Long-Term Persistence of Activated Cytotoxic T Lymphocytes after Viral Infection of the Central Nervous System

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Summary

Mice intranasally inoculated with influenza A/X-31 are protected against a subsequent intracerebral challenge with the neurovirulent influenza A/WSN and this heterotypic protection is mediated by CD8\(^+\) cytotoxic T lymphocytes. We have studied the kinetics of this secondary immune response and found that despite the elimination of replication-competent virus by day 10, we were able to recover activated influenza-specific cytotoxic T lymphocytes (CTLs) that killed freshly ex vivo from the brains of mice for at least 320 d after the intracerebral inoculation. The activated antiviral CTLs expressed high levels of the early activation marker CD69, suggesting continuing TCR signaling despite a lack of viral protein and major histocompatibility complex staining by immunohistochemistry in the brain parenchyma and barely detectable levels of viral nucleic acid by single and two-step reverse transcription PCR. Local persistence of activated lymphocytes may be important for efficient long-term responses to viruses prone to recrudescence in sites of relative immune privilege.

Key words: immunity • virus • central nervous system • influenza virus • mouse

One outcome of inefficient central nervous system (CNS)\(^1\) immunity is virus persistence (for review see reference 1) and the diseases increasingly associated with it (2, 3). Terminally differentiated cells such as neurons are good long-term hosts for viruses since they do not readily undergo either viral or CTL-mediated apoptosis (4) and they can escape CTL detection due to low levels of surface MHC molecules (5). We have recently shown that although influenza virus infection confined to the brain parenchyma is associated with marked inflammation, it is weakly immunogenic even if the virus actively replicates (6, 7). The lack of lymphoid tissue and resident dendritic cells (for review see reference 8), the exclusion of lymphocytes with a naive phenotype (9, 10), and the lack of well-defined lymphatic drainage (for review see reference 11) all reduce the efficiency of immune responses to virus infection in the CNS parenchyma. Control of virus infection largely depends on an influx into the brain of cellular effectors and/or a specific antibody that have been primed systemically. Systemic activation of antiviral CTLs would be expected in acute encephalitis since systemic infection usually precedes or accompanies infection in the CNS. But what provides the stimulus for CNS migration after the peripheral infection subsides is unknown; the activation state of most circulating antiviral effectors wanes after systemic virus has been cleared (indeed some memory cells revert to a more naive phenotype; reference 12).

Persistent antiviral immunity in the CNS could result either from continuing lymphocyte recirculation into the CNS or from persistence of lymphocytes at the site of initial inflammation. Recently, memory CTLs have been shown to persist locally in the lungs after intranasal infection (13). We now show that after an acute monophasic viral encephalitis, activated CTLs remain localized to the site of virus infection long after effective viral clearance. The persistent CD8\(^+\) lymphocytes are not proliferating yet express high levels of the early activation marker CD69, suggesting continuing lymphocyte interaction with specific MHC–peptide complexes, despite an absence of detectable viral protein and undetectable levels of viral nucleic acid.

Materials and Methods

Mice, Viruses, and Inoculation. 6-8-wk-old C57BL/10 mice were used for experiments. Influenza viruses A/WSN (H1N1) and A/X31 (H3N2) were propagated, titered, and inoculated as previously described (14).

\(^1\)Abbreviations used in this paper: CNS, central nervous system; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein.
Virus Titers and Viral RNA. Replication-competent A/WSN virus was recovered from infected organs as previously described (14). cDNA was synthesized from total brain RNA by standard methods. A 489-bp fragment of the influenza A nucleoprotein (NP) gene was amplified using the following oligonucleotide primer pairs and conditions: NP1, 5′-GGAATCAGGCAAATGCTGCTGCAT-3′; NP2, antisense 5′-CTCTGGATTTGCTCTCGGA-3′; 5 min at 95°C, followed by 30 1-min cycles of 57, 70, and 94°C. As a positive control for the cDNA synthesis, we also amplified a 214-bp fragment of murine hypoxanthine guanosine phosphoribosyl transferase cDNA using the following primer pairs and conditions: 5′-GTAATGATCGATCAAGGGGGAC-3′; 5′-CAGAATCTTGACATTTCTCTG3′; 5 min at 95°C, followed by 30 1-min cycles of 55, 70, and 94°C. A 293-bp fragment was amplified from one fifth of each of the first set of NP reactions and their negative, using an additional internal NP primer (NP4, 5′-ATCACAAGGGGCTCTGC-3′, with the above NP2 primer) as for the first round reaction.

Lymphocyte Cultures. Lymphocyte cultures were maintained as in reference 6. The NP 366-374-specific CTL clone was derived from an A/X-31–stimulated short-term line by limiting dilution as in reference 6. The NP 366-374-specific CTL clone was derived from single animals (5%) RPMI 1640 medium (Life Technologies, Paisley, UK)/10% FCS. Organs were harvested into ice-cold 25 mM Hepes-buffered F-10 medium (Life Technologies, Paisley, UK)/10% FCS.

As a positive control for the cDNA synthesis, we also amplified a 214-bp fragment of murine hypoxanthine guanosine phosphoribosyl transferase cDNA using the following primer pairs and conditions: 5′-GTAATGATCGATCAAGGGGGAC-3′; 5′-CAGAATCTTGACATTTCTCTG3′; 5 min at 95°C, followed by 30 1-min cycles of 55, 70, and 94°C. A 293-bp fragment was amplified from one fifth of each of the first set of NP reactions and their negative, using an additional internal NP primer (NP4, 5′-ATCACAAGGGGCTCTGC-3′, with the above NP2 primer) as for the first round reaction.

Flow Cytometry, Cell Sorting, and Immunohistochemistry. Cell surface staining was performed as described in reference 6 using the following primary antibodies: rat anti–mouse: anti-CD4, KT6-PE, IgG2a (Serotec, Kidlington, UK); anti-CD8, KT15-FITC, IgG2a (Serotec); anti-CD62L, biotinylated MEL-14, IgG2a; anti-CD44, biotinylated IM7.8.1, IgG2b; anti-CD49d, biotinylated PS2/1, IgG2b (16); anti-Mac-1, biotinylated M1/70, IgG2b; anti-CD25, Quantum red–conjugated 3C7 (Sigma Immunochemicals, Poole, UK); hamster anti–mouse: anti-CD69, biotinylated H.1.2F3, IgG1 (Pharmingen, San Diego, CA), anti-γ-δ TCR mAb, biotinylated GL3, IgG (Serotec). After a 30-min primary incubation and a wash, the appropriate samples were incubated for 15 min with streptavidin Quantum red (Sigma Immunochemicals). After a further wash the samples were immediately analyzed on a Becton Dickinson (Mountain View, CA) FACSort™ instrument using Cellquest V1.1 software (Becton Dickinson). The combinations of primary antibodies used provided internal specificity controls M1/70 and GL3 staining were negative on brain and spleen samples (not shown). Changes in cell phenotype consequent upon the process of cell purification were excluded by mixing brain cells and splenocytes at the time of homogenization and then demonstrating that naive cells could be purified by density gradient centrifugation (Hawke, S., unpublished data; reference 10). In vivo administration and internal staining of bromodeoxyuridine (BRDU) with anti-BRDU antibody (Becton Dickinson) was performed as described previously (17). After perfusion with heparinized PBS, brains from A/X-31 immune animals were cryopreserved at the following time points after intracranial challenge with A/WSN on days 2, 5, 7, 21, 42, 115, 212, and 320 (three mice at each time point). Immunohistochemistry was performed exactly as in references 6, 7, and 14 using the following primary antibodies rat anti–mouse CD8, YTS 169.4; CD4, GK1.5; MHC class I (H-2K), M1-42.3.9.8; MHC class II, M5/114.15.2; PS/2, IgG2b (16); anti–Mac-1, biotinylated M1/70, IgG2b; anti–Mac-1, biotinylated GL3, IgG (Serotec). After a further wash the samples were immediately analyzed on a Becton Dickinson (Mountain View, CA) FACSort™ instrument using Cellquest V1.1 software (Becton Dickinson). The combinations of primary antibodies used provided internal specificity controls M1/70 and GL3 staining were negative on brain and spleen samples (not shown). Changes in cell phenotype consequent upon the process of cell purification were excluded by mixing brain cells and splenocytes at the time of homogenization and then demonstrating that naive cells could be purified by density gradient centrifugation (Hawke, S., unpublished data; reference 10). In vivo administration and internal staining of bromodeoxyuridine (BRDU) with anti-BRDU antibody (Becton Dickinson) was performed as described previously (17). After perfusion with heparinized PBS, brains from A/X-31 immune animals were cryopreserved at the following time points after intracranial challenge with A/WSN on days 2, 5, 7, 21, 42, 115, 212, and 320 (three mice at each time point). Immunohistochemistry was performed exactly as in references 6, 7, and 14 using the following primary antibodies rat anti–mouse CD8, YTS 169.4; CD4, GK1.5; MHC class I (H-2K), M1-42.3.9.8; MHC class II, M5/114.15.2; B220, RA3-6B2; and rabbit antiinfluenza ribonucleoprotein (sourced as in reference 7).

Results and Discussion

After direct inoculation into the lateral cerebral ventricle of the mouse, the neurovirulent influenza A/WSN (H1N1) replicates in ependymal cells and then spreads into the brain parenchyma where it infects neurons (18). In nonimmune mice, the resulting encephalitis leads to death within 6–8 d. Earlier immunization with intranasal influenza A/X-31 (H3N2) protects 80% of mice and this “heterotypic” protection is mediated by CD8+ lymphocytes (14). A sustained clinical recovery occurs after day 5, and this is coincident with a marked reduction in recoverable replication-competent virus (14).

We purified lymphocytes from the brains of immune mice challenged with A/WSN and found that there was rapid recruitment of CD4+ and CD8+ lymphocytes (Fig. 1a), initially (days 0 and 4) in proportions similar to those found in peripheral blood or spleen. But from day 4 to day 21 there was a 300-fold increase in the number of CD8+ lymphocytes, which then declined exponentially, never reaching normal levels during the follow-up period (320 d). Histological analysis, even as late as 320 d after the intracerebral challenge showed CD8+ lymphocytes in the brain, both within or adjacent to the choroid plexus and scattered...
Figure 1. High frequencies of CD8+ T cells are recoverable from the brain for long after A/WSN infection. (a) Cells were purified from the brains of A/X-31–primed A/WSN–challenged mice at various time points as described in Materials and Methods and used directly in CTL assays without restimulation. Total cell counts (broken line, circles), CD4 (triangles), and CD8 (squares) frequencies are plotted. Each data point represents the means of two separate trials with two to eight mice at each time point, except for day 320, which represents a single assay on brain lymphocytes pooled from eight mice. Standard deviations <10% are not shown. (b) Lysis of virus-infected (circles) and peptide-pulsed (triangles) EL-4 target cells is titratable and antigen specific (squares, untreated target cells). Variation between duplicates was <15%. E/T ratios refer to the ratio of CD8+ cells to target cells.

Figure 2. Cryostat coronal brain sections from mice inoculated 5 (a–f) and 320 (g–l) d previously with intracerebral A/WSN. Day 5: (a) CD8+ cells infiltrating the choroid plexus of the third ventricle (lIlv) and the periaqueductal parenchyma. (b) CD4+ cells (c) markedly increased MHC class I expression in brain parenchyma. (d) Increased MHC class II expression predominantly within the inflammation obliterating the lateral ventricle (Lv), (e) B220+ cells in the lateral recess of the third ventricle, and (f) influenza ribonucleoprotein (RNP) expression surrounding the cerebral aqueduct (Aq). A row, virus-infected cell. Day 320: (g) groups of CD8+ cells adjacent to the corpus callosum, (h) parenchymal CD4+ cell, single CD4+ cells in the brain parenchyma were evident on occasional sections. Lack of MHC class I (i) and II (j) staining in the brain parenchyma. (k) Accumulation of B220+ cells within the choroid plexus of the lateral ventricle, (l) absence of antinfluenza RNP staining (periaqueductal region). The ependymal cell loss seen in h, k, and l is consequent on viral cytopathology. Bar: 50 μm in g and h and 250 μm in the other panels.

Day 5

Day 320

Downloaded from jem.rupress.org on July 9, 2017
Table 1. Long-term Antiviral CTL Response

<table>
<thead>
<tr>
<th>Day after intracranial A/WSN</th>
<th>CD8+ cell frequency (mean ± SD)</th>
<th>M axisum E/T cell ratio (adjusted for CD8+ frequency)</th>
<th>N P 366-374 proportion of maximal CTL lysis mean ± SD</th>
<th>A/WSN proportion of maximal CTL lysis mean ± SD</th>
<th>NIL proportion of maximal CTL lysis mean ± SD</th>
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<td>n*</td>
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Fresh killing from lymphocytes purified from the brain

<table>
<thead>
<tr>
<th></th>
<th>%†</th>
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<tr>
<td>0</td>
<td>4</td>
<td>9.6 ± 2.0</td>
<td>ND</td>
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</tr>
<tr>
<td>4</td>
<td>3</td>
<td>15.8 ± 4.0</td>
<td>3:1</td>
<td>30 ± 13</td>
<td>66 ± 8</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>35.3 ± 2.2</td>
<td>7:1</td>
<td>39 ± 19</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>7.0 ± 4.0</td>
<td>14:1</td>
<td>41 ± 30</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>71.0 ± 5.7</td>
<td>14:1</td>
<td>55 ± 24</td>
<td>35 ± 12</td>
</tr>
<tr>
<td>28</td>
<td>3</td>
<td>69.0 ± 1.1</td>
<td>14:1</td>
<td>58 ± 12</td>
<td>55 ± 23</td>
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<tr>
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<td>47</td>
</tr>
<tr>
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<td>58.4 ± 3.9</td>
<td>12:1</td>
<td>27 ± 9</td>
<td>24 ± 6</td>
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<tr>
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<td>33.5 ± 5.0</td>
<td>6.7:1</td>
<td>38 ± 8</td>
<td>21 ± 5</td>
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<td>2</td>
<td>27.3 ± 10.6</td>
<td>4.3:1</td>
<td>35.2 ± 7</td>
<td>24 ± 6</td>
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<td>20</td>
<td>4:1</td>
<td>17</td>
<td>49</td>
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Fresh killing from lymphocytes purified from the lung

<table>
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<td>7.7 ± 0.5</td>
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<td>&lt;1</td>
</tr>
<tr>
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<td>4</td>
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<td>20.6 ± 5.8</td>
<td>10.2:1</td>
<td>59 ± 34</td>
<td>24 ± 10</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>21.0 ± 6.8</td>
<td>10.5:1</td>
<td>24 ± 1</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>33</td>
<td>4</td>
<td>17.1 ± 8.0</td>
<td>8.5:1</td>
<td>3 ± 3</td>
<td>1.5 ± 0.5</td>
</tr>
</tbody>
</table>

* n, number of assays at each time point. For brain, from days 0 to 56, lymphocytes were purified from individual or pairs of mice before being assayed; from day 115, lymphocytes derived from pools of four to eight mice were assayed. For lung, two separate trials each with two assays at each time point with two mice in each.

† CD8+ frequency was determined by flow cytometry.

‡ Each assay included lysis by an N P 366-374-specific CTL clone (clone 1:3) to allow comparison between each assay. Lysis of peptide- or virus-pulsed targets is expressed as a proportion of maximum killing of peptide- or virus-pulsed targets by clone 1:3.

§ Proportion of maximal lysis by clone 1:3 of peptide-pulsed targets. Average killing over all assays by clone 1:3 of unpulsed, WSN–, and N P 366–374-pulsed targets was 2.2 ± 2.0, 35 ± 13, and 57 ± 13 (mean percent specific lysis ± SD, n = 17), respectively.

We tested the ability of lymphocytes purified from the brain to kill peptide-pulsed and virus-infected target cells. By day 7 when the mice were clinically recovering, we found consistent cytotoxicity immediately ex vivo against the immunodominant D8-restricted influenza A NP 366-274 peptide (21) and virus-pulsed targets. Surprisingly, this fresh killing ex vivo was still demonstrable 320 d after clinical recovery, which was the last time point examined (Fig. 1 b and Table 1). This fresh killing was also remarkably consistent when sufficient cells were recovered to test the response from individual mice. To compare the secondary CTL response in another organ, we intranasally inoculated A/X-31-primed mice with 0.2 hemagglutinating units A/WSN and purified lung lymphocytes by the same method. In contrast to the brain, fresh killing by lung lymphocytes ex vivo was demonstrable from day 4 to day 18, but was absent at day 33 (Table 1), even though previous work has...

throughout the brain parenchyma (Fig. 2 g). In contrast, numbers of CD4+ cells returned to normal within 28 d, although rare CD4+ staining cells were evident in the choroid plexus and even in the brain parenchyma at later time points (Fig. 2 h). Whether CD4+ lymphocytes undergo apoptosis in the brain (19), or eventually migrate out of the brain is unknown. CD4+ cells do not contribute to protection in this system (14). In the acute phase, large aggregates of B220+ cells were present in the choroid plexus and some were scattered throughout the brain parenchyma (Fig. 2 e). At later time points, small aggregations of B220+ cells were evident adjacent to or within the choroid plexus, but not in the brain parenchyma (Fig. 2 k compared with reference 20). MHC class I and II molecules were rapidly upregulated in acute A/WSN encephalitis (Fig. 2, b and c, and reference 14) and then declined to normal levels by days 115 and 320 (Fig. 2, i and j) after inoculation.
inquiring activation (9). We therefore cytofluorometrically cells selectively migrated into the brain, i.e., without re-brally might also exhibit fresh killing, and that the activated cells in the A/X-31–primed mice not challenged intracere-

366-374–specific CD8
cytotoxic lymphocytes was CD69 (Fig. 3 c), indicating that specific viral MHC–peptide complexes persisted and/or that the activation state had not been turned off. Only a small proportion of the CD8+ cells were dividing (as measured by BRDU uptake) whether assessed at 3 (not shown) or 6 (Fig. 3 c) mo, possibly because levels of cytokines such as type 1 interferon (23) had fallen. This contrasted with the large proportion of CD8+ cells that took up BRDU when pulsed for 6 d after the intrace-rebral challenge with A/WSN (Fig. 3 b).

Previous work had demonstrated that after intracerebral inoculation of A/WSN into X-31–primed mice, the virus replicated to high titers for 5 d, but thereafter declined rapidly and was unrecoverable after 6 d (14). In this study we were again unable to recover replication-competent virus from brain homogenates on day 7 and 10 by plaque assay or brain coculture on MDCK cells or by inoculation into embryonated eggs. Finally, infectious virus was also unrecoverable from the brains of lethally irradiated (1,000 cGy) mice 1 and 6 mo after the intracerebral A/WSN challenge. In the lung, replication-competent virus was recoverable from only 30% of mice 3 d after the challenge.

What maintains the persistently activated NP-specific CTL in the brain? Viral antigens have been reported to persist in the CNS (24, 25), but in our model, viral ribonu-cleoproteins were undetectable immunohistochemically af-ter 21 d (days 5 and 320 shown in Fig. 2). We sought viral mRNA from whole brain cDNA using single-step PCR and were consistently unable to amplify the influenza nu-cleoprotein cDNA after 23 d (Fig. 4). This was in contrast to the slower clearance of viral nucleic acid in other experimen-tal CNS virus infections after adoptive transfer of immune effectors (24–26). To increase the sensitivity of de-
tection, we performed two-step seminested PCR (Fig. 4) and identified viral nucleic acid in 7 of 24 mice tested from day 30. At day 115, three of nine mice were positive. However, NP cDNA was never detected in brain cDNA by the two-step, seminested PCR in animals sampled after day 115 or from mice that had received intranasal A/X-31 alone (Table 3).

### Table 2. NP 366-374–specific CTL Precursor/Effecter Cell Frequency

<table>
<thead>
<tr>
<th>Day after intracranial A/WSN</th>
<th>Frequency of NP 366-374-reactive CTL precursor</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1/brain lymphocytes</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>7</td>
<td>8.4</td>
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</tr>
<tr>
<td>42</td>
<td>&lt;30</td>
</tr>
<tr>
<td>56</td>
<td>10</td>
</tr>
<tr>
<td>190</td>
<td>27</td>
</tr>
</tbody>
</table>

Each frequency (individual assays shown above) was determined for lymphocytes purified from duplicate or quadruplicate mice.

*96.5 ± 1.7% of the CD8+ cells purified from the brains of A/WSN - inoculated mice are CD62Llo (mean ± SD; n = 10).

**The apparent high frequency of NP 366-374–reactive precursors at day 4 most probably reflects a combination of specific and nonspecific lysis (see Table 1).
heterotypic lung infection, viral nucleic acid was undetectable with single-step PCR after day 9 (not shown). Others similarly found that viral nucleic acid is not amplifiable with single step PCR 2 wk after primary influenza A/X-31 in the lung (27).

In previous work from other groups, CTLs have been recovered from the brains of animals infected with persistent CNS viruses (28). However, our results indicate that activated specific antiviral CTLs can persist in the brain long after viral clearance. Recently, a population of activated antiviral lymphoblasts was recovered from the spleens of mice after clearance of systemic lymphocytic choriomeningitis virus (LCMV; reference 29). It remains to be determined if our persistent antiviral CTLs in the CNS are analogous to this population, but collectively these data indicate that a component of long-term memory may be persistently activated cells at the site of initial infection. If this

![Figure 3](image)

**Figure 3.** (a) CD8+ cells purified from day 7 (early response) and day 180 (late response) have a similar activated/memory phenotype and CD69 is a better activation marker than CD25. (b) In vivo proliferation of brain CD8+ cells early in the response to A/WSN. (c) Brain CD8+ cells are not proliferating late in the response. A representative one of three experiments is shown. Comparison of BRDU uptake by the splenic and brain CD8+CD44hi populations showed that there was a 2.9 ± 0.17-fold enrichment of BRDU+ cells at day 6 in the brain compared with a 0.48 ± 0.22-fold enrichment at day 180 (mean ± SEM; P < 0.005 using an unpaired t test). A significant reduction in CD8+BRDU+ cells in the late brains compared with their CD8+CD44hi counterparts was also evident. Quadrant frequencies are shown (%).

<table>
<thead>
<tr>
<th>Days after intracranial challenge</th>
<th>Single-step NP1/NP2 PCR</th>
<th>Seminested NP4/NP2 PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>16</td>
<td>5/5</td>
<td>ND</td>
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</tr>
<tr>
<td>196</td>
<td>0/6</td>
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</tr>
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* Number of positive tests/number of tests performed.
population is capable of recirculation after LCMV infection, they may have an important role in immune surveillance in nonlymphoid organs where their state of activation would favor endothelial transmigration. However, in our model where systemic infection is minimal, it is more likely that CTLs recovered from the brain are not recirculating into it, but given the extreme sensitivity of CTLs (30), have been retained by persisting specific MHC class I–viral peptide complexes on neural cells that are below the threshold of detection by immunohistochemistry. It would be of interest if the persistence of CTLs specific for influenza virus reflects virus persistence at the DNA level as has recently been demonstrated for LCMV (31). The persistence of activated CTLs at sites of previous viral infection may be an important component of the response to virus recrudescence.

Figure 4. PCR amplification of nucleoprotein cDNA from mouse brain. Numbers refer to day after intracerebral inoculation. M, HaeIII-digested 4× 174 markers. R1 is a positive from the NP1/NP2 reaction run for size comparison. Representative gels are shown; each lane represents the result from an individual mouse. With seminested PCR, nucleoprotein cDNA was amplified from three of nine animals at day 115 and from none of six animals at day 196 (Table 3). Hypoxanthine guanosine phosphoribosyl transferase PCR was positive in all samples indicating that the cDNA synthesis had been satisfactory. PCR products were visualized on 2% agarose ethidium bromide gels under UV light and photographed on Polaroid film.

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