Cancer Immunol Immunother (1996) 42: 280–284

ORIGINAL ARTICLE

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Bacillus Calmette-Guérin plus interleukin-2 and/or granulocyte/macrophage-colony-stimulating factor enhances immunocompetent cell production of interferon-γ, which inhibits B16F10 melanoma cell growth in vitro

Received: 29 January 1996 / Accepted: 9 April 1996

Abstract Although immunotherapy with bacillus Calmette Guérin (BCG) is an established adjuvant treatment for malignant melanoma, the mechanism of its role in this process is unclear. To investigate the possible contribution of tumor-inhibitory cytokines induced by BCG, B16F10 melanoma cell growth in culture was assessed in response to purified cytokines and conditioned media of BCG-stimulated splenocytes. Interferon-γ (IFNγ) was the most potent single agent ($IC_{50} \approx 50$ pg/ml). Tumor necrosis factor α was substantially weaker (IC50 > 10 ng/ml) but provided synergy with IFNγ. None of the other cytokines such as interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-12, or granulocyte/macrophage-colony-stimulating factor had direct antitumor activity against B16F10 melanoma cells. However, when IL-2 and/or GM-CSF were combined with BCG either by exogenous addition or through endogenous production by novel cytokine-secreting recombinant BCG (rBCG), a substantial increase in INFγ production by splenocytes was observed. Antitumor activity of this conditioned medium directly correlated with IFNγ concentration and was completely blocked by neutralizing antibody to IFNγ. These results suggest that BCG may exert part of its antitumor action on melanoma through the induction of IFNγ, which can be greatly enhanced through the concomitant addition of IL-2 and/or GM-CSF. Furthermore, by utilizing rBCG that secrete these cytokines, it may be possible to potentiate the antitumor effect of BCG directly at the site of BCG inoculation.

Key words BCG · Melanoma · IFNγ · IL-2 · GM-CSF

This work was supported in part by NIH grant R29CA64230

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Introduction

Mycobacterium bovis, bacillus Calmette-Guérin (BCG), has demonstrated clinical activity against melanoma [5, 23]. However, its precise mechanism of action has remained elusive. BCG activates multiple cellular compartments in vivo and in vitro including $CD4^+$ and $CD8^+$ T cells, γδ T cells, B cells, macrophages and natural killer (NK) cells, and involves a complicated network of cytokines [8, 19, 20]. Interferon γ (IFNγ), a cytokine that plays a crucial role in controlling intracellular mycobacterial infection, has also been found to have direct antiproliferative effects against a number of tumor cell lines including melanoma [2, 6, 10, 15]. This study was undertaken to determine whether changes in the cytokine milieu at the time of BCG administration would alter the immune response more favorably in the direction of IFNγ production. It was found that the addition of exogenous interleukin-2 (IL-2) and/or granulocyte/macrophage-colony-stimulating factor (GM-CSF) substantially enhanced the ability of immunocompetent cells to produce IFNγ, which was growth-inhibitory to B16F10 melanoma cells. The potentiating effect of these costimulating cytokines was confirmed by using recombinant BCG species (rBCG) that express and secrete these cytokines. This methodology may provide a means to enhance the immune response directly at the site of BCG inoculation.

Materials and methods

Tumor cell line

B16F10 melanoma cells (a gift of Dr. Glenn Dranoff, Dana-Farber Cancer Institute, Boston, Mass.), were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM glutamine and 50 mg/ml gentamicin at 37 °C in a humidified incubator with 5% CO_2. The TF1 clone of the B16F10 cell line was established by a limiting-dilution method. The conditioned medium of B16F10 (TF1) melanoma cells contained no detectable interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-12, GM-CSF, IFNγ, or tumor necrosis factor α (TNFα).

Recombinant BCG

The development and characterization of mouse recombinant BCG secreting IL-2 (rBCG-IL-2) and its plasmid-transfected control (rBCG-p261) have been previously described [18]. rBCG secreting GM-CSF (rBCG-GM-CSF) was prepared in a similar manner (the gift of Peter Murray, Whitehead Institute, Cambridge, Mass.) [16]. Log-phase cultures of rBCG-IL-2 and RBCG-GM-CSF containing 1.3×10^7 colony-forming units (cfu)/ml (absorbance at 600 nm, A_{600} = 0.5) secreted approximately 2.0×10^4 pg/ml and 4.0×10^3 pg/ml IL-2 and GM-CSF respectively into the culture medium over 48 h.

Recombinant cytokines and antibodies

Recombinant mouse cytokines and capture and detecting antibodies for enzyme-linked immunosorbent assays (ELISA) were obtained commercially (Pharmingen, San Diego, Calif.; Genzyme, Cambridge, Mass.; Endogen, Cambridge, Mass.). Recombinant murine IL-12 and IL-12 antibodies were gifts of the Genetics Institute Inc. (Cambridge, Mass.) The detection limit for cytokine ELISA varied between 5 pg/ml and 20 pg/ml. Neutralizing rat anti-(mouse IFNγ) monoclonal antibody was obtained from Lee Biomolecular (San Diego, Calif.).

In vitro splenocyte cultures

Splenocytes from 6- to 8-week-old C57BL/6 female mice (Charles River, Wilmington, Mass.) were pooled from three to five mice per experiment. Procedures were performed according to NIH guidelines [17]. This protocol was approved by the Beth Israel Hospital Animal Care and Use Committee. Conditioned medium was prepared from 72-h cultures of 2×10^6 splenocytes/ml in the presence or absence of exogenous murine recombinant cytokines and BCG in RPMI-1640 medium supplemented with 10% FCS. Conditioned medium was assayed for IL-2, IL-4, IL-6, IL-10, IL-12, GM-CSF, IFNγ and TNFα by ELISA.

Assay system for antitumor effect

The direct antitumor effects of various cytokines or conditioned media of cultured splenocytes against B16F10 (TF1) melanoma cells were examined using the 4-h colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue, Sigma] assay [14]. B16F10 (TF1) cells were cultured for 72 h in Eagle's MEM supplemented with 10% FCS at a concentration of 1×10^4 cells/well in a volume of 2 ml on 24-well plates. In the screening assay, recombinant cytokine was added to a final concentration of 2.5×10^3 pg/ml. Conditioned medium was diluted at least fivefold with fresh medium to use in the tumor-growth-inhibition assay. The relative tumor growth rate (R_{tg}) and inhibition rate (R_i) were calculated according to the following formula:

$R_{tg} = 100\times[A$ on day 3 of treated sample$-A$ on day 0$)/(A$ on day 3 of untreated sample$-A$ day 0$)]$. $R_i = 100-R_{tg}$.

The concentration of cytokine that produced a 50% R_i was defined as IC_{50}.

Statistical analyses

All values were expressed as the mean ± one standard deviation. Statistical analysis was by Student's *t*-test using Statworks statistical software (Cricket Software Inc., Philadelphia, Pa.). All experiments were done in duplicate a minimum of three times with equivalent results. Figures were derived from representative experiments.

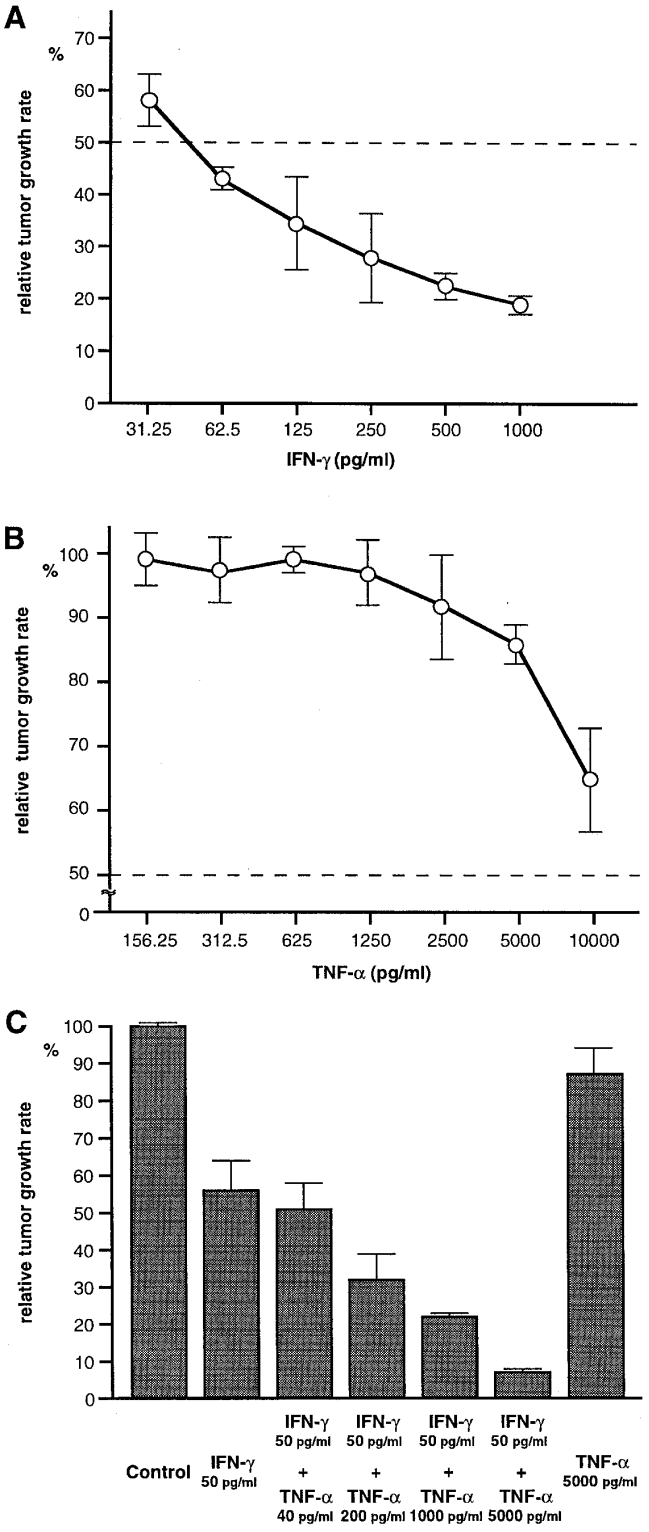

Fig. 1A–C Direct antitumor effect of (**A**) recombinant interferon γ (*IFNγ*), (**B**) tumor necrosis factor α (*TNFα*), and (**C**) the synergistic antitumor effect of IFNγ and TNFα against B16F10 (TF1) melanoma cells. B16F10 (TF1) cells were cultured for 72 h. Tumor cell growth was examined by using a colorimetric MTT assay and the relative tumor growth rate was calculated. Values are expressed as means ±SD from duplicate-well incubations

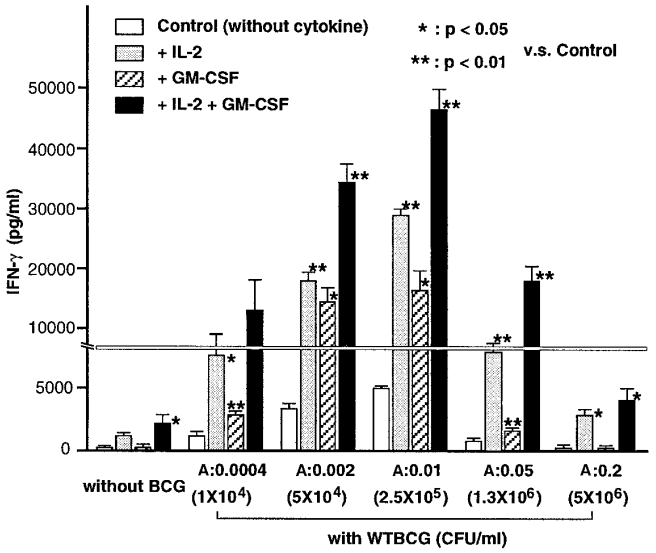

Fig. 2 IFNγ production of cultured splenocytes stimulated with various concentrations of wild-type bacillus Calmette-Guérin (*WTBCG*) and interleukin-2 (*IL-2*; 2000 pg/ml) and/or granulocyte/macrophage-colony-stimulating factor (GM-CSF; 1000 pg/ml). IFNγ production in the conditioned medium of cultured splenocytes was detected by ELISA. Values are expressed as means ± SD from duplicate-well incubations

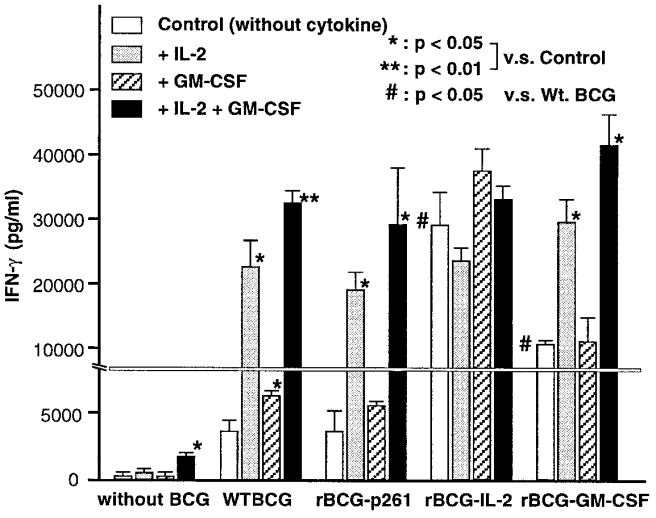

Fig. 3 IFNγ production of cultured splenocytes stimulated with various BCG and IL-2 (2000 pg/ml) and/or GM-CSF (1000 pg/ml). *WTBCG* wild-type BCG, *rBCG-p261* BCG transfected with the non-producer plasmid pMV261, *rBCG-IL-2* rBCG secreting IL-2, *rBCG-GM-CSF* rBCG secreting GM-CSF. Each BCG was used at a concentration giving A_{600} = 0.01 (2.5×10^5 cfu/ml). IFNγ production in the conditioned medium of cultured splenocytes was detected by ELISA. Values are expressed as means ± SD from duplicate-well incubations

Results

Direct antitumor effects of cytokines against B16F10 (TF1) melanoma cells

Among various cytokines, IFNγ was the most potent in inhibiting tumor cell growth. TNFα was only mildly inhibitory. The IC_{50} for IFNγ was 50 pg/ml and that of TNFα was greater than 10 ng/ml (Fig. 1A,B). IL-2, IL-4, IL-6, IL-10, IL-12, and GM-CSF did not directly inhibit tumor cell growth at concentrations as high as 2.5 ng/ml (data not shown). Among the cytokines tested, only TNFα exerted a dose-dependent synergistic inhibitory effect when combined with suboptimal concentrations of IFNγ (Fig. 1C). BCG exerted no significant direct effect on tumor cell growth at concentrations under A_{600} = 0.1 (2.5×10^6 cfu/ml) (data not shown).

BCG-stimulated cytokine production from splenocytes

No significant basal expression was detected from splenocytes in the absence of BCG or exogenous IL-2 or GM-CSF. In the group treated with IL-2 plus GM-CSF without BCG, there was a modest elevation in IFNγ production (Fig. 2). Splenocytes incubated with BCG produced modest amounts of IL-6, IL-10, and IL-12 but little to no IL-2 or IL-4, as has been previously reported [18]. GM-CSF and TNFα concentrations ranged between 100–300 pg/ml and 5–250 pg/ml respectively. IFNγ production followed a parabolic dose/response curve, with optimal production realized when BCG was present at a final concentration of A_{600} = 0.01 (2.5×10^5 cfu/ml). The basis for this parabolic dose/response curve is unknown and has been reported elsewhere [12].

The addition of either exogenous IL-2 or GM-CSF to the splenocyte cultures significantly enhanced BCG-stimulated IFNγ production while preserving the general shape of the dose/response curve. Maximal stimulation of IFNγ release (an approximately ninefold increase) occurred when both IL-2 and GM-CSF were present together and roughly equalled the sum of their solitary contributions. Recombinant BCG secreting IL-2 or GM-CSF produced similar results to those seen with exogenous cytokine addition. The IFNγ production induced by rBCG-IL-2 or RBCG-GM-CSF was equivalent to that induced by the combination of each cytokine and wild-type BCG. Additional exogenous IL-2 or GM-CSF did not further increase IFNγ production, indicating that a saturating level of endogenous stimulation was produced by the rBCG-IL-2 or rBCG-GM-CSF respectively (Fig. 3). In contrast to IFNγ, TNFα production was only minimally increased by adding IL-2 or GM-CSF to the BCG-stimulated splenocytes. There was no increased production of any of the other cytokines evaluated (data not shown).

The antitumor effect of conditioned medium from BCG-stimulated splenocytes

When the conditioned medium from BCG-stimulated splenocytes was used to treat B16F10 (TF1) melanoma cells in culture, tumor growth inhibition was entirely dependent on

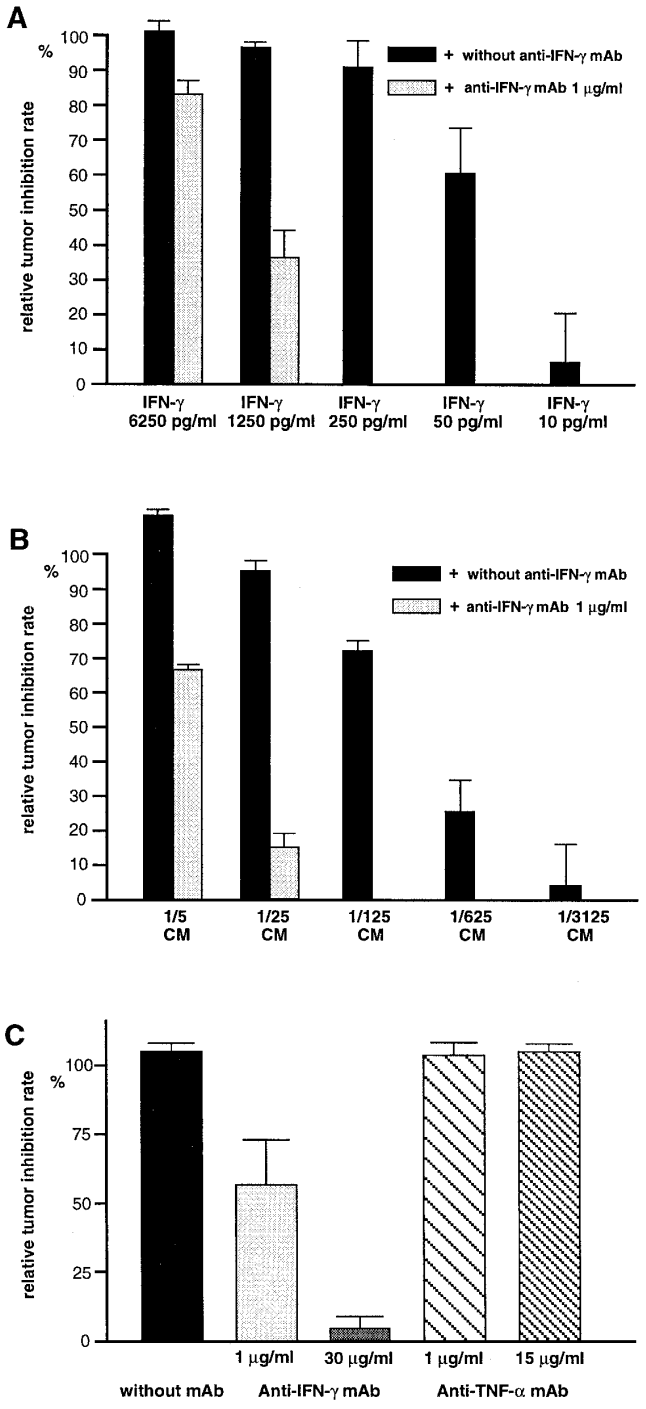

Fig. 4A–C Antiproliferative effect of (**A**) recombinant IFNγ and (**B**) conditioned medium (*CM*) of BCG + IL-2 + GM-CSF-stimulated splenocytes on B16F10 (TF1) melanoma cells with and without neutralizing anti-IFNγ monoclonal antibody. **C** Neutralization of 1/5-diluted conditioned medium with larger amounts of anti-IFNγ was highly effective in reversing tumor-growth inhibition while anti-TNFα antibody was ineffective (3 μg/ml antibody neutralizes approximately 1 ng/ml TNFα in L929 bioassay; data not shown). B16F10 (TF1) cells were cultured for 72 h. Tumor cell growth was examined by using a colorimetric MTT assay and the relative tumor inhibition rate was calculated. Values are expressed as means ± SD from duplicate-well incubations

IFNγ concentration (Fig. 4B) and similar in magnitude to that of recombinant IFNγ (Fig. 4A). This tumor-growth-inhibitory effect was blocked with neutralizing anti-(mouse IFNγ) antibody but not by neutralizing anti-(mouse TNFα) antibody (Fig. 4C).

Discussion

BCG remains an important component of melanoma vaccine strategies [5, 13, 23]. Despite decades of use, the antitumor mechanism of action of BCG has not been clearly elucidated. In this report, the possible role of antitumor cytokines produced by BCG-stimulated immunocompetent cells was examined and induction of IFNγ was identified as a potentially manipulatable antitumor mechanism. Whether local production of IFNγ can be induced in vivo by BCG and cytokines and lead to tumor-growth inhibition is unknown; however, IFNγ has emerged as an important tumor immunomodulator in several melanoma models [1, 2, 9].

After an analysis of an extensive panel of cytokines, it was determined that IFNγ possessed the strongest antiproliferative activity against a cloned subline of the widely used B16 murine melanoma (Fig. 1). The only other cytokine with similar activity was TNFα, which on a weight basis was at least 200 times less effective than IFNγ. The combination of IFNγ and TNFα demonstrated synergy, an observation that has been noted in other tumor systems [2, 25]. The basis for synergy against this particular tumor model remains unknown. Both transcriptional and receptor-mediated pathways in other tumor model systems have been described [4, 21].

While BCG can stimulate the production of several cytokines from immunocompetent cells, it appears that IFNγ is largely responsible for the in vitro activity of BCG against the B16 melanoma (Figs. 1, 4). As with several complex biological response modifiers, the dose/response characteristics are decidedly non-linear and, in the case of BCG, actually parabolic (Fig. 2) [12]. Maneuvers that amplify as well as extend the effective therapeutic dose/response range of BCG are thus likely to improve its efficacy and reliability.

The observed additive amplification of BCG-driven IFNγ production by both IL-2 and GM-CSF reported here (Figs. 2, 3) suggests two separate and independent mechanisms. It is unlikely that IL-2 simply preserves T cell viability during the 3-day incubation period as splenocyte viability and CD4/CD8 subset constitution are not appreciably altered by the presence of IL-2 (O′Donnell, unpublished observations). As IL-2 is normally produced only in appreciable quantity by previously primed T cells upon restimulation, it is noteworthy that dosing of BCG at multiple intervals, which would provoke such a memory response, is required for clinical antitumor efficacy [22]. Whatever the mechanism, it is possible that the early addition of exogenous IL-2 circumvents this requirement. The role of GM-CSF in this process is equally problematic. While GM-CSF

has traditionally been appreciated for its positive role in myeloid development, it has recently been found to be a powerful enhancer of antigen processing and presentation by dendritic cells [3, 11]. Conceivably, by enhancing BCG antigen processing, GM-CSF could magnify the immunostimulating features of BCG. A precedent for similar enhancing effects of GM-CSF on IFN release in cancer patients has been reported [23].

The development of recombinant BCG organisms with the capacity to secrete biologically active cytokines into their immediate microenvironment underscores the need both to define and to consider the therapeutic potential of combining cytokines with complex but effective biological response modifiers such as BCG. By secreting high-dose cytokine into the localized lesion, rBCG secreting cytokines may enhance tumor antigen presentation and local effector functions while avoiding systemic toxicity [7]. Certainly, in vivo studies will be required to determine whether these theories can be realized.

In conclusion, this study has demonstrated that it is possible to enhance the immunostimulatory effect of BCG on IFNγ production by immunocompetent cells by supplying co-stimulator cytokines such as IL-2 and/or GM-CSF. That the effects of IL-2 and GM-CSF appear additive, argues for the inclusion of both cytokines in future cancer therapy protocols involving BCG. Finally, with the advent of rBCG genetically engineered to express and secrete these cytokines, it may be possible to enhance local tumor immunity at the site of BCG inoculation.

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