BCG increase cytotoxicity to a maximum at 7 days (Bohle et al., 1993) but are unable to demonstrate NK-mediated killing of bladder cancer cell lines.

The greater cytotoxic activity of unstimulated lymphocytes compared with unstimulated monocytes/macrophages may be due to cytokine release by effectors in the lymphocyte frac-

| Cytokine | 10 | 100 | 1000 |
|----------|----|-----|------|
| IFN-α2b  | 2.7 ± 1.0 | 4.0 ± 1.0 | 6.4 ± 2.4 |
| IFN-γ    | 3.0 ± 2.0 | 3.7 ± 1.5 | 3.1 ± 1.0 |
| IL-2     | 2.5 ± 1.2 | 6.6 ± 3.2 | Not tested |

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Figure 7  Monocytes (circles) and lymphocytes (squares) isolated from the blood of two pretherapy bladder cancer patients (patient 1, open symbols; patient 2, closed symbols) were stimulated with BCG, but only those from one patient became more cytotoxic towards UCRU-BL-17 tumour cells (monocytes, \( P = 0.009; \) lymphocytes, \( P = 0.0947 \)). As each sample was only available once, the results are expressed as the mean of triplicate wells in a single experiment \( (P < 0.05) \).

Figure 8  The percentage specific release by UCRU-BL-17 target cells co-cultured with PBMCs, lymphocytes, T cells and T cells + 20% NK cells increased with increasing BCG concentration and incubation time, but this was not statistically significant (0 vs 50 μg ml⁻¹ BCG, \( P = 0.2568; \) 0 vs 250 μg ml⁻¹ BCG, \( P = 0.1041; \) 72 h vs 144 h: no BCG, \( P = 0.2101; \) 50 μg ml⁻¹ BCG, \( P = 0.4647; \) 250 μg ml⁻¹ BCG, \( P = 0.3472 \). The results are expressed as the mean of triplicate samples in a single experiment.

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Table 1  Effect of cytokines on percentage specific release of \[^{3}H\]thymidine by UCRU-BL-17 bladder tumour cells
tion. We found that SNs from BCG-activated monocytes/macrophages and lymphocytes increased target cell lysis, indicating that BCG cytotoxicity is largely cytokine mediated. Possible candidate cytokines include tumor necrosis factor alpha (TNF-α), IFN-γ, IL-1β and IL-6 (Kurisu et al., 1995). This is supported by clinical studies which show that BCG therapy induces the presence of IL-1, IL-2, IL-6, TNF-α (De Boer et al., 1992) and IFN-γ (Frescott et al., 1990) in urine, probably derived from activated lymphocytes and, similarly, IFN-γ, IL-2, TNF-α and TNF-β are found in SNs of BCG-activated PBMCs in vitro (Thanhauser et al., 1993). In other studies, CD8+/-CD56+ lymphocytes, but not CD4+ cells or macrophages, have been found to be responsible for BCG-induced cytolsis (Thanhauser et al., 1993).

Both IFN-α2b and IL-2, but not IFN-γ, caused a small increase in the percentage specific release by UCRL-BL-17 cells. The mechanism underlying these effects was not clear, although IFN-α has proven effectiveness in vivo (Torti et al., 1988). Others have shown that IL-2 at 10 U ml⁻¹ induces LAK activity against bladder cancer cell lines after 3 days, but that maximum activity requires 6 days with 1000 U ml⁻¹ (Jackson et al., 1992). Tumour cells modified to express IL-2 and injected into tumour-bearing mice have been shown to give better anti-tumour effects than cisplatin or IFN-γ-producing cells against MBT2 mouse tumours, but no memory was established in 'cured' mice (Connor et al., 1993).

IL-2 also contributes to the maturation of effector cells and stimulates IFN-γ production, inducing activation of macrophages and cytotoxic T cells (Ikemoto et al., 1990). The increase to maximum cytotoxicity by monocytes incubated with IL-2 occurs at 8–16 days (Higashi et al., 1992). The assay time which we used may not be sufficient to demonstrate maximum IL-2-mediated increases in effector cytotoxicity.

In the assay used, BCG was more efficient than cytokines in stimulating effector cells alone. The response of macrophages has been shown to depend on the activating agent.

Thus, lymphokine-triggered cytotoxic activity in bone marrow-derived phagocytes declines after 24 h, but that elicited by bacteria persists (Keller et al., 1990). Furthermore, the secretion of IL-6 and prostaglandin E2 (PGE₂) is enhanced by bacteria but not by lymphokines. In our experiments, a combination of BCG and cytokines was not supra-additive at the concentrations tested and an isobolographic analysis was not therefore justified (Goldstein et al., 1989).

The bladder cancer lines used in this study differed in susceptibility to cytotoxic effects of BCG. Preincubation with BCG induced a dose-dependent increase in cytotoxic activity towards UCRL-BL-17, but not towards 5637, T24 or 382 bladder cancer cells. Others have also found that effector cell killing is independent of the histological grade of the parent tumour and the donor of the effector cells (Jackson et al., 1992). Possible explanations for differences in target cell susceptibility relate to expression of cytokine receptors or tumour-associated antigens. The UCRL-BL-17 line has been established much more recently than the other cell lines, which may have undergone changes in resistance over their long period of tissue culture.

The mechanisms of BCG action are unclear but involve both direct effects possibly mediated via internalised bacteria and indirect immune effects involving immune infiltrates found in the bladder wall and urine of patients treated with BCG instillations. In vitro, the cytotoxic effect of monocytes/macrophages and lymphocytes is significantly enhanced by BCG, indicating that this could be an important indirect immune effect of BCG. Further work is needed to clarify the role of effector cells, using specific defined leucocyte subpopulations, and to understand the differential sensitivity of target cells to cytotoxic effects.

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