Low-Dose Oral Type I Interferons Reduce Early Virus Replication of Murine Cytomegalovirus In Vivo

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ABSTRACT

Immunity to viral infections involves both innate and antigen-specific immune responses. The antiviral properties of interferons (IFNs) are part of the innate immune response. Low doses of type I IFNs (IFN-α and IFN-β) administered daily (10 IU per mouse) by the oral route significantly reduced the early replication of murine cytomegalovirus (MCMV) in both the spleen and liver of MCMV-infected susceptible BALB/c mice. Significant inhibition of virus replication was observed for two different inoculum doses of virus (2 × 10^4 pfu per mouse [0.6 LD50] and 2 × 10^5 pfu per mouse [0.8 LD50]). Analysis of IFN retention, using [35S]-labeled IFN-α1 compared with the nonreceptor binding mutant IFN-α1 (R33M) administered orally to mice, revealed binding of wild-type IFN-α1 to several tissues. In particular, IFN was retained by tissues proximal to lymphoid regions, including the posterior nasal cavity, posterior tongue, small intestine, and rectum. These findings suggest that type I IFNs may inhibit MCMV replication by distal binding of the orally administered IFN to various tissues, which in turn augment the primary immune response to virus infection.

INTRODUCTION

Human interferon (IFN) is currently licensed for the treatment of several clinical disorders, including hairy cell leukemia, hepatitis C, condyloma acuminatum, multiple sclerosis, and Kaposi’s sarcoma.1-6 Most treatment regimens have used high doses of 10^6 IU IFN, which cause undesirable influenza-like side effects immediately after IFN injection and, in 35%-45% of patients, induces the production of neutralizing antibodies after prolonged treatment.7,8 Clearly, new advances in IFN therapy need to be made to improve the clinical efficacy. Administration of low doses of IFN orally has provided a safe alternative to injectable high doses of IFN and has been used effectively in treating HIV-1-positive patients,7,8 although the efficacy remains controversial following similar clinical trials showing no IFN efficacy.9,10

More recently, the oral mode of administration of IFN showed proven efficacy in an animal model of chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE).11 Mice immunized with spinal cord homogenate develop an acute-phase EAE. However, 10 IU IFN administered orally to mice three times a week optimally suppressed clinical symptoms.

We present in this study for the first time the clinical efficacy of low-dose oral IFN for a natural virus infection. We have used the well-established murine model for cytomegalovirus infection. Susceptible BALB/c mice infected l.p. with sublethal doses of murine cytomegalovirus (MCMV) produce high virus titers in the major target organs, spleen and liver, during the acute phase of infection.12,13 We demonstrate that mice orally administered low-dose (10 IU) type I IFNs daily for 1 week before MCMV infection could markedly limit virus replication in both the spleen and liver at day 2 postinfection. As expected, a single l.p. injection of high-dose IFN (20,000 IU) on the day of virus inoculation also significantly reduced the virus load in both spleen and liver. We have further characterized that orally administered IFN is retained in various tissue sites of uninfected normal mice, as determined by [35S]methionine labeling experiments using IFN-α1 compared with a receptor nonbinding mutant IFN-α1 (R33M). Specifically, the posterior nasal cavity, posterior tongue, small intestine, and rectum were found to significantly bind and retain wild-type IFN, which may repre-

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MATERIALS AND METHODS

Mice

C57BL/6 and BALB/c female mice (6–7 weeks old) were purchased from Animal Resources Centre (Murdoch, Western Australia). BALB/c mice were infected with virus at 8 weeks of age.

Virus

The virus stock of MCMV (K181 strain) was prepared as a 20% salivary gland homogenate from infected weanling female BALB/c mice and stored in the gas phase of liquid nitrogen. MCMV was titrated in mouse embryo fibroblasts by plaque assay and expressed as pfu/ml as described previously. Virus inoculum was diluted in pyrogen-free phosphate-buffered saline (PBS).

Synthesis of radiolabeled IFNs

[35S]Methionine-labeled IFNs (wild-type mouse IFN-α1 and mutant mouse IFN-α1 R33M) were synthesized from expression plasmid vectors as previously described using a coupled transcription and translation kit according to the manufacturer's instructions (Promega). Unincorporated [35S]methionine was removed by dialysis against mouse omolarily buffer saline over 5 days at 4°C.

Polyethylene glycol-8000 was used to concentrate the radiolabeled-IFNs, which were then stored at 4°C. Purity was checked by SDS-PAGE and autoradiography. Biologic activity was determined in a bioassay using reduction in cytopathic effect of encephalomyocarditis virus-infected L929 cells. The IFN-α1 had an activity of 4000 IU/ml, whereas the mutant IFN-α1 R33M had no detectable activity, as expected.

To assess the concentrations of the IFNs, relevant bands were cut out from the gel following electrophoresis, solubilized with tissue solubilizer (NCS, Amersham), and counted using a beta-counter. The wild-type and the mutant IFN preparations were adjusted to equal counts of radioactivity (100,000 cpm/7 μl, i.e., 28 IU IFN-α1) before oral administration to mice.

IFN administration and virus inoculation

BALB/c mice were administered 10 IU IFN-α/β (Lee Biomedical) in 7 μl daily by the oral route and stayed from food and water for 2 h after treatment. The volume was delivered using a micropipette to the anterior tongue of the mice while they were restrained as for i.p. injection. Mice were infected with MCMV (2 × 10^6 or 2 × 10^4 pfu per mouse) by the i.p. route after 7 days of low-dose oral IFN treatment. These virus-infected mice were administered low-dose oral IFN for a further 2 days before organ harvest.

A separate group of BALB/c mice received 20,000 IU IFN-α/β by the i.p. route 6 h before MCMV inoculation (2 × 10^4 or 2 × 10^4 pfu per mouse), also by the i.p. route on the same day. Another group of BALB/c mice were not treated with oral IFN but received an equal volume of normal saline. They were similarly infected with MCMV and served as controls. All groups contained 5 mice.

In the retention experiments, C57BL/6 mice (3–5 per group) were given 100,000 cpm of either the radiolabeled wild-type IFN-α1 or IFN-α1 R33M solutions in a 7 μl volume directly into the mouth using a micropipette. No food or water was available to the mice after oral IFN administration for the duration of the 8 h experiment.

Determination of virus titers in spleens and livers

Individual organ homogenates (10% w/v spleen, 20% w/v liver) were titrated in the plaque assay. Virus titers are expressed as mean pfu/ml ± SE from individual organ homogenates (corrected to 100% w/v) obtained from 5 mice prepared 2 days postinfection with MCMV. Organ homogenates from which virus was not recovered were assigned a titer of 50 pfu/ml, the lowest limit of detectable virus.

Determination of binding of radiolabeled IFN

After 1, 2, 4, or 8 h following radiolabeled IFN administration by the oral route, tissues were microsurgically extracted (anterior tongue, posterior tongue, anterior nasal cavity, posterior nasal cavity, farynx and trachea, esophagus, stomach, small intestine, rectum, spleen, and a portion of the mastatory muscle). The nasal cavity was severed along the plane where the nasal rugae terminate into anterior and posterior portions. Tissue samples were blotted to remove excess fluid and solubilized with 6X (w/v) tissue solubilizer (NCS, Amersham). Radioactivity (cpm/g of tissue) was determined in a beta-counter.

Statistical analysis

Levels of significance were determined by the unpaired t-test assuming unequal variance between the means.

RESULTS

Low-dose oral IFN reduces MCMV replication in spleen and liver

BALB/c mice given 10 IU IFN-α/β daily for 1 week before a sublethal infection with MCMV could significantly reduce early virus replication in two major target organs (Fig. 1). Mice infected with 2 × 10^4 pfu MCMV (0.6 LD50) and treated with low-dose oral IFN showed a significant (p < 0.001, 3.5-fold) reduction in virus replication in the spleen at day 2 postinfection compared with control mice that were not administered IFN (Fig. 1A). These control mice had received 1 week prior administration of the same volume of IFN vehicle only (saline). In a further control group, mice given a single injection of high-dose IFN (20,000 IU) by the i.p. route on the day of virus inoculation demonstrated a significant reduction (p < 0.001, 3-fold) in virus replication. Furthermore, a significant (p < 0.01, 5.5-fold) reduction in virus replication in the liver was observed at day 2 postinfection for mice receiving low-dose oral IFN compared with untreated control mice (Fig. 1B). Again, mice given high-dose IFN i.p. also displayed a significant (p < 0.01, 8-fold) reduction in virus titer in the liver at day 2 postinfection.
Low-dose IFN was also assessed using an increased virus load of $2 \times 10^4$ pfu MCMV (0.8 LD$_{50}$) in a similar experimental design. BALB/c mice given low-dose oral IFN daily for 1 week before infection with MCMV were also able to effectively reduce high virus titers obtained from the spleen (Fig. 1C) ($p < 0.003$, 5-fold) and the liver (Fig. 1D) ($p < 0.035$, 2-fold) relative to untreated control mice (IFN vehicle only). As expected, an i.p. injection of 20,000 IU IFN on the day of virus inoculation significantly lowered virus titers in both spleen (Fig. 1C) ($p < 0.03$, 2-fold) and liver (Fig. 1D) ($p < 0.006$, 3-fold).

Other IFN doses covering a range from 0.1 to 1000 IU per mouse have been used in our protocol of orally administered IFN to test the effect of varying the IFN dose. Preliminary data have shown that mice given 10 IU had the greatest reduction in MCMV replication, that is, a U-shaped dose-response curve, with 10 IU being near optimal (data not shown).

Retention analysis of radiolabeled IFN administered orally

The mutant IFN-α1 (R33M) is unable to bind to its cognate receptor, and because binding and internalizing of the IFN-receptor complex is essential for IFN-induced biologic effects, the IFN-α1 (R33M), as expected, failed to confer antiviral effects to the L929 cells used in the bioassay (data not shown). This mutant IFN preparation was used as a control for comparison of IFN binding with wild-type IFN, which possesses full biologic activity.

Mice were orally administered either wild-type [35S]methionine-labeled IFN-α1 or [35S]methionine-labeled mutant IFN-α1 (R33M), and various tissues were assessed for IFN binding at 1, 2, 4, and 8 h following treatment. Specific binding of wild-type IFN-α1 over the mutant was evident for the posterior tongue (Fig. 2A), posterior nasal cavity (Fig. 2B), small intestine (Fig. 2C), and rectum (Fig. 2D) but was not ob-
served for various other tissues, including the masticatory muscle (Fig. 2E), anterior tongue, anterior nasal cavity, larynx and trachea, esophagus, stomach, and spleen (data not shown).

The specific IFN retention is apparent in the posterior tongue (Fig. 2A) at the 4 h time point, whereas by 8 h, an equilibrium is reached. A similar pattern is seen for the posterior nasal cavity (Fig. 2B). The counts per gram of tissue are relatively low for the posterior nasal cavity, although this is to be expected given that this tissue sample contained a relatively large amount of bone. Significant IFN-specific binding is occurring in the small intestine (Fig. 2C). Similarly, in the rectum (Fig. 2D), counts for the IFN-α1 (R33M) control remain relatively stable over the time course, whereas wild-type IFN-α1 accumulates at 8 h. The masticatory muscle (Fig. 2E), although proximal to the oropharyngeal cavity, does not directly contact the oropharyngeal lumen. Thus, this tissue represents a control for nonspecific [35S]methionine accumulation. As expected, the pattern of IFN-α1 and IFN-α1 (R33M) retention is not significantly different for the masticatory muscle.

**DISCUSSION**

**Low-dose oral IFN therapy**

We describe the first reported evidence of the efficacy of low-dose, orally administered type I IFN in reducing herpes virus replication in vivo. Specifically, the viral load in the spleens and livers of susceptible mice infected with MCMV was significantly reduced following prophylactic treatment with low doses of oral type I IFN. Previous work in the field of low-dose oral administration of IFNs has only tangentially examined this mode of IFN administration, examined artificially induced disease models, or analyzed systemic pharmacokinetic parameters. The present model uses the natural mouse pathogen, MCMV, and allows further investigations into the possible mechanisms of action of orally administered low doses of type I IFNs. Because of the dynamic nature of the in vivo retention study, the data obtained can only be suggestive of the trends of the binding behavior of orally administered IFN-α. Overall, there appeared to be a tendency for higher retention levels in certain tissues that were proximal to major lymph tissues.

Proximal to the posterior tongue, a lymph tissue called Waldeyer’s ring is present. The apparent IFN retention seen in the posterior tongue may represent IFN binding to intraepithelial leukocytes or dendritic cells located within the mucosa. Following the binding of the IFN, such cells may home to the Waldeyer’s ring. The posterior nasal cavity tissue sample also contains a lymph tissue, the nasal-associated lymph tissue (NALT). However, any of the administered IFN solution reaching the posterior nasal cavity must first flow past the posterior tongue. As there is apparent retention of IFN occurring in the posterior tongue, less of the IFN-α1 actually reaches the posterior nasal cavity. This implies a fortisit retention of IFN-α1 compared with the IFN-α1 (R33M) control, which may be occurring by possible nasal reflux with solutions initially present in the oropharyngeal region before swallowing. The results from the small intestine support the proposal that oral administration of IFNs is a viable alternative route of administration. The unusual acid stability of IFN-αβ to pH 2.0 may possibly allow the orally administered IFN to retain biologic activity after passage through the acidic environment of the stomach to contact intraepithelial lymphocytes or dendritic cells (or both) present in the small intestine proximal to local lymph tissues, the Peyer’s patches. The accumulation of IFN over the time course in the rectum is to be expected given that the IFN solution was administered orally. However, the higher accumulation of IFN-α1 vs. IFN-α1 (R33M) suggests that IFN-α1 is being specifically retained. The rectum contains lymph nodules scattered throughout its mucosal surface, which may be the specific location of IFN-receptor binding. If IFN receptors are located in the rectum, IFN-containing suppositories may also be an efficacious mode of delivery.

Brod and Khan oral administration of IFN via a 20-gauge ballpoint needle directly into the distal esophagus. Thus, the posterior tongue and posterior nasal cavity were completely bypassed, yet the IFN conferred a strong reduction in clinical symptom score. It is implied from the CR-EAE study and from our present MCMV study that orally administered IFN-α may bind to type I IFN receptors present on cells throughout the gastrointestinal tract, particularly in regions proximal to known lymph tissues.

**Evolutionary perspectives of oral IFN**

Two evolutionary explanations that are not mutually exclusive may explain the phenomenon of IFN in the gut lumen binding to receptors on cells located proximal to the Peyer’s patches. First is the postnatal drip phenomenon. A mammal that has encountered an upper respiratory tract virus will produce IFN in the nasal secretions as part of the innate immune response. These secretions will be swallowed and contact IFN receptors located elsewhere in the gastrointestinal tract. Migration of the mucosa patrolling cells back to proximal lymph tissue following IFN binding could be the trigger for systemic immune status alterations. For example, the systemic immune response can be primed toward an antiviral state in anticipation of a particularly virulent virus possibly infecting other tissues. Second, cytokine networks could be activated by oral IFN to potentiate other systemic responses.

**Mechanism of action of oral low-dose IFN-αβ**

Binding of orally administered IFN to type I IFN receptors throughout the gastrointestinal tract may induce signal transduction to underlying lymphoid tissue. The overall result may be an altered immune status for the animal, which is less favorable for viral replication (Th1). Type I IFN production is an initial event occurring after virus infections and thus may act as an early warning signal of the innate immune response that then predisposes or alters the acquired immune response.

Tough et al., have recently reported that type I IFN can induce bystander T cell proliferation, especially of the CD8+CD44+ T cell subset (activated and memory phenotypes), which are the lymphocytes that show marked expansion during virus infections. Furthermore, type I IFNs have profound effects on lymphocyte trafficking. We postulate that low-dose orally administered type I IFNs may have the ability to induce T cell proliferation and regulate the mobilization patterns of the specific immune response. Thus, periodic stimulation with ei-
FIG. 2. IFN is specifically retained in the posterior tongue, posterior nasal cavity, small intestine, and rectum following oral administration. C57BL/6 mice were orally administered 100,000 cpm of either [35S]methionine-labeled wild-type Mu-IFN-ε (black bars) or mutant IFN-ε (R33M, receptor nonbinding) (hashed bars). Mice (n = 3–5) were killed after 1, 2, 4, or 8 h, and tissues were microsurgically extracted, blotted, and weighed. Tissues were solubilized, and radioactivity was detected in a beta-counter. Counts were converted to cpm/g of tissue, and the averages and standard errors are shown for the posterior tongue (A), posterior nasal cavity (B), small intestine (C), rectum (D), and masticatory muscle (E).
their low-dose orally administered IFN or mucosal IFN secretions naturally produced during intermittent viral infections may assist the generation and maintenance of memory T cell pools. The work described in this article may thus be another example of the close and effective interaction of the innate and acquired immune responses. Our findings of low-dose orally administered IFN in the experimental mouse model for CMV infection open investigations into delineating the mechanisms of action of low-dose oral type I IFN administration.

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