Modulation of Peripheral Leukocyte Counts in Mice by Oral Administration of Interferons

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Abstract. **The ability of interferons (IFN) to exert a systemic effect following their oral administration was evaluated. One systemic effect of parenteral interferon administration has been shown to be a suppression of the number of peripheral white blood cells both in man and in mouse models. Using the mouse model of peripheral white blood cell suppression, the relative systemic effects of orally and subcutaneously administered interferons were determined. Murine IFN-β, murine IFN-γ and cross-reactive recombinant human IFN-αA/D were examined. The oral administrations of each of the three interferons were found to cause a dose-dependent suppression of the peripheral white blood cell counts. Significant levels of suppression were seen with as little as 5 units/day of murine IFN-β and with 500 units/day of recombinant human IFN-αA/D and murine IFN-γ. The dose-response curves obtained with orally administered interferons were much more shallow than those obtained with subcutaneously administered interferons. The results demonstrate that oral administration of interferons can provide a significant systemic effect. Further, the results support the possibility that the oral administration of interferons may have therapeutic potential.** [P.S.E.B.M. 1991, Vol 197]

Interferons have shown some promise for the treatment of several cancers (1–5), but the majority of the responses to interferon therapy have been partial responses. A major challenge for the future is to develop new treatment protocols that increase the antitumor activity of interferon while reducing or moderating its side effects. One approach is to develop a new method for the administration of interferons.

There are several limitations with the current methods of intravenous, intramuscular, or subcutaneous administration of interferons (IFN), particularly of IFN-β and IFN-γ. Interferons differ greatly in their ability to be distributed systemically following intramuscular or subcutaneous injection (6). IFN-α is readily transported from the local site of injection and achieves a systemic distribution. Indeed, intramuscular and subcutaneous injections are the methods of choice for the clinical administration of IFN-α. However, IFN-β and IFN-γ appear to be retained at the local site of intramuscular or subcutaneous injection and are not as well distributed systemically as IFN-α (6). This problem has largely blocked the clinical use of IFN-β and has limited the clinical use of IFN-γ. Intravenous bolus is the current method of choice for the clinical administration of IFN-γ. This method of administration does provide a better systemic distribution of IFN-γ than does subcutaneous or intramuscular administration (7), but is inconvenient to employ. Thus, a method for the administration of IFN-β and IFN-γ is required that is convenient and that also provides a good systemic distribution of the interferons.

There is some evidence in the literature to support the therapeutic usefulness of orally administered IFN-α. Several of these studies have shown that the oral (or nasal) administration of human (Hu) IFN-α in humans and in calves or murine (Mu) IFN-α/β in mice can establish a local protective effect against virus infections that are also initiated through the oral (or nasal) route (8–15). Other studies have shown that oral administration of IFN-α can establish a systemic protective effect

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that extends the efficacy of the interferon beyond the local site of oral administration. For example, oral administration of HuIFN-α has been shown to protect cats from the pathogenic effects of feline leukemia virus that was administered by intravenous inoculation (16). Also, two reports suggest that oral administration of HuIFN-α may have utility in humans (17–18).

In this study, the oral route has been evaluated in a mouse model for its suitability as an efficient method for the administration of rHuIFN-αA/D, MuIFN-β, and MuIFN-γ. To determine whether the interferons exerted a systemic effect following oral administration, suppression of the peripheral white blood cell count was monitored. We show that the oral administration of each of these interferons can cause a significant suppression of the peripheral white blood cell count. Suppression of the peripheral white blood cell count has previously been shown to be a side effect of the systemic distribution of interferons. Thus, the oral route appears to be a suitable method for generating a systemic effect of interferons.

Materials and Methods

Mice. Pathogen-free female C57BL/6 mice 6–8 weeks old were purchased from Harlan Sprague Dawley (Indianapolis, IN). 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.

Mouse Bleeding. Mice were bled from the retro-orbital venous plexus at 1 PM on the days of bleeding.

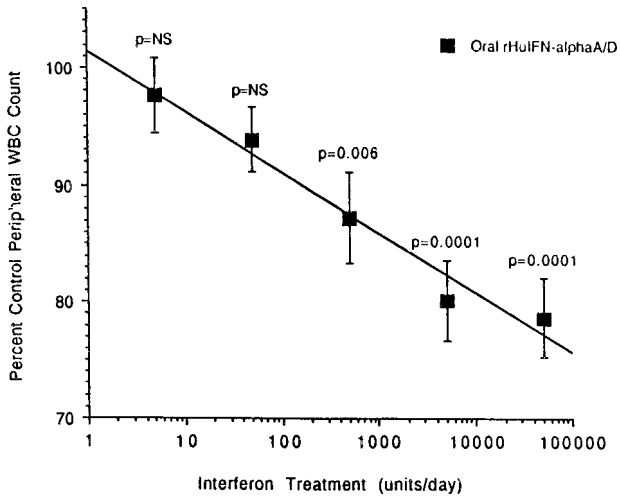

Figure 1. Effect of oral administration of rHuIFN-αA/D on peripheral white blood cell counts. Mice were provided with various concentrations of rHuIFN-αA/D in a gelatin/water solution (0.1% w/v) for 3 days. White blood cell (WBC) counts were made 1 day before initiation of rHuIFN-αA/D treatment (Day 0) and after 3 days of rHuIFN-αA/D treatment (Day 4). The data were calculated as percentage of Day 0 values and expressed as the percentage of control peripheral WBC count. The percentage of control peripheral WBC count was plotted versus rHuIFN-αA/D treatment. Each data point represents the mean ± SE of WBC counts from two experiments (15–16 mice).

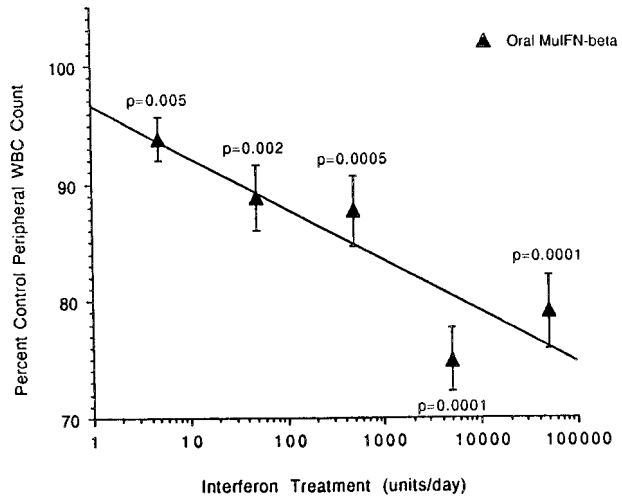

Figure 2. Effect of oral administration of MuIFN-β on peripheral white blood cell counts. Mice were provided with various concentrations of MuIFN-β in a gelatin/water solution (0.1%) for 3 days. White blood cell (WBC) counts were made 1 day before MuIFN-β treatment (Day 0) and after 3 days of MuIFN-β treatment (Day 4). The data were calculated as percentage of Day 0 values and expressed as the percentage of control peripheral WBC count. The percentage of control peripheral WBC count was plotted versus MuIFN-β treatment. Each data point represents the mean ± SE of WBC counts from three experiments (19–27 mice).

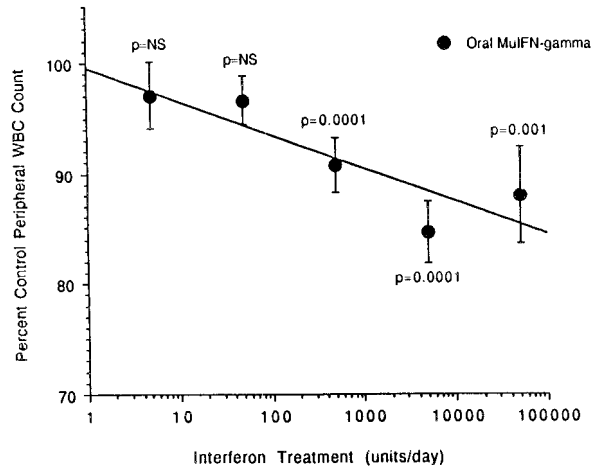

Figure 3. Effect of oral administration of MuIFN-γ on peripheral white blood cell counts. Mice were provided with various concentrations of MuIFN-γ in a gelatin/water solution (0.1%) for 3 days. White blood cell (WBC) counts were made 1 day before MuIFN-γ treatment (Day 0) and after 3 days of MuIFN-γ treatment (Day 4). The data were calculated as percentage of Day 0 values and expressed as the percentage of control peripheral WBC count. The percentage of control peripheral WBC count was plotted versus MuIFN-γ treatment. Each data point represents the mean ± SE of WBC counts from two experiments (10–18 mice).

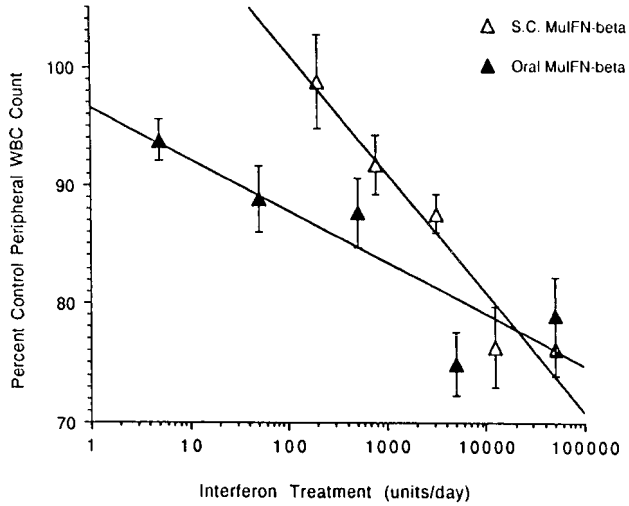

Figure 4. Comparative effects of subcutaneous administration of MuIFN-β and of oral administration of MuIFN-β on peripheral white blood cell counts. Mice were injected subcutaneously with various concentrations of MuIFN-β for 3 days. White blood cell (WBC) counts were made 1 day before MuIFN-β treatment (Day 0) and after 3 days of MuIFN-β treatment (Day 4). The data were calculated as percentage of Day 0 values and expressed as the percentage of control peripheral WBC count. The percentage of control peripheral WBC count was plotted versus MuIFN-β treatment. Each data point represents the mean ± SE of WBC counts. The data for orally administered MuIFN-β from Figure 2 are overlaid on this figure for illustrative purposes.

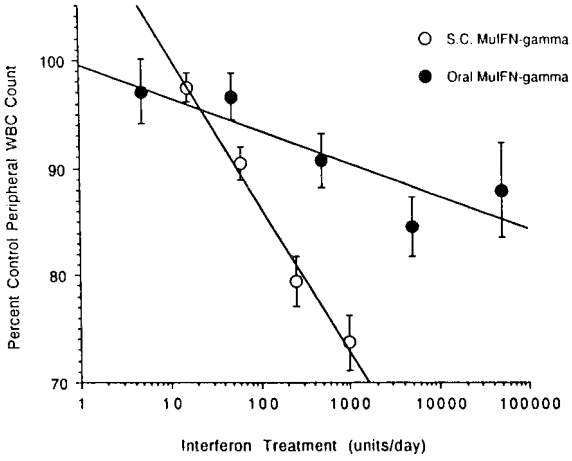

Figure 5. Comparative effects of subcutaneous and oral administration of MuIFN-γ on peripheral white blood cell counts. Mice were injected subcutaneously with various concentrations of MuIFN-γ for 3 days. White blood cell (WBC) counts were made 1 day before MuIFN-γ treatment (Day 0) and after 3 days of MuIFN-γ treatment (Day 4). The data were calculated as percentage of Day 0 values and expressed as the percentage of control peripheral WBC count. The percentage of control peripheral WBC count was plotted versus MuIFN-γ treatment. Each data point represents the mean ± SE of WBC counts. The data for orally administered MuIFN-γ from Figure 3 are overlaid on this figure for illustrative purposes.

A capillary tube that had been dipped in EDTA (10%; Sigma Chemical Co., St. Louis, MO) and dried was filled to approximately 100 μl with blood. The blood was then thoroughly mixed with 5 μl of EDTA. Next, the blood was diluted 1/20 in phosphate-buffered saline (250 μl of final volume). Red blood cells were lysed by adding 1 drop of ZAP-OGLOBIN (Coulter Diagnostics, Hialeah, FL). Total white blood cell (WBC) counts were made in a hemocytometer. Each count was based on the average of four counting areas. Statistical analyses of the data were done using Student's *t* test. Prebleeding the day before initiation of treatment 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.

Interferons. Recombinant DNA-derived rHuIFN-αA/D was kindly provided by Dr. Michael Brunda (Hoffman-LaRoche, Nutley, NJ). MuIFN-β (10^7 units/mg of protein) was purchased from Lee Biomolecular Research Laboratories (San Diego, CA). MuIFN-γ (>95% pure) was prepared in L12R4 cells according to the method of Gribaudo *et al.* (19) and purified by antibody affinity chromatography using a monoclonal antibody to MuIFN-γ (20).

Interferon titers were determined in a microtiter plaque reduction assay (21), compared with the appropriate National Institutes of Health International Reference Standards, and expressed as International Reference Units/ml.

Protocol for Oral Administration of Interferons. Mice were bled one day before interferon treatment to obtain baseline peripheral white blood cell counts. The mice were divided into groups of 5–10 mice per treatment group. Then, mice were orally treated with various concentrations of interferons. Oral treatment was accomplished by including the interferons together with 0.1% gelatin (w/v) in their water supply. Control mice received 0.1% gelatin in their water supply. Interferon/gelatin/water solutions were prepared prior to the initiation of each experiment and were stored frozen at −70°C until needed. Storage at −70°C had no effect on interferon titer. Fresh interferon/gelatin/water solutions were supplied each day for the mice. After 3 days of treatment, the mice were bled, and white blood cell counts were determined and expressed for each individual mouse as the percentage of the prebleed white blood cell count.

The amount of water consumed per day by each treatment group was monitored. Neither the inclusion of 0.1% gelatin alone nor the inclusion of interferon +0.1% gelatin in the water supply affected the amount of water consumed per day (approximately 5 mls/mouse/day). Also, the inclusion of 0.1% gelatin in the water supply did not affect the white blood cell counts of control mice (103.3 ± 4.0%, 99.1 ± 3.0%, and 99.9 ± 2.7% of the prebleed white blood cell count for the control mice from the rHuIFN-αA/D, MuIFN-β, and MuIFN-γ experiments, respectively).

The amount of interferon activity remaining in the drinking water was monitored. The percentage residual interferon activity (mean ± SD) remaining in the inter-

feron/gelatin/water preparations after 24 hr averaged 22.3 ± 11.0% for rHuIFN-αA/D, 51.5 ± 10.7% for MuIFN-β, and 58.8 ± 6.2% for MuIFN-γ.

Results

Effect of Oral Administration of rHuIFN-αA/D. The effect of oral administration of rHuIFN-αA/D on the peripheral white blood cell count was examined. Five concentrations of rHuIFN-αA/D ranging from 1 unit/ml to 10,000 units/ml were provided for the mice in a gelatin/water solution. This represented a daily dose of 5 units/day to 50,000 units/day, respectively. The averaged results of two experiments are shown in Figure 1. It can be seen that oral treatment with rHuIFN-αA/D caused a dose-dependent reduction in the number of peripheral white blood cells. Treatment with 5, 50, 500, 5,000, and 50,000 units/day reduced peripheral white blood cell counts on Day 4 to 97.6%, 93.9%, 87.3%, 80.2%, and 78.7% of the control peripheral white blood cell count, respectively. The slope of the dose-response curve was −5.2. Oral administration of rHuIFN-αA/D at a treatment level as low as 500 units/day (100 units/ml) caused a significant (P = 0.006) reduction in peripheral white blood cell counts (87.3 ± 3.3% of the control white blood cell count). Peripheral white blood cell counts of control mice were not significantly affected by treatment of the mice with the gelatin/water solution (103.3 ± 4.0% of the Day 0 white blood cell count).

Effect of Oral Administration of MuIFN-β. The effect of oral administration of MuIFN-β on the peripheral white blood cell count was examined. The averaged results of three experiments are shown in Figure 2. Five concentrations of MuIFN-β ranging from 5 units/day to 50,000 units/day were employed. It can be seen that oral treatment with MuIFN-β caused a dose-dependent reduction in the number of peripheral white blood cells. Treatment with 5, 50, 500, 5,000, and 50,000 units/day of MuIFN-β reduced peripheral white blood cell counts to 93.8%, 88.8%, 87.7%, 74.9%, and 79.0% of the control peripheral white blood cell count. The slope of the dose-response curve was −4.4. Oral administration of MuIFN-β at a treatment level as low as 5 units/day (1 unit/ml) caused a significant (P = 0.005) reduction in peripheral white blood cell counts (93.8 ± 1.8% of the control white blood cell count).

Peripheral white blood cell counts of control mice were not significantly affected by treatment of the mice with the gelatin/water solution (100.6 ± 2.4% of the Day 0 white blood cell count).

A comparison of Figures 1 and 2 shows that the relative potency of orally administered MuIFN-β was similar to, though slightly more than, that of rHuIFN-αA/D. It may be that the slightly greater potency of MuIFN-β was a result of the approximately 2-fold greater stability of MuIFN-β in the water/gelatin preparations (51.5% recovery of MuIFN-β after 24 hr versus 22.3% recovery of rHuIFN-αA/D). Thus, the results obtained with MuIFN-β paralleled those obtained with rHuIFN-αA/D.

Effect of Oral Administration of MuIFN-γ. The effect of oral administration of MuIFN-γ on the peripheral white blood cell count was examined. The averaged results of two experiments are shown in Figure 3. Four concentrations of MuIFN-γ ranging from 5 units/day to 50,000 units/day were employed. It can be seen that oral treatment with MuIFN-γ caused a dose-dependent reduction in the number of peripheral white blood cells. Treatment with 5, 50, 500, 5,000, and 50,000 units/day of MuIFN-γ reduced peripheral white blood cell counts to 97.1%, 96.7%, 90.8%, 84.6%, and 88.0% of the control white blood cell count. The slope of the dose-response curve was −3.0. Oral administration of MuIFN-γ at a treatment level as low as 500 units/day (100 units/ml) caused a significant (P = 0.0001) reduction in peripheral white blood cell counts (90.8 ± 2.5% of the control white blood cell count). A comparison of Figure 3 with Figures 1 and 2 shows that the relative potency of orally administered MuIFN-γ was slightly lower than those of rHuIFN-αA/D and MuIFN-β. Nonetheless, the results obtained with MuIFN-γ paralleled those obtained with rHuIFN-αA/D and MuIFN-β. Peripheral white blood cell counts of control mice were not significantly affected by treatment of the mice with the gelatin/water solution (99.8 ± 1.7% of the Day 0 white blood cell count).

Comparative Effects of Oral Administration and Subcutaneous Administration of Interferons. Linear regression curves generated for orally administered interferons were compared with linear regression curves generated for subcutaneously administered interferons (Fig. 4 and 5). Figure 4 presents a comparison of orally administered MuIFN-β with subcutaneously administered MuIFN-β. The dose-response curve of subcutaneously administered MuIFN-β is similar to that published previously (22–23) and is steeper than that of orally administered MuIFN-β. However, a significant level of supression of peripheral white blood cell counts occurs with a lower dose of orally delivered MuIFN-β.

Figure 5 presents a comparison of orally administered MuIFN-γ with subcutaneously administered MuIFN-γ. The dose-response curve of subcutaneously administered MuIFN-γ is similar to that published previously (22–23) and is steeper that that of orally administered MuIFN-γ. Also, a significant level of supression of peripheral white blood cell counts occurs with a lower dose of subcutaneously delivered MuIFN-γ.

Thus, the dose-response curves of orally administered and subcutaneously administered interferons are quite different. The dose-response curves of subcutaneously administered interferons are steeper than the

those of the orally administered interferons. Also, while subcutaneously administered MuIFN-γ is much more potent than subcutaneously administered MuIFN-β, orally administered MuIFN-γ has approximately the same potency as orally administered MuIFN-β.

Discussion

Interferons exert a number of systemic effects when they are administered subcutaneously, intramuscularly, or intravenously. This study examined the ability of orally administered interferons to exert a specific systemic effect, the suppression of peripheral white blood cell counts. The use of a reduction in the number of peripheral white blood cells as an indicator of the systemic effect of the interferons was based on previous observations that parenterally administered interferon both in humans (24) and in the mouse (25–26) causes a suppression of the peripheral white blood cell count.

The effect of rHuIFN-αA/D on the peripheral white blood cell count was examined. rHuIFN-αA/D was chosen because it had been shown to cross species barriers and to be effective in the murine system. The data presented showed that the oral administration of rHuIFN-αA/D caused a dose-dependent suppression of peripheral white blood cell counts. This observation suggested that the oral administration of rHuIFN-αA/D exerted a systemic effect. It was perhaps not surprising to find that rHuIFN-αA/D could exert a systemic effect, since IFN-α has been shown to be efficiently distributed systemically when it is administered by a variety of routes (6). Also, others have shown previously that oral (or nasal) administration of IFN-α can protect against systemically administered virus in cats (16) and can exert a systemic effect in humans (17–18).

It was important to determine whether the peripheral white blood cell suppression observed with oral administration of rHuIFN-αA/D was unique to IFN-α or part of a more general phenomenon. First, the effect of oral administration of MuIFN-β on the peripheral white blood cell count was examined. The data presented showed that the oral administration of MuIFN-β caused a dose-dependent suppression of peripheral white blood cell counts. Moreover, the potency of orally administered MuIFN-β was similar to that observed with orally administered rHuIFN-αA/D. Second, it was important to determine whether the peripheral white blood cell suppression was limited to oral administration of Type I interferons (IFN-α and IFN-β) or whether it was also induced with Type II interferon (IFN-γ). To address this question, the effect of oral administration of MuIFN-γ on the peripheral white blood cell count was examined. The data presented showed that the oral administration of MuIFN-γ caused a dose-dependent suppression of peripheral white blood cell counts. Further, the potency of orally administered MuIFN-γ was close to that observed with orally administered rHuIFN-αA/D and MuIFN-β. The results indicated that oral administration was an effective route of administration for each of the three types of interferons.

The mechanism by which interferon induces the suppression of peripheral white blood cell counts is unknown. The white blood cell suppression may reflect an increased rate of recruitment of white blood cells out of the circulation and into the lymphatic system. On the other hand, it may also reflect a decreased rate of white blood cell production mediated by a suppression of the bone marrow. Studies to investigate this mechanism further are in progress. Whatever the mechanism, these results indicate that oral administration of rHuIFN-αA/D, MuIFN-β, or MuIFN-γ can cause a systemic effect, as measured by a reduction in the peripheral white blood cell counts.

The mechanism by which orally administered interferons exert their systemic effect is also unknown. It may be that orally administered interferons exert their systemic effect after becoming systemically distributed. In this regard, it is unlikely that the interferons are absorbed through the intestinal system, given the sensitivity of MuIFN-γ to low pH. On the other hand, the interferons may be absorbed through the mucosal tissue of the oropharynx and esophagus. However, it has not been possible to reproducibly detect significant levels of interferons in the serum of mice during the oral administration of MuIFN-β or MuIFN-γ at concentrations as high as 10,000 units/ml (50,000 units/day; data not shown). A previous study has shown that, following nasal administration of 20,000 units of HuIFN-β, only 95 units/ml (0.64%) were detectable in the serum of mice 30 min after administration (27). It should be noted that this reported level of HuIFN-β detected in the serum following nasal administration (0.64%) is similar to the level of rHuIFN-αA/D that has been reported to be detected in the serum following subcutaneous administration (1.5% after 1 hr and 0.4% after 4 hr) (28). In the unlikely event that the mice would have drunk 1 ml of water containing 10,000 units/ml of interferon 30 min before bleeding, the maximal amount of interferon in the serum might have approached 60 units/ml. Thus, given the sensitivity of the assay (>10 units/ml) and the low amount of interferon imbibed at any given time, it may not be surprising that significant levels of interferon were not detected in the serum following oral administration.

It may also be that the orally administered interferons exert their systemic effect through a cell-mediated effect. In this regard, it could be proposed that the interferons interact with and activate leukocytic cells that line the oropharynx. These activated leukocytic cells might then travel to distant sites where they might transfer their activation signals to adjacent cells

in the manner described by Blalock and his colleagues (29–32).

Finally, it may be that orally administered interferons exert their systemic effect though the interferon-induced production of biological regulatory substances such as lymphokines or cytokines. Studies to distinguish among these possible mechanisms are in progress.

Whatever the mechanism by which orally administered interferons exert their systemic effects, the three interferons were found to have relatively similar potencies in their peripheral white blood cell suppressive effects. This observation was unexpected, since IFN-β and IFN-γ have been reported to be less readily distributed systemically than IFN-α (6). It can be noted, however, that the relative potencies of MuIFN-β and MuIFN-γ differ for the two routes of administration, with the slopes of the dose-response curves obtained with oral administration of interferons being much more shallow than the dose-response curves obtained with subcutaneous administration of interferons. This observation suggests the possibility that oral interferon administration might provide a broader therapeutic range with less severe side effects than subcutaneous interferon administration.

The results of this study support the concept that oral administration of each of the three types of interferons may have promise for clinical use. Oral administration of HuIFN-α has already been shown to protect cats against systemic viral infection (16). Thus, it will be important to determine and quantitate other possible systemic effects (such as antiviral activity and antitumor activity) that may be mediated by oral administration of the three interferons, particularly of IFN-β and IFN-γ.

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