Interplay between Alpha/Beta and Gamma Interferons with B, T, and Natural Killer Cells in the Defense against Herpes Simplex Virus Type 1

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Received 10 September 2003/Accepted 23 December 2003

The main immunological elements that control infection with herpes simplex virus type 1 (HSV-1) include interferon (IFN), NK cells, and specific T and B cells. Both IFN-α/β and IFN-γ have been shown to be essential for virus control (33). In addition to their direct antiviral properties, IFNs are potent regulators of cell growth and thus indirectly influence virus replication. Global gene expression analysis has estimated that several hundred genes are regulated within a single cell following IFN stimulation in vitro. The complexity of the “IFN transcriptome” will likely increase when different cell populations are analyzed and global gene expression of organs or even whole organisms can be achieved (24). Besides effects on cell regulation, inflammation, and stress, IFNs dominantly influence cells that belong to the innate and adaptive immune system (2). However, it is not known at what time point after viral infection IFN-α/β, IFN-γ, and NK, T, and B cells are required; how these immune elements cooperate; or whether they are functionally redundant.

Natural and gene-targeted mice are useful for assigning biological roles for genes in vivo. We have concentrated on gene deletions that inactivate the receptor for IFN-α/β and -γ and thus abrogate the function of IFN-α/β and -γ; the common cytokine receptor gamma chain (γc) for interleukin-2 (IL-2), -4, -7, -9, -15, and -21 that is required for the development of NK cells; and the recombination activating gene (RAG) required for the development of mature T and B cells. Our main findings are that (i) IFN-α/β is able to control acute HSV-1 infection in the absence of NK cells or specific immunity, (ii) IFN-α/β and -γ systems participate to eliminate HSV-1 without the need for NK cells or specific immunity, and (iii) lymphocytes (either B, T, or NK cells) appear to be required to control persistent virus.

**MATERIALS AND METHODS**

**Animals and virus.** Six- to 8-week-old 129Sv/Ev or C57BL/6 mice and congenic strains with gene-targeted disruptions of the IFN-α/β receptor, IFN-γ receptor, recombination activating gene (RAG), as well as combinations thereof obtained by breeding were used (Table 1) (18, 25, 47). Mice bred on the 129Sv/Ev genetic background contain "129" as part of the name (Table 1). All other mice are on a C57BL/6 genetic background. In p40-/- mice, p35-/- IL-12 and IL-23 are inactivated (4, 46). In mice deficient in RAG and the common cytokine receptor chain (γc; [RAG-/-c -/-]), mature T and B cells as well as NK cells are absent, and IL-2, -4, -7, -9, -15, and -21 are nonfunctional (7, 22). All gene-altered C57BL/6 mice were backcrossed at least 10 generations. Mice of both sexes were used for the experiments. The animals were bred and maintained under specific-pathogen-free conditions in the Laboratory, Universit鋞 Zurich, Zurich, Switzerland.

HSV-1 strain F was originally obtained from B. Roizman (University of Chicago) and propagated on Vero cells (11, 44). For all experiments, virus particles were used after purification by ultracentrifugation on a sucrose density gradient, and the virus titer (PFU) was determined as described previously (44).

Animals were infected by intraperitoneal inoculation of 100 μl of virus suspension. The 50% lethal dose (LD₅₀) was calculated as described previously (23). We euthanized infected animals when they were terminally ill or at 21 days postinfection, unless stated otherwise. In some experiments, mice were treated with neutralizing monoclonal antibody (MAb) 10F6 (specific for IL-12 p40), which neutralizes IL-12-dependent bioactivity (29). Neutralizing MAb XMG1.2 specific for murine IFN-γ was used (29) until animals were terminally ill. All experiments were conducted at least twice, and different doses of virus were used where appropriate.

**Dissection of trigeminal and spinal ganglia and PCR.** Trigeminal ganglia were separated from brain tissue after opening and removing the upper skull. Spinal ganglia were surgically removed by opening the spinal cord under microscopic observation. To detect HSV-1 genomic sequences in spleen, liver, lung, kidney, or brain, DNA was isolated by standard procedures, and HSV-specific genes were amplified by PCR. Primers were directed to the HSV-1 glycoprotein B (gB) gene. The forward primer was 5'-TCCCCGTATCGAAAGCCAG, and the reverse primer was 5'-AGCAGGCGCGTGGTCTCGTG. The conditions for PCR and sensitivity testing were described previously (39, 48).

**RESULTS**

IFN-α/β controls high doses of HSV-1 for a short time in the absence of NK cells and mature T and B cells. IFN-α/β is considered crucial for the control of acute viral infections (33). However, IFN-γ can cooperate with IFN-γ and regulate
lymphocytes of innate or specific immunity (24). In order to analyze the effect of the IFN-α/β system in the absence of T and B cells, we infected GR129 mice (Table 1), which lack IFN-γ receptors as well as mature T and B cells, with $10^5$ PFU of HSV-1. The infected mice survived the early phase of infection (up to 15 days) but succumbed after this time (Fig. 1).

Infection of GR129 mice with higher HSV-1 doses ($10^5$ PFU) was lethal for all mice within 9 days (data not shown). Mice lacking both IFN-α/β and γ systems had no appreciable resistance against infection with HSV-1 (Fig. 2). These data highlight the contribution of the IFN-α/β system in the defense against HSV-1 but left unanswered the question of the importance of NK cells in the control of viral replication.

To evaluate the role of NK cells during acute HSV-1 infection, we utilized RAG$^{-/-}$ mice, which are devoid of all lymphocyte subsets (B, T, and NK cells, Table 1) (7). In this experiment, RAG$^{-/-}$ mice were treated with neutralizing antibodies against IFN-γ (29). Under these conditions, mice have an intact IFN-α/β system, lack all lymphocytes, and have no capacity to respond to IFN-γ. RAG$^{-/-}$ mice infected with $10^5$ PFU of HSV-1 in the presence of neutralizing antibodies specific to IFN-γ also died within 15 days (Fig. 1). These data indicated that NK cells do not contribute to survival under the experimental conditions used. To further substantiate these data, RAG$^{-/-}$ mice were also treated with neutralizing antibodies specific to IFN-γ and infected with $10^5$ PFU of HSV-1. Again, none of the infected mice survived longer than 16 days (data not shown). Naïve untreated RAG$^{-/-}$ mice survived infection with $10^5$ PFU of HSV-1 (Table 2) (data not shown). Therefore, data from three different mouse strains indicated that the IFN-α/β system alone can control infections with HSV-1 for several days without the need for NK cells and mature B and T cells.

**In the absence of IFN-α/β, IL-12 is required for the defense against HSV-1.** We next analyzed whether mice without an IFN-α/β system but with intact IFN-γ and mature B and T cells (A129, Table 1) were also able to control infection with $10^5$ PFU of HSV-1. Surprisingly, about 30% of them survived the infection, and the mice stayed healthy for more than 6 months (Fig. 3 and Table 3). The presence of neutralizing antibody specific to IL-12 during infection abolished the protective effect completely (Fig. 3), as seen previously with AR129 mice (48). As expected, neutralizing antibodies specific to IFN-γ present during virus challenge led to a lethal outcome for the infected A129 mice (data not shown). Furthermore, mice without functional IFN (AG129 mice, Table 1) did not resist infective doses as low as 50 to 100 PFU of virus. Interestingly immunization (16) or transfer of neutralizing antibodies against HSV-1 (48) prolonged survival but did not lead to protection against viral infection. Therefore, the lack of IFN-α/β responses during the early period after HSV-1 infection can be compensated for, to some extent, by elements of specific immunity (48), provided that IL-12 is functional (Fig. 3). In the

**FIG. 2. IFN is essential for protection against HSV-1.** C57BL/6, AGR129, and AG129 mice were infected with $10^5$ PFU of HSV-1. The survival of mice after viral infection is shown.

**TABLE 2. HSV-1-specific LD$_{50}$s for different mouse strains**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>IL-12</th>
<th>IFN γ</th>
<th>RAG</th>
<th>LD$_{50}$ (PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>$5 	imes 10^6$</td>
</tr>
<tr>
<td>IL-12</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>$5 	imes 10^6$</td>
</tr>
<tr>
<td>G129 (F)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>$5 	imes 10^6$</td>
</tr>
<tr>
<td>G129 (M)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>$5 	imes 10^6$</td>
</tr>
<tr>
<td>RAG$^{-/-}$</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>$5 	imes 10^6$</td>
</tr>
</tbody>
</table>

a At least 20 mice of each sex were used. F, female; M, male.

b Statistical difference from wild-type animals at $P < 0.05$ (Mann-Whitney U test).
presence of functional IFN-α/β and γ, the absence of IL-12 had no deleterious effect (Table 2).

NK cells or mature T and B cells are not required to resist infection with HSV-1. Components of the adaptive immune system, including antibodies and in particular CD8⁺ T cells, have been postulated to be essential for long-term protection against HSV-1 infection (35). RAG-deficient mice, which are unable to generate mature T and B cells, were 10 times more susceptible to infection with HSV-1 than C57BL/6 or 129Sv/Ev mice (Tables 1 and 2). However, HSV-1-infected mice that controlled virus during the first 10 to 14 days survived for several months until the experiment was terminated. To investigate the role of NK cells in this process, RAG⁻/⁻/γc⁻/⁻ mice that have no significant numbers of these cells were infected with virus. Interestingly, 20 to 40% of RAG⁻/⁻/γc⁻/⁻ mice infected with 10³ or 10⁴ PFU of HSV-1 survived the infection for more than 6 months, the longest period analyzed (Fig. 4 and Table 3).

Immunological requirements to detect persistent viral DNA in neuronal tissue. We have previously shown that persistent virus can be controlled in the absence of both specific immunity and IFN-α/β (48). Therefore, it was interesting to see whether the different gene-deleted animals that had survived infection harbored viral DNA, indicating the presence of persistent virus (Tables 1 and 3). Trigeminal and spinal ganglia were removed at least 3 weeks after infection with a viral dose that led to 30 to 50% survival (Table 3). HSV-1 DNA was detected in ganglia of mice with the IFN-α/β and γ genes deleted as well as in C57BL/6 wild-type mice. Interestingly, HSV-1 gB-specific DNA was only detected in four of eight RAG gene-deficient mice but none of the nine RAG⁻/⁻/γc⁻/⁻ mice (Table 3). Therefore, the presence of mature T and B cells or NK cells is correlated with the persistence of viral DNA. It would therefore appear that RAG⁻/⁻/γc⁻/⁻ mice, which do not have these cells, either eliminate the virus during the acute phase or succumb to infection.

**DISCUSSION**

**IFN in acute infection of HSV-1.** It is well established that treatment with IFN-α, IFN-β, and IFN-γ is very effective against HSV-1 infections in mice (34). To further understand the plethora of interactions between IFN and various immune elements, we monitored animals with defects in IFN receptors, NK cells, and/or mature T and B cells during the course of infection with HSV-1.

We have shown that the IFN-α/β system is the most effective immune element able to control acute HSV-1 infection and can operate in the absence of NK cells and mature T and B cells (Fig. 1). In the presence of IFN-α/β, treatment of mice with neutralizing antibody to IL-12 p40 did not influence the course of the infection (data not shown). Therefore, after infection with HSV-1, IFN-α/β, IL-12, and possibly direct cell-cell contact (14) may not activate NK cells. The role of NK cells in acute infections with HSV-1 in mice is still controversial (1, 3, 5, 15, 19, 38), and the role of NK cells in chronic infections should be evaluated separately (see below). Evaluation of NK cell function in vivo by eliminating cells with antibodies to NK1.1 or asialo-GM1 is difficult if not impossible to assess. NK1.1 is not expressed on all NK cells (37). In contrast, NK1.1 and asialo-GM1 are not only expressed on NK cells, but also on macrophages (30) and some dendritic cells (DC) (41). This is important to note, because IFN produced during infection may prime these cells for enhanced resistance and control of HSV-1 replication (20, 24).

This early IFN-α/β production may control virus replication long enough to enable initiation of specific immunity. Indeed, priming of HSV-1-specific CD8⁺ T cells in C57BL/6 mice

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**TABLE 3. Persistence of HSV-1 DNA in neuronal tissue**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Viral dose (PFU)</th>
<th>Survival (no. that survived/total)</th>
<th>No. of mice with viral DNA/survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5 × 10⁶</td>
<td>5/12</td>
<td>5/5</td>
</tr>
<tr>
<td>A129</td>
<td>1 × 10³</td>
<td>6/18</td>
<td>5/6</td>
</tr>
<tr>
<td>G129⁻/⁻</td>
<td>5 × 10⁵</td>
<td>4/12</td>
<td>4/4</td>
</tr>
<tr>
<td>RAG⁻/⁻</td>
<td>5 × 10⁵</td>
<td>8/14</td>
<td>4/8</td>
</tr>
<tr>
<td>RAG⁻/γc⁻/⁻</td>
<td>1 × 10⁴</td>
<td>5/15</td>
<td>0/5</td>
</tr>
<tr>
<td>RAG⁻/γc⁻</td>
<td>1 × 10³</td>
<td>4/10</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*See Table 1 for details.

The amount of HSV-1 used was adjusted for each mouse strain (Tables 1 and 2).

Animals that survived the viral challenge for more than 21 days.

* HSV-1 gB-specific gene segments amplified from DNA isolated from brain and ganglia (Materials and Methods).
requires approximately 30 h (32). Significant virus-specific cytototoxic T-cell activity in draining lymph nodes is detectable within 2 to 3 days (32). IFN-α/β controls infection with 10^5 PFU of HSV-1 for the first 6 to 9 days. This time appears sufficient for T-cell priming and expansion to induce a protective HSV-1-specific immune response in the absence of IFN-γ (Table 2).

In the absence of IFN-α/β, mice are very susceptible to infection with HSV-1. The remaining protection was strictly IL-12 dependent. In the absence of IFN-α/β, IL-12 may activate NK cells, macrophages, and possibly T cells (14, 16, 48).

**Synergies with IFN for long-term survival after HSV-1 infection.** In the absence of mature T and B cells and either IFN system (GR129 or AR129, Table 1) mice did not survive infection with HSV-1 (Fig. 1). Synergistic actions between innate and specific immune elements were required for long-term survival. Neutralizing antibodies specific to HSV-1 transferred to AR129 mice (48) or GR129 mice (data not shown) and the presence of adaptive immunity represented such elements (Table 2 and Fig. 3).

Mice without mature B and T cells (RAG−/− mice, Table 1) but intact IFN systems were remarkably resistant against infection with HSV-1 (Table 2). Direct cooperative effects between the two IFN system as well as NK cells may explain this observation (1, 40). To directly analyze the effect of NK cells, we infected mice that have no significant numbers of NK cells and lack RAG (RAG−/−/γc−/− mice, Table 1). In addition, the IL-2, IL-15, and possibly IL-21 systems in these mice required for efficient activation of NK cells are nonfunctional (7, 8, 10, 17). Some of the RAG−/−/γc−/− mice survived infection with HSV-1 (Fig. 4 and Table 3). Because treatment of RAG−/− or RAG−/− mice with neutralizing antibodies to IFN-γ aborted long-term survival after HSV-1 infection (Fig. 1), we suggest that in these mice, the cooperative effect between the two IFN systems is mandatory, whereas the contribution of NK cells is synergistic (Table 2, Table 3, and Fig. 4).

**Requirements for persistent viral DNA in CNS.** “True” latent HSV-1 infections in humans and in animal models, including mice, have been extensively studied, and the need for immune cells and cytokines—in particular IFN-γ—was postulated (6, 12, 13, 27, 31, 45, 49). Latent HSV-1 infections have three separable phases: establishment, maintenance, and reactivation. In this report, we have determined the general potential of the various mice to maintain the HSV-1 genome in tissues of the central nervous system (CNS). The presence of HSV-1 gB DNA was analyzed by PCR following more than 3 weeks of infection (Table 3). After infection with high doses of HSV-1, both wild-type mice and mice with the genes coding for the IFN-α/β or γ receptor deleted cannot harbor viral DNA. In only four of eight RAG−/− mice infected with different doses of HSV-1, no virus-specific DNA was detected. The role of T cells, NK cells, and macrophages in persistent viral infection has been discussed previously (26, 28, 42, 43, 48). In RAG−/−/γc−/− mice, which have no mature T and B cells and no significant numbers of NK cells, the absence of viral DNA may indicate that persistent virus is incompatible with survival. HSV-1-infected RAG−/−/γc−/− mice either eliminate the virus or die. The elimination of virus is most likely the result of a cooperative effect between IFN-α/β and γc, as shown previously in vitro (40). The cells able to produce IFN-γ in RAG−/−/γc−/− mice have yet to be determined. One possible source may be DC (21).

The mice described in this article may be the basis for a detailed in situ analysis of the immune elements required to control viral replication, the route of viral spread, and the sites and conditions of virus maintenance as well as reactivation.

**ACKNOWLEDGMENTS**

We thank the team of the Laboritkurse, University of Zurich, headed by Frank Bootz and Martin Moritz, for breeding the animals and maintaining the correct genetic background of each strain. Bogdana Salathé carefully monitored the infection experiments. We appreciate the comments and suggestions by Hans Peter Hefl from our institute.

This work was supported by the Kanton of Zürich and a grant from the European Union (ERBFMRXCT960053) financed by the Swiss Federal Office for Education and Research.

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