

Effect of interleukin 12 on tumor induction by 3-methylcholanthrene

(chemically induced tumors/interferon γ /nonspecific immunotherapy)

YUJI NOGUCHI, ACHIM JUNGLUTH, ELIZABETH C. RICHARDS, AND LLOYD J. OLD*

Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

Contributed by Lloyd J. Old, July 30, 1996

ABSTRACT Interleukin (IL)-12 has strong antitumor activity in transplantable tumor systems in the mouse. The present study was designed to determine whether tumor induction by 3-methylcholanthrene (3-MC), a carcinogenic hydrocarbon, can be inhibited by IL-12. BALB/cBy mice were injected subcutaneously with 25 μ g or 100 μ g of 3-MC and treated with 100 ng, 10 ng, or 1 ng of IL-12 for 5 days a week for 18 weeks, with a schedule of 3 weeks on and 1 week off. In mice injected with 25 μ g of 3-MC, treatment with 100 ng of IL-12 delayed tumor appearance and reduced tumor incidence. Tumor appearance was also delayed in mice injected with 100 μ g of 3-MC and treated with 100 ng of IL-12, but the final tumor incidence was the same as in non-IL-12-treated mice. In contrast to the characteristically round, hard, well-circumscribed, and protruding tumor induced by 3-MC, a percentage of tumors induced in IL-12-treated mice had atypical characteristics: flat, soft, and invasive. Atypical tumors had a longer latent period and were more frequently seen in mice injected with 100 μ g of 3-MC and treated with 100 ng of IL-12. Interferon γ , IL-10, and tumor necrosis factor could be induced throughout the treatment period by IL-12, indicating that repeated injections of IL-12 do not induce a state of tachyphylaxis. High production of interferon γ by CD8 T cells and a TH₂ \rightarrow TH₁ or TH₀ shift in the cytokine secretion profile of CD4 T cells were also seen in the IL-12-treated mice. IL-12 provides a powerful new way to explore the defensive role of the immune system in tumorigenesis.

Chemically induced tumors in inbred mice, particularly sarcomas induced by 3-methylcholanthrene (3-MC) or other carcinogenic hydrocarbons, have been a favorite model for tumor immunologists since the discovery of their antigenic properties by Gross (1), Foley (2), and Prehn and Main (3). The fundamental observation is that prior immunization with viable or irradiated tumor cells renders mice resistant to subsequent challenge with the same tumor, with the degree of immunogenicity of different tumors ranging from very strong to weak (4). One of the most striking features of chemically induced tumors (in contrast to virus-induced tumors) is their antigenic individuality, each tumor appearing to have its own distinctive antigen or complex of antigens not shared with other tumors similarly induced in the same inbred mice and even in the same mouse (5, 6). The molecular identity of the individually distinct antigens of chemically-induced tumors remains unknown, although a mutational origin, comparable to the tum⁻ antigens of mutagenized tumor cells (7), seems likely. Because of the strong immunogenicity of chemically induced tumors, it might be expected that their induction could be influenced by modulating immune reactivity. In fact, nonspecific activation of the immune system with *Mycobacterium bovis* bacillus Calmette-Guérin prolonged the latent period for tumor induction by 3-MC (8). However, the prediction that tumor

appearance would be faster and the frequency higher in immunosuppressed mice—e.g., nu/nu mice—has been difficult to prove, with a number of studies showing no differences between immunosuppressed and normal mice (9). The discovery of cytokines with strong immunomodulating activity has provided another approach to defining immunological influences on tumor induction. Studies with transplanted tumors have shown that interleukin (IL) 12 is one of the most powerful anti-tumor cytokines identified to date. In a number of tumor systems, systemic administration of IL-12 causes regression of established tumor transplants in a high proportion of mice (10–12). In the present study, we investigated the influence of IL-12 on the induction of 3-MC induced tumors in BALB/cBy mice.

MATERIALS AND METHODS

Mice. BALB/cBy female mice were purchased from The Jackson Laboratory and CB-17 female severe combined immunodeficient (SCID) mice were obtained from the animal facility at Memorial Sloan-Kettering Cancer Center.

Recombinant Murine IL-12. Recombinant murine IL-12 was kindly provided by Genetic Institute (Cambridge, MA). The IL-12 was diluted with PBS containing 1% syngeneic mouse serum and injected intraperitoneally in a volume of 0.2 ml 5 days a week, with an injection schedule of 3 weeks on and 1 week off for 18 weeks, or 15 weeks in the case of SCID mice.

Induction of Tumors by 3-Methylcholanthrene (3-MC). 3-MC (Sigma) was dissolved in peanut oil at a concentration of 125 μ g/ml or 500 μ g/ml and injected subcutaneously in a volume of 0.2 ml.

T-Cell Culture. CD4 and CD8 T cells were isolated from the spleen using magnetic beads (Dynal, Great Neck, NY), and isolated T cells (1×10^5 /ml) were cultured with 10 μ g of ConA per ml at a total volume of 200 μ l in 96-well plates for 3 days at 37° in 5% CO₂. Supernatants were then harvested and assayed for cytokines.

Cytokine and Nitrite Assays. Serum tumor necrosis factor (TNF)- α and interferon (IFN)- γ were assayed with ELISA kits from Genzyme (Cambridge, MA), serum IL-10 was assayed with an ELISA kit from R & D Systems, and serum nitrite was assayed with a detection kit from Alexis (San Diego). Serum IL-12 was assayed using two rat anti-mouse IL-12 mAbs (kindly provided by G. Trinchieri, Wistar Institute) as capture antibodies and a biotinylated rabbit polyclonal antibody against mouse IL-12 (kindly provided by M. K. Gately, Hoffmann-LaRoche) as a detection antibody. Culture supernatants were assayed for IL-4 and IFN- γ using protocols and reagents from PharMingen.

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Abbreviations: 3-MC, 3-methylcholanthrene; IL, interleukin; TNF, tumor necrosis factor; IFN- γ , interferon γ ; SCID, severe combined immunodeficiency.

*To whom reprint requests should be addressed.

RESULTS

Influence of IL-12 on Tumor Induction. Fig. 1 *a* and *b* show the effect of IL-12 on the induction of tumors in BALB/cBy mice injected with 25 μ g or 100 μ g of 3-MC. A significant delay in tumor appearance and a lower tumor incidence were seen

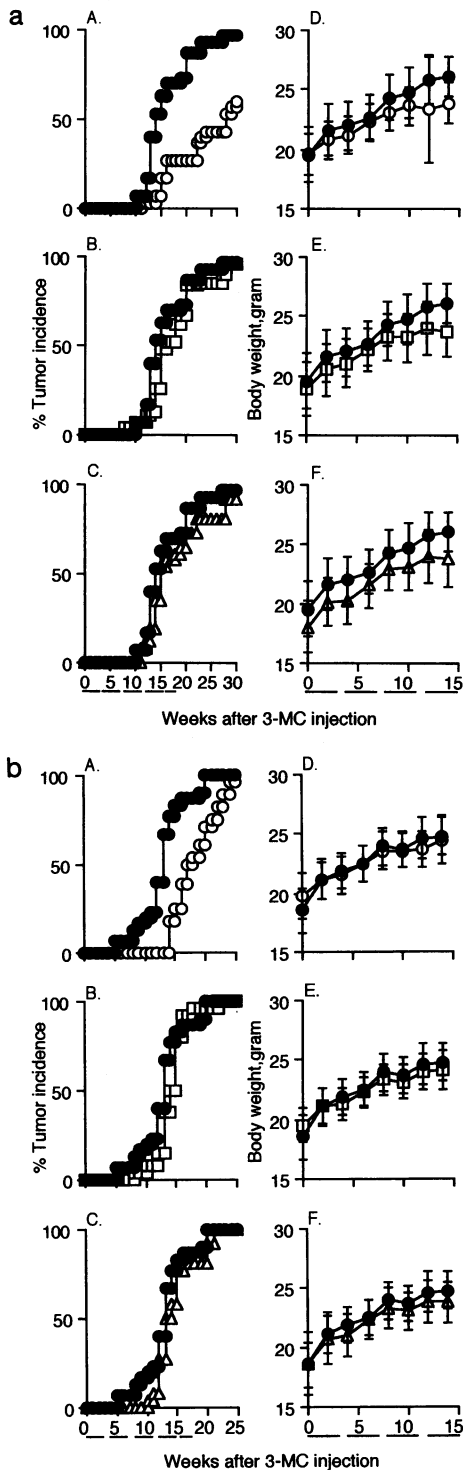


FIG. 1. Effect of IL-12 on tumor induction by 3-MC and on body weight. BALB/cBy female mice were injected with 25 μ g (*a*) or 100 μ g (*b*) of 3-MC and treated with 100 ng of IL-12 (○; *A* and *D*), 10 ng of IL-12 (□; *B* and *E*), or 1 ng of IL-12 (△; *C* and *F*). Control mice were injected with 25 μ g or 100 μ g of 3-MC and received no further injections (●). Each group consisted of 30 mice. Mice were injected intraperitoneally with IL-12, with an injection schedule of 3 weeks on and 1 week off. Lines at the bottom of figures indicate IL-12 treatment periods.

in mice injected with 25 μ g of 3-MC and treated with 100 ng of IL-12. In mice injected with 100 μ g of 3-MC, treatment with 100 ng of IL-12 delayed tumor appearance but did not reduce tumor incidence. Treatment with lower doses of IL-12 (10 ng and 1 ng) had little or no effect on tumor induction. The influence of the 100-ng IL-12 dose on 3-MC tumor induction was confirmed in a second experiment (Fig. 2). Mice treated with the various doses of IL-12 appeared active and healthy and showed weight gains comparable to untreated mice (Fig. 1 *a* and *b*). No difference in latent period or final tumor incidence was seen in CB-17 SCID mice injected with 25 μ g of 3-MC and treated with 100 ng of IL-12 (Fig. 3), indicating the importance of T cells in the protective effect of IL-12 on 3-MC tumor induction.

Appearance of Atypical Tumors in IL-12-Treated Mice. Tumors induced after subcutaneous injection of 3-MC are characteristically round or oblong and hard, have well-demarcated borders, and protrude up from the skin. An atypical pattern of tumor growth was observed in IL-12-treated mice, most frequently in mice treated with 100 μ g of 3-MC and injected with 100 ng of IL-12 (Table 1). These atypical tumors were not circumscribed, spreading laterally rather than protruding outward, were soft to palpation and were frequently ulcerated, and invaded the underlying peritoneum and peritoneal cavity. Atypical tumors tended to have longer latent periods than typical tumors and were the predominant tumor type occurring after stopping IL-12 treatment. Histological examination showed that tumors in IL-12-treated and untreated mice were sarcomas with no differences in tumor cell morphology noted between typical and atypical tumors.

Cytokine Production in IL-12-Treated Mice. Serum specimens from BALB/cBy mice injected with 25 μ g of 3-MC and treated with IL-12 were assayed for IL-12, IFN- γ , IL-10, TNF, and nitrite (Fig. 4). IL-12 increased to the same level during each course of 100-ng IL-12 treatment, indicating the absence of an antibody response to the recombinant IL-12 product. The highest level of serum IFN- γ was observed during the initial course of treatment with 100 ng of IL-12, but significant levels of IFN- γ continued to be induced by subsequent IL-12 injections. IL-10 remained elevated throughout the entire treatment course with 100 ng of IL-12. The 10-ng and 1-ng doses

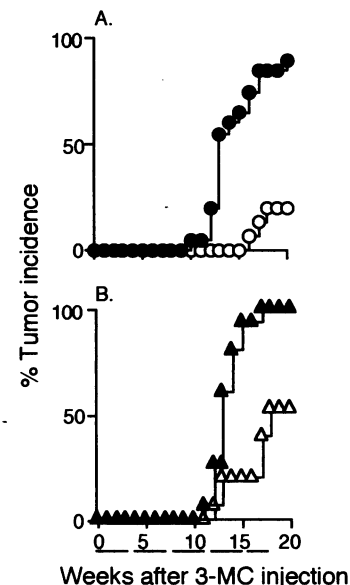


FIG. 2. Effect of IL-12 on tumor induction by 3-MC; results of a second experiment. BALB/cBy female mice were injected with 25 μ g (*A*) or 100 μ g (*B*) of 3-MC and treated with 100 ng of IL-12 (○ and △) or no treatment (● and ▲). Each group consists of 15 mice.

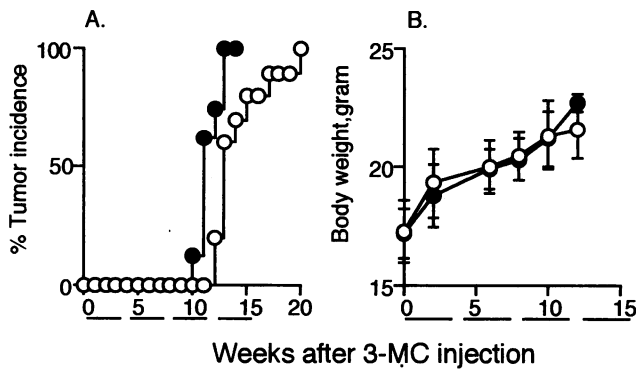


FIG. 3. Effect of 100 ng of IL-12 on tumor induction by 3-MC and on body weight in CB-17 SCID mice. CB-17 female SCID mice were injected with 25 μ g of 3-MC and treated with 100 ng of IL-12 (○) or no treatment (●). Each group consisted of 10 mice. Tumor frequency and body weights are shown in A and B, respectively.

of IL-12 had minimal or no effect on IFN- γ or IL-10 levels. No increase in serum TNF was seen during the initial course of 100-ng IL-12 treatment, but subsequent courses induced comparable peaks of TNF. Nitrite levels peaked during each course of IL-12 treatment.

Fig. 5 shows the influence of IL-12 treatment on cytokine production by separated CD4 and CD8 T cells from the spleen. Following a 3-day culture period with 10 μ g of Con A per ml, supernatants from the CD4 cultures were tested for IFN- γ and IL-4, and supernatants from the CD8 cultures were tested for IFN- γ . The cytokine secretion profile of CD4 cells from non-IL-12-treated mice is clearly a TH₂ pattern [low IFN- γ (3.4 \pm 0.6 units/ml) and high IL-4 (2030 \pm 689 pg/ml)], and CD8 cells from untreated mice produce very low levels of

Table 1. Frequency of atypical tumors in BALB/cBy mice injected with 3-MC and treated with various doses of IL-12

Dose of 3-MC, μ g	Dose of IL-12, ng	Atypical tumors, %
25	0	0
	1	15
	10	19
100	100	23
	0	0
	1	12
	10	8
	100	72

Data are from mice shown in Fig. 1 a and b. Observation period was 30 weeks. In IL-12-treated mice, the latent period was 14 \pm 2 weeks for typical tumors and 20 \pm 2 weeks for atypical tumors.

IFN- γ (16.6 \pm 6.5 units/ml). Treatment with 100 ng of IL-12 markedly increased IFN- γ production and decreased IL-4 production by CD4 cells, shifting the cytokine secretion profile from a TH₂ to a TH₁ pattern. Lower doses of IL-12 had a similar but generally less pronounced effect on IFN- γ and IL-4 production. IFN- γ production by CD8 cells was significantly augmented by IL-12, particularly the 100-ng dose.

DISCUSSION

Nonspecific approaches to cancer immunotherapy have their origin in the repeated observation that microbes and microbial products have strong antitumor activity (13). Three stages are distinguishable in the early history of nonspecific approaches: (i) Coley (14) initiated the approach with his clinical studies of mixed bacterial vaccines; (ii) Shear (15) took the next step by identifying endotoxin as the active component in Gram-negative bacteria responsible for tumor hemorrhagic necrosis;

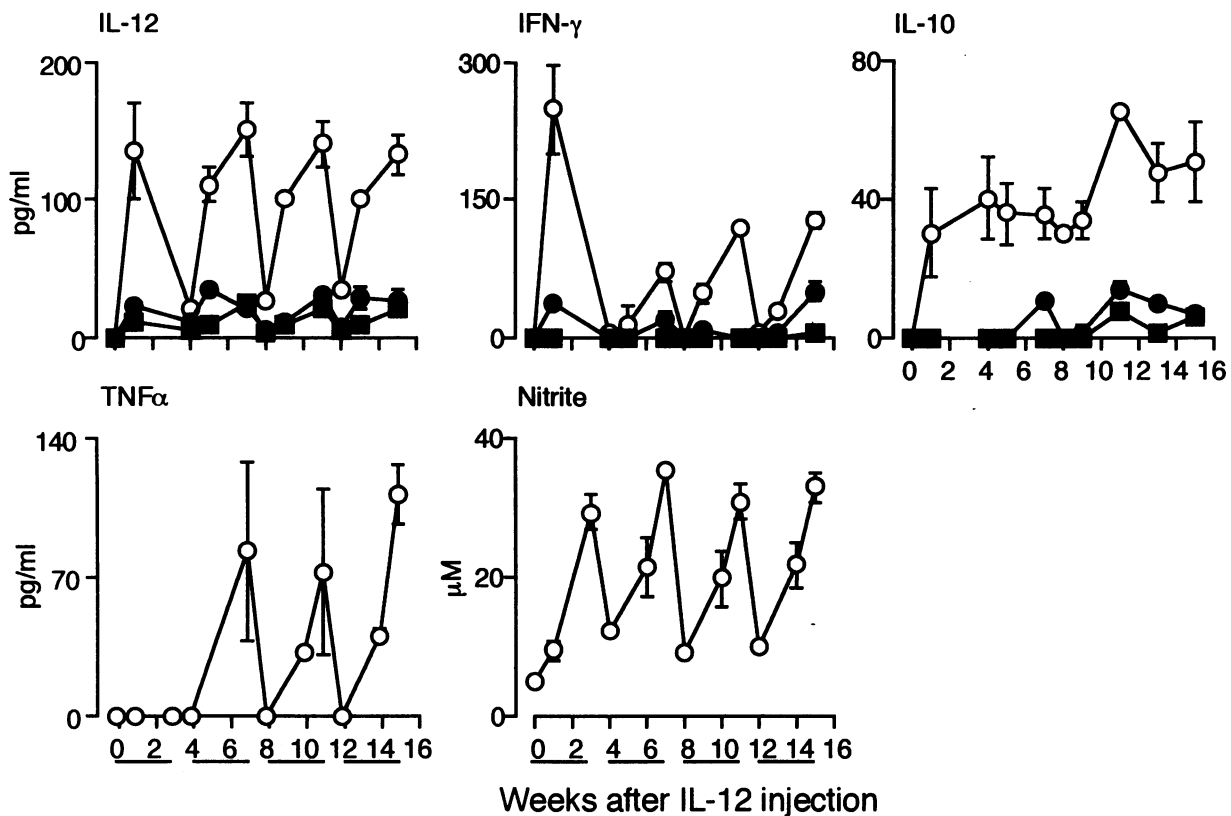


FIG. 4. Effect of IL-12 on serum cytokine and nitrite levels. BALB/cBy female mice were injected with 25 μ g of 3-MC and treated with 100 ng of IL-12 (○), 10 ng of IL-12 (●), or 1 ng of IL-12 (■). Each data point represents the average value of two mice. Serum samples were taken 2-3 hr after IL-12 injection.

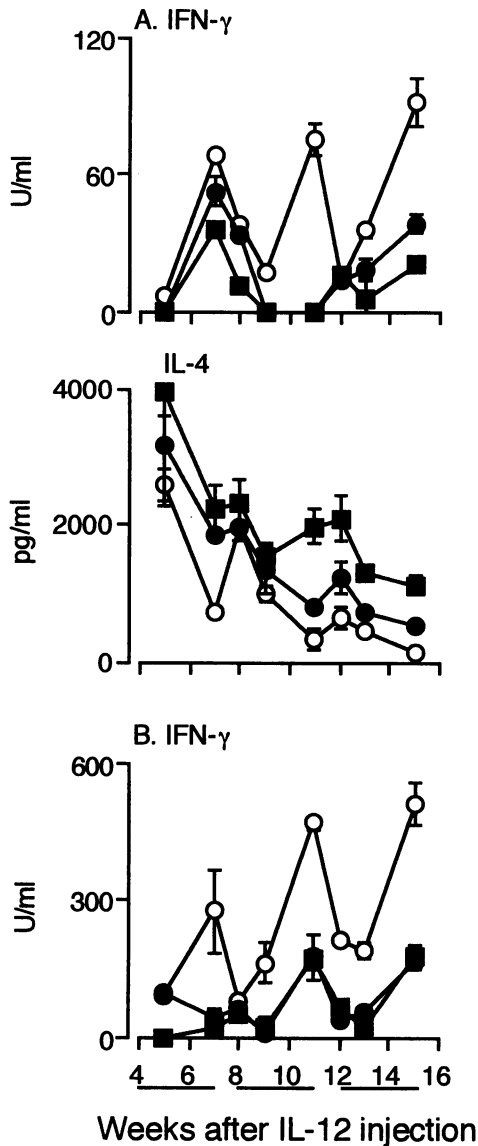


FIG. 5. Effect of IL-12 on cytokine production by isolated CD4 and CD8 T cells. BALB/cBy female mice were injected with 25 μ g of 3-MC and treated with 100 ng of IL-12 (○), 10 ng of IL-12 (●), or 1 ng of IL-12 (■). CD4 and CD8 T cells were separated from the spleens by magnetic beads and cultured with 10 μ g of Con A per ml for 3 days. The supernatants of CD4 cells were assayed for INF- γ and IL-4 (A) and the supernatants of CD8 cells were assayed for INF- γ (B). Each data point represents the average value of two to three mice.

and (iii) the third stage began with the finding that bacillus Calmette-Guérin infection increases resistance to tumor growth in the mouse (16). With the discovery of cytokines and their key role in orchestrating inflammation and immunity, there has been a shift away from the use of microbial products for cancer therapy and an intensive exploration of the antitumor activity of cloned cytokines, particularly IFNs, TNF, IL-2, and IL-12. Of these, IL-12 has the most striking antitumor activity in transplanted tumor systems. IL-12, a heterodimeric cytokine produced mainly by macrophages/monocytes, has potent IFN- γ -inducing properties, and IFN- γ appears to be a mediator of most IL-12 activities, including its antitumor activity in transplanted mouse systems (17). A number of pathways are undoubtedly involved in the protective activity of IL-12 demonstrated in microbial, viral, parasite, and tumor models (10, 18–20). These include an increase in IFN- γ production, principally by CD8 T cells, a shift in the cytokine

secretion profile of CD4 T cells from a TH₂ to a TH₁ pattern, and the activation of macrophages, resulting in increased TNF and nitrite production. In the present study, these immunomodulating activities of IL-12 persisted throughout the entire course of IL-12 treatment, indicating that repeated injections of IL-12 do not induce a tachyphylactic state. With regard to tumor systems, the IL-12-mediated TH₂ \rightarrow TH₁ shift may be of particular importance, by preventing the loss of TH₁ cells accompanying tumor growth (21) and inhibiting the development of CD4 cells with specific immunosuppressive properties (22). The anti-angiogenesis activity of IFN-inducible protein-10 induced by IL-12/IFN- γ may also play a role in tumor inhibition (23), and this possibility needs careful attention.

In addition to the effects of IL-12/IFN- γ on the host, recent studies have highlighted the critical role of a direct action of IFN- γ on tumor cells (24). A line of IFN- γ -insensitive tumor cells was generated by transfection with a dominant-negative IFN- γ receptor construct, and tumors derived from these transfected cells were resistant to lipopolysaccharide-induced (IFN- γ -mediated) rejection. In addition, these IFN- γ -unresponsive tumor cells grew at lower cell numbers than did wild-type tumor cells and were less immunogenic in terms of inducing immunity to wild-type tumor cells (24). The atypical tumors observed in IL-12-treated mice in the present study may also represent tumor cell variants that are unresponsive to IFN- γ signals, extreme examples of IFN- γ escape variants selected by the strong IFN- γ pressure exerted by prolonged IL-12 treatment.

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