Orally Administered Interferons Suppress Bone Marrow Function (43646)

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Abstract. **The accepted routes of interferon (IFN) administration in clinical applications are intramuscular, subcutaneous, intraperitoneal, intratumor, and intravenous. Recently, oral administration of interferons has been shown to cause a suppression of peripheral white blood cell (WBC) counts. Moreover, orally administered interferons mediate their peripheral WBC suppression via a different mechanism than that of intraperitoneally administered interferons. This study extends the previous studies to show that the peripheral WBC suppression induced by oral interferon treatment reflects an actual bone marrow suppression. The bone marrow-suppressive effects of orally and subcutaneously administered recombinant human IFN-αA/D (rHuIFN-αA/D) have been partially characterized in kinetics studies and compared with the peripheral WBC-suppressive effects of orally and subcutaneously administered rHuIFN-αA/D. Oral and subcutaneous administrations of rHuIFN-αA/D cause a significant suppression of peripheral WBC counts with 1 day of rHuIFN-αA/D administration. This suppression reaches its maximum level with 3 days of rHuIFN-αA/D administration and plateaus over a 12-day treatment time. Similarly, oral and subcutaneous administrations of rHuIFN-αA/D cause a significant suppression of bone marrow function with 1 day of rHuIFN-αA/D administration. This suppression reaches its maximum level with 3 days of rHuIFN-αA/D administration and plateaus over a 12-day treatment time. Thus, the WBC-suppressive and bone marrow-suppressive effects of rHuIFN-αA/D administered either orally or subcutaneously parallel each other. The peripheral WBC-suppressive activities of orally and subcutaneously administered rHuIFN-αA/D diminish at the same rate, after cessation of rHuIFN-αA/D treatment. Peripheral WBC suppression is lost by 5 days after cessation of rHuIFN-αA/D treatment. The mechanisms by which orally and subcutaneously administered interferons exert their bone marrow-suppressive effects differ, however. Bone marrow suppression mediated by subcutaneous administration of murine IFN-α/β (MuIFN-α/β) is blocked by the presence of circulating antibodies to MuIFN-α/β. In contrast, the bone marrow suppression mediated by oral administration of MuIFN-α/β occurs even in the presence of circulating antibodies to MuIFN-α/β. These results continue to support a potential clinical role for oral administration of interferons, particularly for the control of diseases of myelogenous origin.** [P.S.E.B.M. 1993, Vol 204]

Interferons (IFN-α, IFN-β, and IFN-γ) are potent antiviral, antitumor, and immunomodulatory agents. They have been approved for clinical use for a number of diseases (reviewed in Ref. 1). IFN-α has been approved in various countries for treatment of hairy cell leukemia, chronic myelogenous leukemia, multiple myeloma, non-Hodgkin's lymphoma, cutaneous T cell lymphoma, laryngeal papillomatosis, condyloma acuminatum, carcinoid, malignant melanoma, renal cell carcinoma, Kaposi's sarcoma in the acquired immunodeficiency syndrome, hepatitis B, and non-A, non-B viral hepatitis. IFN-β has been approved for the treatment of cervical intraepithelial neoplasia. IFN-γ has been approved for treatment of chronic granulomatous disease. These clinical uses of interferons employ the subcutaneous or intramuscular route of interferon administration.

Several studies have addressed whether the oral route of interferon administration might also be an

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effective route of interferon delivery. These studies have suggested that oral administration of interferon may provide local (2–9), and perhaps even systemic (10–12), antiviral protection.

Recently, using a mouse model, oral administration of each of the three interferons has been shown to suppress the peripheral white blood cell (WBC) count in a dose-dependent manner (13). Further studies have shown that the peripheral WBC-suppressive effect of orally administered interferons occurs by a novel mechanism (14). Peripheral WBC suppression induced by orally administered interferons is not blocked by the presence of circulating antibody to the interferons, while the peripheral WBC-suppressive effects of subcutaneously administered interferons are blocked. The peripheral WBC-suppressive effects were also seen to be adoptively transferred. Recipient mice receiving blood cells from donor mice showed a significant degree of peripheral WBC suppression which equaled that seen in the donor mice themselves.

It was not known whether the peripheral WBC-suppressive effect of orally administered interferon was reflective of a suppression of bone marrow function or due to an alteration in the trafficking of WBC. This study focuses on that question.

Materials and Methods

Mice. Pathogen-free, 6- to 8-week-old female C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were maintained in cages with autoclaved bedding. The cages were bathed in sterile air from horizontal laminar flow animal stations (Germfree Laboratories, Inc., Miami, FL). They were given autoclaved food and water *ad libitum*. Mice were monitored for exposure to mouse pathogens and were confirmed to remain pathogen-free during the course of the experiments by specific antibody testing for mouse hepatitis virus, minute virus of mice, Sendai virus, pneumonia virus of mice, GD-7, and *Mycoplasma pulmonis*.

Interferons. Recombinant DNA-derived human IFN-αA/D (rHuIFN-αA/D; $10^{7.8}$ units/mg protein) was kindly provided by Drs. Michael Brunda and Peter Sorter (Hoffmann-LaRoche, Nutley, NJ). rHuIFN-αA/D is a recombinant molecular hybrid of subtypes of rHuIFN-α, which exerts antiviral and myelotoxic activities in mice (15, 16). Natural murine IFN-α/β (MuIFN-α/β; $10^{8.3}$ units/mg protein) was purchased from Lee Biomolecular Research Laboratories (San Diego, CA). Interferon titers were determined in a microtiter plaque reduction assay (17) and compared with the appropriate NIH Reference Standards. Interferon titers are expressed as International Reference Units/ml. Interferons for oral administration were prepared in a 3 mg/ml bovine serum albumin in water solution (BSA/water). Interferons for subcutaneous administration were prepared in a 3 mg/ml bovine serum albumin in phosphate-buffered saline solution (BSA/PBS).

Protocol for Oral Administration of Interferons. Mice received oral interferon treatment in their drinking water. This interferon preparation was supplied *ad libitum*, with approximately 3.5 ml consumed per mouse per day. For example, mice treated with 10,000 units/day of orally administered rHuIFN-αA/D drank approximately 3.5 ml of an rHuIFN-αA/D/BSA/water solution that was prepared to contain 3,000 units/ml of rHuIFN-αA/D. Control mice received 3 mg/ml bovine serum albumin in their water supply. Interferon/BSA/water solutions were prepared before the initiation of each experiment and were stored frozen at −70°C until used in experiments. Storage at −70°C had no effect on interferon titer. Freshly thawed interferon/BSA/water solutions were supplied each day for the mice. After the indicated number of days of interferon treatment, the mice were bled; WBC counts were determined, and the WBC counts were expressed for each individual mouse as the percentage of the average WBC count of BSA/water-treated mice.

Mouse Bleeding. Anesthetized mice (Ketalar, 2.5 mg/mouse; Parke-Davis, Morris Plains, NJ) were bled from the retro-orbital venous plexus. For routine bleeding experiments, 100 μl of blood were collected in a disposable Micro-pipet (Fisher Scientific Co., Pittsburgh, PA) that had been dipped in EDTA (2%; Sigma Chemical Co., St. Louis, MO) and dried. The blood was delivered to a round-bottom polypropylene tube (Falcon, 12 × 75 mm; Becton Dickinson and Co., Lincoln Park, NJ) containing 5 μl of EDTA and thoroughly mixed. Next, the blood was diluted 1/30 in phosphate-buffered saline (300 μl final volume). Red blood cells were lysed by adding 1 drop of ZAP-OGLOBIN (Coulter Diagnostics, Hialeah, FL). Total WBC counts were made in a hemocytometer. Each count was based on the average counts of two counting chambers. Prebleeding the day before initiation of the experiment permitted the recognition and elimination from the experiments of mice that had high and low white blood cell counts, thus giving a more narrow standard deviation.

Hematopoietic Growth Factor. Recombinant DNA-derived murine granulocyte/macrophage colony-stimulating factor (rMuGM-CSF) was purchased from Genzyme (Cambridge, MA). It had a specific activity of 1×10^6 units/mg protein. The rMuGM-CSF activity was measured in a standard 7-day bone marrow colony assay (18).

***In Vitro* Colony-Forming Assay.** Bone marrow function was measured in a granulocyte/macrophage colony-forming unit assay (18). Briefly, after the determination of peripheral WBC counts, three mice from each group (the three closest to the average peripheral

WBC count for the group) were sacrificed by cervical dislocation. Bone marrow cells were harvested from the femurs of the three mice in each group and pooled. Red blood cells were lysed by treatment at 4°C for 2 min with 0.83% NH_4Cl. Nucleated cells were washed twice in MEM alpha medium (GIBCO, Grand Island, NY), counted in a hemocytometer, and resuspended at a concentration of 9×10^4 cells/ml in MEM alpha medium supplemented with 15% newborn bovine serum (Hazelton Dutchland, Denver, PA), 5×10^{-5} *M* 2-mercaptoethanol (Sigma), antibiotics (penicillin: 100 units/ml, Pfizer, New York, NY; streptomycin: 100 μg/ml, Sigma; gentamycin: 11 μg/ml, Elkins-Sinn, Inc., Cherry Hill, NY), and 0.35% agarose (Sigma). The resuspended nucleated cells were then plated (0.9 ml/plate) onto 35-mm plastic gridded tissue culture dishes (Sarstedt, Newton, NC) containing various concentrations of rMuGM-CSF (0.1 ml/plate). The cells were incubated at 37°C in a humidified atmosphere of 7% CO_2 in air (NapCo 5300 CO_2 incubator; National Appliance Corp., Portland, OR). After 7 days, colonies containing 50 or more cells were enumerated at ×40 magnification using an inverted microscope (Leitz Diavert; E. Leitz, Inc., Rockleigh, NJ). Three dishes were counted for each experimental point and the results were averaged.

Antibodies to Interferons. Polyclonal antibody to MuIFN-α/β (30,000 units/ml) was purchased from Lee Biomolecular Research Laboratories. The MuIFN-α/β antibody titer was determined in neutralization tests, which were performed as defined by the World Health Organization. In this method, 1 unit of antibody is the amount of antibody required to reduce 10 units of interferon to 1 unit. Antibodies to MuIFN-α/β were diluted and administered as a 0.1 ml intraperitoneal injection (1,000 units/mouse) 1 day before MuIFN-α/β administration.

On the day that the mice were bled for peripheral WBC counts and after bleeding for WBC counts, the mice were exsanguinated without using EDTA. Their blood was allowed to clot and the cells were spun out of the serum using a microcentrifuge (Fisher Scientific). The serum was decanted and titered for antibody. Mice that exhibited less than 30 units/ml of antibody to MuIFN-α/β (3 of 48 antibody-treated mice) were excluded from the experiments. The antibody titers of all of the mice included in the experiments exceeded 90 units/ml of serum.

Statistical Analyses. The data were evaluated for significance using Student's *t* test for comparison of data points and using one-factor analysis of variance of least squares means for comparison of curves (SuperAnova; Abacus Concepts, Inc., Berkeley, CA).

Results

Effect of Orally Administered rHuIFN-αA/D on Bone Marrow Function. The relative abilities of orally and intraperitoneally administered rHuIFN-αA/D to cause peripheral WBC suppression and bone marrow suppression were monitored. Previous studies in the murine system have shown that orally administered interferons, like interferons administered via other routes, exert similar degrees of suppression on each of the three major types of leukocytes: lymphocytes, neutrophils, and monocytes (14). Therefore, bone marrow suppression was monitored for suppression of neutrophil and monocyte progenitors by measuring the responsiveness of cultured bone marrow cells to GM-CSF. Mice were divided into eight groups. Four groups were treated orally with BSA/water or with three concentrations of rHuIFN-αA/D in BSA/water (300 units/day, 3,000 units/day, and 30,000 units/day). Four groups were treated subcutaneously with BSA/PBS or with three concentrations of rHuIFN-αA/D in BSA/PBS (300 units/day, 3,000 units/day, and 30,000 units/day). After 3 days of treatment, the peripheral WBC counts and the relative number of granulocyte/macrophage colony-forming units present in the bone marrows of each group of mice were determined.

The relative peripheral WBC-suppressive effects of orally and subcutaneously administered rHuIFN-αA/D are shown in Figure 1 as the averaged results of two essentially identical experiments. The data show that both orally and subcutaneously administered rHuIFN-αA/D caused a dose-dependent suppression of peripheral WBC counts. Furthermore, the relative potencies of orally and subcutaneously administered rHuIFN-αA/D were similar. The data confirm previously published observations (13).

The relative bone marrow-suppressive effects of orally and subcutaneously administered rHuIFN-αA/D are shown in Figure 2 as the averaged results of two essentially identical experiments. It was noted that, as expected, the bone marrow colonies that developed after GM-CSF stimulation consisted of granulocyte colonies, macrophage colonies, and mixed granulocyte/macrophage colonies. The relative proportions of these colonies were not remarkably different with the different treatments. The data in Figure 2A show that subcutaneously administered rHuIFN-αA/D exerted a dose-dependent suppression of the bone marrow, with incremental doses of rHuIFN-αA/D causing significantly greater levels of bone marrow suppression. The data of Figure 2B show that orally administered rHuIFN-αA/D also exerted a dose-dependent suppression of the bone marrow, with incremental doses of rHuIFN-αA/D causing significantly greater levels of bone marrow suppression. Comparison of the curves of Figure 2A and Figure 2B shows that oral and subcuta-

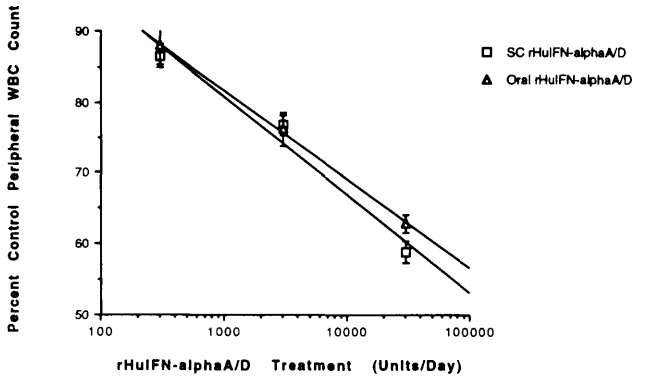

Figure 1. Effect of oral and subcutaneous administration of rHuIFN-αA/D on peripheral white blood cell counts. Orally treated C57BL/6 mice were given rHuIFN-αA/D in a BSA/water solution for 3 days at a concentration of 0 units/day, 300 units/day, 3,000 units/day, or 30,000 units/day. Subcutaneously treated mice were inoculated with rHuIFN-αA/D in a BSA/PBS solution for 3 days at concentrations of 0 units/day, 300 units/day, 3,000 units/day, or 30,000 units/day. WBC counts were made on Day 4 after 3 days of rHuIFN-αA/D treatment. Control white blood cell counts for these experiments averaged 11,738 ± 330 (mean ± SE). The data were plotted as the percentage of control peripheral WBC count versus rHuIFN-αA/D treatment. Each data point represents the mean ± SE of WBC counts from two experiments (20 mice/group). Statistical analysis of the data gave the following results: BSA/PBS versus 300 units/day oral interferon, $P = 0.02$; BSA/PBS versus 3,000 units/day oral interferon, $P = 0.0001$; BSA/PBS versus 30,000 units/day oral interferon, $P = 0.0001$; BSA/PBS versus 300 units/day subcutaneous interferon, $P = 0.0002$; BSA/PBS versus 3,000 units/day subcutaneous interferon, $P = 0.0001$; BSA/PBS versus 30,000 units/day subcutaneous interferon, $P = 0.0001$.

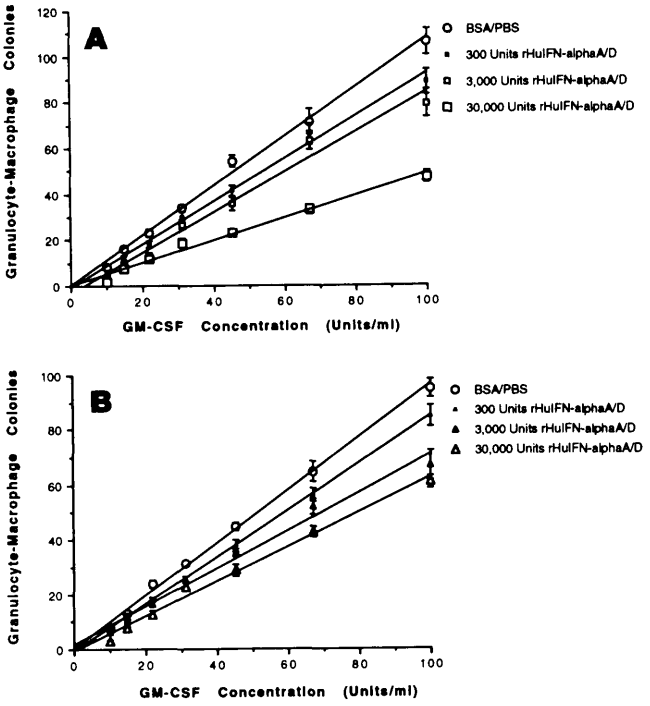

Figure 2. Effect of oral and subcutaneous administration of rHuIFN-αA/D on formation of granulocyte/macrophage (GM) colonies *in vitro*. C57BL/6 mice were orally or subcutaneously treated with rHuIFN-αA/D (0 units/day, 300 units/day, 3,000 units/day, or 30,000 units/day) for 3 days as described in Figure 1. On Day 4, after 3 days of rHuIFN-αA/D treatment, three mice from each treatment group were sacrificed and their bone marrow cells were harvested and pooled. The bone marrow cells were cultured in triplicate in soft agarose with different concentrations of rMuGM-CSF. The number of GM colonies were enumerated with an inverted microscope. The results are plotted as the number of GM colonies versus rMuGM-CSF concentration. (A) Subcutaneously administered rHuIFN-αA/D. (B) Orally administered rHuIFN-αA/D. Each data point represents the mean ± SE of the number of GM colonies from two experiments. Statistical analysis of the curves of 2A gave the following results: BSA/PBS versus 300 units rHuIFN-αA/D, $P = 0.006$; BSA/PBS versus 3,000 units rHuIFN-αA/D, $P = 0.0001$; BSA/PBS versus 30,000 units rHuIFN-αA/D, $P = 0.0001$; 300 units rHuIFN-αA/D versus 3,000 units rHuIFN-αA/D, $P = 0.0228$; 300 units rHuIFN-αA/D versus 30,000 units rHuIFN-αA/D, $P = 0.0001$; and 3,000 units rHuIFN-αA/D versus 30,000 units rHuIFN-αA/D, $P = 0.0001$. Statistical analysis of the curves of 2B gave the following results: BSA/PBS versus 300 units rHuIFN-αA/D, $P = 0.0001$; BSA/PBS versus 3,000 units rHuIFN-αA/D, $P = 0.0001$; BSA/PBS versus 30,000 units rHuIFN-αA/D, $P = 0.0001$; 300 units rHuIFN-αA/D versus 3,000 units rHuIFN-αA/D, $P = 0.0009$; 300 units rHuIFN-αA/D versus 30,000 units rHuIFN-αA/D, $P = 0.0001$; and 3,000 units rHuIFN-αA/D versus 30,000 units rHuIFN-αA/D, $P = 0.0009$.

neous administration of rHuIFN-αA/D gave similar levels of bone marrow suppression when administered at 300 units/day and 3,000 units/day. However, with 30,000 units/day of rHuIFN-αA/D, subcutaneous administration of rHuIFN-αA/D appeared to cause a more potent bone marrow suppression than oral administration.

Kinetics of Development of Peripheral WBC Suppression after Oral and Subcutaneous Administration of rHuIFN-αA/D. Mice were divided into three groups and treated daily for 12 days with 10,000 units/day of rHuIFN-αA/D administered subcutaneously, with 10,000 units/day of rHuIFN-αA/D administered orally, or with BSA/PBS administered both subcutaneously and orally. Mice from each group were bled on Days 0, 1, 3, 6, and 12. Each mouse was bled only once. Figure 3 represents the averaged results of two essentially identical experiments. The data show that the peripheral WBC counts of BSA/PBS-treated mice were relatively constant over the course of the experiments. Subcutaneous administration of rHuIFN-αA/D caused a peripheral WBC suppression that reached its maximal level by Day 1 after initiation of rHuIFN-αA/D administration. In contrast, in each of the two experiments, oral administration of rHuIFN-αA/D caused a peripheral WBC suppression that reached its maximal level by Day 3 after rHuIFN-αA/D administration. For each route of rHuIFN-αA/D administration, the peripheral WBC suppression was maintained at its maximal level through 12 days of treatment. Thus, neither a refractory state nor a tolerance to rHuIFN-αA/D treatment appeared to develop.

Kinetics of Development of Bone Marrow Suppression after Oral and Subcutaneous Administration of rHuIFN-αA/D. Mice from the experiments described above were sacrificed and their bone marrows

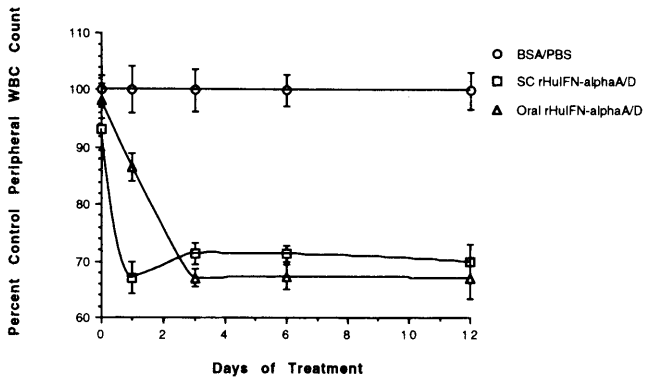

Figure 3. Kinetics of development of peripheral WBC suppression after oral and subcutaneous rHuIFN-αA/D administration. Orally treated C57BL/6 mice were given rHuIFN-αA/D (0 units/day or 10,000 units/day) in a BSA/water solution, with different subsets being treated for various periods of time. Subcutaneously treated mice were inoculated with rHuIFN-αA/D (0 units/day or 10,000 units/day) in a BSA/PBS solution, with different subsets being treated for various periods of time. White blood cell counts were performed on the different subsets of mice at 0, 1, 3, 6, or 12 days after administration of rHuIFN-αA/D (note: Day 0 was actually 3-hr after administration of rHuIFN-αA/D treatment). Control white blood cell counts for these experiments averaged 11,355 ± 299 (mean ± SE). The data were plotted as percentage of control peripheral WBC count versus days of rHuIFN-αA/D treatment. Each data point represents the mean ± SE of WBC counts from two experiments (19 mice/subset). Statistical analysis of the data gave the following results: Day 0 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = NS and P = NS, respectively; Day 1 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = 0.0079 and P = 0.0001, respectively; Day 3 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = 0.0001 and P = 0.0001, respectively; Day 6 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = 0.0001 and P = 0.0001, respectively; Day 12 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = 0.0001 and P = 0.0001, respectively.

were used for granulocyte/macrophage colony assays. The kinetics of development of bone marrow suppression by subcutaneously administered rHuIFN-αA/D are presented in Figure 4 as the averaged results of two essentially identical experiments. The data of Figure 4A show the number of granulocyte/macrophage colonies developing from bone marrows of mice sacrificed on Day 1 after initiation of subcutaneous rHuIFN-αA/D administration. While the curves are similar for BSA/PBS- and rHuIFN-αA/D-treated mice, there is a statistically significant level of suppression (P = 0.034). The data of Figure 4, B, C, and D show a much greater level of suppression, with the number of granulocyte/macrophage colonies being reduced by essentially identical levels on Days 3, 6, and 12 of subcutaneous rHuIFN-αA/D administration. The data for Day 0 are not presented. However, no suppression was observed (P = 0.48).

The kinetics of development of bone marrow suppression by orally administered rHuIFN-αA/D are presented in Figure 5 as the averaged results of two essentially identical experiments. The data of Figure 5A show the number of granulocyte/macrophage colonies developing from bone marrows of mice sacrificed on Day 1 after initiation of oral rHuIFN-αA/D administration. While the curves are similar for BSA/PBS- and rHuIFN-αA/D-treated mice, there is a statistically significant level of suppression (P = 0.013). The data of Figure 5, B, C, and D show a much greater level of suppression, with the number of granulocyte/macrophage colonies being reduced by essentially identical levels on Days 3, 6, and 12 of oral rHuIFN-αA/D administration. The data for Day 0 are not presented. However, no suppression was observed (P = 0.18).

Thus, bone marrow suppression was demonstrable by Day 1 and plateaued by Day 3 after initiation of both subcutaneous and oral rHuIFN-αA/D administration. The maximum level of bone marrow suppression was maintained through 12 days of subcutaneous or oral rHuIFN-αA/D treatment.

Kinetics of Loss of Peripheral WBC Suppression after Cessation of Orally and Subcutaneously Administered rHuIFN-αA/D. Mice were divided into three groups and treated daily for 3 or 6 days with 10,000 units/day of rHuIFN-αA/D administered subcutaneously, with 10,000 units/day of rHuIFN-αA/D administered orally, or with BSA/PBS administered both subcutaneously and orally. Mice from each group were bled 1 day, 3 days, and 5 days after the cessation of rHuIFN-αA/D treatment (Days 0, 2, and 5, respectively). Each mouse was bled only once. Figure 6 presents the averaged results of two essentially identical experiments. The data show that the peripheral WBC-suppressive effects of both orally and subcutaneously administered rHuIFN-αA/D are gradually lost after cessation of rHuIFN-αA/D treatment. Furthermore, since the slopes of the curves for oral and subcutaneous treatment are essentially identical, the rates of decay of the peripheral WBC-suppressive effects were similar.

Effect of Circulating Antibody to MuIFN-α/β on the Bone Marrow-Suppressive Effects of Subcutaneously and Orally Administered MuIFN-α/β. Mice were divided into two groups and inoculated intraperitoneally with either 1000 units of antibody to MuIFN-α/β or phosphate-buffered saline. The two groups were then subdivided into three groups each. The six subgroups were treated for 3 days with subcutaneously administered MuIFN-α/β, orally administered MuIFN-α/β, or both subcutaneously and orally with BSA/PBS. Mice were treated with MuIFN-α/β rather than with rHuIFN-αA/D because of the greater availability of antibody to MuIFN-α/β. Mice were bled to determine peripheral WBC counts on Day 4 after 3 days of interferon treatment. Only those mice treated with antibody to MuIFN-α/β that had circulating antibody titers to MuIFN-α/β of 30 units/ml or greater were evaluated. Figure 7 presents the averaged results of two essentially identical experiments. The data show that administration of antibody to MuIFN-α/β had no effect on the

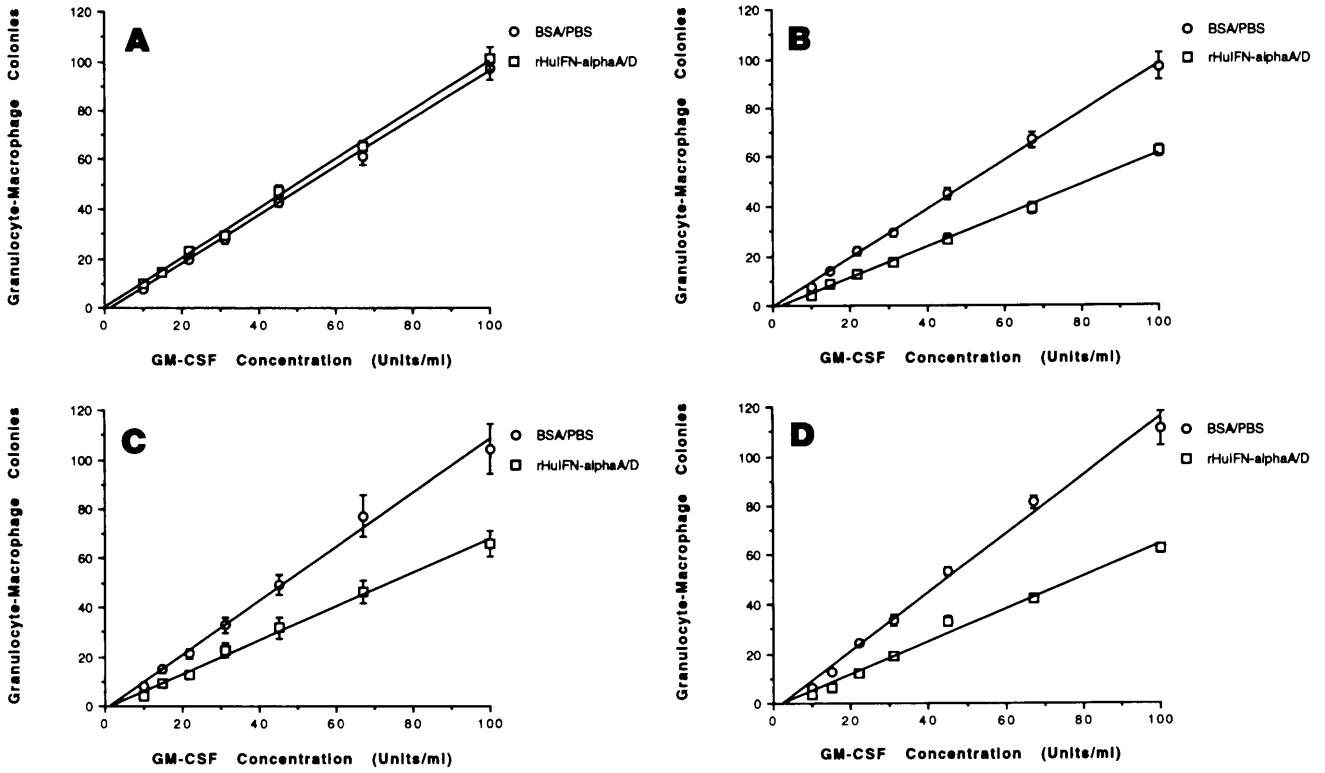

Figure 4. Kinetics of development of bone marrow suppression after subcutaneous rHuIFN-αA/D administration. C57BL/6 mice were subcutaneously inoculated with rHuIFN-αA/D (10,000 units/day) or with interferon carrier for various numbers of days as described in Figure 3. Three mice from each treatment group were sacrificed and their bone marrow cells were harvested and pooled. The bone marrow cells were cultured in soft agarose with different concentrations of rMuGM-CSF. The number of granulocyte/macrophage (GM) colonies were enumerated with an inverted microscope. The results are plotted as the number of GM colonies versus rMuGM-CSF concentration. (A) After 1 day of rHuIFN-αA/D treatment. (B) After 3 days of rHuIFN-αA/D treatment. (C) After 6 days of rHuIFN-αA/D treatment. (D) After 12 days of rHuIFN-αA/D treatment. Each data point represents the mean ± SE of the number of GM colonies from two experiments that were performed in triplicate. Statistical analysis of the curves gave the following results: (A) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.034$; (B) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.0001$; (C) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.0001$; and (D) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.0001$.

peripheral WBC counts of BSA/PBS-treated mice. As expected (14), the peripheral WBC-suppressive activity of subcutaneously administered MuIFN-α/β was blocked by circulating antibody to MuIFN-α/β. Also as shown previously (14), the data show that the peripheral WBC-suppressive activity of orally administered MuIFN-α/β was unaffected by circulating antibody to MuIFN-α/β.

Bone marrow cultures were made from the pooled bone marrow cells of each of the groups of mice described above. Figure 8 presents the averaged results of two essentially identical experiments. The data of Figure 8A show that antibody to MuIFN-α/β had little or no effect on the formation of granulocyte-macrophage colonies of BSA/PBS-treated mice. In parallel to the peripheral WBC suppression data, the bone marrow-suppressive activity of subcutaneously administered MuIFN-α/β was blocked by circulating antibody to MuIFN-α/β. The data of Figure 8B show that, in parallel to the peripheral WBC suppression data, the bone marrow-suppressive activity of orally administered MuIFN-α/β was unaffected by circulating antibody to MuIFN-α/β.

Discussion

Oral administration of interferons has been shown to suppress the peripheral WBC count in mice (13). However, the study did not distinguish between a peripheral WBC suppression that was due to an actual suppression of bone marrow function or suppression that was due to an alteration in the trafficking of the WBC. Therefore, this study was undertaken to determine the relative bone marrow-suppressive effects of orally and subcutaneously administered interferons.

Mice were treated orally or subcutaneously with rHuIFN-αA/D and monitored for both peripheral WBC counts and bone marrow function, as determined by a granulocyte-macrophage colony-forming assay. Bone marrow suppression developed in mice treated both orally and subcutaneously, with both routes of rHuIFN-αA/D administration demonstrating an rHuIFN-αA/D dose-dependent bone marrow suppres-

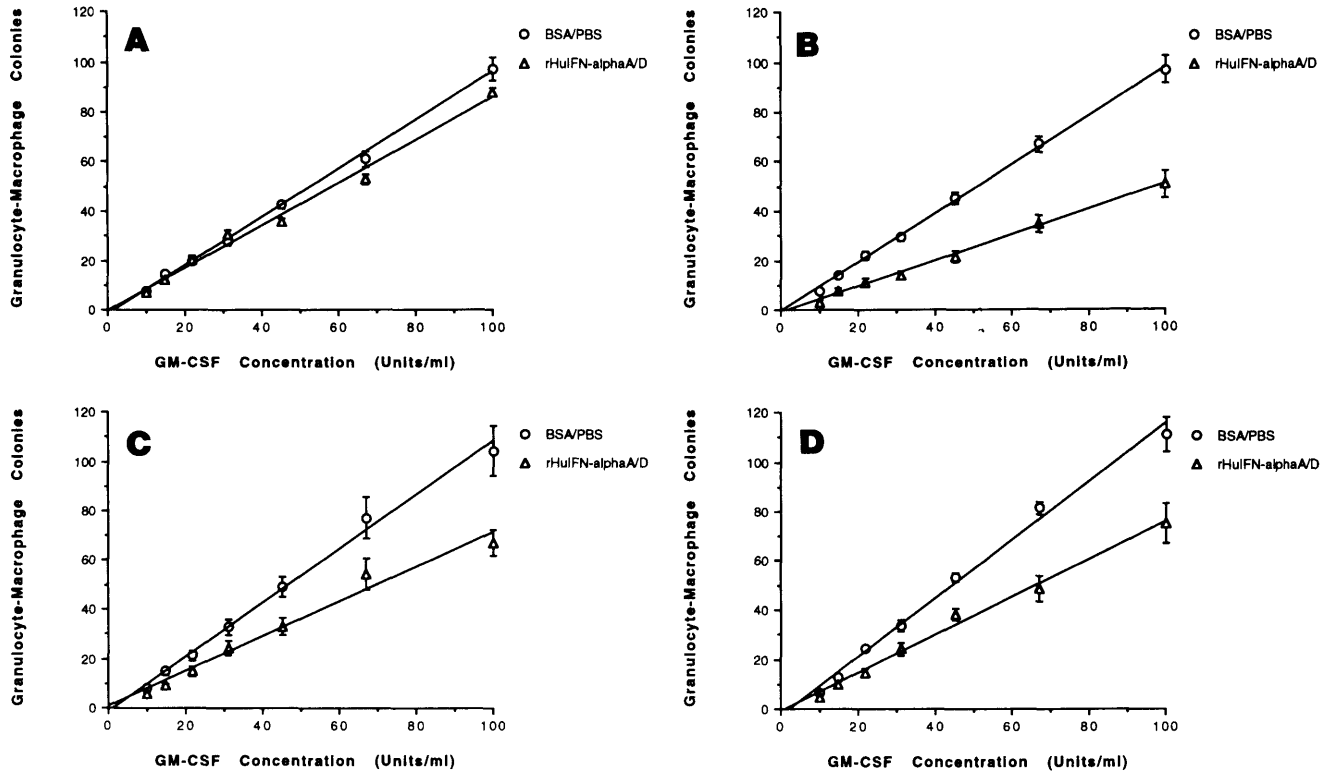

Figure 5. Kinetics of development of bone marrow suppression after oral rHuIFN-αA/D administration. C57BL/6 mice were orally treated with rHuIFN-αA/D (10,000 units/day) or with BSA/water for various numbers of days as described in Figure 3. Three mice from each treatment group were sacrificed and their bone marrow cells were harvested and pooled. The bone marrow cells were cultured in soft agarose with different concentrations of rMuGM-CSF. The number of granulocyte/macrophage (GM) colonies was enumerated with an inverted microscope. The results are plotted as the number of GM colonies versus rMuGM-CSF concentration. (A) After 1 day of rHuIFN-αA/D treatment. (B) After 3 days of rHuIFN-αA/D treatment. (C) After 6 days of rHuIFN-αA/D treatment. (D) After 12 days of rHuIFN-αA/D treatment. Each data point represents the mean ± SE of the number of GM colonies from two experiments that were performed in triplicate. Statistical analysis of the curves gave the following results: (A) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.013$; (B) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.0001$; (C) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.0001$; and (D) BSA/PBS versus rHuIFN-αA/D treatment, $P = 0.0001$.

sion. Significant peripheral WBC suppression and bone marrow suppression were seen with treatment of as little as 300 units/day of rHuIFN-αA/D. This dosage for a mouse would correspond to a dosage of about 1 million units/day for a human.

The characteristics of the peripheral WBC suppression and the bone marrow suppression established by oral and subcutaneous administration of rHuIFN-αA/D were examined. The kinetics of development of the peripheral WBC suppression and the bone marrow suppression were determined first. Both rHuIFN-αA/D treatment routes caused a significant level of peripheral WBC suppression with just 1 day of rHuIFN-αA/D treatment, with maximal levels being achieved by 3 days of rHuIFN-αA/D treatment. It can be noted that the kinetics of peripheral WBC suppression by orally administered rHuIFN-αA/D lagged slightly behind those of subcutaneously administered rHuIFN-αA/D, with an intermediate level of WBC suppression occurring by Day 1 and maximal suppression occurring by Day 3 compared with maximal suppression occurring by Day 1, respectively. This difference may be reflective of the different mechanisms by which peripheral WBC suppression is achieved (cell mediated for orally administered interferon versus plasma mediated for subcutaneously administered interferon [14]). Also, in the absence of a determination for level of WBC suppression on Day 2, it is possible that the differences represent differences in actual time of treatment, with subcutaneously administered interferon being administered at a definite time and orally administered interferon being administered over a 24-hr period. The level of peripheral WBC suppression plateaued after 3 days, showing neither the development of a refractory state nor the establishment of tolerance over the 12-day treatment period. In a parallel manner, both rHuIFN-αA/D treatment routes caused a significant level of bone marrow suppression with just 1 day of rHuIFN-αA/D treatment, with maximal levels being achieved by 3 days of rHuIFN-αA/D treatment. The level of bone marrow suppression plateaued after 3 days, showing neither the development of a refractory state nor tolerance over the 12-day treatment period. Thus, both rHuIFN-αA/D treatment routes caused essentially identical effects on both peripheral WBC suppression and bone marrow suppression.

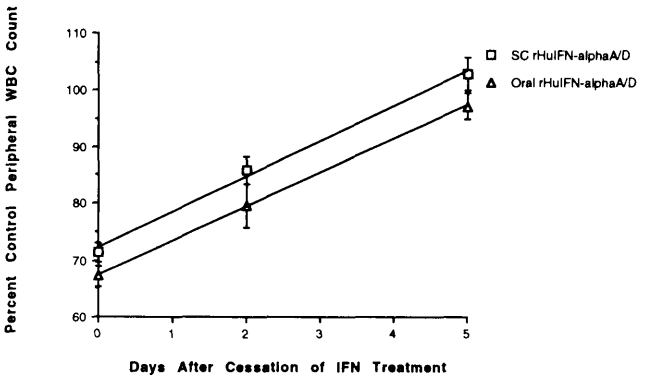

Figure 6. Kinetics of loss of peripheral WBC suppression after cessation of orally and subcutaneously administered rHuIFN-αA/D. Mice that were orally or subcutaneously treated for 3 to 6 days with rHuIFN-αA/D (10,000 units/day, as described for Figure 3) or with interferon carrier were removed from further rHuIFN-αA/D treatment. White blood cell counts were made 1 day, 3 days, and 6 days after the last rHuIFN-αA/D treatment (Days 0, 2, and 5, respectively, in the Figure). Control white blood cell counts for these experiments averaged 12,331 ± 362 (mean ± SE). The data were expressed as the percentage of control peripheral WBC count versus days after cessation of rHuIFN-αA/D treatment. Each data point represents the mean ± SE of WBC counts from two experiments (19 mice/group). Statistical analysis of the data gave the following results: Day 0 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = 0.0001 and P = 0.0001, respectively; Day 2 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = 0.0003 and P = 0.001, respectively; Day 5 BSA/PBS versus oral interferon and versus subcutaneous interferon, P = NS and P = NS, respectively.

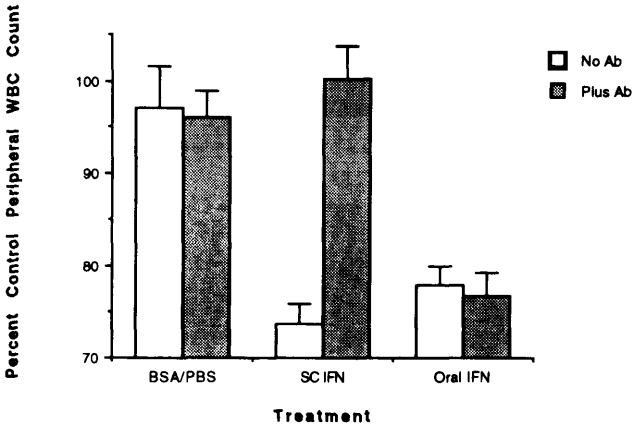

Figure 7. Effect of antibodies to MuIFN-α/β on the peripheral WBC-suppressive activity of MuIFN-α/β. Groups of mice were inoculated intraperitoneally with antibody to MuIFN-α/β (1,000 units/mouse) or with phosphate-buffered saline. The groups of mice were subdivided and were orally or subcutaneously treated with MuIFN-α/β or with interferon carrier for 3 days (10,000 units/day as described for Figure 1). White blood cell counts were made on Day 4 after 3 days of MuIFN-α/β treatment. The data are plotted as the percentage of control peripheral WBC count versus MuIFN-α/β treatment. Each data point represents the mean ± SE of WBC counts from two experiments (16 mice/group). Statistical analysis of the data gave the following results: BSA/PBS versus BSA/PBS + antibody, P = NS; BSA/PBS versus subcutaneous interferon treatment, P = 0.0001; BSA/PBS + antibody versus subcutaneous interferon treatment + antibody, P = NS; subcutaneous interferon treatment versus subcutaneous interferon treatment + antibody, P = 0.0001; BSA/PBS versus oral interferon treatment, P = 0.0005; BSA/PBS + antibody versus oral interferon treatment + antibody, P = 0.0001; oral interferon treatment versus oral interferon treatment + antibody, P = NS.

The persistence of peripheral WBC suppression after cessation of rHuIFN-αA/D treatment was monitored. Peripheral WBC suppression was found to be gradually lost over approximately a 5-day period, with similar rates of recovery of peripheral WBC counts observed for both orally and subcutaneously administered rHuIFN-αA/D (as shown by the similar slopes of the recovery curves). Given the correlation of peripheral WBC suppression and bone marrow suppression seen in the kinetics studies cited above, this recovery of peripheral WBC counts was presumably due to the recovery of bone marrow function, through it was not directly assessed.

It has been reported previously that the mechanism of peripheral WBC suppression established by orally administered interferons differed from that established by intraperitoneally administered interferons (14). In that study, it was shown for both MuIFN-β and rMuIFN-γ that the presence of circulating antibody to the interferons could block the peripheral WBC suppression mediated by intraperitoneally administered interferons but not by orally administered interferons.

Therefore, the effects of antibody to MuIFN-α/β on the peripheral WBC-suppressive and bone marrow-suppressive activities of orally and subcutaneously administered MuIFN-α/β were determined. First, the presence of circulating antibody to MuIFN-α/β was evaluated for its ability to block the peripheral WBC suppression mediated by orally and subcutaneously administered MuIFN-α/β. As was shown in previous studies with intraperitoneally administered MuIFN-β (14), subcutaneously administered MuIFN-α/β did not cause peripheral WBC suppression in mice in the presence of circulating antibodies to MuIFN-α/β. In contrast to the observations with intraperitoneally administered MuIFN-β and in confirmation of the previous studies, orally administered MuIFN-α/β suppressed the peripheral WBC count even in the presence of circulating antibodies to MuIFN-α/β.

Next, the ability of circulating antibodies to block the bone marrow-suppressive activities of orally and subcutaneously administered MuIFN-α/β was determined. The presence of circulating antibody to MuIFN-α/β blocked the bone marrow-suppressive activity of subcutaneously administered MuIFN-α/β. In parallel to the observations with the peripheral WBC-suppressive activity of MuIFN-α/β, the presence of circulating antibody to MuIFN-α/β did not affect the bone marrow-suppressive activity of orally administered MuIFN-α/β. Thus, the bone marrow suppression mediated by orally administered MuIFN-α/β was mediated by a different mechanism than the bone marrow suppression mediated by subcutaneously administered MuIFN-α/β.

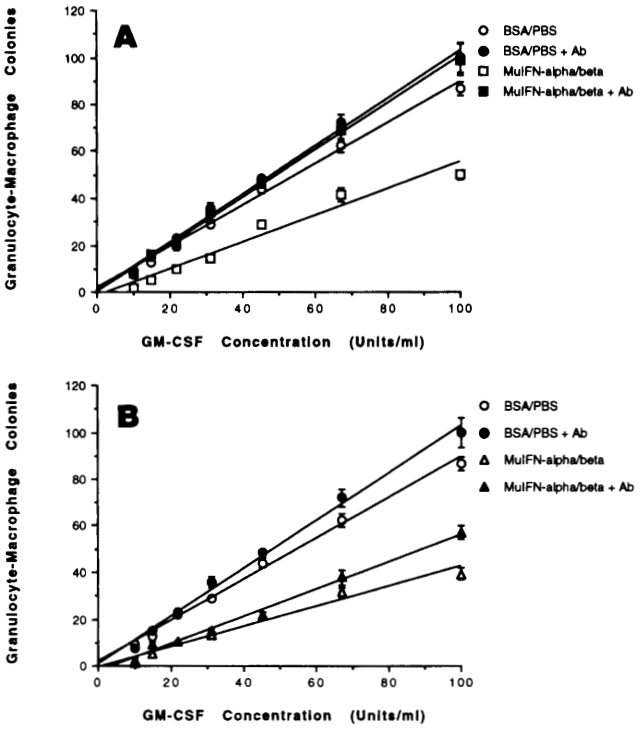

Figure 8. Effect of antibodies to MuIFN-α/β on the peripheral WBC-suppressive activity of MuIFN-α/β. Three mice from each treatment group described in Figure 7 were sacrificed and their bone marrow cells were harvested and pooled. The bone marrow cells were cultured in soft agarose with different concentrations of rMuGM-CSF. The number of granulocyte/macrophage (GM) colonies were enumerated with an inverted microscope. The results are plotted as the number of GM colonies versus rMuGM-CSF concentration. (A) Subcutaneously administered MuIFN-α/β ± antibody to MuIFN-α/β. (B) Orally administered MuIFN-α/β ± antibody to MuIFN-α/β. Each data point represents the mean ± SE of the number of GM colonies from two experiments that were performed in triplicate. Statistical analysis of the curves gave the following results. (A) BSA/PBS versus subcutaneous MuIFN-α/β treatment, $P = 0.0001$; BSA/PBS + antibody to MuIFN-α/β versus subcutaneous MuIFN-α/β + antibody treatment, P = NS. (B) BSA/PBS versus oral MuIFN-α/β treatment, $P = 0.0001$; BSA/PBS + antibody to MuIFN-α/β versus oral MuIFN-α/β + antibody treatment, $P = 0.0001$.

Taken together, these observations indicate that there was a bone marrow suppression after oral administration of rHuIFN-αA/D or MuIFN-α/β. Furthermore, they show that the kinetics and the mechanism of bone marrow suppression by orally administered interferons mirror the kinetics and the mechanism of peripheral WBC suppression by orally administered interferons. Thus, while changes in trafficking of WBC may play a role in the suppression of peripheral WBC counts, it appears to be most probable that the bulk of the peripheral WBC suppression observed after oral administration of interferon was due to a bone marrow suppression rather than an alteration in the trafficking of WBC.

Furthermore, previous studies had shown that the peripheral WBC-suppressive effects of orally administered interferons could be adoptively transferred from one mouse to another (14), indicating that the orally administered interferons were exerting a true systemic effect. The present studies, which show an actual bone marrow suppression by orally administered interferons, support this concept of a systemic effect of orally administered interferons.

Finally, the results of this study support a potential therapeutic role for the oral administration of interferons. In particular, it would be most interesting to determine whether the oral administration of HuIFN-γ could be as effective as other routes of administration of interferon in the control of diseases of myelogenous origin.

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