Gamma Interferon (IFN-γ) Receptor Null-Mutant Mice Are More Susceptible to Herpes Simplex Virus Type 1 Infection than IFN-γ Ligand Null-Mutant Mice

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Mouse strains with null mutations in the gamma interferon gene (Ifng) or the gamma interferon receptor gene (Ifngr) have been engineered. The use of these strains as animal models of viral and bacterial infections has enhanced our understanding of the role of gamma interferon (IFN-γ) in the host immune response. However, direct comparisons between Ifng−/− (GKO) and Ifngr−/− (RGKO) mice have been problematic because previously available strains of these mice have had different genetic backgrounds (i.e., C57BL/6 and BALB/c for GKO mice and 129/Sv/Ev for RGKO mice). To enable direct comparison of herpes simplex virus type 1 (HSV-1) infections in GKO and RGKO mice, we introduced the IFN-γ null mutation into the 129/Sv/Ev background. We report that, after HSV-1 inoculation, mortality was significantly greater in RGKO mice than in GKO mice (38 versus 23%, P = 0.0001). Similarly, the mortality from vaccinia virus challenge was significantly greater in RGKO mice than in GKO mice. With differences in genetic background excluded as a confounding issue, these results are consistent with the existence of an alternative ligand(s) for the IFN-γ receptor that is also capable of mediating protection against viral challenge.

Gamma interferon (IFN-γ) was originally referred to as the immune interferon in recognition of its antiviral activity. However, IFN-γ is currently better known for the remarkable spectrum of pleiotropic effects it elicits in diverse tissues and particularly for its unique immunoregulatory properties which distinguish it from IFN-α/β, other antiviral proteins in the interferon family. The antiviral effects of IFN-γ are not as well understood, but they appear to be especially important for long-term control of viral infections (4, 38). IFN-γ also mediates protection against intracellular microbes, including certain viruses, by activating macrophages and other mononuclear phagocytes to produce nitrous oxide (2, 4). IFN-γ and IFN-α/β bind to distinct receptors, and no cross-reactivity has been observed. IFN-γ binds to the α chain of the high-affinity IFN-γ receptor that is ubiquitously expressed on different cell types, and it is the only known ligand for this receptor. A low-affinity IFN-γ receptor has been identified on human macrophages, but its physiological significance is unknown (1, 13).

Recent studies of null-mutant mice have confirmed the importance of IFN-γ in the host immune responses to several bacterial and viral pathogens, including herpes simplex virus type 1 (HSV-1), vaccinia virus, measles virus, and Theiler’s virus (5, 9, 16, 17, 24). Studies with these IFN-γ mutant strains have also provided evidence, albeit controversial at times, supporting and refuting the involvement of IFN-γ in autoimmune diseases such as experimental allergic encephalomyelitis, experimental autoimmune myasthenia gravis, and insulin-dependent diabetes mellitus (2, 15, 25, 40). Controversy has arisen from the fact that different results have been obtained from various studies depending on whether the null mutation is in the IFN-γ gene (Ifng) (GKO mice) (10) or in the IFN-γ receptor gene (Ifngr) (RGKO mice) (24). Previously, GKO and RGKO mice have been available only in different genetic backgrounds (i.e., C57BL/6 and BALB/c for GKO and 129/Sv/Ev for RGKO).

Previously we reported a prolonged inflammatory response in the trigeminal ganglion after ocular inoculation with HSV-1 (7). We showed the CD4+ and CD8+ T cells associated with the large amounts of IFN-γ which were tightly surrounding neurons (some of which were latently infected with HSV-1 as shown by in situ hybridization). These observations led to speculation that IFN-γ might play a role during HSV-1 infection, and we proposed the use of GKO and RGKO strains to directly test this hypothesis. However, the strain restrictions of these mice were problematic, since it is known that different mouse strains vary in susceptibility to HSV-1, with the C57BL/6 strain being relatively resistant to the virus (26). To circumvent the influence on experimental outcome of the different genetic backgrounds of the mutant mice, the IFN-γ null mutation was introduced into the 129/Sv/Ev background. This enabled the direct comparison of the outcomes of HSV-1 infections of GKO and RGKO mice, which differed genetically only at the mutant locus.

Isolation of the IFN-γ null mutation in the 129/Sv/Ev background. The AB-1 embryonic stem (ES) cell clone 97E of the AB-1 background (22) was microinjected into blastocysts of the C57BL/6 strain. Chimeric founder mice were mated to 129S/SvTac female mice (Taconic Farms, Germantown, N.Y.) to generate 129/Sv/Ev mice heterozygous for the IFN-γ null mutation. Offspring resulting from germ line transmission of the ES cell genome were identified by glucose phosphate isomerase-1 (Gpi-1) isoforms: type A (derived from type AB) and type B (derived from GKO), and of these selected mice, those carrying the mutation were identified by PCR. The IFN-γ mutation was thereby retained in the 129/Sv/Ev genetic background. Ifng−/− heterozygous
mice were intercrossed to produce Ifng^{−/−} homozygous, GKO mutant mice which were identified by PCR analysis of DNA extracted from their tails for IFN-γ and Neor gene sequences. In Fig. 1a, PCR genotyping results for several offspring from matings of heterozygous 129/Sv/Ev mice were analyzed by PCR for IFN-γ and Neor gene sequences to identify mice homozygous for the IFN-γ null mutation. The upper band is the Neor gene amplification product, and the lower band is the IFN-γ gene amplification product. PCR primers for IFN-γ gene are as follows: forward, 5'-AGAAGTAAGTGGAAGGGCCCAGAAG, and reverse, 5'-AGGGAAATCGGTAGAAGGGCCCGAAG, giving a 220-bp product. Primers for the Neor gene are as follows: forward, 5'-TCACGGCGAGGGCGCCCGTTCT TT, and reverse, 5'-TXGGCTCGAAGCCGCTCCATCGGA, giving a 375-bp product. (b) RT-PCR for Ifng mRNA. cDNA preparations from ConA-stimulated spleen cells isolated from 129/Sv/Ev mice (lanes 1 and 2), GKO mice (lanes 3 and 4), and RGKO mice (lanes 5 and 6) were amplified with Ifng-specific primers. M, markers; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Breeding pairs of mice homozygous for the null mutation in Ifng were intercrossed to produce Ifng^{−/−} homozygous, GKO mice. Prior studies with normal mice and mice unresponsive to IFN-γ due to a null mutation in Ifng or Ifnrg have established that interferon is crucial for survival after inoculation with poxviruses such as vaccinia virus (31). To confirm the susceptibility of GKO mice with the 129/Sv/Ev background to vaccinia virus and to directly compare the response of the GKO and RGKO strains, mutant and control male mice were challenged with vaccinia virus by tail vein inoculation. The results presented in Fig. 2 confirm that the control 129/Sv/Ev mice were completely resistant to vaccinia virus infection as expected (9, 31, 39). Compared to the mortality rates of control mice, the mortality rates in vaccinia virus-infected GKO mice were high (56%) (P = 0.002) and even higher (92%) in RGKO mice (P = 0.0001). The difference in mortality rates between RGKO and GKO mice was significant (P = 0.03) and corresponded to a 3.3-fold increase in the relative risk of death for RGKO mice inoculated with HSV-1 compared to similarly inoculated GKO mice (95% confidence limit).

Mortality of HSV-1 Infected GKO, RGKO, and control mice. To determine the role of IFN-γ during the acute stage of HSV-1 infection, deeply anesthetized, 6- to 8-week-old male mice were inoculated intravenously with 10^{7} PFU of vaccinia virus. There were 12 mice in each group, except that the GKO group had 9 mice. Mice were monitored daily over a 20-day period, and mice with symptoms of life-threatening disease were euthanatized.

FIG. 2. Survival of vaccinia virus-infected 129/Sv/Ev, GKO, and RGKO mice. Deeply anesthetized, 6- to 8-week-old male mice were inoculated intravenously with 10^{7} PFU of vaccinia virus. There were 12 mice in each group, except that the GKO group had 9 mice. Mice were monitored daily over a 20-day period, and mice with symptoms of life-threatening disease were euthanatized.

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FIG. 1. (a) PCR genotyping of GKO mice. Tail DNA from offspring of matings of heterozygous 129/Sv/Ev mice were analyzed by PCR for IFN-γ and Neor gene sequences to identify mice homozygous for the IFN-γ null mutation. The upper band is the Neor gene amplification product, and the lower band is the IFN-γ gene amplification product. PCR primers for IFN-γ gene are as follows: forward, 5'-AGAAGTAAGTGGAAGGGCCCAGAAG, and reverse, 5'-AGGGAAATCGGTAGAAGGGCCCGAAG, giving a 220-bp product. Primers for the Neor gene are as follows: forward, 5'-TCACGGCGAGGGCGCCCGTTCT TT, and reverse, 5'-TXGGCTCGAAGCCGCTCCATCGGA, giving a 375-bp product. (b) RT-PCR for Ifng mRNA. cDNA preparations from ConA-stimulated spleen cells isolated from 129/Sv/Ev mice (lanes 1 and 2), GKO mice (lanes 3 and 4), and RGKO mice (lanes 5 and 6) were amplified with Ifng-specific primers. M, markers; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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in the trigeminal ganglia and brain stem cells of RGKO than in
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tion and/or persistence of HSV-1 in the RGKO mice. We have
anticipated with a significant protective effect.

These results show that IFN-γ fails to confer protection against
mortality induced by HSV-1 infection, while, in contrast, the
presence of the IFN-γ receptor (as in GKO mice) was associ-
ated with a significant protective effect.

**Time course of HSV-1 infection in RGKO and GKO mice.**
The increased mortality of RGKO compared to GKO and
129/Sv/Ev mice suggested the possibility of enhanced replica-
cation and/or persistence of HSV-1 in the RGKO mice. We have
previously reported a statistically significant higher mean titer in
the trigeminal ganglia and brain stem cells of RGKO than in
129/Sv/Ev mice 3 to 4 days after HSV-1 inoculation; however, the
difference in titer was slight (<0.5 log) (7). More recently, we
determined the daily viral titers in trigeminal ganglia and
brain stem cells from days 2 to 7 after HSV-1 inoculation. The
results showed that, contrary to expectation, there was no
significant difference in either the kinetics of HSV-1 replica-
cation or clearance in the trigeminal ganglia of RGKO, GKO,
and 129/Sv/Ev mice (6). More relevant to the mortality results
presented here, Fig. 4 shows a time course of daily brain stem
HSV-1 titers in the RGKO, GKO, and 129/Sv/Ev mice. For
statistical comparison of HSV-1 titers, the total plaque counts
for the three mouse groups were fitted to an overdispersed
Poisson model. Statistical analysis of HSV-1 titers in the eye
indicated a significantly greater likelihood of HSV-1 persis-
tence in the eyes of GKO and RGKO mice than in 129/Sv/Ev
mice (not shown), similar to the study of Bouley et al., which
showed persistence of HSV-1 in the tear film of GKO, but not
control, mice (5). Persistence of HSV-1 in the eyes of IFN-γ
mutant mice shows that IFN-γ-dependent mechanisms are cru-
cial for clearance of HSV-1 from the eye, as was previously
shown for skin infections (21, 35). However, we found no
significant difference in brain stem viral titers over the course
of the infection (Fig. 4). All the mice surviving inoculation with
HSV-1 were confirmed as being latently infected.

At face value, the equivalent titers are surprising, since cell
culture studies have demonstrated inhibition of HSV-1 by
IFN-γ (11, 14). However, the results from our time course
study are in agreement with an earlier study of GKO mice from
another group which showed no difference in mean HSV-1
titers in nervous system tissue at set times after HSV-1 inocu-
lation (19). Indeed, other studies using an IFN-γ-transgenic
model showed that IFN-γ protected against HSV-1-induced
mortality without significantly affecting HSV-1 titers in the
nervous system of transgenic, compared to nontransgenic, mice
(18). Similarly, in studies with murine gamma herpesvirus 68,
there was no difference in viral load or rate of virus clearance
in the lungs of infected RGKO mice compared to 129/Sv/Ev
mice (12). In contrast to the apparent dispensability of IFN-γ
in the control of murine gamma herpesvirus 68 in the lung,
severe structural damage and elevated latency loads were seen
in the spleens of RGKO, but not 129/Sv/Ev, mice.

Contrasting its role in acute infection, we have recently dem-
onstrated a role for IFN-γ in the control of reactivated HSV-1

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**FIG. 3. Survival of HSV-1-infected 129/Sv/Ev, GKO, and RGKO mice.**
Male, 6- to 8-week-old mice were inoculated in the right eye by ocular scarifi-
cation with doses of HSV-1 ranging from $10^5$ to $10^7$ PFU. Mice were monitored
daily over a 20-day period for signs of encephalitis, and mice with pronounced
symptoms indicative of imminent death were euthanatized. As no statistically
significant difference was found between groups given inocula of $10^7$, $10^6$, or $10^5$
PFU, the data from these three groups were pooled and analyzed together.

**FIG. 4. Time course of HSV-1 infection in 129/Sv/Ev, GKO, and RGKO mice.**
The right eyes of 6- to 8-week-old male mice were inoculated with $10^6$ PFU
of HSV-1 by ocular scarification. Three mice from each group were sacrificed
daily from day 2 through day 7, and HSV-1 titers in brain stem cells were
determined. The whole-tissue HSV-1 titers were plotted against day postinocu-
lation for 129/Sv/Ev (strain T), GKO (strain G), and RGKO (strain R) mice.
There was no statistically significant difference in HSV-1 titers in the brain stem
cells for the three groups of mice.
Possible explanations for the phenotypic difference between RGKO and GKO mice. We do not think that minor differences in genetic background can explain the higher mortality rates of RGKO compared to GKO mice (3, 20). This is because the GKO strain used in this study was derived from the same 129/Sv/Ev background as was the RGKO strain. Although the 129/Sv/Ev strain used to derive the GKO strain was Gpis1s/Gpis1s, we selected Gpis1s/Gpis1s animals for propagation because RGKO mice, also derived with AB-1 ES cells, are Gpis1s/Gpis1s (unpublished observation). For the GKO strain, the Gpis1s allele originated from the AB-1 ES cell clone that carried the targeted mutation of Ifng (24). We typed two independent AB-1 ES clones, including the clone used here, and found that they were both Gpis1s/Gpis1s and not Gpis1s/Gpis1s, as previously reported for AB-1 ES cells (34). Based on this analysis, we believe the GKO and RGKO strains to be isogenic except for the mutant locus and to differ from the control 129/Sv/Ev strain at Gpis. Such minor differences in histocompatibility loci between the IFN-γ knockout strains (GKO and RGKO mice) and 129/Sv/Ev would not be expected to influence the results reported here. However, prior studies of HSV-1 infection in GKO mice of different genetic backgrounds have produced dramatically different results. Thus, Geiger et al. (19) reported no mortality in C57BL/6 control or C57BL/6 GKO mice inoculated with HSV-1 strain F, while, in contrast, Bouley et al. (5) reported that BALB/c GKO mice died after inoculation with a dose of HSV-1 which was sublethal when routinely used to induce herpes stromal keratitis in control BALB/c mice. Aside from differences in the genetic background of GKO strain studied, differences in HSV-1 strain, route of inoculation, age, and gender of the mice used could all contribute to the discrepant mortality rates of the different GKO strains (3, 34). For example, we found that gender strongly influenced the outcome of HSV-1 infection in both IFN-γ mutant and control mice, with males being more susceptible than females (20a).

The interpretation that we favor for these results is that some novel ligand induced by viral infection elicits a protective response against HSV-1 and vaccinia virus infections in GKO mice by virtue of its interaction with the IFN-γ receptor which is functional in GKO, but not in RGKO, mice (9, 24). Although not formally excluded, the notion that the mere presence of the receptor, independent of ligand binding, can somehow protect against viral challenge is much less attractive, because it is difficult to envisage how such a mechanism could affect virus infection. We considered potential developmental effects of the Ifng<sup>−/−</sup> and Ifngr<sup>−/−</sup> mutations but could not conceive an explanation that could account for our results without the presence of an alternative ligand for the receptor. As an explanation for our results, we also considered the possibility that IFN-γ signaling through the low-affinity IFN-γ receptor (1, 13) somehow enhances susceptibility to viral infections in RGKO mice. But, even if this mechanism exists, it would not explain the relative resistance to viral infection that we observed in GKO mice. We even considered the possibility that a prior, apparent, bacterial or viral infection in our IFN-γ mutant colonies could, through effects on the host immune response, have altered the outcome of a subsequent virus infection, as was recently shown for selected virus pairs (33). Since both colonies of mice were housed together, it is likely that both would have been infected with the unknown agent (if such an infection had occurred), and hence, it is difficult to explain the relative resistance of GKO mice compared to RGKO mice on this basis. Finally, our hypothesis is consistent with the conclusions drawn in other systems where similar phenotypic discrepancies between ligand and receptor null mutants have been found. Invariably, the proposed, and in some instances verified, explanation for the discrepancy has been that the receptor was indeed being utilized by another, closely related but previously unrecognized, ligand(s) (10, 28, 29).

A candidate for the novel ligand in the GKO mice is the neuronal IFN-γ immunoreactive molecule (N-IFN-γ), which has recently been purified from rat trigeminal ganglia by using two monoclonal antibodies (DB1 and DB16) that recognize distinct epitopes in rat IFN-γ (30). Although distinct from authentic IFN-γ, N-IFN-γ shares many of its biological properties, including induction of major histocompatibility complex class I and II antigens on macrophages and cultured skeletal muscle cells, direct antiviral activity, and growth-stimulatory activity for Trypanosoma brucei. The proposed existence of N-IFN-γ is controversial, though, because no other reports confirming the isolation of this protein have been made. However, a recent independent study, combining whole-cell patch-clamp electrophysiology with single-cell RT-PCR and confocal laser immunocytochemistry, has demonstrated the expression of IFN-γ in cultured, fetal-rat, dorsal root ganglion neurons (27). Because the same DB1 antibody was used for immunostaining, this neuron-expressed IFN-γ may be identical to N-IFN-γ (30). The putative N-IFN-γ is an attractive candidate as the novel ligand for the IFN-γ receptor, since it is thought to be expressed in sensory ganglionic neurons, the primary site of HSV-1 replication and latency (32, 36). After ocular inoculation, HSV-1 spreads by retrograde axonal transport to sensory ganglionic and central nervous system neurons, where compensatory upregulation of inducible N-IFN-γ expression may occur, conferring resistance to infection to GKO mice. The validity of this hypothesis can be tested once the putative N-IFN-γ gene is isolated, as detailed characterization and elucidation of the pattern of expression of the protein will then be possible. Derivation of the IFN-γ null mutation in the same 129/Sv/Ev background as the IFN-γ receptor null mutation should prove useful for examining the diverse biological roles of IFN-γ. In particular, these strains can be used to determine the mechanisms underlying the differences in susceptibilities to autoimmune diseases and infections previously reported for IFN-γ null mutant mice (15, 25, 37, 41).

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