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On the mode of action of intravesical bacillus Calmette-Guérin: in vitro characterization of BCG-activated killer cells

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Abstract Previously we had shown that, upon activation with viable bacillus Calmette-Guérin (BCG), peripheral blood mononuclear cells (PBMNC) could be rendered cytotoxic against otherwise insensitive natural killer (NK)-resistant bladder cancer cell lines. This phenomenon had been termed the BCG-activated killer (BAK) cell phenomenon. By means of depletion and enrichment procedures of mononuclear cell subpopulations derived from BCG-activated PBMNC we further characterized the cytolytic BAK effector cells functionally in an in vitro cytotoxicity assay against the bladder carcinoma cell line BT-A and phenotypically in their pathway of activation. Neither macrophages nor $CD4^+$ T-helper/inducer cells exerted cytotoxic BAK activity. This cytotoxicity was restricted to the $CD8^-CD56^+$ subpopulation of T-cytotoxic/NK cells. Furthermore, activation of BAK cells via interferon gamma (IFN-γ) was evidenced by the complete inhibition of BAK cell generation with an IFN-γ antibody.

Key words BCG vaccine · Immunotherapy · Bladder cancer · Cytotoxicity · NK-cells

Several phenomena of intravesical BCG immunotherapy against recurrent superficial bladder cancer in man have been described, such as mononuclear cell infiltration of the bladder wall [1, 3, 8, 10, 21, 26, 29] and secretion of cytokines into the urine [4, 13, 23, 28]. Evolving from these observations, several possible cytotoxic effector mechanisms against urothelial carcinoma cells have been considered as operative during BCG immunotherapy against bladder carcinoma and have consequently been analyzed in vitro. As one of these cytotoxic mechanisms lymphokine-activated killer (LAK) cell cytotoxicity, which is induced in mononuclear cells by interleukin-2 (IL-2), was proposed by our group [5] and by Jackson et al. [15]. Recently we reported that not only activation with IL-2, but also activation with viable BCG, could render peripheral blood mononuclear cells (PBMNC) cytotoxic against natural killer (NK)-resistant cells of bladder carcinoma cell lines [6]. By analogy with the LAK cell system [27], this phenomenon was termed the BCG-activated killer (BAK) cell phenomenon [6]. Here, we extend these in vitro observations and further characterize the BCG-induced cytolytic effector cells with regard to their phenotype and their pathway of activation.

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Materials and methods

Cytotoxicity assay

A modified cytotoxicity assay was performed as described recently [6, 16]. Briefly, target cells were radiolabelled with L-[^{3}H]methionine, seeded in flat-bottom microtiter plates at 5000 cells/200 µl, and allowed to adhere for 20 h. With regard to LAK cell-mediated cytotoxicity, labelling of target cells with radioactive L-methionine yields similar results to the standard 4 h ^{51}Cr-release assay [16]. Before addition of effector cells the medium was completely renewed. Effector cells were added at effector/target ratios varying from 40:1 to 10:1 for PBMNC and, due to the restricted number of purified cells, 25:1 to 5:1 for positively selected fractions. All assays were performed in triplicate. After coincubation of effector and target cells for 20 h, plates were centrifuged (1500 rpm/15 min), supernatants were harvested and radioactivity was determined in a β-counter (Canberra-Packard, Frankfurt, Germany). Spontaneous release was determined after culturing of target cells in RPMI alone, and maximum release was measured after complete lysis of target cells in SDS/Triton-X-100 (Sigma, Deisenhofen, Germany). Specific cytolysis was determined by the formula:

$$\text{specific lysis (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

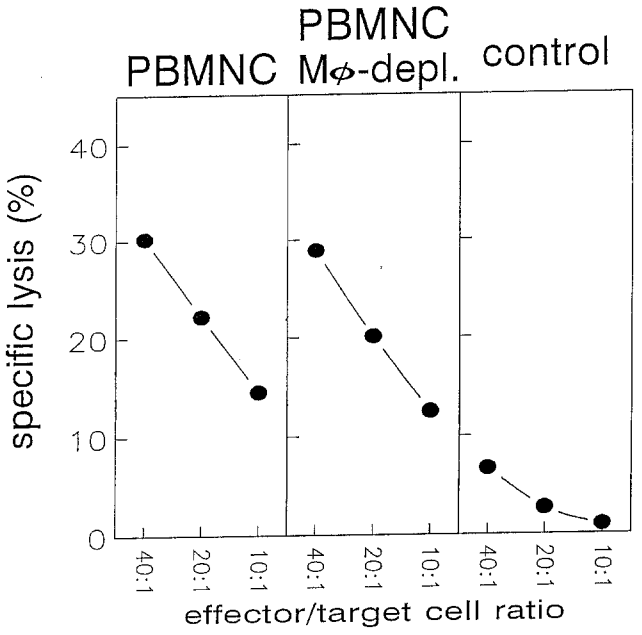

Fig. 1 Cytotoxicity of bacillus Calmette-Guérin (BCG)-activated killer (BAK) cells after macrophage depletion. Peripheral blood mononuclear cells (PBMNC) were cultured with or without BCG for an activation period of 7 days. Cytotoxicity of these BAK cells against BT-A bladder cancer cells was determined before (*PBMNC*) and after removal of macrophages by iron phagocytosis (*Mø-depl.*). Unstimulated PBMNC were used as controls. Each value plotted is the mean of three independent cultures, SD less than 15%

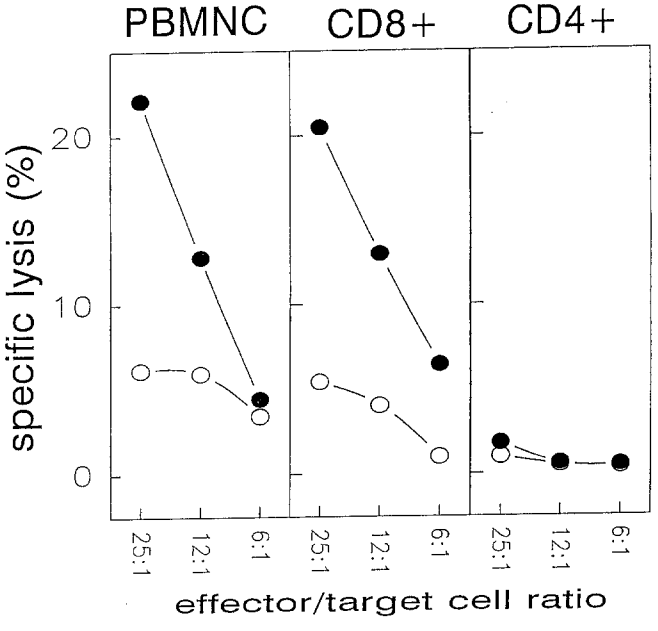

Fig. 2 Cytotoxicity of T-cell subpopulations against bladder carcinoma cells. PBMNC were cultured with (●) or without (○, control) BCG for 7 days. Cytotoxicity of these BAK cells was determined within the complete population (PBMNC), after positive selection (enrichment) of $CD8^+$ (suppressor/cytotoxic) T-cells ($CD8^+$), and after positive selection (enrichment) of $CD4^+$ (helper/inducer) T-cells ($CD4^+$). Target cells: BT-A; each value plotted is the mean of three independent determinations, SD less than 15%

Bladder carcinoma target cell line

A bladder carcinoma line (BT-A) derived from a patient with grade 3 transitional cell carcinoma was kindly provided by Dr. v.d. Bosch, Forschungsinstitut Borstel, Germany.

Effector cells

PBMNC were isolated from the peripheral blood of 15 normal PPD-positive healthy donors by density gradient centrifugation, washed repeatedly, and adjusted to a concentration of 1×10^6 cells/ml for stimulation. To exclude inter-individual differences between donors, each experiment was repeated three times with PBMNC of a different donor. The figures refer to one representative experiment out of these three.

Stimulation procedures

Stimulation procedures have been described in detail previously [6]. Briefly, stimulation of PBMNC was performed by coincubation over 7 days in medium (RPMI-1640 supplemented with 10% FCS) with viable BCG (Pasteur strain, Institut Mérieux, Leimen, Germany) at a concentration of 3.75×10^4 colony-forming units (cfu) per ml or with 100 U/ml recombinant human IFN-γ (kindly provided by Boehringer, Mannheim, Germany) at 37°C and 5% CO_2. To further test the involvement of IFN-γ in our assay system, a monoclonal antibody (mAb) neutralizing IFN-γ (clone GZ 4, Boehringer, Mannheim, Germany) was added at 20 µg/ml. Cytotoxicity of PBMNC cultured for 7 days without stimulus was referred to as control cytotoxicity.

Preparation of subpopulations of effector cells

Macrophages were magnetically depleted from PBMNC by the carbonyl-iron phagocytosis method, resulting in a purity of lymphocytes of more than 98%. $CD4^+$ (T helper/inducer) cells and $CD8^+$ (T suppressor/cytotoxicity) cells were enriched by positive selection using magnetic beads against these determinants (Dynabeads M-450-CD4, and M-450-CD8, Dynal, Hamburg, Germany), and detachment was performed by the detachabead system (Dynal). The enriched fractions were labelled (Dako, Glostrup, Denmark), and analyzed by flow cytometry (50H, Ortho Diagnostics, Neckargemünd, Germany). Purity of the enriched fraction was > 95%.

Negative selection (depletion) of $CD8^+$ cells was performed in the same way as described above from stimulated PBMNC, resulting in a concentration of < 5% of $CD8^+$ cells in the remaining population. Depletion of $CD56^+$ cells was performed by incubation with mouse anti-CD56 (Leu 19, Becton Dickinson, Heidelberg, Germany) followed by magnetic depletion using goat anti-mouse antibodies (Dynabeads M-450, Dynal), resulting in a concentration of less than 4% of $CD56^+$ cells in the remaining population.

Results

Coincubation of PBMNC with viable BCG resulted in the generation of cytotoxic effector cells against NK cell-resistant bladder carcinoma target cells, as had been shown previously [6]. The following in vitro experiments were performed to identify the subpopulations within PBMNC responsible for this BAK activity.

Analyses of subpopulations of PBMNC

Crude PBMNC were stimulated with BCG, and subpopulations were subsequently depleted from these cells and

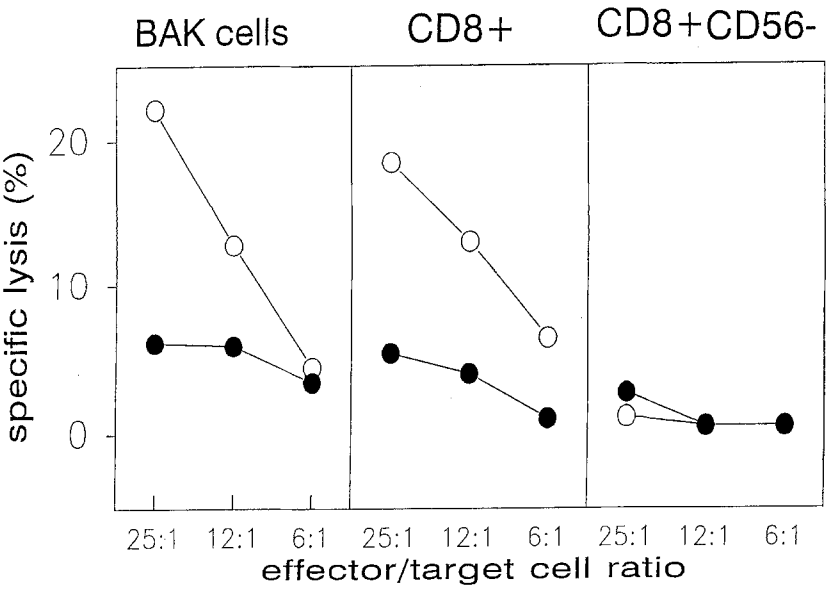

Fig. 3 Cytotoxicity of BAK cells in $CD8^+$ subpopulation. PBMNC were cultured with (○) or without BCG for 7 days. BCG-activated cytotoxicity of complete PBMNC population (BAK), within the $CD8^+$ subpopulation after enrichment ($CD8^+$), and after depletion of $CD56^+$ cells from this subpopulation ($CD8^+CD56^-$). Unstimulated PBMNC served as controls (●). Target cells: BT-A; each value plotted is the mean of three independent determinations, SD less than 15%

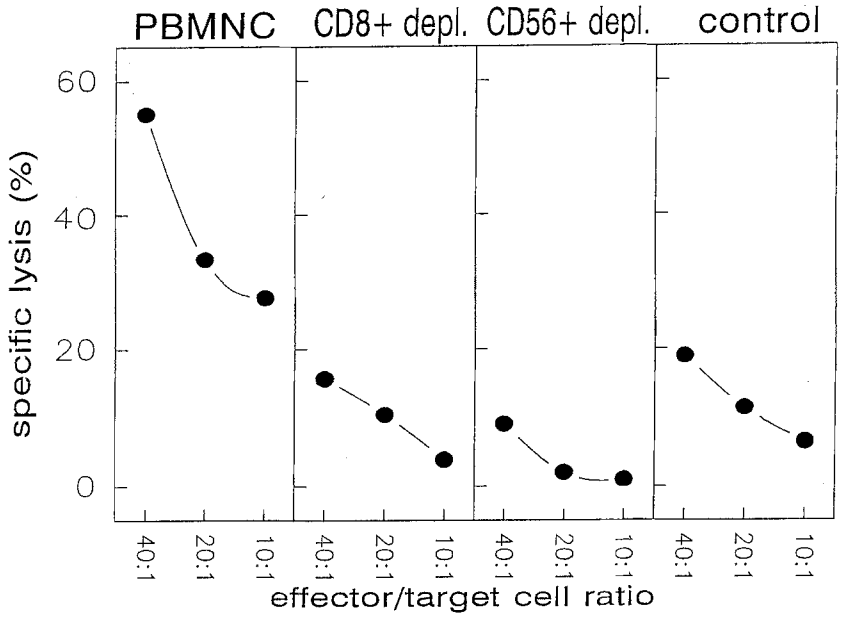

Fig. 4 Cytotoxicity of BAK cells after $CD8^+$ cell depletion (*$CD8^+$ depl.*) and after $CD56^+$ cell depletion (*$CD56^-$ depl.*) and within undepleted PBMNC population (*PBMNC*). Natural cytotoxic activity of unstimulated PBMNC is also shown for comparison (*control*). Target cells: BT-A; each value plotted is the mean of three independent determinations, SD less than 15%

analyzed for their cytolytic potential against bladder carcinoma targets. Cells from the control culture always showed a specific cytotoxicity of about 5–10% at an E/T ratio of 20 (25):1, depending on the donors used (Figs. 1–6). Depletion of macrophages from PBMNC after stimulation with BCG for 7 days revealed no decrease in BCG-induced cytotoxicity (Fig. 1). Furthermore, T-cell subpopulations were examined for their BAK potential against bladder cancer target cells. Positive selection (enrichment) of T-helper/inducer ($CD4^+$) and T-suppressor/cytotoxic ($CD8^+$) cells was performed and cytotoxicity was analyzed in each fraction. $CD4^+$ cells were not cytotoxic against BT-A tumor cells (Fig. 2), but within the $CD8^+$ fraction the cytolytic activity was detectable (Fig. 2). Further characterization of this subpopulation of cytotoxic cells was performed by depletion of $CD56^+$ cells from the $CD8^+$ subpopulation (Fig. 3). Removal of this $CD56^+$ fraction within the $CD8^+$ population abolished cytotoxicity, suggesting that the BAK effector cell bears both surface markers ($CD8^+$ and $CD56^+$). This was tested in a further experiment in which either $CD8^+$ or $CD56^+$ cells were removed from PBMNC after stimulation with BCG. Both procedures resulted in almost complete elimination of cytotoxicity against the bladder cancer cell line BT-A, confirming that the BAK effector cell is $CD8^+CD56^+$ (Fig. 4).

Further experiments showed that specific inhibition of IFN-γ by an antibody against IFN-γ, GZ-4, was able to interfere with the generation of BAK cells. The addition of GZ-4 at the beginning of coculture of PBMNC and BCG

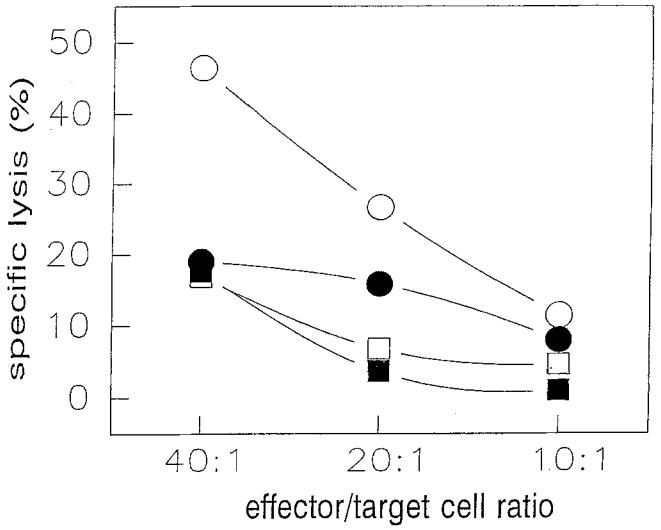

Fig. 5 Inhibition of BAK cell generation by gamma interferon antibody (anti-IFNγ). PBMNC were cultured with (○, BCG) or without (■, control) BCG in presence or absence of an antibody neutralizing interferon-γ [BCG + anti-IFN-γ (●), control + anti-IFN-γ) (□)]. After 7 days, cytotoxicity against BT-A bladder cancer cells was measured. Each value represents the mean of three independent determinations, SD less than 15%

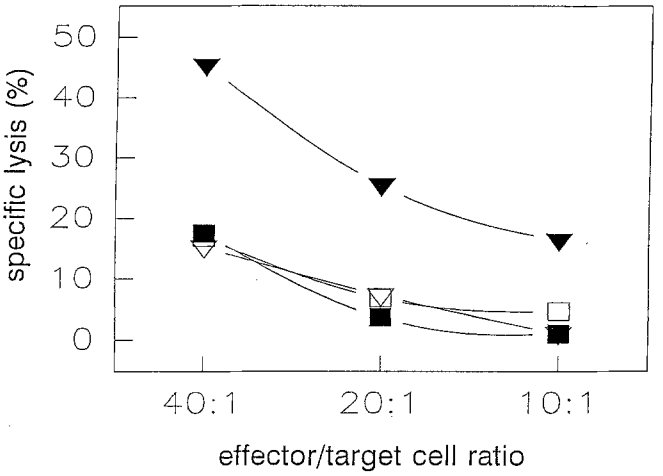

Fig. 6 Cytotoxicity induced by IFNγ and inhibition by IFNγ antibody. PBMNC were cultured with (▼)or without (■, control) IFNγ in presence or absence of an antibody neutralizing IFNγ [IFNγ + anti-IFNγ (▽), control + anti-IFNγ (□)]. After 7 days, cytotoxicity against BT-A bladder cancer cells was measured. Each value plotted is the mean of three independent determinations, SD less than 15%

significantly reduced BAK cytotoxicity against the bladder cancer cell line BT-A (Fig. 5). Stimulation of PBMNC with IFN-γ itself led to the induction of LAK cells against the NK-resistant bladder tumor cell line BT-A. This cytotoxicity was completely inhibited by GZ-4 (Fig. 6). The antibody itself had no stimulatory or inhibitory effects on PBMNC.

Discussion

During intravesical BCG immunotherapy, cytokines are released into the urine [4, 13, 23, 28]. Furthermore, several immunocompetent cell populations have been identified in the urine and in the bladder wall of patients, e.g., macrophages, T-helper/inducer cells, T-suppressor/cytotoxicity cells, and B-cells [1, 3, 8, 10, 21, 26, 29]. Recently, the degree of infiltration with immune cells has been correlated to a favorable response to therapy [24].

In a previous contribution we had begun to dissect this complex immune response in an in vitro model with regard to the relevance for the induction of cytotoxicity against bladder cancer cells [5, 6, 32]. We had shown that the cytokines found in the urine were unable to lyse bladder tumor cells. However, upon activation with viable BCG for 7 days, mononuclear cells became cytotoxic against NK cell-resistant bladder cancer cells. This cytotoxic effect has been termed the BAK cell phenomenon [6]. Whether BAK cells exert only tumor-specific cell-mediated lysis without damaging normal bladder urothelial cells remains to be investigated, but depends on normal urothelial cells growing in vitro. Unfortunately, they have not been established in our laboratory up to now. The generation of BAK cells can clearly be distinguished from other cytotoxic mechanisms mediated by BCG, such as in vitro boosting of NK-activity [17] or from NK and LAK cell activity by their target cell specificity and their prolonged time of activation which has been shown recently [6]. In continuation of these aforementioned studies, we tried to characterize the effector cells of the BAK cell phenomenon. We found that neither macrophages nor $CD4^+$ T-cells are effector cells. The cells responsible for BCG-induced cytotoxicity are T-cells bearing both $CD8^+$ and $CD56^-$ on their surface. This has been shown by depleting either cell population from BCG-induced PBMNC and, furthermore, by removal of the $CD56^+$ subfraction from the $CD8^+$ cells (Fig. 3, Fig. 4). These results are in accordance with both in vitro long-term cultures of highly cytotoxic LAK cells, in which exactly this phenotypic pattern predominates [12], and with in vivo results, where only responsive tumors of IL-2-treated patients showed a pronounced infiltration with $CD8^+$ cells [7]. Furthermore, it is well established that the $CD56^+$ NK cell is the predominant LAK effector cell from peripheral blood during systemic high-dose IL-2 therapy [19, 20, 22, 33]. However, our in vitro data need further confirmation by therapy accompanying immunohistological studies of bladder biopsies from BCG-treated patients using double-staining techniques. An interesting approach in this direction has been published by de Boer et al. [2], who reported that the $CD56^+$ lymphocyte population was present in small amounts in the urine of patients after intravesical BCG therapy.

In an animal model using immunocompetent mice, Ratliff et al. [25] showed that depletion of either $CD4^+$ or $CD8^+$ cells abrogated the BCG-induced antitumor response. In this system, $CD4^+$ cells seem to be necessary for the generation of the antitumor response. In our system, however, crude PBMNC were stimulated with BCG, and subsequently subpopulations were isolated and analyzed for their cytolytic potential against bladder carcinoma cells. This approach focuses on the characterization of the ultimate cytolytic effector cells and disregards any effects of other subpopulations during the initial process of activation. Thus, we cannot determine the function of macrophages or T-helper/inducer cells during the initial period of activation. Macrophages may be restricted to antigen presentation and/or production of proinflammatory cytokines. In the second line, activated $CD4^+$ cells might also produce cytokines, such as IFN-γ. Indeed, in a further step this cytokine was shown to be involved in the generation of BAK cells, as inhibition by a specific antibody abrogated BAK cell generation in our assay system. This IFN-γ-induced cytolytic activity is not mediated by macrophages, but by BAK cells; macrophages are not involved in BAK cell cytotoxicity (Fig. 1). Clinically, IFN-γ was demonstrated in the urine of patients after intravesical BCG [23], which underscores the importance of our in vitro findings. IFN-γ plays an important part in the generation of LAK cells in vitro and in vivo [9, 14], but also in the generation of BAK cells. Furthermore, it seems to be crucial for the host defense against mycobacteria [11, 18, 30, 31]. It is suggested, therefore, that this cytokine may represent the link between the immune reaction of the host against BCG and the generation of antitumor activity. However, our data are derived from a cell culture system and need further confirmation from in vivo treatment studies.

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