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Cytostatic effect of different strains of Bacillus Calmette-Guérin on human bladder cancer cells in vitro alone and in combination with mitomycin C and Interferon-α

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Summary. The cytostatic activity of five Bacillus Calmette-Guérin (BCG) strains (Pasteur, Evans, Tice, RIVM and Connaught) on human transitional cell cancer T24 cells was examined. A striking effect was noted even in 2-day cultures, and the effect was more pronounced when the cells were incubated for 5 days with different BCG strains alone. The concentrations needed were about the same as those used in clinical practice (10^9 colony-forming units of Pasteur strain in 100 ml buffered saline solution). Combination with mitomycin C or interferon-α-2b potentiated the cytostatic effect. A slight difference in cytostatic activity between different BCG strains was found.

Key words: Bladder cancer – BCG – Interferon-α – Cytostatic activity in vitro

The data that have accumulated since the publication of the preliminary reports [4, 6] demonstrate an antitumour activity of different BCG strains on superficial transitional cell carcinoma of the bladder [2]. Immunology plays an essential role in the action the exact mechanism of which, however, still remains distinct [2, 5, 7]. Besides specific and nonspecific responses to tumour antigens, a direct cytotoxic effect of BCG has been assumed but not demonstrated by many authors [2, 5, 7]. On the other hand, interactions between intravesical immunoagents and cytostatics might be possible when these are used in combinations or alternately. In the present study the direct in vitro cytostatic effects of five BCG strains both alone and combined with mitomycin C and interferon-α-2b were investigated.

Materials and methods

The method of estimating antitumour activity is based on luminometric measuring of the adenosine triphosphate (ATP) activity of viable cells in a suspension described by Kangas et al. in 1984 [3]. The method of measuring intracellular ATP has been proven to be accurate when correlated with changes in cell count and (^{3}H)-thymidine incorporation [3]. The method has also been used to compare the activities of different cytostatics on the Walker 256 carcinosarcoma cell line [1]. In the preliminary study BCG strain Pasteur (Rhone-Poulenc) in concentrations of 150, 300, 600, 1200 and 2400 μg/ml was incubated with T24 cells for 5 days and the cytostatic activity was measured with bioluminescence (Fig. 1). On the basis of the results, concentrations equal to 1200 μg/ml, 2400 μg/ml and 4800 μg/ml were chosen for the final study. The five BCG strains, Pasteur (Rhone-Poulenc), Evans (Evans), Tice (Organon), RIVM (H. Lundbeck) and Connaught (Connaught) were suspended in and diluted with physiologic saline solution. Concentrations equal to 10^7, 2×10^7 and 4×10^7 colony forming units (CFU)/ml (equal to 1200, 2400 and 4800 μg/ml BCG strain Pasteur) were prepared. Because of technical difficulties in diluting the Evans strain it was possible to make two concentrations only, namely 10^7 and 2×10^7 CFU/ml. Mitomycin C (Mutamycin, Bristol Myers) was used at a concentration of 0.1 μg/ml and recombinant interferon-α-2b (Intron A, Schering Plough), at 500000 IU/ml. A cell suspension of human transitional cell cancer cells (T24) was prepared. With 35000 cells/ml medium RPMI (Biological Industries) and 10% fetal bovine serum. The cells were incubated in microtitre plates in the

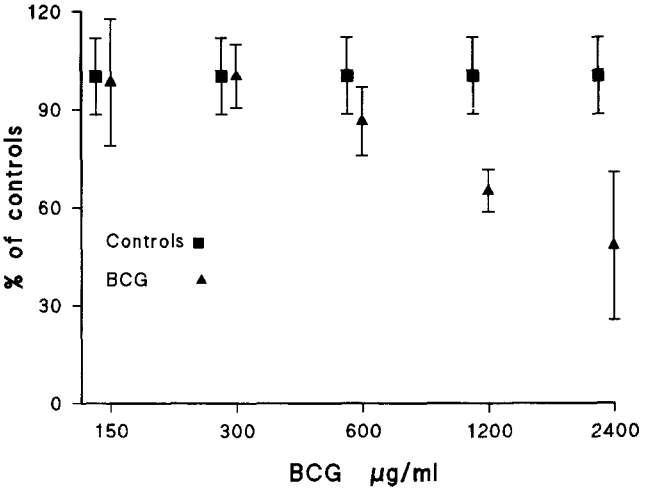

Fig. 1. Preliminary study of the dose-dependence of the cytostatic effect of BCG strain Pasteur on T24 cells in 5-day cultures. Concentrations are given as micrograms per millilitre. *Error bars,* 95% confidence limits

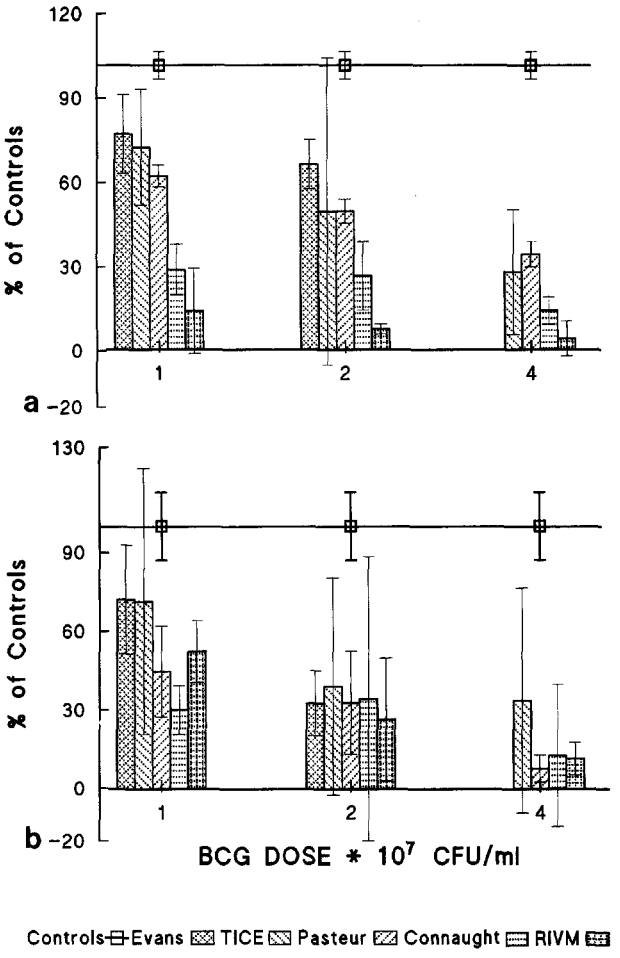

Fig. 2a, b. Cytostatic effect of five different strains of BCG on T24 cells in a 2-day and b 5-day cultures. *Error bars,* 95% confidence limits

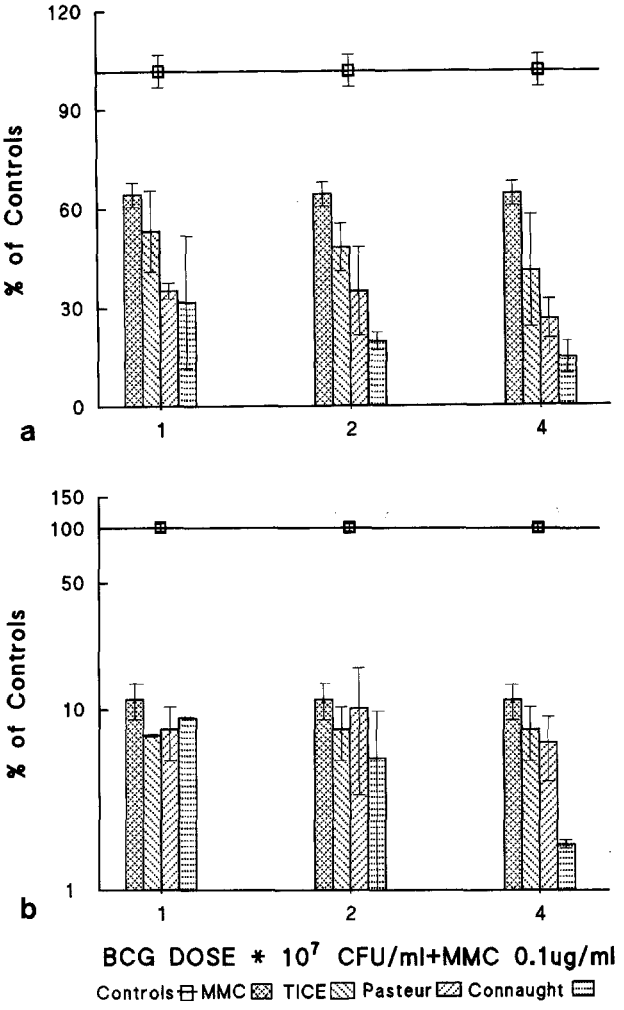

Fig. 3a, b. Cytostatic effect of BCG strains combined with mitomycin C 0.1 μg/ml on T24 cells a 2-day and b 5-day cultures. MMC, Mitomycin C alone; *error bars,* 95% confidence limits. To illustrate differences between the strains the scale is logarithmic

incubator for 2 and 5 days: alone as controls, with three different concentrations of BCG strains, and with combinations of BCG + mitomycin C and BCG + interferon-α. All BCG strains were incubated with medium and 10% serum for 2 and 5 days as controls. Cell suspension was extracted for ATP by 1% trichloracetic acid solution and mixed with purified firefly luciferase. The bioluminescence was read with luminometer 1250 (LKB-Wallac, Turku, Finland). Of each concentration and combination were made three parallel measurements. Statistical analysis was made using a paired *t*-test.

Results

The results are shown in Figs. 2–4. The growth of control cells is marked as 100%, and the error bars show the 95% confidence limits. BCG inhibited cell growth and the inhibition was concentration-dependent (Fig. 2). The cytostatic activity was stronger with Pasteur 4×10^7 CFU/ml than with 1×10^7 CFU/ml ($P<0.001$), and significant differences were also noted between the highest and the lowest concentrations of TICE ($P<0.05$) and Connaught ($P<0.05$). Different BCG strains had similar kinds of inhibitory effects. In 2-day cultures the BCG strains Connaught and RIVM seem to have a stronger inhibitory effect on cell growth than the others ($P<0.05$); the differences in the 5-day cultures, however, are smaller. In 5-day cultures the BCG strain Tice has a slightly weaker cytostatic effect than the other four strains, although only the difference from Connaught is significant ($P<0.05$). The cytostatic activity was significantly lower in 2-day cultures than in 5-day cultures in strains Pasteur ($P<0.01$) and Evans ($P<0.05$), while in the case of strain RIVM the 2-day culture had the more pronounced effect ($P<0.05$). Combination with mitomycin C had a cytostatic effect that was greater than the effect of BCG or mitomycin C alone, significant differences being noted in Pasteur ($P<0.01$) and Connaught ($P<0.05$) strains (Fig. 3). The combinations of 4×10^7 CFU/ml different BCG strains (2×10^7 CFU/ml Evans strain) with 500000 IU/ml interferon α caused additive inhibition of tumour cell growth (Fig. 4). Statistically significant differences were seen only with combinations of RIVM strain with interferon against RIVM alone ($P<0.05$) or interferon alone ($P<0.01$). Cell death was verified by microscopic examination although a striking amount of dead bacilli and cellular debris was

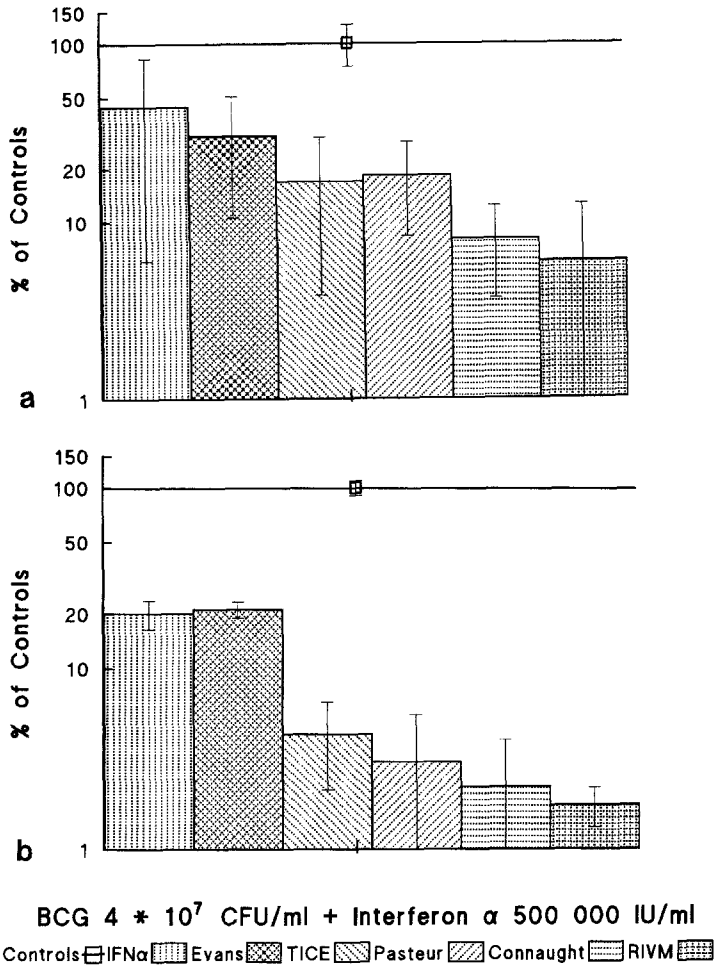

Fig. 4a, b. Cytostatic effect of BCG strains (4×10^7 CFU/ml, 2×10^7 CFU/ml Evans strain) combined with 500000 IU/ml interferon-α on T24 cells in **a** 2-day and **b** 5-day cultures. IFN, Interferon-α alone; *error bars,* 95% confidence limits. Logarithmic scale

present. Different BCG strains incubated with serum but without cells showed no ATP activity in either in 2-day or 5-day cultures.

Discussion

At present there are no data to support the hypothesis that BCG has a direct cytostatic effect in addition to the immunological mechanism that mediates the anti-tumour activities of BCG. In the present study we have shown a direct cytostatic activity of BCG in vitro. The concentrations of viable bacteria were about the same as those used in clinical practice. The concentrations in CFUs are not exact, because there may be variations in the amount of viable bacteria. Because of this it is better to compare CFU rather than microgram amounts when preparing the solutions of different BCG strains. The comparison of CFUs has also been recommended when different BCG strains are used in clinical practice [5, 7]. It is difficult to compare different BCG strains and express the differences in exact figures; in any case the three parallel measurements in each strain varied only slightly. The amount of debris varies in different BCG strains, depending on the method of production. As we are dealing with biological material, the results obtained in the 2-day and 5-day cultures are variable. The BCG strains RIVM and Connaught had greater cytostatic activity in 2-day than in 5-day cultures. This could mean that the cytostatic activity is reversible and the cell growth increased after 2 days' inhibition.

The combination of BCG with mitomycin C or interferon-α seem to increase the cytostatic effect in vitro. In this study the incubation times were much longer than the 2 h generally used in clinical practice. Nevertheless, it was not possible to incubate cells for 2 h with BCG and then culture them for 5 days without BCG to see the long-term effects of this incubation time.

It can be concluded that the antitumour effect of BCG may be the combination of cytostatic and immunological effects. Our results further indicate that combination of BCG with mitomycin C or with interferon does not decrease the cytostatic effect. On the contrary, there may be an additive effect, and in this connection intravesical treatment of superficial bladder cancer with combined or alternating drugs also seems rational. Before clinical trials are initiated the possible toxic or immunological side-effects of the combinations must be carefully investigated.

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