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Induction of bacillus-Calmette-Guérin-activated killer cells from human peripheral blood mononuclear cells against human bladder carcinoma cell lines in vitro

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Abstract. Cytotoxicity against two human bladder carcinoma cell lines (BT-A and BT-B) was investigated using human peripheral blood mononuclear cells (PBMC) stimulated with viable bacillus Calmette-Guérin (BCG) or sonicated BCG (s-BCG). We applied a cytotoxicity assay based on radioactive labelling of tumour cells by incorporation of L[^{3}H]methionine. The results were compared with the cytotoxicity exerted by lymphokine-activated killer (LAK) cells generated by interleukin-2 (IL-2) and interferon γ (IFNγ). BCG-stimulated PBMC showed a cytotoxic potential against BT-A and BT-B comparable to that of IFNγ-generated LAK cells, but this did not reach the level of IL-2-generated LAK cells. We termed these cytotoxic effectors BCG-activated killer (BAK) cells. In contrast to their cytotoxicity against bladder tumour cells, BAK cells did not differ from unstimulated PBMC in the killing of K562 cells. Only viable but not sonicated BCG was able to induce cytotoxicity against BT-A and BT-B. We could demonstrate the presence of the cytokines IFNγ, IL-2, tumour necrosis factor α (TNFα) and TNFβ in the supernatants harvested during the generation of BAK cells. Monoclonal antibodies neutralizing IFNγ were able to inhibit BCG-mediated cytotoxicity, giving evidence of the involvement of IFNγ in the induction of BAK cells. Furthermore, we performed experiments to investigate the cytotoxic potential of distinct cell populations. The cells effective in BCG-activated killing of bladder tumour cells could be localized within the CD8$^+$/CD56$^+$ lymphocyte subset. CD4$^+$ cells and macrophages did not exhibit cytolytic activity. Our findings imply that the activation by BCG of CD8$^+$/CD56$^+$ killer cells might be an important antitumoral mechanism during BCG therapy against superficial urothelial bladder cancer.

Key words: Bacillus Calmette-Guérin (BCG) – Cellular cytotoxicity – Bladder cancer

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Introduction

Intravesical instillation of bacillus Calmette-Guérin (BCG) is an established postsurgical therapy against recurrent superficial urothelial bladder cancer [15]. Compared to other agents used for therapy against bladder cancer, like thiotepa or doxorubicin, BCG immunotherapy has proved to be superior in long-term studies [17]. In contrast to this efficiency of BCG therapy in clinical application, the immunological mechanisms involved in its antitumoral actions are still far from being understood. In experiments with nude athymic mice the involvement of T cells was observed: adoptive transfer of BCG-sensitized mouse splenocytes into nude athymic mice revealed anti-bladder-tumour activity in these animals [28]. Lymphocytes [7] as well as cytokines like interleukin-1 (IL-1), IL-2 and tumour necrosis factor α (TNFα) [5] are known to be released into the patient's urine after BCG instillation, whereas the appearance of urinary interferon γ (IFNγ) is controversial [8, 27]. Previous studies by ourselves [36] and others [19] have shown that natural-killer(NK)-cell-resistant cell lines from bladder tumours were killed by IL-2-generated lymphokine-activated killer (LAK) cells. LAK cells can be distinguished from NK cells by their extended target pattern. Their cytotoxicity is not restricted by the major histocompatibility complex (MHC) [26]. LAK cells are generated under the influence of cytokines, predominantly by high concentrations of IL-2 [13, 30, 38]. They do not represent a homogeneous population characterized by the expression of a common phenotype. In spite of this, LAK cells show different markers typical for NK cells, like CD16 and CD56, or for T cells, like CD3 and CD8 [12, 25].

We have tested whether or not stimulation with BCG can activate LAK-like killer cells from peripheral blood mononuclear cells (PBMC) against bladder tumour cells in vitro. We will demonstrate the generation of BCG-activated killer cells (BAK cells) after stimulation of PBMC with viable but not sonicated BCG. These BAK cells are CD8$^+$/CD56$^+$ T cells and, thus, differ in their cytotoxic pattern from NK cells and LAK cells.

Materials and methods

Preparation of effector cells. Peripheral blood mononuclear cells (PBMC) from healthy human donors were isolated by density-gradient centrifugation [34] on Ficoll-Paque (Pharmacia, Uppsala, Sweden). After repeated washing in Hanks' balanced salt solution (HBSS), cells were counted and adjusted to a concentration of 2×10^6 in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). This medium was used for the culture of all effector and target cells described.

After determination of the optimal stimulation time for the generation of BAK cells, the following stimulation protocol was applied. One fraction of freshly isolated PBMC was tested for cytotoxicity to measure NK cell activity. Other fractions of PBMC were cultured at a cell concentration of 1×10^6 cells/ml for 7 days in medium with or without stimulus to investigate the cytotoxic activity after this time. For the induction of LAK cells PBMC were cultured for a period of 7 days in the presence of 800 U/ml native human IL-2 (a kind gift from Dr. H. Mohr, Blood Transfusion Service of Lower Saxony, Springe, Germany) or 100 U/ml recombinant human IFNγ (kindly provided by Boehringer, Mannheim, Germany) in six-well microtiter plates (Nunc, Roskilde, Denmark) at 37° C and 5% CO_2.

Other fractions of PBMC were cultured with viable BCG strain Pasteur (Institut Mérieux, Leimen, Germany) at a concentration of 100 nl/ml or sonicated BCG (s-BCG) at 10 μg/ml for 7 days. The sonification of BCG was performed according to the method of Harboe et al. [16]. Briefly, bacteria were sonicated at 4° C for 20 min using a Branson sonifier cell disrupter (Branson Sonic Power Co., Heusenstamm, Germany). Insoluble membrane components were removed by ultracentrifugation. Supernatants were collected, treated with 1 μg/ml DNase (Sigma, Deisenhofen, Germany), filtered through 0.2-μm Millipore filters, and stored at −20° C. The protein content was determined using a micro-protein assay (Bio-Rad Laboratories, München, Germany).

After 7 days of stimulation, PBMC were harvested by means of a rubber policeman, washed in HBSS, and adjusted to the concentrations needed for the cytotoxicity assay. To exclude macrophages in target cell killing, macrophages were removed by iron phagocytosis. In brief: after harvesting, BCG-stimulated PBMC were adjusted to a cell concentration of 5×10^6 cells/ml and incubated in RPMI-1640 medium supplemented with 20% FCS and 40 mg/ml iron powder (Fluka, Buchs, Switzerland) for 30 min. PBMC were then depleted of cells phagocytosing iron particles by means of a magnet.

To test the involvement of IFNγ during induction of cytotoxicity, a monoclonal antibody neutralizing IFNγ (clone GZ4, Boehringer, Mannheim, Germany) was added at a concentration of 20 μg/ml at the beginning of culture. Unstimulated PBMC and PBMC cultured with GZ4 alone were used as controls.

For the characterization of the effector cell populations, $CD4^+$ and $CD8^+$ cells were prepared from the BCG-stimulated PBMC and the unstimulated control, respectively, by positive selection. These experiments were performed with magnetic beads (Dynabeads M-450, CD4 and Dynabeads M-450, CD8, Dynal, Hamburg, Germany). Beads were detached from the positive fractions by the Detachabead system (Dynal, Hamburg, Germany). Cell samples of the derived populations were fluorescein-isothiocyanate(FITC)- labelled with Dako CD4-FITC and Dako CD8-FITC (Dako A/S, Glostrup, Denmark), respectively, and analyzed by flow cytofluorometry (50H, Ortho Diagnostic Systems, Neckargemünd, Germany). $CD4^+$ and $CD8^+$ cells were then used for the cytotoxicity assay at effector/target ratios from 25 : 1 to 6 : 1.

BCG-stimulated cells were depleted of $CD8^+$ cells with magnetic beads (Dynabeads M-450, CD8, Dynal, Hamburg, Germany), the success of depletion being controlled by cytofluorometry.

PBMC were depleted of $CD56^+$ cells by incubation with anti-CD56 (Leu19, Becton Dickinson, Heidelberg, Germany) followed by magnetic beads coated with goat anti-(mouse Ig) antibodies (Dynabeads M-450, Dynal, Hamburg). $CD56^+$ cells were determined before and after depletion by cytofluorometry.

Target cells. Cells of two NK-cell-resistant, LAK-cell-sensitive [36] human bladder tumour cell lines, BT-A and BT-B (kindly provided by Dr. J. van der Bosch, Forschungsinstitut Borstel), were chosen as target cells. Both cell lines were established directly from a surgical specimen [35]. We also used as targets cells of the NK-cell-sensitive, LAK-cell-sensitive human erythroleukaemia cell line K562.

Cytotoxicity assay. The cytotoxicity assay applied by us was a modification of the method described by Leibold and Bridge [22], in which target cells were labelled with L-[^{3}H]methionine instead of L-[^{45}Se]selenomethionine. In brief: target cells at a concentration of 4×10^5/ml were radiolabelled by incubation with L-[^{3}H]methionine (specific activity 70–85 Ci/mmol, Amersham Buchler, Braunschweig, Germany) in L-methionine-free RPMI-1640 medium, supplemented with 10% FCS, for 4 h at 37° C and 5% CO_2. Following several washes, bladder tumour cells were seeded out at 5000 cells/200 μl in 96-well flat-bottom microtiter plates (Falcon 3072, Becton Dickinson, Heidelberg, Germany) and allowed to adhere for 20 h. Culture medium was completely removed before effector cells were added. K562 cells were labelled in the same way as bladder tumour cells, incubated for 20 h, washed and transferred to round-bottom microtiter plates (Greiner, Nürtingen, Germany).

Effector cells were added at effector/target ratios from 40 : 1 to 10 : 1 in 200 μl/well. All assays were performed in triplicate. To determine the spontaneous release, target cells were cultured in RPMI medium alone, the maximum release being measured after lysis of target cells in sodium dodecyl sulphate/Triton X-100 (Sigma, Deisenhofen, Germany).

Following a coincubation time of 20 h, plates were spun down at 1500 rpm for 15 min. A 100-μl sample of the supernatant of each well was harvested and transferred into counting vials (Pony-Vials, 6 ml, Canberra-Packard, Frankfurt/Main, Germany) containing 2 ml scintillation cocktail (Rotiscint eco plus, Carl Roth, Karlsruhe, Germany). Radioactivity was measured in a beta counter (Canberra-Packard) and specific lysis determined according to the following formula:

$$\text{specific lysis (\%)} = (r_{exp} - r_{spon}) \times 100/(r_{max} - r_{spon})$$

where r_{exp} is the experimental release, r_{spon} the spontaneous release and r_{max} the maximum release.

Induction of cytokines

Supernatants of BCG-stimulated PBMC were harvested on days 1–7 of culture and investigated for the cytokines IL-2 and TNFα + TNFβ by bioassay, and IFNγ and TNFβ by enzyme-linked immunosorbent assay (ELISA). All assays were performed by established methods described previously. In brief: IL-2 activity was determined in an assay based on IL-2-dependent proliferation of CTL-6 cells (murine T lymphoma cells) [6]. IFNγ was determined by an ELISA kindly supplied by Dr. Gallati (Hoffmann-La Roche, Basel, Switzerland) [9]. Determination of TNF activity was based on the cytopathic effect of both TNFα and TNFβ on L929 cells (murine fibrosarcoma cells) (detection limit 0.4–1.2 U/ml) [10].

To distinguish between the effect of TNFα and TNFβ, an ELISA for TNFβ (Serva, Heidelberg, Germany) was performed (detection limit 15 pg/ml) [2].

To investigate whether these cytokines are cytotoxic to BT-A or BT-B on their own, we tested them in the cytotoxicity assay described above. For that, radioactive labelled bladder tumour cells were coincubated with native IL-2, (a kind gift from Dr. H. Mohr, Blood Transfusion Service of Lower Saxony, Springe, Germany), recombinant IFNγ (kindly provided by Boehringer, Mannheim, Germany), TNFα and TNFβ (Boehringer, Mannheim, Germany) in concentrations from 63 U/ml up to 500 U/ml. After 20 h, supernatants were harvested and the specific release was determined.

Results

Cytotoxicity of PBMC, LAK cells and BAK cells

Freshly isolated PBMC and PBMC cultured with or without stimulus were tested for their cytotoxicity

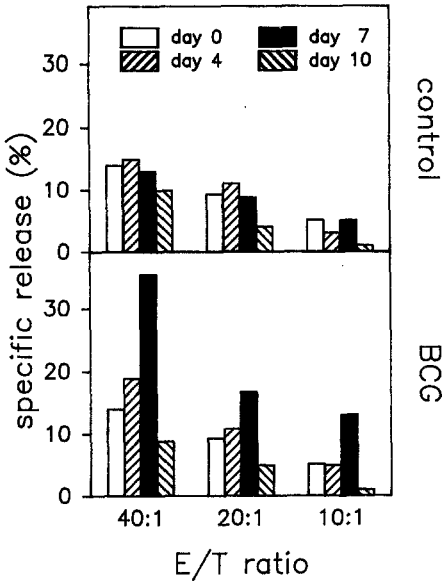

Fig. 1. Kinetics of cytotoxicity of bacillus-Calmette-Guérin(BCG)-stimulated peripheral blood mononuclear cells (PBMC) against the bladder tumour cell line BT-A. PBMC were cultured in the presence or absence of BCG for 10 days. On days 0, 3, 5, 7 and 10, PBMC were harvested and tested for cytotoxicity against bladder tumour cells at different effector/target ratios. Each value represents the mean of triplicate cultures, SD less than 15%

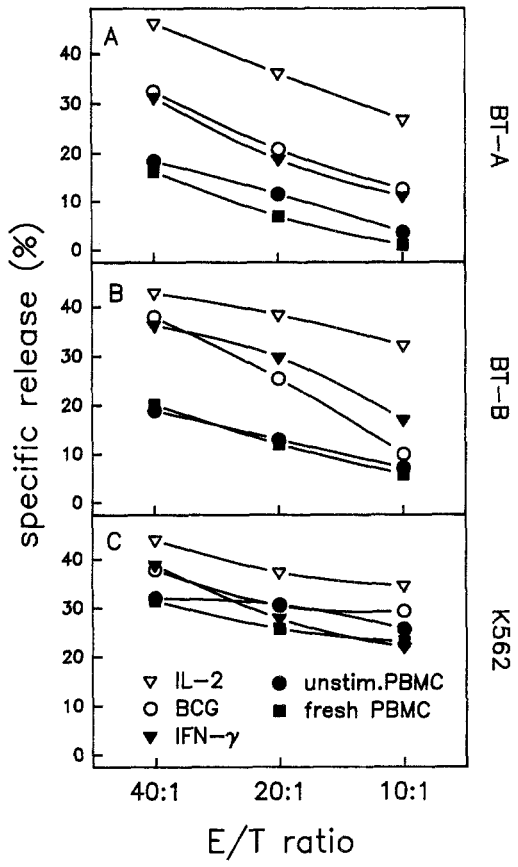

Fig. 2 A–C. Target cell pattern of natural killer (NK), lymphokine-activated killer (LAK) and BCG-activated killer, (BAK) cells. Fresh PBMC and PBMC cultured for 7 days with or without stimulus were tested for cytotoxicity in the L-[^{3}H]methionine assay using different target cell populations. Interleukin-2 (IL-2), interferonγ (IFNγ) and BCG were used as stimuli. Effector and target cells were coincubated for 20 h at effector/target ratios from 40 : 1 to 10 : 1. Data are taken from one experiment representative for others. Each value represents the mean of triplicate cultures, SD less than 15%. Target cells in **A** were BT-A; **B**, BT-B; **C**, K562

against bladder tumour cells and K562 cells in the L-[^{3}H]methionine-dependent cytotoxicity assay. Stimuli used for the induction of cytotoxicity were IL-2, IFNγ, BCG, and s-BCG. A culture time of 7 days was found to be optimal for generating BAK cells (Fig. 1).

Fresh PBMC and PBMC cultured for 7 days without stimulus were only slightly or not at all able to kill cells of the BT-A or BT-B line, whereas both populations were cytotoxic to K562 cells (Fig. 2A–C).

A significant increase of cytotoxicity against BT-A and BT-B could be achieved by generating LAK cells cultured over 7 days. In this respect IL-2 was more effective than IFNγ (Fig. 2A, B). IL-2 slightly enhanced cytotoxicity against K562 after a stimulation period of 7 days, whereas IFNγ did not (Fig. 2C). It should be noted that IL-2- and IFNγ-generated LAK cells were likewise cultured over 7 days and still showed potent cytotoxicity against cells of BT-A and BT-B. This point has to be kept in mind when looking at the cytotoxicity against K562 cells: LAK-cell-mediated cytotoxicity against K562 cells is well known to reach its maximum on days 3 and 4 of culture [14, 33] and to decrease afterwards.

Furthermore, we tested whether BCG induced the generation of cytotoxic cells against cells of the bladder tumour lines BT-A and BT-B. The degree of cell lysis mediated by BCG reached the level of IFNγ-generated LAK cells (Fig. 2A, B). An increase of cytotoxicity against K562 cells was not observed, even at the time of optimal induction (Fig. 2C). Unlike viable bacteria, s-BCG failed to generate cytotoxicity against bladder tumour cells. Stimulation of PBMC with s-BCG did not enhance the cytotoxicity level of the unstimulated control cells (Fig. 3).

Depletion of macrophages to less than 2% by iron phagocytosis after stimulation did not affect BCG-mediated cytotoxicity on bladder tumour cells, suggesting that BCG did not activate macrophages for cytotoxicity against bladder tumour cells (Fig. 4).

Addition of the monoclonal antibody GZ4, which neutralizes IFNγ, resulted in a strong decrease of IFNγ-generated LAK cell cytotoxicity (Fig. 5). GZ4 also reduced the induction of BAK cells (Fig. 5). In control experiments the antibody itself had no influence on the induction of cytotoxicity of PBMC against BT-A and BT-B (Fig. 5).

To characterize the cytotoxic effector cells generated by BCG, CD4$^+$ and CD8$^+$ cells were isolated with magnetic beads. After isolation the beads were detached again. Fluorescence-activated cell-sorting (FACS) analysis revealed that this way of positive selection leed to CD4$^+$ and CD8$^+$ populations with a purity of more than 95%. The data show that CD4$^+$ cells were not able to kill target cells from BT-A and BT-B lines (Fig. 6D), whereas the CD8$^+$ fraction exerted a strong cytotoxicity against these cells (Fig. 6B). Accordingly, depletion of CD8$^+$ cells to below

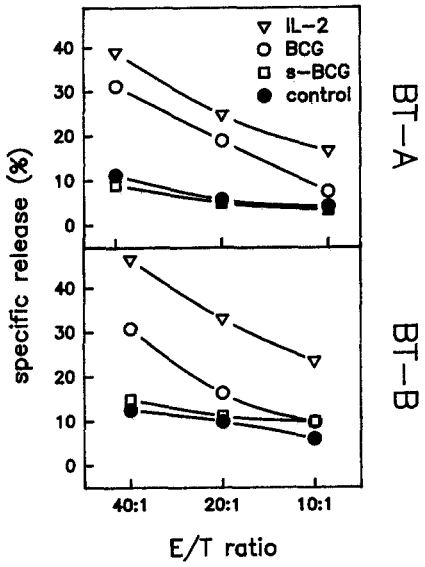

Fig. 3. Cytotoxicity of PBMC stimulated with BCG and sonicated BCG (s-BCG) against BT-A and BT-B. IL-2-induced LAK cells and unstimulated PBMC were used as controls. For further details see Fig. 2. Each value represents the mean of triplicate cultures, SD less than 15%

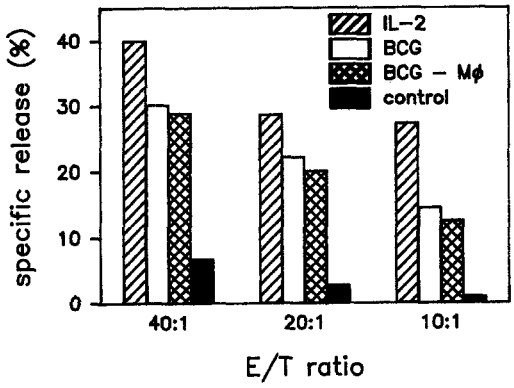

Fig. 4. Cytotoxicity of BAK cells before and after depletion of macrophages. PBMC were cultured in the presence of BCG. Cells were harvested, and cytotoxicity against BT-A was compared with the same population after depletion of macrophages by iron phagocytosis. IL-2-generated LAK cells and PBMC without stimulus were used as positive and negative controls. Each value represents the mean of triplicate cultures, SD less than 15%

5% from BCG-stimulated PBMC led to a strong decrease of cytotoxicity (Fig. 7). The involvement of CD56+ cells was proven in two experiments: depletion of either PBMC (Fig. 7) or CD8+ cells (Fig. 6C) of CD56+ cells to below 5% abolished the cytotoxicity of BAK cells against cells of the bladder tumour cell lines BT-A and BT-B.

Detection of cytokines

PBMC were stimulated by BCG over 7 days, and supernatants were harvested on each day of culture and assayed for the cytokines IL-2, IFNγ, TNFα, and TNFβ.

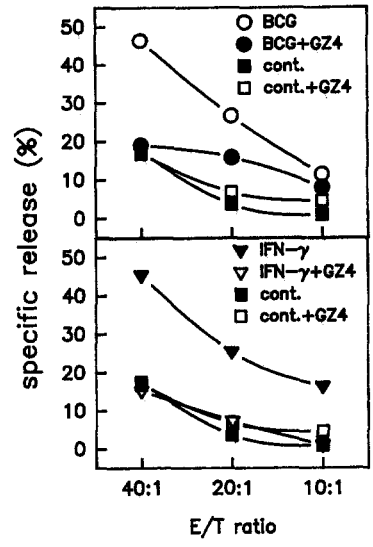

Fig. 5. Inhibition of induction of BAK cells by monoclonal antibodies against IFNγ. PBMC were stimulated with BCG in the presence or absence of monoclonal anti-IFNγ antibody GZ4. After 7 days cells were harvested and tested for cytotoxicity against BT-A cells. PBMC cultures in medium with or without GZ4, and IFNγ-induced LAK cells incubated with GZ4 were used as controls

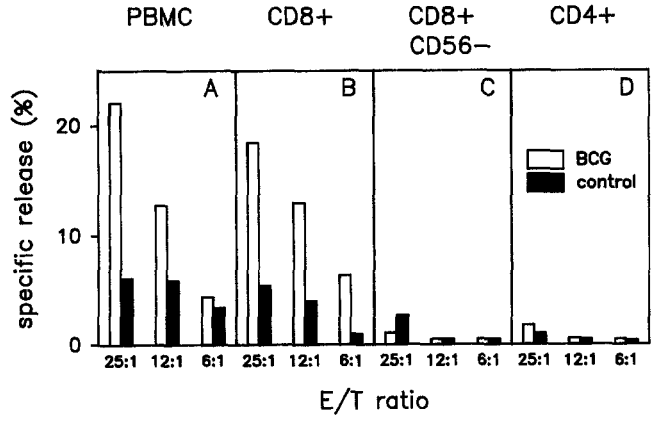

Fig. 6A–D. Cytotoxicity of the CD8+ and the CD4+ fractions of BAK cells against BT-A cells. CD8+ and CD4+ lymphocytes were obtained by positive selection with magnetic beads from BAK cells (BCG) and PBMC cultured without stimulus (control). Their cytotoxicity was compared to unseparated PBMC of both populations by incubating effector and target cells at ratios from 25:1 to 6:1. The CD8+ fraction was depleted of CD56+ cells by magnetic beads. The remaining cells were also checked for cytotoxicity. SD less than 15%; for further details see Fig. 2. **A** Unseparated PBMC; **B** CD8+ cells; **C** CD8+/CD56− cells; **D** CD4+ cells

IL-2 was detectable in very low concentrations in the supernatants only on day 1 after BCG stimulation (Fig. 8A). IFNγ could be detected first in low concentrations on day 1; it subsequently increased and accumulated in the culture until day 5 to a concentration of up to nearly 100 ng/ml. On days 6 and 7 a decrease was observed (Fig. 8B).

The occurrence of TNF activity in the L-929 bioassay showed a typical pattern with one peak on day 2 and a

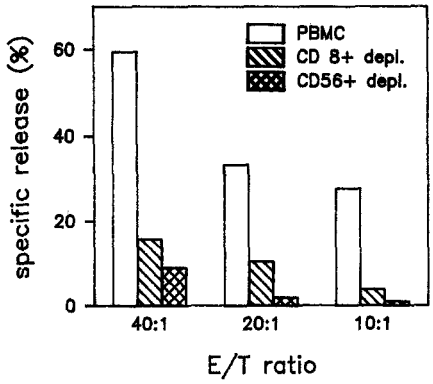

Fig. 7. Cytotoxicity of BAK cells before and after depletion of $CD8^+$ or $CD56^+$ cells. BAK cells were depleted of $CD8^+$ or $CD56^+$ cells using magnetic beads. The starting and the remaining population were analysed by flow cytometry. The fraction of $CD8^+$ or $CD56^+$ cells decreased to less than 5%. Cytotoxicity was tested against BT-A cells. SD less than 15%; for further details see Fig. 2

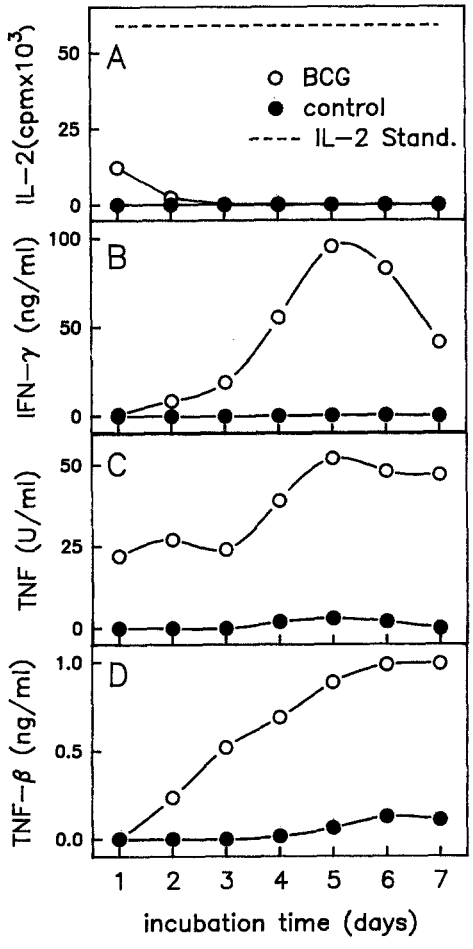

Fig. 8A–D. Induction of cytokines by BCG, PBMC were stimulated with BCG for 7 days. On each day supernatants were harvested, and the release of the cytokines IL-2, IFNγ, tumour necrosis factor α (TNFα) and TNFβ was determined. **A** Release of radioactivity in an IL-2 bioassay compared to an IL-2 standard (68 U/ml). **B** Release of IFNγ measured by enzyme-linked immunosorbent assay (ELISA). **C** Release of TNF (α and β) measured by L929 bioassay. **D** Release of TNFβ determined by ELISA. All samples were measured as duplicates, SD less than 10%

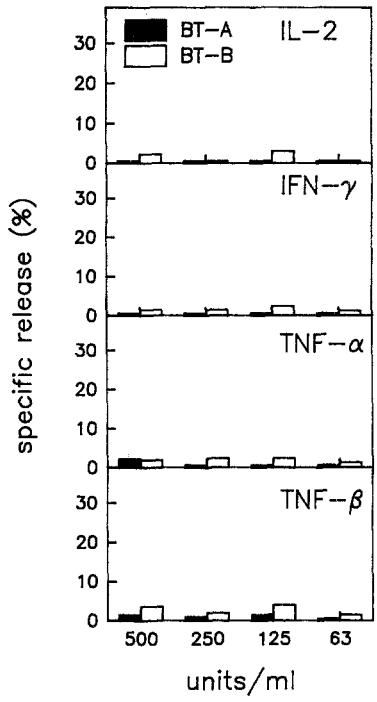

Fig. 9. Cytotoxicity of cytokines against BT-A and BT-B. Radioactive labelled bladder tumour cells were coincubated with different concentrations of the cytokines IL-2, IFNγ, TNFα, and TNFβ for 20 h. Supernatants were harvested and specific release was determined

plateau of high activity from days 5 to 7 (Fig. 8C). TNFβ, as detected by ELISA, started to rise on day 2 and reached its highest level on days 5–7 (Fig. 8D).

None of these four cytokines was found to be cytotoxic by itself to BT-A or BT-B cells (Fig. 9).

Discussion

Previously we had reported that LAK cells are able to kill cells of NK-cell-resistant bladder tumour cell lines [36]. Therefore, it was of interest to investigate whether activation of PBMC by BCG is a means of generating LAK-like activity against these cell lines. Our studies indeed show a significant increase of cytotoxicity following stimulation with BCG for 7 days compared to the low cytotoxicity of freshly isolated and cultured unstimulated PBMC (Fig. 2A, B). These fresh or cultured unstimulated cells exhibit a strong cytotoxic potential against the NK-cell-sensitive target cells of the K562 cell line (Fig. 2C), which was comparable to that of BAK cells, implying that different cell populations are responsible for the killing of these different targets. When examining the cytokines produced during the generation of BAK cells, we could detect TNFα, TNFβ, and IFNγ in considerable amounts, whereas IL-2 appeared only in very low amounts on day 1 of culture (Fig. 8). Furthermore, we could show that there is no direct cytotoxic effect of these cytokines against our bladder tumour cell lines (Fig. 9). IFNγ is important with respect to the induction of LAK-like cells by BCG in vitro. This is reflected by the following: IFNγ is produced in high

amounts after BCG stimulation (Fig. 8B). The level of cytotoxicity reached by BCG-activated cells is comparable to that induced by IFNγ-activated cells (Fig. 2A, B). BCG-induced cytotoxicity was strongly inhibited by the presence of anti-IFNγ antibody GZ4 (Fig. 5). These results are in accordance with studies of others, which demonstrate the importance of IFNγ for the generation of LAK cells [11, 18]. On the other hand, low IFNγ also occurs in supernatants of PBMC stimulated with s-BCG (data not shown). Thus, stimulation of IFNγ release by s-BCG does not seem to be sufficient to generate cytotoxicity against bladder tumour cells, since s-BCG is unable to induce cytotoxicity against BT-A and BT-B (Fig. 3). IL-2 could be detected only in very low amounts on day 1 of culture (Fig. 8A). The production of this cytokine is known to be an initial signal for IFNγ release [23], but IL-2-independent pathways for IFNγ release have also been discussed [20]. In our in vitro system the fast and complete consumption of IL-2 could be the reason for its minimal presence in the culture medium.

It has already been reported by others that TNFα can be involved synergistically with IL-2 in LAK cell generation [19, 21]. This cytokine and also TNFβ were found in the supernatants of our cultures (Fig. 8C, D). However, further studies are necessary to understand the role of the TNFα and TNFβ that are released during BCG stimulation in our in vitro system.

Looking at the cell populations exerting cytotoxic reactions after BCG stimulation, we could exclude the possibility that the cytotoxic reactions mediated by PBMC after 7 days of stimulation by BCG are due to residual macrophages (Fig. 4). Macrophage-mediated cytotoxicity has to be considered in our system for the following reasons. It has been shown that BCG-activated macrophages mediate cytotoxicity against different tumour cells [1] including bladder tumour cells [24]. Others have been able to demonstrate that IFNγ, which was detected by us after stimulation of PBMC with BCG (Fig. 8), increases the antitumour cytotoxicity of human blood monocytes [31]. In our experiments, however, the depletion of macrophages from BCG-stimulated PBMC by iron phagocytosis had no effect on the cytotoxicity, which suggests that the role of macrophages might be restricted to antigen presentation and cytokine production during the induction of BAK cells in our system.

Furthermore, we could demonstrate that CD4+ cells did not take part in the active killing of target cells (Fig. 6D). Studies on LAK cell phenotypes performed by others also confirm that CD4+ cells disappear in long-term LAK cell cultures [12]. Histochemical studies show that in long-term BCG-treated bladder cancer patients the number of CD4+ cells is increased in the bladder wall [3]. Therefore, CD4+ cells may be important for maintaining long-lasting inflammatory reactions, possibly by the production of IFNγ, but are not cytotoxic effector cells themselves. The involvement of CD8+ and CD56+ cells in the cytotoxicity of BCG-stimulated PBMC was demonstrated in various experiments. Depletion of CD8+ cells from BAK cells led to a dramatic decrease of cytotoxicity against BT-A or BT-B cells (Fig. 7). Furthermore, positively selected CD8+ cells showed a strong killing potential against bladder tumour cells (Fig. 6B). Likewise of interest was the subset expressing the NK cell marker CD56, which was shown to be also a marker of LAK cells [12, 25]. Since depletion of these cells either from BCG-stimulated PBMC (Fig. 7) or from preselected CD8+ cells (Fig. 6C) led to the disappearance of the cytolytic effect, we conclude that the BCG-activated effector cells are characterized by the coexpression of CD8 and CD56. Cells with this phenotype are known to increase in long-term LAK cell cultures [12]. They also appear to be responsible for antitumour activity in cancer patients treated with IL-2 [37]. These in vitro findings might help us to understand the success of BCG therapy in suppressing superficial bladder cancer recurrences. In clinical studies of cytokine release into the urine of BCG-treated bladder cancer patients, the early appearance of TNFα, IL-1, and IL-2 [4] was observed, whereas different results were obtained for IFNγ [8, 27]. In our results we found evidence of an important role for IFNγ during the induction of BAK cells (Fig. 5). However, because the generation of LAK cells depends on a concerted action of various cytokines like IL-2 [30], TNFα [21], and IL-7 [32], other cytokines might be active during the induction of BAK cells. Immunohistochemical investigations of BCG-treated bladder carcinoma patients have revealed that CD4+ T cells and macrophages predominate in the infiltrate of the bladder wall [3]. The competence of macrophages in killing BT-A and BT-B cells after activation by BCG has also been reported by our group [29]. The present in vitro study, however, demonstrates CD8+ CD56+ killer cells to be the active population in eliminating (allogeneic) bladder tumour cells. One conclusion of these data must be the possibility of a cooperation of those phenotypically different cell types. Macrophages are important both for the killing of tumour cells and for maintaining the production of proinflammatory cytokines. The role of CD4+ cells indeed appears to be restricted to a helper/inducer function. In patients with superficial bladder cancer, BCG-activated killer (BAK) cells (CD8+/CD56+ cells) might play an important role in the maintenance of the relapse-free state.

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