

# IFN- $\alpha$ 2B Enhances Th1 Cytokine Responses in Bladder Cancer Patients Receiving *Mycobacterium bovis* Bacillus Calmette-Guérin Immunotherapy<sup>1</sup>

Yi Luo, Xiaohong Chen, Tracy M. Downs, William C. DeWolf, and Michael A. O'Donnell<sup>2</sup>

Combination therapy with intravesical bacillus Calmette-Guérin (BCG) plus IFN- $\alpha$  for superficial bladder cancer has been demonstrated to be more effective than either single agent alone in animal studies and of suggested greater efficacy in clinical studies. However, the mechanism by which IFN- $\alpha$  enhances BCG-mediated antitumor activity is poorly understood. Using PBMCs from bladder cancer patients, IFN- $\alpha$  was found to substantially enhance the efficacy of BCG to induce IFN- $\gamma$  production. Among 34 patients tested, 80% showed >4-fold increase. This effect of IFN- $\alpha$  was observed in both initial and memory responses to BCG. In addition, IFN- $\alpha$  up-regulated BCG-induced IL-12 and TNF- $\alpha$  and down-regulated BCG-induced IL-10. Neutralizing endogenous IL-10 or adding exogenous IL-12 provided further synergy for IFN- $\gamma$  production. In clinical practice, intravesical IFN- $\alpha$  2B (50 million units (MU)/dose) was observed to accelerate urinary IFN- $\gamma$  production to low-dose BCG (one-tenth or one-third of a full dose) in patients treated with combination therapy compared with BCG alone. These results suggest that IFN- $\alpha$  is a potent BCG enhancer that polarizes the BCG-induced immune response toward the cellular immune pathway by promoting Th1 cytokine expression and reducing Th2 cytokine expression. This study provides an immunological basis for future rational use of IFN- $\alpha$  in conjunction with intravesical BCG for bladder cancer immunotherapy. *The Journal of Immunology*, 1999, 162: 2399–2405.

Bladder cancer is the fifth most common malignant disease in the United States with an annual incidence of ~53,000 new cases and 12,000 deaths, respectively (1). Although intravesical administration of bacillus Calmette-Guérin (BCG)<sup>3</sup> has been accepted as the most effective therapy for superficial bladder cancer and carcinoma in situ of the bladder, ~25–40% of patients never respond to BCG therapy. Furthermore, long term remission (>5 yr) is only achieved in 50% of patients (2–5). Toxicity associated with the BCG therapy is frequent with occurrence of severe adverse effects in 5% of patients and life-threatening symptoms in 0.5% of patients (6, 7).

Intravesical IFN- $\alpha$  administered at doses of 50–100 million units (MU) in 50 ml of physiologic saline has been demonstrated to possess clinical efficacy with minimal local and systemic toxicity in treating superficial bladder cancer; however, its efficacy and durability are clearly inferior to BCG alone (8–11). Because of these limitations, an alternative schedule with concomitant administration of low-dose BCG plus IFN- $\alpha$  was recently proposed. Bercoff et al. (12) evaluated the safety and efficacy of the combination therapy in a double arm randomized study of 36 patients treated with one-half dose BCG and 10 MU of IFN- $\alpha$  2B vs full-dose BCG and observed that the combination therapy had less side effects but similar efficacy. Stricker et al. (13) conducted a similar study of 12 patients and reported that combination therapy was

well tolerated and yielded a high complete response rate. Safety and efficacy studies of combination therapy in murine models are also very encouraging. BCG plus IFN- $\alpha$  or BCG plus the IFN inducer broprimine were observed to be more effective than either agent alone (14, 15). The limited pilot clinical trials and animal studies have shown that adding IFN- $\alpha$  to BCG bladder cancer immunotherapy could lower BCG toxicity due to the reduced BCG dose while at the same time preserving or enhancing BCG activity against tumors.

As a potent immune stimulator, BCG has revealed its ability to efficiently elicit both humoral and cellular immune responses. However, antitumor immunity induced by intravesical BCG in bladder cancer therapy appears to require involvement of the cellular immune pathway (16). Studies on animal bladder tumor models have shown that the ability to either prevent or retard the outgrowth of tumor with BCG relies on T lymphocytes. BCG is unable to render antitumor activity in athymic mice unless T lymphocytes are reconstituted from immunocompetent animals before BCG therapy (17). Similarly, BCG loses its ability to induce antitumor activity in immunocompetent mice if they are first depleted in vivo of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells (18). In humans, the immunological basis for BCG anti-bladder cancer activity is also manifested. A typical delayed type hypersensitivity response in the bladder can be immunohistologically observed after clinical BCG instillation, showing a mononuclear cell infiltration into the superficial layers of the bladder (19, 20). The major types of infiltrating cells consist of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages, and NK cells. Elevated expression of MHC class I and II Ags on the urothelium is also apparent (21). This phenomenon is associated with the expression of IFN- $\gamma$  and TNF- $\alpha$  (21). Coincident with the prominent T cell infiltration, a massive burst of Th1 cytokines (IFN- $\gamma$ , IL-2, IL-12, and TNF- $\alpha$ ) appears in patients' urine after BCG instillation (22–25), suggesting that an active cellular immune response is occurring in the bladder. In contrast to the clinical responders, patients who fail BCG treatment show in their serum higher Ab responses to BCG heat shock proteins and a

Division of Urology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

Received for publication April 28, 1998. Accepted for publication October 22, 1998.

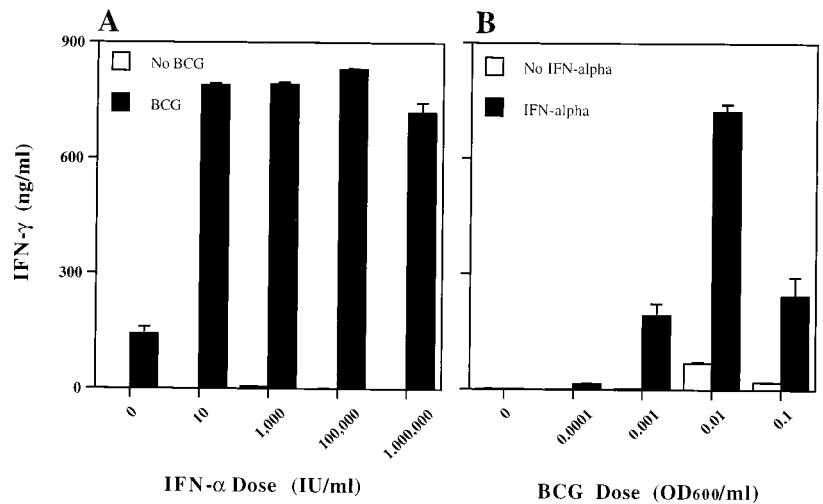
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by National Institutes of Health Grants R29CA64230 and RR01032, and a grant from Schering Corporation.

<sup>2</sup> Address correspondence and reprint requests to Dr. Michael A. O'Donnell, Division of Urology, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. E-mail address: Modonnell@bidmc.harvard.edu

<sup>3</sup> Abbreviation used in this paper: BCG, bacillus Calmette-Guérin.

**FIGURE 1.** Dose response of IFN- $\gamma$  production in human PBMC culture. *A*, PBMCs were incubated with various doses of IFN- $\alpha$  in the presence or absence of BCG (0.01 OD<sub>600</sub>/ml). *B*, PBMCs were incubated with various doses of BCG in the presence or absence of IFN- $\alpha$  ( $1 \times 10^5$  IU/ml). IFN- $\gamma$  in the cultures was quantitated and the values were expressed as means  $\pm$  SD from triplicate-well incubations.



urinary cytokine profile with a higher level of IL-6 and/or IL-10, the restricted Th2 cytokines that inhibit the Th1 immune response by orienting it toward a humoral immune pathway (25–27).

Although both clinical trials and animal studies that use combination therapy have shown advantages, the mechanism by which IFN- $\alpha$  enhances BCG-mediated antitumor activity is poorly understood. IFN- $\alpha$ , a cytokine initially discovered as an antiviral agent (28), has proven itself to be effective in antitumor immunity (29, 30). Its antitumor activity is thought to be associated with its ability to promote Th1 immune response (31–34). Previously, we reported that IFN- $\alpha$  was pharmacologically compatible with intravesical preparations of BCG (35). In this study, we explore the mechanism through which IFN- $\alpha$  acts on BCG for the induction of enhanced antitumor immunity by focusing on its regulation of Th1 cytokine production. Because IFN- $\gamma$  is the predominant Th1 cytokine observed in clinical responders undergoing BCG therapy (25), the expression of this cytokine was evaluated in this study to define the acquisition of a Th1 immune response.

## Materials and Methods

### BCG and IFN- $\alpha$

MV261 BCG, a Pasteur strain previously transfected with the kanamycin resistance plasmid pMV261 (36), was used in the *in vitro* experiments. This strain had been shown to possess very similar immunostimulatory properties to that of commercial lyophilized BCG preparations. This BCG strain was routinely kept at 37°C in 7H9 Middlebrook broth (Difco, Detroit, MI) supplemented with 10% albumin dextrose concentrate (5% BSA, 2% dextrose and 0.85% NaCl), 0.05% Tween 80 (Sigma, St. Louis, MO), and 30  $\mu$ g of kanamycin/ml. One unit of absorbance at 600 nm for the BCG culture was calculated as  $2.5 \times 10^7$  CFU. For clinical intravesical therapy, lyophilized preparations of BCG (TheraCys; Connaught Pasteur Merieux, Ontario, Canada) was used. Human rIFN- $\alpha$  2B (Intron A, Schering, Kenilworth, NJ) was used for both *in vitro* and *in vivo* studies.

### PBMC culture

In accordance with the approved clinical protocol at our institution, blood samples were collected from bladder cancer patients before intravesical BCG therapy and immediately before the 6th intravesical dose. PBMCs were prepared from buffy coat leukocytes purified on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Viability by trypan blue exclusion usually exceeded 95%. PBMCs were suspended in RPMI 1640 medium containing 10% FCS and 30  $\mu$ g/ml of kanamycin, and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator at a density of  $8 \times 10^5$  cells/200  $\mu$ l/well in 96-well tissue culture plates in the presence or absence of designated doses of BCG, IFN- $\alpha$  2B, or both. In most experiments, an IFN- $\alpha$  2B concentration of  $1 \times 10^5$  IU/ml was selected to approximate the concentration applied clinically into the bladder. In some experiments human recombinant cytokines IL-12 (Genetics Institute, Cambridge, MA) and IL-10 (PharMingen, San Diego,

CA) or neutralizing Abs: goat anti-human IL-12 (R&D Systems, Minneapolis, MN), mouse anti-human IL-10 (R&D Systems), and rabbit anti-human IFN- $\alpha$  (Pepro Tech, Rocky Hill, NJ) were further used to determine the immune pathway of IFN- $\alpha$  action. The plates were incubated for 72 h and then frozen at  $-70^\circ\text{C}$  until cytokine ELISA assays were performed.

### Urine samples

Previously, we assayed voided urine samples collected from different times after intravesical BCG therapy for IFN- $\gamma$  and found that >90% of urinary IFN- $\gamma$  appeared within the first 2–12 h after therapy. Thus, voided urine during that period of time was collected and pooled for later analysis. Urine samples were stabilized during patient collection with a concentrated buffer containing 2 M Tris-HCl (pH 7.6), 5% BSA, 0.1% sodium azide, and four protease inhibitors (aprotinin, pepstatin and leupeptin at 0.01 mg/ml for each and 4-(2-amino ethyl)benzenesulfonyl fluoride (AEBSF) at 0.1 mg/ml; all purchased from Sigma). At the end of collection, the volume of the 10-h urine was recorded. A 10-ml sample was further preserved by the addition of a protease inhibitor mixture tablet (Boehringer Mannheim, Mannheim, Germany) and then stored at  $-70^\circ\text{C}$  before batch analysis for cytokines by ELISA.

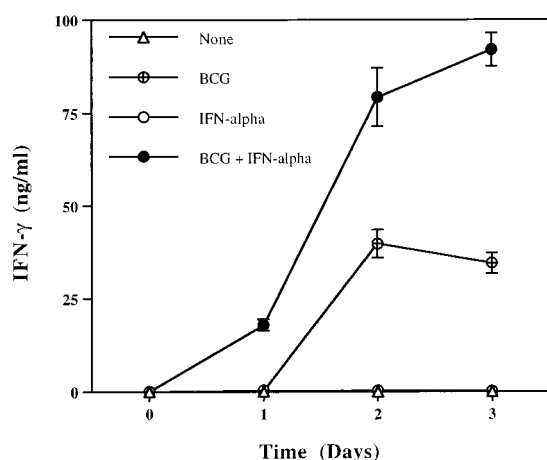
### ELISA assays and reagents

ELISA reagents including recombinant human cytokines and paired monoclonal capture and detecting Abs for the cytokines were obtained from Endogen (Cambridge, MA) for IFN- $\gamma$ , from Genetics Institute for IL-12, and from PharMingen for TNF- $\alpha$ , IL-6, and IL-10. Samples of conditioned PBMC cultures and urine collections were assayed by ELISA using a sandwich format according to the manufacturer's instructions. Cytokine concentrations were calculated in standard mass/volume format using standard curves derived from purified recombinant cytokine standards. For all of the above measured cytokines, one IU is equal to  $\sim 50$ –100 pg of purified cytokine.

## Results

### Enhancement of BCG-induced IFN- $\gamma$ production by IFN- $\alpha$ 2B

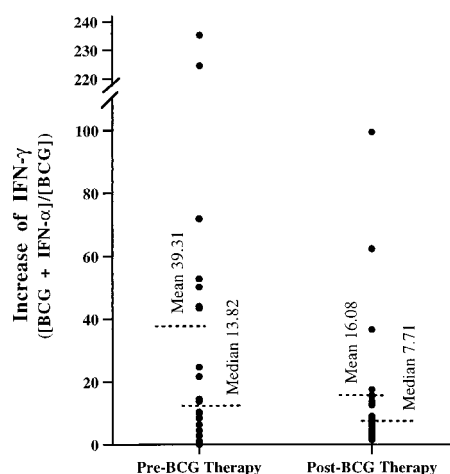
BCG has the ability to stimulate PBMCs in culture to produce IFN- $\gamma$ ; however, the quantity of IFN- $\gamma$  produced depends on the applied BCG dose and the individual patient's sensitivity to BCG. Although IFN- $\alpha$  had minimal ability by itself to induce IFN- $\gamma$  from PBMCs, it synergistically increased IFN- $\gamma$  production induced by BCG (Fig. 1A). This effect of IFN- $\alpha$  on BCG-stimulated PBMCs was so potent that even small doses of IFN- $\alpha$  (10 IU/ml) could significantly augment the capacity of BCG to induce IFN- $\gamma$  production (5-fold increase). Several other experiments using different patient PBMCs confirmed that saturation routinely occurs between 10 and 100 IU/ml of IFN- $\alpha$ . IFN- $\alpha$  exerted its effect in a BCG dose-dependent manner, preserving the bell-shaped dose-response curve seen with BCG alone whereby the optimum BCG



**FIGURE 2.** Time course of IFN- $\gamma$  production in human PBMC cultures. PBMCs were incubated with medium, BCG ( $0.01 \text{ OD}_{600}/\text{ml}$ ), IFN- $\alpha$  ( $1 \times 10^5 \text{ IU}/\text{ml}$ ), or BCG plus IFN- $\alpha$  for up to 3 days. IFN- $\gamma$  in the cultures was quantitated and the values were expressed as means  $\pm$  SD from triplicate-well incubations.

dose also yielded the maximum production of IFN- $\gamma$  for combination therapy (Fig. 1B). A kinetic study of IFN- $\gamma$  production showed an accelerated onset of IFN- $\gamma$  production which was maintained throughout the 3-day culture (Fig. 2).

The effect of IFN- $\alpha$  on BCG-induced IFN- $\gamma$  production in PBMC culture was further evaluated in 34 patients. Among them, 22 patients furnished PBMCs from both pre-BCG therapy and preweekly treatment number 6. To compare individual patients to one another, the values were numerated as a ratio of BCG plus IFN- $\alpha$  to BCG alone (Fig. 3). Eighteen of 22 patients (82%) in the pre-BCG therapy group showed  $>4$ -fold increase. The overall mean of increase was 39.3-fold with median of 13.8-fold. Similarly, 17 of 22 patients (77%) in post-BCG therapy group showed  $>4$ -fold increase. However, the overall mean of increase in this group was 16.1-fold with median of 7.7-fold. The lower fold increase of IFN- $\gamma$  in the latter group was due to the increased sensitivity of immune competent cells to BCG alone after repeated intravesical BCG therapy and a maximum limit to total IFN- $\gamma$  production. Only three patients in the pre-BCG group showed no IFN- $\alpha$  effect and one patient in the post-BCG group showed  $<2$ -fold increase. Analysis of IFN- $\gamma$  production in the remaining 12 unpaired PBMC cultures further agreed with this positive effect of IFN- $\alpha$  with an overall mean increase of 12.0-fold and median of 5.0-fold (data not shown).



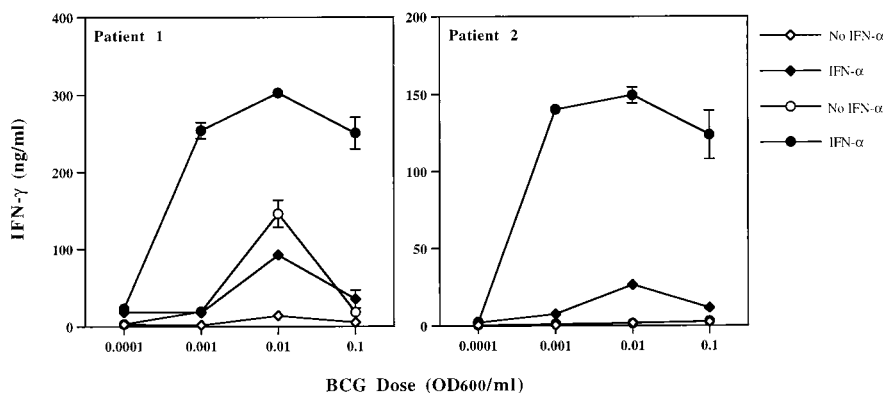
**FIGURE 3.** Effect of IFN- $\alpha$  on BCG-induced IFN- $\gamma$  production in human PBMC cultures. Human PBMCs obtained from 22 patients before intravesical BCG therapy and after five doses of BCG were incubated with BCG alone ( $0.01 \text{ OD}_{600}/\text{ml}$ ) or BCG (same dose) plus IFN- $\alpha$  ( $1 \times 10^5 \text{ IU}/\text{ml}$ ). The quantity of IFN- $\gamma$  in the cultures was measured and represented as the ratio of [BCG + IFN- $\alpha$ ] to [BCG]. Means and medians are indicated for each group.

#### Augmentation of IFN- $\gamma$ production by IFN- $\alpha$ 2B during the memory response to BCG

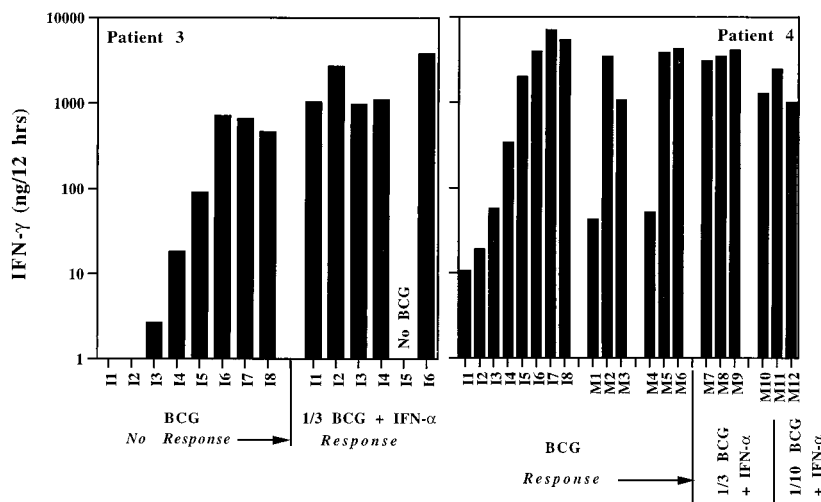
IFN- $\alpha$  has been observed to affect both initial and memory responses to BCG stimulation. As shown in Fig. 4, PBMCs obtained from two patients before initial BCG therapy and after the fifth dose were compared for their IFN- $\gamma$  production in response to escalating doses of BCG alone or BCG plus IFN- $\alpha$ . In both cases, the addition of IFN- $\alpha$  to BCG during the naive period generated a response similar to or higher than that achieved during the later memory period with BCG alone. Furthermore, the addition of IFN- $\alpha$  to BCG during the later period not only markedly increased IFN- $\gamma$  but also broadened the shape of the dose-response curve such that even very low doses of BCG ( $\text{OD}_{600} = 0.001$ ) were equipotent to doses 100 times higher.

Analysis of urinary IFN- $\gamma$  production agreed with the observations found from PBMC cultures. Two patients had urinary cytokine monitoring during the induction phase of intravesical BCG alone therapy and then were switched over to combination therapy during either a repeat induction phase (patient 3) or during a maintenance phase (patient 4). Maintenance was given as three doses once a week per course at interval of 3–6 mo (Fig. 5). Typically, urinary IFN- $\gamma$  production gradually increases during the initiation

**FIGURE 4.** Effect of IFN- $\alpha$  on the induction of IFN- $\gamma$  in a memory response to BCG. Human PBMCs obtained from two patients before intravesical BCG therapy (diamond) and after five doses of BCG (circle) were incubated with various doses of BCG with (solid) or without (open) IFN- $\alpha$  ( $1 \times 10^5 \text{ IU}/\text{ml}$ ). IFN- $\gamma$  in the cultures was quantitated and the values are represented as means  $\pm$  SD from triplicate-well incubations.



**FIGURE 5.** Effect of IFN- $\alpha$  on urinary IFN- $\gamma$  production in response to BCG. The urine of two patients was collected for the first 2–12 h after each intravesical treatment and assayed for IFN- $\gamma$ . The values are represented as the total IFN- $\gamma$  mass produced during each of these 12-h periods. Letters I and M stand for induction and maintenance therapy, respectively. The clinical status of the patients in response to therapy is indicated in italics.



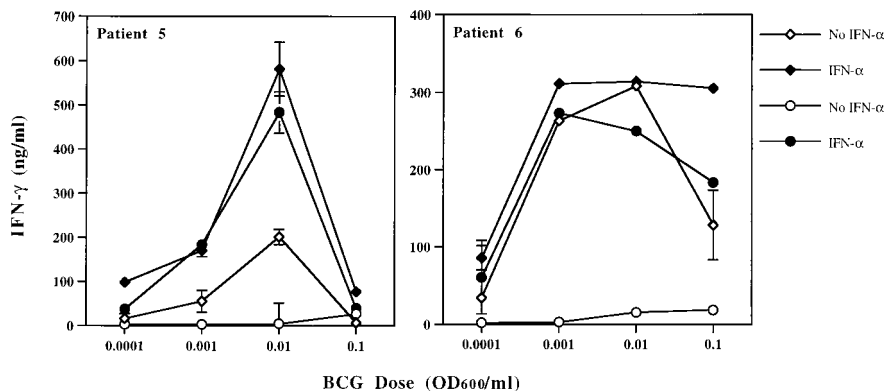
of induction therapy. Adding IFN- $\alpha$  to subsequent therapy “jump” started the induction of IFN- $\gamma$  at the first retreatment, even at reduced BCG doses of one-third or one-tenth of standard dose. It is noteworthy from the analysis of patient 3 that IFN- $\alpha$  favored induction of IFN- $\gamma$  only when combined with BCG (2nd I5 treatment with IFN- $\alpha$  alone) supporting the conceptual synergy effect of IFN- $\alpha$  on BCG stimulation. The failure of IFN- $\alpha$  to induce urinary IFN- $\gamma$  has subsequently been confirmed in three other patients receiving IFN- $\alpha$  monotherapy (data not shown).

#### *Restoration of IFN- $\gamma$ production by IFN- $\alpha$ -2B in response to BCG tachyphylaxis*

In this study, we observed that about 20% of patients displayed an early peak production of urinary IFN- $\gamma$ , which then diminished progressively during the remainder of therapy. Such developed immune resistance to BCG (tachyphylaxis) may result from an exaggerated sensitivity to repetitive BCG exposure (37). In accordance with this idea, PBMCs from several of these patients already showed high sensitivity to BCG and produced large amounts of IFN- $\gamma$  in culture before BCG therapy (Fig. 6). Patient 6, for instance, was also known to be purified protein derivative positive on skin testing before BCG treatment. However, by the completion of BCG therapy IFN- $\gamma$  production by these PBMCs was actually suppressed. IFN- $\alpha$  was observed to attenuate this suppression and restore the immune response in most cases.

#### *Accentuation of Th1 immune response to BCG by IFN- $\alpha$ 2B*

In addition to IFN- $\gamma$ , IFN- $\alpha$  enhanced other Th1 cytokine (IL-12 and TNF- $\alpha$ ) production from BCG-stimulated PBMCs (Fig. 7).

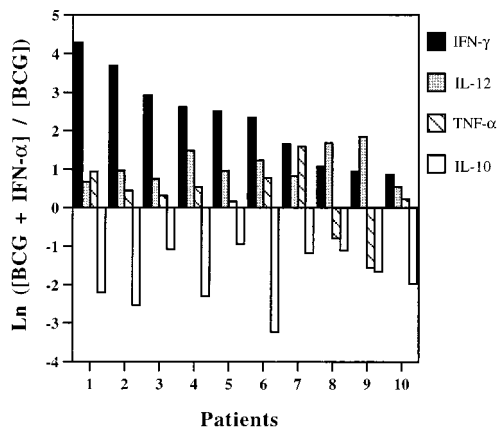


**FIGURE 6.** Effect of IFN- $\alpha$  on restoration of IFN- $\gamma$  production in response to BCG tachyphylaxis. Human PBMCs obtained from two patients before intravesical BCG therapy (diamond) and after five doses of BCG in the presence (solid) or absence (open) of IFN- $\alpha$  ( $1 \times 10^5$  IU/ml). IFN- $\gamma$  in the cultures was quantitated and the values are illustrated as means  $\pm$  SD from triplicate-well incubations.

IL-12 increased in all 10 patients tested (100%) and TNF- $\alpha$  increased in 8 of the 10 patients (80%). Expression of IL-10, an antagonist of the Th1 response, was suppressed by the action of IFN- $\alpha$  in all 10 patients. In a separate experiment involving five other patient PBMCs, IL-6, another Th2 cytokine, was observed to decrease correlatively with IL-10 in the presence of IFN- $\alpha$ . The extent of reduction for IL-6 averaged between 20 and 30% compared with the more substantial drop of 80–90% for IL-10. This reciprocal effect on Th1 vs Th2 cytokines indicated that adding IFN- $\alpha$  polarized the BCG immune response toward Th1. To confirm that such polarization of immune response resulted from IFN- $\alpha$  addition, a neutralizing Ab to IFN- $\alpha$  was used (Fig. 8). Clearly, the Ab could block IFN- $\alpha$  effects on BCG-induced IFN- $\gamma$  and IL-10. The down-regulatory effects of IL-10 and up-regulatory effects of IL-12 on IFN- $\gamma$  production was further demonstrated by experiments in which exogenous cytokines (IL-10 or IL-12) or neutralizing Abs (anti-IL-10 or anti-IL-12) were added (Fig. 9). Surprisingly, although additional IL-12 significantly augmented IFN- $\gamma$  production, neutralizing anti-IL-12 Ab had minimal effect. This suggests that there may be another non-IL-12-dependent pathway involved in the up-regulation of IFN- $\gamma$  production from BCG-stimulated human PBMCs.

## Discussion

Combination therapy with intravesical BCG plus IFN- $\alpha$  has been demonstrated to be as effective as, if not more than, either single agent alone in both clinical and animal studies (12–15). These two biologicals appear to work synergistically via different pathways. Intravesical BCG usually results in massive pyuria coincident with



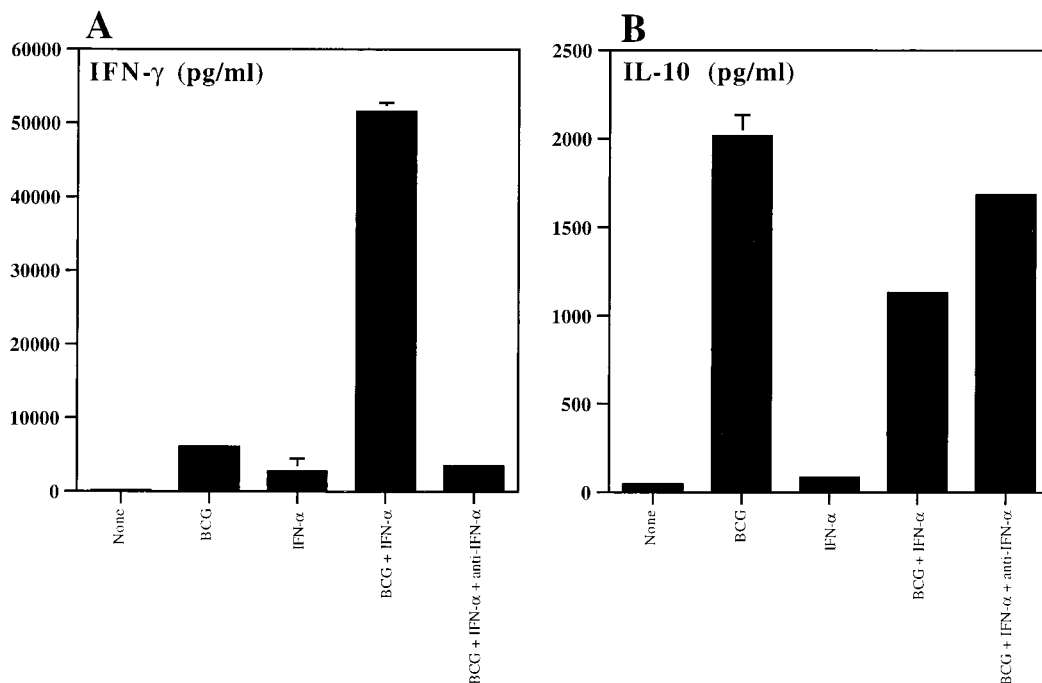
**FIGURE 7.** Effect of IFN- $\alpha$  on BCG-induced Th1 and Th2 cytokine responses. Human PBMCs from 10 patients were incubated with BCG alone (0.01 OD<sub>600</sub>/ml) or BCG (same dose) plus IFN- $\alpha$  ( $1 \times 10^5$  IU/ml). Cytokines IFN- $\gamma$ , IL-12, TNF- $\alpha$ , and IL-10 in the cultures were quantitated and their values are represented as Ln ([BCG + IFN- $\alpha$ ]/[BCG]).

the release of various cytokines into the bladder (19, 24, 38, 39). In contrast, IFN- $\alpha$  is notably absent in causing these phenomena (38). IFN- $\alpha$  has been observed to decrease tumor proliferation (40, 41), up-regulate MHC class I expression on target cells (42), activate local NK cells (43), increase both IFN- $\gamma$  producing and cytolytic CD4<sup>+</sup> T cells (31, 32), and suppress Th2 cells (32, 33). In the present study, we investigated the mechanism by which IFN- $\alpha$  enhances BCG-mediated antitumor activity. We observed that IFN- $\alpha$  polarized the immune response to BCG in the direction of the Th1 pathway by up-regulating the production of the Th1 cytokines IFN- $\gamma$ , IL-12, and TNF- $\alpha$  and by down-regulating the expression of Th2 cytokines IL-6 and IL-10. This observation may

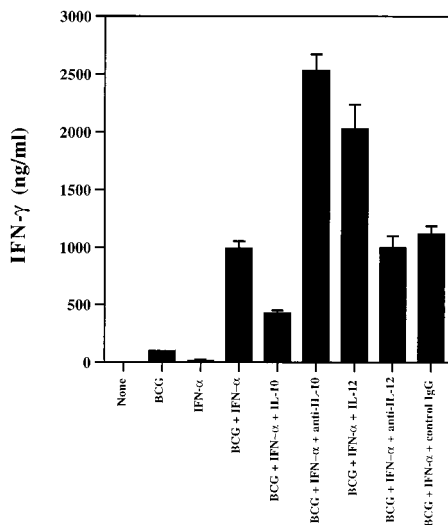
provide an immunological basis for future rational drug use and development.

In acquired immunity, Th lymphocytes orchestrate an immune response toward the cellular or humoral pathway under the instruction of immune modulators (44). Despite the key roles of IL-12 and IL-4 in regulation of these two distinct responses, IFN- $\gamma$  and IL-10, two antagonistic cytokines, also actively participate in controlling Th cell development (45–47). IFN- $\gamma$  activates the Th1 response and inhibits the Th2 response, whereas IL-10 blocks the Th1 response and favors the Th2 response. Dominance of one cytokine over the other critically influences the ultimate direction of the immune pathway. Because intravesical BCG therapy for bladder cancer is felt to be strongly influenced by cellular and humoral T cell responses, accentuation of IFN- $\gamma$  expression and/or curtailment of IL-10 production may increase the efficacy of this therapy.

In this study, IFN- $\alpha$  showed a potent effect on BCG-induced IFN- $\gamma$  production, although it had no obvious effect on induction of this cytokine by itself. This up-regulated IFN- $\gamma$  production was associated with elevation in IL-12 induction. IL-12 is known to originate primarily from macrophages and acts as an upstream positive regulator for IFN- $\gamma$  production from NK and Th1 cells (48–52). However, neutralizing Ab to IL-12 was essentially ineffective in reversing IFN- $\alpha$ -induced synergy with BCG. There are several possible explanations for this paradox. 1) Our IL-12 ELISA does not discriminate between the active IL-12 p70 heterodimer and the inactive or inhibitory p40 homodimer; therefore, biostimulatory p70 may not have been induced. 2) The level of induction of IL-12 may have been insufficient to be responsible for the observed synergy. In most cases the IL-12 measured was in the range of 5–40 pg/ml, close to its limit of bioactivity. 3) The Ab may not have been sufficient to block the majority of endogenous IL-12. The Ab did effectively block exogenous human rIL-12 even



**FIGURE 8.** Blockage of IFN- $\alpha$  effects on BCG-induced IFN- $\gamma$  and IL-10 by neutralizing anti-IFN- $\alpha$  Ab. Human PBMCs were incubated with BCG (0.01 OD<sub>600</sub>/ml) plus IFN- $\alpha$  (10 IU/ml) in the presence or absence of anti-IFN- $\alpha$  Ab (5  $\mu$ g/ml). As controls for the costimulation, PBMCs were incubated with medium, BCG (same dose) or IFN- $\alpha$  (same dose). IFN- $\gamma$  and IL-10 in the cultures were quantitated and the values are illustrated as means  $\pm$  SD from triplicate-well incubations.



**FIGURE 9.** Effects of IL-10 and IL-12 on IFN- $\gamma$  production in response to costimulation with BCG plus IFN- $\alpha$ . Human PBMCs were incubated with BCG (0.01 OD<sub>600</sub>/ml) plus IFN- $\alpha$  ( $1 \times 10^5$  IU/ml) in the presence or absence of IL-10 (5,000 pg/ml), anti-IL-10 Ab (3  $\mu$ g/ml), IL-12 (200 pg/ml), anti-IL-12 Ab (3  $\mu$ g/ml), or control IgG (3  $\mu$ g/ml). As controls for the costimulation, PBMCs were incubated with medium, BCG (same dose) or IFN- $\alpha$  (same dose). IFN- $\gamma$  in the cultures was quantitated and the values are illustrated as means  $\pm$  SD from triplicate-well incubations.

at doses of IL-12 10 times that endogenously produced (data not shown); however, the Ab may still have allowed enough endogenous IL-12 to function especially if very short distance paracrine effects were responsible. Overall, the most likely explanation is that a non-IL-12 pathway may be responsible. For instance, IFN- $\gamma$ -inducing factor, now also referred to as IL-18 (53, 54), is capable of substantially enhancing the efficacy of BCG to induce IFN- $\gamma$  production from a mouse splenocyte culture system (our unpublished observations).

Down-regulation of the Th2 cytokine IL-10 by the costimulation of BCG plus IFN- $\alpha$  is also likely to have contributed to the enhanced IFN- $\gamma$  production. This finding was supported by the observation that adding exogenous IL-10 in the costimulation PBMC cultures could reduce IFN- $\gamma$  production, whereas further depleting endogenous IL-10 by adding neutralizing Ab could increase IFN- $\gamma$  production. In both experimental cell culture and clinical intravesical therapy, BCG has been observed to induce IL-10 amplification from immune cells after induction of IFN- $\gamma$  (25, 55). Dominance of IL-10 over IFN- $\gamma$  results in suppression of BCG-mediated antitumor activity as observed in clinical nonresponders of bladder cancer to intravesical BCG (25). A heightened BCG antitumor effect along with increased IFN- $\gamma$  release is also seen in genetically deficient IL-10 knockout mice bearing transplantable bladder tumors (T. L. Ratliff, unpublished data).

Our limited clinical observations, although as yet insufficient to prove Th1 polarization occurs during combination treatment because of coincident reductions in BCG dose, are nonetheless consistent with this effect. For the two patients represented in Fig. 5, upon the switch to combination BCG plus IFN- $\alpha$  therapy, the mean ratio of IFN- $\gamma$  to IL-10 increased from 25.8 to 529 for patient 3 and from 7.6 to 9.4 for patient 4. Additionally, we have also previously reported a statistically significant increase in IFN- $\gamma$  to IL-10 ratios for 20 nonrandomized patients sequentially treated (10 each) with BCG plus IFN- $\alpha$  vs BCG alone (56). A double-blind, randomized clinical trial comparing BCG with BCG plus IFN- $\alpha$  is underway to validate these preliminary observations.

Further exploration into the detailed mechanism through which IFN- $\alpha$  regulates immune response to BCG is currently under investigation. Our preliminary results with immune subset isolation and reconstitution suggest that adherent PBMCs (primarily macrophages) by themselves are responsible for the bulk of IL-10 production after BCG exposure and that IFN- $\alpha$  can directly down-regulate this production. However, only mixed cultures of macrophages with either CD4<sup>+</sup> or CD8<sup>+</sup> cells have the ability to produce IFN- $\gamma$  after BCG and/or BCG plus IFN- $\alpha$  stimulation; purified T cell subsets are incapable. Furthermore, the augmenting effect of IFN- $\alpha$  is markedly attenuated if it is given 24 h before or after BCG exposure. This need for cellular cooperation between the macrophage and the T cell compartments for generating IFN- $\gamma$  is not altogether surprising because similar observations have previously been noted for another bacterially derived Th1-polarizing product, streptococcal cell wall preparation OK432 (57).

Although the evidence that IFN- $\alpha$  enhances Th1 immune responses to BCG in vitro is fairly clear, it is not known if these laboratory observations will translate into meaningfully improved clinical responses. Our pilot clinical trial using Intron A plus BCG for the treatment of aggressive superficial bladder cancer is currently underway and the preliminary data from our open-label combination therapy program is encouraging with >70% complete response rates for BCG failures (58). However, a portion of these patients remain recalcitrant to immunotherapy. How to circumvent their insensitivity remains to be explored.

## References

- Parker, S. L., T. Tong, S. Bolden, and P. A. Wingo. 1996. Cancer statistics, 1996. *CA-Cancer J. Clin.* 46:5.
- Kavoussi, L. R., R. J. Torrence, D. P. Gillen, M. A. Hudson, E. O. Haaff, S. M. Dresner, T. L. Ratliff, and W. J. Catalona. 1988. Results of 6 weekly intravesical bacillus Calmette-Guérin instillations on the treatment of superficial bladder tumors. *J. Urol.* 139:935.
- Dejager, R., P. Guinan, and D. Lamm. 1991. Long-term complete remission in bladder carcinoma in situ with intravesical Tice bacillus Calmette-Guérin. *Urology* 38:507.
- Herr, H. W., C. M. Pinsky, and W. F. Whitmore. 1986. Long-term effect of intravesical bacillus Calmette-Guérin on flat carcinoma in situ of the bladder. *J. Urol.* 135:265.
- Lamm, D. L. 1992. Long-term results of intravesical therapy for superficial bladder cancer. *Urol. Clin. North Am.* 19:573.
- Steg, A., S. Adjiman, and B. Debre. 1992. BCG therapy in superficial bladder tumours: complications and precautions. *Eur. Urol.* 21 (Suppl. 2):35.
- Lamm, D. L., A. D. P. M. Van Der Meijden, A. Morales, S. A. Brosman, W. J. Catalona, H. W. Herr, M. S. Soloway, A. Steg, and F. M. J. Debruyne. 1992. Incidence and treatment of complications of bacillus Calmette-Guérin intravesical therapy in superficial bladder cancer. *J. Urol.* 147:596.
- Torti, F. M., L. D. Shortliffe, R. D. Williams, W. C. Pitts, R. L. Kempson, J. C. Ross, J. Palmer, F. Meyers, M. Ferrari, J. Hannigan, et al. 1988.  $\alpha$ -Interferon in superficial bladder cancer: a Northern California Oncology Group study. *J. Clin. Oncol.* 6:476.
- Glashan, R. W. 1990. A randomized controlled study of intravesical  $\alpha$ -2b-interferon in carcinoma in situ of the bladder. *J. Urol.* 144:658.
- Williams, R. D. 1988. Intravesical interferon  $\alpha$  in the treatment of superficial bladder cancer. *Semin. Oncol.* 15:10.
- Chodak, G. 1989. Intravesical interferon treatment of superficial bladder cancer. *Urology* 34:84.
- Bercovich, E., M. Deriu, F. Manferrari, and G. Irianni. 1995. BCG vs. BCG plus recombinant  $\alpha$ -interferon 2b in superficial tumors of the bladder. *Arch. Ital. Urol. Androl.* 67:257.
- Stricker, P., K. Pryor, T. Nicholson, D. Goldstein, D. Golovsky, R. Ferguson, P. Nash, S. Ehsman, J. Rumma, G. Mammen, and R. Penny. 1996. *Bacillus Calmette-Guérin* plus intravesical interferon  $\alpha$ -2b in patients with superficial bladder cancer. *Urology* 48:957.
- Sarosdy, M. F., and C. A. Kierum. 1989. Combination immunotherapy of murine transitional cell cancer using BCG and an interferon-inducing pyrimidinone. *J. Urol.* 142:1376.
- Riggs, D. R., W. F. Tarry, J. I. DeHaven, J. Sosnowski, and D. L. Lamm. 1992. Immunotherapy of murine transitional cell carcinoma of the bladder using  $\alpha$  and  $\gamma$  interferon in combination with other forms of immunotherapy. *J. Urol.* 147:212.
- Ratliff, T. L. 1992. Role of the immune response in BCG for bladder cancer. *Eur. Urol.* 21 (Suppl. 2):17.
- Ratliff, T. L., D. P. Gillen, and W. J. Catalona. 1987. Requirement of a thymus dependent immune response for BCG-mediated antitumor activity. *J. Urol.* 137:155.

18. Ratliff, T. L., J. K. Ritchey, J. J. Yuan, G. L. Andriole, and W. J. Catalona. 1993. T-cell subsets required for intravesical BCG immunotherapy for bladder cancer. *J. Urol.* 150:1018.
19. Peuchmaur, M., G. Benoit, A. Viellefond, A. Chevalier, G. Lemaigre, E. D. Martin, and A. Jardin. 1989. Analysis of mucosal bladder leukocyte subpopulations in patients treated with intravesical bacillus Calmette-Guérin. *Urol. Res.* 17:299.
20. Kelley, D. R., E. O. Haaff, M. Becich, J. Lage, W. C. Bauer, S. M. Dresner, W. J. Catalona, and T. L. Ratliff. 1986. Prognostic value of PPD skin test and granuloma formation in patients treated with intravesical bacillus Calmette-Guérin. *J. Urol.* 135:268.
21. Prescott, S., K. James, T. B. Hargreave, G. D. Chisholm, and J. F. Smyth. 1992. Intravesical Evans strain BCG therapy: quantitative immunohistochemical analysis of the immune response within the bladder wall. *J. Urol.* 147:1636.
22. de Reijke, T. M., E. C. de Boer, K. H. Kurth, and D. H. Schamhart. 1996. Urinary cytokines during intravesical bacillus Calmette-Guérin therapy for superficial bladder cancer: processing, stability and prognostic value. *J. Urol.* 155:477.
23. Bohle, A., C. Nowc, A. J. Ulmer, J. Musehold, J. Gerdes, A. G. Hofstetter, and H. D. Flad. 1990. Elevations of cytokines interleukin-1, interleukin-2 and tumor necrosis factor in the urine of patients after intravesical bacillus Calmette-Guérin immunotherapy. *J. Urol.* 144:59.
24. Jackson, A.M., A.B. Alexandroff, R.W. Kelly, A. Skibinska, K. Esuvaranathan, S. Prescott, G.D. Chisholm, and K. James. 1995. Changes in urinary cytokines and soluble intercellular adhesion molecule-1 (ICAM-1) in bladder cancer patients after *Bacillus Calmette-Guérin* (BCG) immunotherapy. *Clin. Exp. Immunol.* 99:369.
25. O'Donnell, M. A., X. Chen, and W. C. DeWolf. 1996. Maturation of the cytokine immune response to BCG in the bladder: implications for treatment schedules. *J. Urol.* 155:1030A.
26. Esuvaranathan, K., A. B. Alexandroff, M. McIntyre, A. M. Jackson, S. Prescott, G. D. Chisholm, and K. James. 1995. Interleukin-6 production by bladder tumors is upregulated by BCG immunotherapy. *J. Urol.* 154:572.
27. Zlotta, A. R., A. Drowart, K. Huygen, J. De Bruyn, H. Shekarsarai, M. Decock, M. Pirson, F. Jurion, K. Palfiet, O. Denis, et al. 1997. Humoral response against heat shock proteins and other mycobacterial antigens after intravesical treatment with bacille Calmette-Guérin (BCG) in patients with superficial bladder cancer. *Clin. Exp. Immunol.* 109:157.
28. Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proc. R. Soc. London Ser. B.* 147:258.
29. Baron, S., S. K. Tying, W. R. Fleischmann Jr., D. H. Coppenhaver, D. W. Niesel, G. R. Klimpel, G. J. Stanton, and T. K. Hughes. 1991. The interferons: mechanisms of action and clinical applications. *JAMA* 266:1375.
30. Pestka, S., J. A. Langer, K. C. Zoon, and C. E. Samuel. 1987. Interferons and their actions. *Annu. Rev. Biochem.* 56:727.
31. Brinkmann, V., T. Geiger, S. Alkan, and C. H. Heusser. 1993. Interferon  $\alpha$  increases the frequency of interferon  $\gamma$ -producing human CD4<sup>+</sup> T cells. *J. Exp. Med.* 178:1655.
32. Parronchi, P., M. De Carli, R. Manetti, C. Simonelli, S. Sampognaro, M. Piccinni, D. Macchia, E. Maggi, G. del Prete, and S. Romagnani. 1992. IL-4 and IFN ( $\alpha$  and  $\gamma$ ) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. *J. Immunol.* 149:2977.
33. Brinkmann, V., C. H. Heusser, J. Baer, E. Kilchherr, and F. Erard. 1992. Interferon  $\alpha$  suppresses the capacity of T cells to help antibody production by human B cells. *J. Interferon Res.* 12:267.
34. Wenner, C. A., M. L. Guler, S. E. Macatonia, A. O'Garra, and K. M. Murphy. 1996. Roles of IFN- $\gamma$  and IFN- $\alpha$  in IL-12-induced T helper cell-1 development. *J. Immunol.* 156:1442.
35. Downs, T. M., A. Szilvasi, and M. A. O'Donnell. 1997. Pharmacological biocompatibility between intravesical preparations of BCG and interferon- $\alpha$  2B. *J. Urol.* 158:1.
36. Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, et al. 1991. New use of BCG for recombinant vaccines. *Nature* 351:456.
37. Ratliff, T. L., and W. J. Catalona. 1989. Depressed proliferative responses in patients treated with 12 weeks of intravesical BCG. *J. Urol.* 141:230A (abstr.).
38. Keeley, J., F.X., E. Lattime, P. McCue, and L. G. Gomella. 1994. A comparison of the local immune response to intravesical  $\alpha$ -interferon and bacillus Calmette-Guérin (BCG) in patients with superficial bladder cancer. *J. Urol.* 151:473A (abstr.).
39. De Boer, E. C., W. H. De Jong, P. A. Steerenberg, L. A. Aarden, E. Tetteroo, E. R. De Groot, A. P. Van der Meijden, P. D. Veegt, F. M. Debruyne, and E. J. Ruitenberg. 1992. Induction of urinary interleukin-1 (IL-1), IL-2, IL-6 and tumor necrosis factor during intravesical immunotherapy with bacillus Calmette-Guérin in superficial bladder cancer. *Cancer Immunol. Immunother.* 34:306.
40. Pryor, K., P. Stricker, P. Russell, D. Golovsky, and R. Penny. 1995. Antiproliferative effects of bacillus Calmette-Guérin and interferon  $\alpha$ 2b on human bladder cancer cells in vitro. *Cancer Immunol. Immunother.* 41:309.
41. Borden, E. C., D. S. Groveman, T. Nasu, C. Renzikoff, and G. T. Bryan. 1984. Antiproliferative activities of interferons against human bladder carcinoma cell lines in vitro. *J. Urol.* 132:800.
42. Tzai, T.-S., and S.-N. Lin. 1992. Interferon- $\alpha$  can alter the lytic susceptibility of murine bladder transitional cell carcinoma (MBT-2) by their original poor specific cytotoxic tumor infiltrating lymphocytes. *J. Urol.* 147:523.
43. Trotta, P. 1985. Interferon  $\alpha$  2: summary of pre-clinical data. *Dev. Oncol.* 27:13.
44. Fearon, D. T., and R. M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. *Science* 272:50.
45. Sieling, P. A., J. S. Abrams, M. Yamamura, P. Salgame, B. R. Bloom, T. H. Rea, and R. L. Modlin. 1993. Immunosuppressive roles for IL-10 and IL-4 in human infection: in vitro modulation of T cell responses in leprosy. *J. Immunol.* 150:5501.
46. Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley. 1989. Reciprocal expression of interferon  $\gamma$  or interleukin 4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* 169:59.
47. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170:2081.
48. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 13:251.
49. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Scherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827.
50. Chan, S. H., B. Perussia, J. W. Gupta, M. M. P. Kobayashi, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri. 1991. Induction of IFN- $\gamma$  production by NK cell stimulatory factor (NKSF): characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869.
51. Scharton, T. M., and P. Scott. 1993. Natural killer cells are a source of interferon  $\gamma$  that drives differentiation of CD4<sup>+</sup> T cell subsets and induces early resistance to *Leishmania major* in mice. *J. Exp. Med.* 178:567.
52. Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on CD4<sup>+</sup> T cells to enhance priming for interferon- $\gamma$  production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 90:10188.
53. Okamura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, et al. 1995. Cloning of a new cytokine that induces IFN- $\gamma$  production by T cells. *Nature* 378:88.
54. Ushio, S., M. Namba, T. Okura, K. Hattori, Y. Nukada, K. Akita, F. Tanabe, K. Konishi, M. Micallef, M. Fujii, et al. 1996. Cloning of the cDNA for human IFN- $\gamma$  inducing factor, expression in *Escherichia coli* and studies on the biological activities of the protein. *J. Immunol.* 156:4274.
55. Sander, B., U. Skansen-Saphir, O. Damm, L. Hakansson, J. Andersson, and U. Andersson. 1995. Sequential production of Th1 and Th2 cytokines in response to live bacillus Calmette-Guérin. *Immunology* 86:512.
56. Downs, T. M., X. Chen, W. C. DeWolf, and M. A. O'Donnell. 1997. Urinary cytokine expression following intravesical BCG treatment and its correlation with patient symptoms. *Proc. 66th Annu. Meet. N. Engl. Sect. Am. Urol. Assn.* 66:118.
57. Fujimoto, T., R. B. Duda, A. Szilvasi, X. Chen, M. Mai, and M. A. O'Donnell. 1997. Streptococcal preparation OK-432 is a potent inducer of interleukin-12 and a Th1 dominant state. *J. Immunol.* 158:5619.
58. Belledgrun, A. S., J. R. Franklin, M. A. O'Donnell, L. G. Gomella, E. Klein, R. Neri, U. O. Nseyo, T. L. Ratliff, and R. D. Williams. 1998. Superficial bladder cancer: the role of interferon  $\alpha$ . *J. Urol.* 159:1793.