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HYBRID RECOMBINANT HUMAN LEUKOCYTE INTERFERON INHIBITS DIFFERENTIATION IN MURINE B-16 MELANOMA CELLS

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We have investigated the effects of recombinant human leukocyte interferons (IFN-αA and IFN-αD) and various hybrid recombinant human leukocyte interferons on differentiation in B-16 mouse melanoma cells. Inhibition of both spontaneous and melanocyte hormone stimulated differentiation was observed with one hybrid construct, IFN-αA/D (Bgl) consisting of amino acids 1 to 62 from IFN-αA and amino acids 64 to 166 from IFN-αD. In contrast, the parental human interferons, IFN-αA and IFN-αD, when used alone or in combination, as well as other hybrid human leukocyte interferons, did not cause significant inhibition of melanogenesis in B-16 mouse cells. The tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) also inhibited B-16 differentiation and the combination of TPA with IFN-αA/D (Bgl) or mouse L-cell interferon was synergistic in delaying melanogenesis. These studies indicate that the IFN-αA/D (Bgl) hybrid that exhibits antiviral activity on mouse cells can also inhibit differentiation of murine cells.

The ability of various interferon preparations to inhibit the development and growth of specific tumors in vivo is often attributed to its capacity to augment immune defense mechanisms of the host (1-3) as well as its ability to alter directly the growth capacity of normal and tumor cells (1-9). Recent observations indicating that interferon can modulate the differentiation of tumor cells in vitro suggest that in certain situations the antitumor activity may be related to effects on differentiation (10-16). In general, both the antiproliferative and antiviral activities of interferons are somewhat species

ABBREVIATIONS USED: IFN, interferon; IFN-αA and IFN-αD, recombinant human leukocyte interferons; IFN-αA/D (Bgl) and IFN-αA/D (Pvu), recombinant hybrid human leukocyte interferons; MSH, α-melanocyte-stimulating hormone; TPA, 12-0-tetradecanoyl-phorbol-13-acetate

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specific: physiological levels of human interferons will not induce a biochemical response in mouse cells and vice versa (for review see 17). However, by recombinant DNA techniques it has been possible to construct hybrid human leukocyte interferons that induce antiviral and antiproliferative effects in both mouse and human cells (17-19). In the B-16 mouse melanoma cell culture system, in which melanogenesis can be modulated by a variety of agents including mouse L-cell interferon (14,20,21) we find that a hybrid human leukocyte interferon also influences differentiation of murine cells.

MATERIALS AND METHODS

Cell Culture: A subclone of the C_3 clone of B-16 mouse melanoma cells, B-16-S3 (21), was used to analyze the effect of various interferon preparations on melanogenesis. Cells were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% bovine serum (DMEM-10)(Grand Island Biological Company, Grand Island, NY). Cells were passaged prior to differentiating as previously described (20,21) with 0.2% versene in phosphate-buffered saline (0.01 M phosphate pH 7.4 and 0.15 M NaCl).

Experimental Design: B-16 cells were seeded at 2.5 X 10^5 cells/35 mm dish and 3 hr after plating the compounds to be tested were added in fresh DMEM-10. Cell counts were determined by removing cells from plates with 0.2% versene in phosphate-buffered saline, diluting cell suspensions with isotonic diluent (Fisher Scientific Co., Fairlawn, NJ) and counting with an electronic model Z_f Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

Melanin Determinations: The melanin content of the cell culture medium was determined spectrophotometrically as previously described (20,21).

Interferons: The plasmid coding for the expression of human leukocyte interferons (IFN-αA and IFN-αD) and various recombinant hybrid human leukocyte interferons were produced in Escherichia coli as previously described (17,19,22). Hybrid interferon recombinants were constructed as described (19). These recombinants coded for the following hybrid interferon molecules: IFN-αA/D (Pvu II); IFN-αA/D (Bgl II); IFN-αD/A (Pvu II); IFN-αD/A (Bgl II); and IFN-αA/D/A (Bgl II - Pvu II), the designation in parentheses indicates the restriction site at which the interferon genes were joined. These hybrid interferons were purified to homogeneity with the use of a monoclonal antibody to human leukocyte interferon (19,23). The interferon titers were determined by a cytopathic effect inhibition assay with vesicular stomatitis virus on a bovine kidney cell line (MDBK) or human fibroblast AG-1732 cells (24). Interferon preparations were stored in 2 ml vials at -80°C, thawed immediately prior to use and diluted to the appropriate concentrations in DMEM-10. Mouse L-cell interferon was produced and titered as previously described (14).

Phorbol Ester Tumor Promoters: TPA was purchased from Consolidated Midland, Brewster, NY. Stock solutions of 1 mg/ml in dimethyl sulfoxide were divided into small portions and stored at -20°C.

RESULTS

In previous studies we demonstrated that crude mouse L-cell interferon (0.03 to 30 units/ml) results in a concentration-dependent inhibition of both

spontaneous and MSH-induced differentiation in B-16 melanoma cells (14). The inhibitory effect of mouse L-cell interferon was eliminated when interferon preparations were heated (60 min at 60°C) or trypsinized (5 mg/ml for 30 min at 37°C). On the other hand, crude human leukocyte interferon (100 to 5000 units/ml) did not alter the pattern of growth or melanin synthesis in B-16 cultures (14). When assayed for antiviral activity on mouse L-cells, recombinant human IFN-αA was inactive and IFN-αD displayed very low activity (18,19). In contrast, the hybrid recombinant human leukocyte interferons, IFN-αA/D (Bgl) and IFN-αA/D (Pvu), containing the amino-terminal portion of IFN-αA combined with the carboxy-terminal region of IFN-αD, displayed significant antiviral activity in mouse cells (18,19). The specificity of this effect is indicated by the fact that the other hybrid interferons, IFN-αD/A (Bgl), IFN-αD/A (Pvu) and IFNαA/D/A (Bgl-Pvu), exhibited little or no antiviral activity on mouse cells (19). It has been shown that the IFN-αA/D (Bgl) hybrid displays antiviral activity on other non-human cells (18,19). As can be seen in Fig. 1, the hybrid IFN-αA/D (Bgl) was capable of suppressing spontaneous melanogenesis in B-16 cells, whereas the parental leukocyte interferons, IFN-αA and IFN-αD, and the hybrid IFN-αA/D (Pvu) were not active in inhibiting differentiation, even when tested at 6000 units/ml. Combinations of IFN-αA plus IFN-αD and hydrid constructs IFN-αD/A (Bgl), IFN-αD/A (Pvu) or IFN-αA/D/A (Bgl-Pvu) were

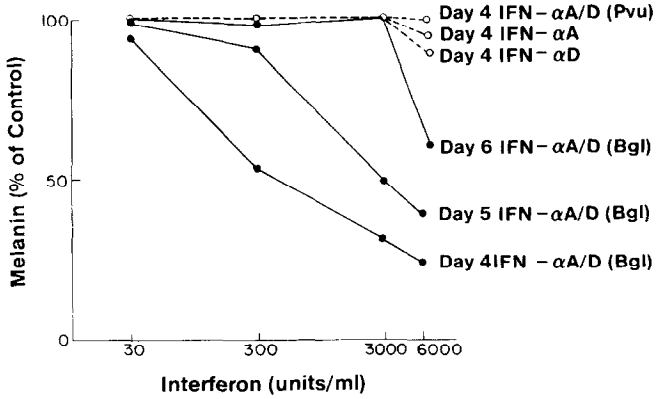
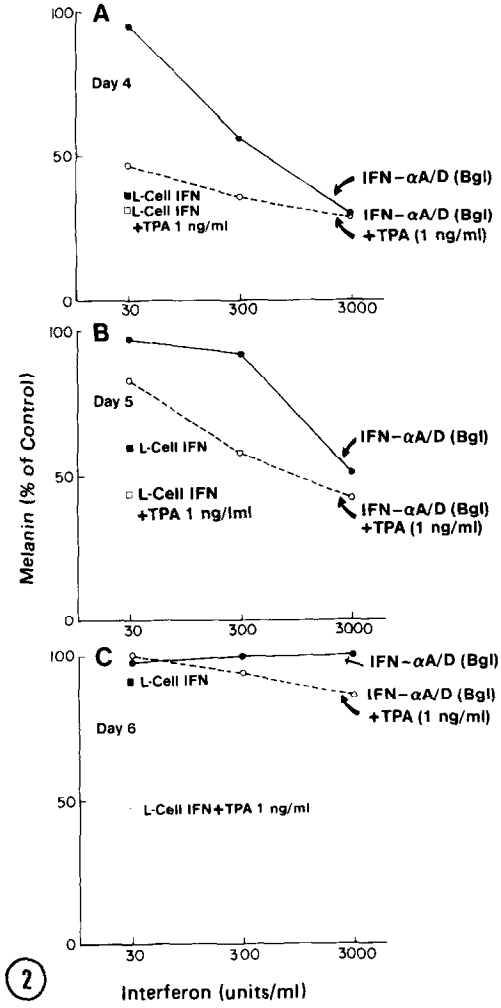


Figure 1. Effects of recombinant and hybrid recombinant human leukocyte interferon on spontaneous melanogenesis in B-16 mouse melanoma cells.

also devoid of activity when tested in the B-16 melanoma system (data not shown). This ability of IFN-αA/D (Bgl) to inhibit melanogenesis in B-16 cells was concentration-dependent and occurred when 100 units/ml or higher levels of the purified hybrid protein were used (Fig. 1 and 2). In previous studies, concentrations of crude mouse L-cell interferon as low as 0.03 units/ml inhibited melanogenesis (14). The requirement for much higher levels of the hybrid interferon may reflect a lower affinity of the human hybrid interferon for receptors on mouse cells that modulate differentiation. Alternatively, it is possible that crude mouse L-cell interferon contains other substances, such as lymphokines, which inhibit the differentiation of B-16 cells. With both mouse and hybrid human leukocyte interferon, the inhibition of melanogenesis in B-16 cells was not associated with significant alterations in the growth kinetics of treated cells (data not shown). When mouse L-cell interferon or IFN-αA/D (Bgl) were combined with the tumor promoting agent TPA, both the extent of inhibition of differentiation and the delay in escape from inhibition of differentiation were increased (Fig. 2). The combination of mouse L-cell interferon plus TPA was more active than IFN-αA/D (Bgl) plus TPA in altering the program of differentiation in B-16 cells.

Although our subclone of B-16 cells undergoes spontaneous melanogenesis as the culture reaches confluence, melanogenesis can be accelerated by addition of α-MSH (5×10^{-7}M). Among the human interferons only the hybrid IFN-αA/D (Bgl) was active in inhibiting α-MSH-induced melanogenesis by these cells (Fig. 3). With time in culture, B-16 cells eventually escaped from the inhibition of melanogenesis caused by the hybrid human interferon and terminally differentiated (Fig. 3). This escape phenomenon has also been observed in B-16 cells incubated with mouse L-cell interferon and phorbol ester tumor promoters (14,20,21 and Fig. 2). Both the degree and duration of inhibition of MSH-induced differentiation by IFN-αA/D (Bgl) were markedly enhanced when the cells were simultaneously incubated with TPA (Fig. 3). The ability of these cells to eventually escape and terminally differentiate does not appear to result from degradation of interferon or its depletion from the medium, since biologically



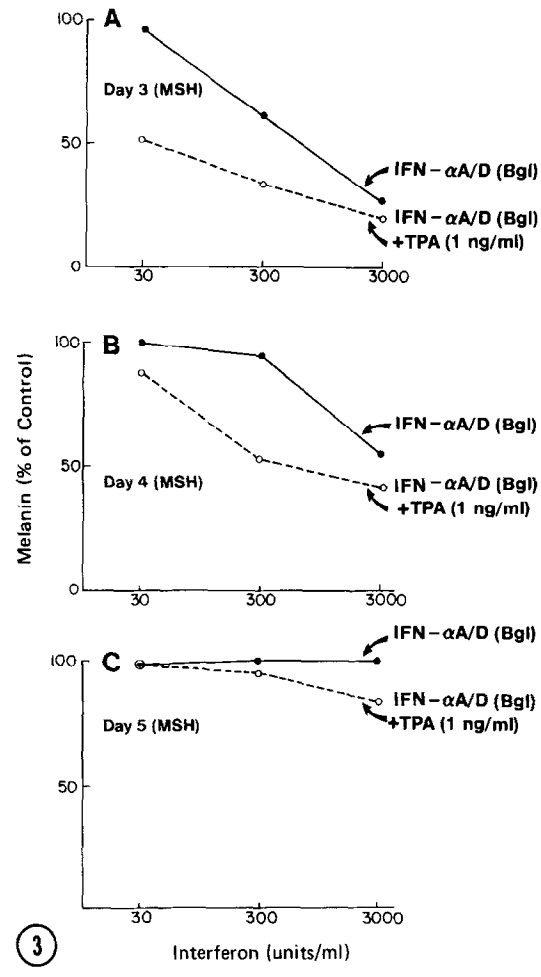


Figure 2. Effects of hybrid recombinant human leukocyte interferon (IFN-αA/D (Bgl), mouse L-cell interferon and 12-0-tetradecanoyl-phorbol-13-acetate (TPA), used alone and in combination, on spontaneous melanogenesis in B-16 mouse melanoma cells. ●, IFN-αA/D (Bgl); ○, IFN-αA/D (Bgl) + TPA; ■ , L-cell IFN; □ , L-cell IFN + TPA. When exposed to 1 ng/ml TPA, melanin levels were 85, 97 and 100% of control cultures when assayed at day 4, 5 and 6, respectively.

Figure 3. Effect of hybrid recombinant human leukocyte interferon IFN-αA/D (Bgl) and TPA, used alone and in combination, on α-melanocyte stimulating hormone (MSH) induced melanogenesis in B-16 mouse melanoma cells. ●, IFN-αA/D (Bgl); ○, IFN-αA/D (Bgl) + TPA. When exposed to 1 ng/ml TPA, melanin levels were 82, 96 and 100% of control cultures when assayed at day 3, 4 and 5, respectively.

active interferon could still be isolated from the medium and the addition of fresh interferon did not prevent this escape (data not shown).

DISCUSSION

The ability of hybrid human interferon molecules to inhibit melanogenesis in B-16 mouse melanoma cells does not directly correlate with their antiviral

activity on mouse cells, since IFN-αA/D (Pvu) did not modify melanogenesis in B-16 cells (Fig. 1), although it induces an antiviral state in mouse L-cells (18,19). Recent studies employing ^{125}I-labeled preparations of mouse and human interferons indicate the existence of specific and saturatable high-affinity cell surface receptors for interferon (25-31). In several experimental systems, response of specific cell types to interferon, as assayed by antiviral and/or antiproliferative activity, correlates with the specific binding of interferon to these cells (25,27,29). Competitive binding assays (26,27,30) and biological assays (32,33) also suggest the existence of more than one class of interferon receptors on a given cell type. Alternatively, the interferon receptor may be a complex one containing multiple triggers (32,33). It will be of interest, therefore, to determine if the hybrid IFN-αA/D (Bgl) binds specifically to mouse interferon receptors and if it occupies a class of receptors distinct from that of the hybrid IFN-αA/D (Pvu). Regardless of the mechanism, the ability to construct hybrid human interferon molecules which exert biological activity in murine systems should facilitate a comparison of the effects of interferon in murine and human tumor cell systems.

Although the mechanisms by which interferons induce an antitumor effect are not presently known, recent studies have indicated that crude and partially purified interferons can inhibit: (a) spontaneous as well as α-MSH-induced melanogenesis in B-16 melanoma cells (14); (b) DMSO-induced differentiation in Friend erythroleukemia cells (12,13); (c) spontaneous and insulin-stimulated adipocyte conversion in mouse 3T3 cells (10,11); and (d) myogenesis in chicken myoblasts (15). In contrast, crude mouse interferon has been shown to enhance differentiation in myeloid leukemic cells simultaneously treated with certain inducers of differentiation, either lipopolysaccharide or polyinosinic acid (34). Similarly, although partially purified human IFN-α and IFN-β do not modify differentiation in human promyelocytic leukemia (HL-60) cells when added alone, these interferons do enhance differentiation induced by retinoic acid or phorbol esters (35,36). In addition, recent investigations with human myoblast cultures derived from mature skeletal muscle indicate that IFN-αA can by itself

stimulate the differentiation of these cells, whereas phorbol esters inhibit their differentiation as indicated by both biochemical and morphological endpoints (16). These examples suggest the interesting possibility that in certain systems interferon may induce an antitumor effect by altering the program of differentiation of tumor cells, thus reducing the growth potential of the cells and increasing their immunogenicity (37,38). By combining interferon with other types of agents that modulate differentiation it may, therefore, be possible to increase the clinical utility of interferon as an antitumor agent.

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