Replication of Lymphocytic Choriomeningitis Virus Is Restricted in Terminally Differentiated Neurons†

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We have investigated the replication of lymphocytic choriomeningitis virus (LCMV) before and after the nerve growth factor (NGF)-induced transdifferentiation of PC12 cells from the chromaffin to the neuron-like phenotype. Untreated and NGF-treated cells were equally susceptible to LCMV infection; however, the viral yield was found to be 1,000-fold lower in NGF-differentiated PC12 cells. The reduced viral yield correlated with restricted LCMV replication and transcription within the infected cell, which was not caused by the lack of cell proliferation in the NGF-treated cells but rather was related to the induction or changes in expression levels of specific gene product(s) associated with the cell commitment to a neuronal phenotype. The return to the chromaffin phenotype after withdrawal of NGF restored normal LCMV yields as well as levels of viral replication and transcription. The finding of reduced viral replication in terminally differentiated neuronal cells has important implications for understanding the mechanism by which neurotropic viruses, such as LCMV, are able to establish a long-term persistent infection in the central nervous system in the absence of severe pathological changes.

The mechanisms which enable viruses to infect and persist in differentiated cells, and the specific interactions established between the persisting virus and its host cell, are issues of paramount importance for the understanding of viral pathogenesis. Lymphocytic choriomeningitis virus (LCMV), a member of the arenavirus family (23), has been used extensively as a paradigm for the study of both mechanisms and biological consequences of viral persistence (reviewed in references 5, 14, 37, 38, 44, and 45). LCMV can infect its natural host, the mouse, either acutely or persistently, depending on the immune status of the mouse, the route of infection, and the strain of virus used. Infection of neonatal mice with as few as 2 PFU of LCMV leads to the establishment of a lifelong persistent tolerant infection involving most major organs, including the brain, in the absence of any significant tissue and cellular structural damage (reviewed in references 14, 38, and 44).

Despite the absence of inflammation and necrosis within the central nervous system (CNS), persistently infected mice show subtle neurological deficits (30) which have been linked to neurochemical abnormalities (41). Ultrastructural localization of viral antigens (51) and in situ hybridization studies (22) have documented the distribution of LCMV in the CNS restricted almost exclusively to neurons (22, 51).

Virus can be cleared from persistently infected mice by adoptive transfer of immune memory cytotoxic T lymphocytes (CTL) (Thy1.2⁺ CD8⁺ CD4[−]) which completely clear virus within 7 to 15 days after the adoptive transfer from most sites except neurons in the brain parenchyma, where clearance takes 60 to 120 days (46). Although the mechanism by which virus is cleared from neurons is not understood, it seems unlikely to occur via cell-mediated destruction of infected cells, as no detectable cytopathology or immune infiltrates into the brain parenchyma are found (46). However, the accumulation

of specific major histocompatibility complex-restricted CTL in the Virchow-Robbin spaces suggests that viral antigens are being recognized (46), which could lead to the release by the CTL of a soluble factor(s) with the ability to cross the blood-brain barrier and interfere with LCMV replication in neurons. Alternatively, it would be possible that LCMV infection of neurons is mostly unproductive and transient. Thus, the maintenance of viral load within the brain parenchyma during persistent LCMV infection would require a continuous reinfection of neurons from a productive source. Under either of these two scenarios, a restricted viral replication in terminally differentiated neurons would contribute to the process of viral clearance. Consequently, to gain further understanding about LCMV clearance from the CNS, we have examined the ability of LCMV to infect and replicate in terminally differentiated neurons and whether such cells may restrict the production of LCMV infectious particles.

For these studies, we used the PC12 cell line, a pheochromocytoma clone (25) which has been used extensively as a model of neuronal differentiation (25, 26). These cells can be induced to undergo morphological and physiological differentiation, characterized by a lack of cell division, extension of functional processes, and expression of a variety of neuronal cell-specific markers, following exposure to low doses of nerve growth factor (NGF) (25, 26). It has been previously reported that NGF differentiation of PC12 cells persistently infected with herpes simplex virus (HSV) type 1 delays the transition from early to late HSV polypeptide synthesis (13), while other groups have shown that HSV infection of differentiated PC12 cells leads to transient decreases in levels of enzymes involved in acetylcholine metabolism (54, 55).

In this report, we address the ability of LCMV to infect and replicate in undifferentiated and differentiated PC12 cells. We show that both cell types are readily infectable, but viral yields are 1,000-fold less in NGF-differentiated PC12 cells. This decreased viral yield is at the level of LCMV replication and transcription in NGF-treated cells and is unrelated to a lack of cell division or changes in the expression levels of the LCMV

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receptor. Interestingly, the ability of vesicular stomatitis virus (VSV), another RNA virus, to replicate in PC12 cells was unaffected by the addition of NGF. These results suggest that the cellular environment associated with neuronal differentiation may lead to restricted LCMV replication. Implications for the understanding of persistent viral infection in the CNS are discussed.

MATERIALS AND METHODS

Cells and viruses. The ARM 53b strain of LCMV is a triple-plaque-purified clone from ARM CA 1371 (20). ARM 53b LCMV clonal pool stock was prepared by infection of baby hamster kidney (BHK-21) cells at a multiplicity of infection (MOI) of 0.1 PFU per cell. Forty-eight hours after infection, the supernatant was clarified from cell debris, and aliquots were made and stored at -70°C . VSV, Mudd-Summers strain of the Indiana serotype, was grown in BHK-21 cells and purified as described previously (16).

Clone 13 is a triple-plaque-purified variant of LCMV ARM 53b, derived from spleen cells of an adult BALB/WEHI mouse persistently infected from birth with ARM 53b (1). The two viruses bound and infected susceptible cells at similar levels (3). Only the virus overlay protein blot assays (VOPBA) described were carried out with clone 13; ARM 53b was used in all other experiments.

Virus titers were determined by plaque assay on Vero cells (20). Virus stocks were free of mycoplasma contamination, as determined by Hoechst staining of cells growing in antibiotic-free medium at 48 h after virus infection.

BHK-21 cells were grown in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, 1% glutamine, 0.7% glucose, 5% $2\times$ tryptose phosphate broth, and 3.5% heat-inactivated fetal bovine serum (FBS) plus 3.5% heat-inactivated calf serum. PC12 cells were grown in RPMI 1640 medium supplemented with penicillin, streptomycin, and 1% glutamine plus 10% heat-inactivated horse serum and 5% FBS. For experiments involving induction of the neuron-like phenotype by treatment with NGF (100 ng/ml), cells were grown on collagen-coated dishes ($6\text{ }\mu\text{g}/\text{cm}^2$) and serum concentration was reduced to 2% heat-inactivated horse serum. During NGF treatment, medium was replaced every 48 h. NIH 3T3 fibroblasts were grown in RPMI 1640 medium containing penicillin, streptomycin, and 1% glutamine plus 7% heat-inactivated FBS.

Quantitation of infected cells by infectious center assays and immunofluorescence. The number of cells productively infected with LCMV was quantitated using an infectious center assay previously described (18). Cells expressing the viral nucleoprotein (NP) antigen were determined by immunofluorescence. Cells were fixed with 4% paraformaldehyde-phosphate-buffered saline (PBS) (pH 7.1) to preserve neurite outgrowth in NGF-treated PC12 cells and permeabilized with 0.1% Triton X-100 in PBS for 2 min. Staining was achieved by successive incubations for 45 min at room temperature with mouse monoclonal antibody 1-1.3 to the viral NP (4) (undiluted hybridoma tissue culture fluid) and a goat anti-mouse fluorescein isothiocyanate-labeled second antibody (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (diluted 1:50 in PBS plus 2% bovine serum albumin [BSA]), with five washes (5 min each) in PBS plus 2% BSA–1% Triton X-100 after each addition of antibody. The slides were mounted and analyzed with an Olympus BH-2 fluorescence microscope. The percentage of NP-positive cells was determined by counting the number of NP-positive and total cells in several fields. A

total number of 500 cells, involving 5 to 12 different fields, were analyzed for each sample.

LCMV binding to untreated and NGF-treated PC12 cells. For each assay, 2×10^5 PFU of LCMV (volume, 150 μl) was added to a monolayer of 5×10^5 cells at 0 to 4°C . At the indicated times, the monolayers were washed five times with cold PBS and the cells were resuspended in 0.5 ml of PBS containing 2% FBS. Cells were homogenized, and cell debris was removed by centrifugation at $5,000 \times g$ for 5 min at 4°C . Virus titers in clarified supernatants were determined by plaque assay on Vero cells (20).

VOPBA. Membranes were prepared from subconfluent cell monolayers as previously described (3). Briefly, cells were washed once in homogenization buffer (250 mM sucrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 1 mM EDTA, 100 U of aprotinin per ml) and homogenized by four passages through a ball-bearing homogenizer. Debris was eliminated by centrifugation at $1,000 \times g$ for 10 min at 4°C , and membranes were pelleted from the clarified supernatant by a further spin at 32,500 rpm for 1 h at 4°C in a Beckman SW41 rotor. Membrane pellets were resuspended in 10 mM Tris HCl buffer (pH 7.5), and their protein concentrations were determined by the Bradford assay (7). Membrane proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (57) under reducing conditions (3). Six percent separating and 3% stacking gels were used, and 50 to 100 μg of membrane proteins was run per well. Gels were run at 15 V/cm, and prestained high-molecular-weight SDS-PAGE protein standards (Bio-Rad, Richmond, Calif.) were used to estimate protein molecular sizes. Proteins separated by SDS-PAGE were transferred onto nitrocellulose (0.45- μm pore size; Schleicher & Schuell, Keene, N.H.) (3). The membrane was blocked overnight at 4°C in 2% nonfat milk–0.2% Tween 20–PBS. The membrane was then incubated successively with (i) LCMV clone 13 concentrated and partly purified by precipitation with polyethylene glycol and Renografin density gradient centrifugation, at approximately 10^{10} PFU/ml, diluted 1:10 in PBS plus 0.3% BSA; (ii) monoclonal antibodies specific for the LCMV glycoproteins (GP) (4), undiluted tissue culture fluid from the hybridomas WE-36.1 (GP-1 specific) and WE-33.1 (GP-2 specific) mixed 1:1; (iii) rabbit anti-mouse immunoglobulins (Dakopatts, Carpinteria, Calif.) diluted 1:5,000 in the milk buffer described above; and (iv) ^{125}I -labeled protein A (100 $\mu\text{Ci}/\text{ml}$; specific activity, 30 mCi/mg; Amersham, Arlington Heights, Ill.) diluted 1:100 in the milk buffer. The first incubation was for 1.5 h, and the others were for 1 h each, all at 4°C on a rocking platform. After each incubation, the membrane was washed in milk buffer (three changes, 10 min each) at 4°C . After the final washes, the membrane was rocked overnight at 4°C in milk buffer and then exposed to Kodak XAR-5 film.

Northern (RNA) blot hybridization experiments. Total RNA was extracted from cultured cells by the guanidinium isothiocyanate-acid phenol procedure (10). RNA was quantified on the basis of optical density at 260 nm/optical density at 280 nm and size fractionated on a 1% agarose formaldehyde gel (57). After electrophoresis, gels were briefly washed in H_2O (15 min, with two changes) and then with 10 mM sodium phosphate (pH 6.5) for 20 min at 40°C . RNA was transferred to nylon membrane by capillary action (57). RNA was UV cross-linked by using a total of $1.2 \times 10^5\text{ }\mu\text{J}$ (UV Stratalinker 1800; Stratagene, La Jolla, Calif.), and the blots were hybridized with the indicated probes. Probes were labeled by using an oligolabeling kit from Pharmacia (catalog no. 27-9250-01). Routinely, we used a 1- $\mu\text{g}/\text{ml}$ concentration of probe with a specific activity of 5×10^8 to 10×10^8 cpm/ μg . Hybridizations

FIG. 1. Reduced LCMV yield in NGF-treated PC12 cells. Untreated and NGF-treated (100 ng/ml for 72 h) PC12 cells were infected with ARM 53b LCMV at low (0.1 PFU per cell) and high (5 PFU per cell) MOI. After adsorption for 60 min at 37°C in a volume of 250 μ l/60-mm-diameter dish, the viral inoculum was removed, cell monolayers were washed five times, and then 4 ml of fresh medium with or without NGF was added. At the indicated time points, supernatant virus titers were determined by plaque assay on Vero cells as described previously (19). Results presented represent average values from three independent experiments, with standard deviations indicated by bars. Symbols: ○, MOI = 0.1 PFU per cell, no NGF; ●, MOI = 5 PFU per cell, and no NGF; □, MOI = 0.1 PFU per cell, NGF treatment; ■, MOI = 5 PFU per cell, NGF treatment.

were done at 60°C for 4 h. When necessary, stripping of the probe was done by boiling five times (10 min each) in 10 mM Tris HCl (pH 8.5)–5 mM EDTA–0.5% SDS. When indicated, signals corresponding to RNA hybridization were quantitated by densitometric analysis of underexposed Northern blot autoradiograms with an LKB Ultrascan XL laser densitometer.

RESULTS

Decreased LCMV replication after induction of neuronal differentiation by NGF in PC12 cells. As presented in Fig. 1, replicating chromaffin-like PC12 cells were susceptible to LCMV infection and allowed high viral yield (~100 PFU per cell). In contrast, LCMV infection of the neuron-like PC12 cell population generated by exposure to NGF for 72 h produced a very low viral yield (~0.1 PFU per cell). Similar results were obtained regardless of whether a high (5 PFU per cell) or low (0.1 PFU per cell) MOI was used.

This decreased LCMV yield found in NGF-treated PC12 cells could be explained if the exposure to NGF rendered only a minor fraction of the cell population susceptible to LCMV infection. The proportion of cells susceptible to LCMV infection in untreated and NGF-treated PC12 cells was examined by using infectious center assays and immunofluorescence procedures. As shown in Fig. 2, untreated and NGF-treated PC12 cells were equally susceptible to LCMV infection, reflected by the finding that 100% of the cells in the population, untreated or NGF treated, scored as positive infectious centers at 6, 24,

FIG. 2. Quantitation of LCMV-infected cells. Untreated and NGF-treated cells were infected with ARM 53b LCMV at MOIs of 0.1 (■), 0.5 (□), and 5 (▨) PFU per cell. At the indicated time points postinfection, single cell suspensions were prepared by trypsin treatment and the number of cells productively infected was determined in an infectious center assay as described previously (18). Values represent means with standard deviations of two independent experiments.

FIG. 3. Expression of the NP viral antigen. Untreated and NGF-treated (100 ng/ml for 72 h) PC12 cells were infected with LCMV at an MOI of 5 PFU per cell and 48 h later fixed and analyzed by indirect immunofluorescence as described in Materials and Methods. For each sample, the same field was also analyzed by differential interference contrast Nomarski microscopy (B, D, F, and H). To determine the percentage of cells expressing NP levels detectable by immunofluorescence, a total of 200 cells from several independent fields were counted under Nomarski microscopy, and those positive for NP staining were scored under fluorescence microscopy. Uninfected PC12 cells did not express NP antigen (A and B). A high percentage ($86.7\% \pm 8.2\%$) of LCMV-infected undifferentiated PC12 cells were NP positive (C and D), whereas only $8.0\% \pm 3.3\%$ of the NGF-treated PC12 cells were NP positive 48 h after LCMV infection (E and F). Removal of NGF restored NP expression levels detectable by immunofluorescence in a high percentage ($81.5\% \pm 9.5\%$) of the cells in the population (G and H).

and 48 h after infection with LCMV at high MOI (5 PFU per cell). The 10% of positive infectious centers found in untreated PC12 cells 6 h after LCMV infection at low MOI (0.1 PFU per cell) increased to 70% 24 h after infection and reached 100% 48 h postinfection, reflecting the spread of LCMV within the cell population through the second and third rounds of infection. In contrast, in the case of NGF-treated cells, LCMV infection at low MOI (0.1 PFU per cell) was not followed by significant spread of LCMV within the population during a 48-h period of time, with a similar 10% of positive infectious centers at 6, 24, and 48 h after infection.

Most of the cells in the LCMV-infected PC12 population were positive for the NP viral antigen (Fig. 3C and D), whereas

in the NGF-treated PC12 cells, only a small fraction of the population displayed NP-positive staining after LCMV infection at an MOI of 5 PFU per cell. Quantitation of immunofluorescence studies indicated that $86.7\% \pm 8.2\%$ and $8.0\% \pm 3.3\%$ of the untreated and NGF-treated PC12 cells, respectively, were positive for the viral NP antigen 48 h after LCMV infection (Fig. 3). In the NGF-treated cells, no correlation was found between the stage of differentiation, determined by morphological criteria in the treated cells, and the positive staining for NP antigen. Removal of NGF restored levels of NP expression detectable by immunofluorescence in most of the cells ($81.5\% \pm 9.5\%$) in the population (Fig. 3G and H). The increased cytoplasmic staining displayed by NGF-treated PC12

FIG. 4. (A) Untreated and NGF-treated PC12 cells display similar kinetics of LCMV binding. The percentage of LCMV PFU bound to untreated (○) and NGF-treated (●) PC12 cells was determined at the indicated times as described in Materials and Methods. Values represent the average of two independent experiments with standard deviations. The LCMV infection-resistant lymphocyte cell line WIL2 (3) showed $12.5\% \pm 4.5\%$ of PFU attached after a 60-min adsorption time (not shown). (B) NGF treatment does not affect the level of expression of the putative LCMV receptor on PC12 cells. Membrane proteins were prepared from the indicated cell lines, and 100 μ g of each was analyzed for LCMV-binding proteins, using a VOPBA as described previously (3).

cells compared with untreated PC12 cells is likely to be a consequence of the morphological cellular changes induced by NGF in PC12 cells.

In accordance with a similar susceptibility to LCMV infec-

FIG. 5. Induction of neuronal-like phenotype by NGF treatment is associated with restricted LCMV replication and transcription in PC12 cells. Untreated (–) or NGF-treated (+) PC12 cells were infected with LCMV at an MOI of 5 PFU per cell; at the indicated times postinfection (p.i.), total cellular RNA was extracted and equal amounts of RNA from each sample (10 μ g) were analyzed by Northern blot hybridization. The blot was first hybridized to an NP cDNA probe, stripped, and then hybridized to a cDNA probe for the housekeeping gene encoding cyclophilin. Procedures were as described in Materials and Methods. S and NP indicate the positions of the LCMV genomic S segment (3.5 kb) and the NP mRNA (1.6 kb), respectively.

FIG. 6. Proliferating and nonproliferating PC12 cells display similar levels of LCMV replication. (A) Virus production in proliferating and nonproliferating PC12 cells. Cells cultured under the specified conditions were infected with LCMV ARM 53b at an MOI of 5 PFU per cell. At the indicated time points postinfection (p.i.), supernatant virus titers were determined as described in Materials and Methods. Values represent averages with standard deviations of two independent experiments. When not shown, standard deviations were smaller than 5% of the average value. Numbers of viable cells were counted at zero h, and the percentages of cell survival under each experimental condition determined at 72 h after infection were 205 ± 21 (serum), 92 ± 12 (1 mM insulin), 103 ± 11 (10 μ M forskolin), 74 ± 12 (no serum), and 117 ± 16 (100 ng of NGF per ml). (B) Levels of LCMV replication and transcription in PC12 cells are not significantly affected by lack of proliferation. Proliferating (10% serum) and nonproliferating (1 mM insulin and no serum) PC12 cells were infected with LCMV ARM 53b at an MOI of 5 PFU per cell; at the indicated time points postinfection (p.i.), total cellular RNA was extracted and analyzed by Northern blot hybridization using an LCMV NP-specific probe as described in Materials and Methods. The bottom panel is the ethidium bromide staining of the gel showing similar loading (10 μ g of total RNA) in all cases as determined by the intensity of the 28S and 18S rRNA bands.

FIG. 7. Quiescent fibroblast cells also display a small decrease in LCMV yield without significant reduction in the levels of LCMV replication and transcription. (A) Reduced LCMV yield in quiescent 3T3 fibroblast cells. Quiescent and exponentially growing 3T3 cells were infected with LCMV ARM 53b at an MOI of 5 PFU per cell; at the indicated time points after infection, virus titers in the supernatant were determined as described in Materials and Methods. Values represent averages with standard deviations of three independent experiments. (B) Quiescent and proliferating 3T3 cells show similar levels of LCMV replication and transcription. Quiescent and proliferating 3T3 cells were infected as described for panel A; at the indicated time points postinfection (p.i.), total cellular RNA was extracted and analyzed by Northern blot hybridization as described for Fig. 6B.

tion, untreated and NGF-treated PC12 cells displayed similar kinetics of LCMV binding (Fig. 4A). Moreover, NGF treatment did not affect the level of expression of the putative LCMV receptor on PC12 cells, as determined by using a VOPBA (3). As shown in Fig. 4B, untreated and NGF-treated PC12 cells expressed similar levels of the ca. 116-kDa polypeptide also found in BHK and MC57 cells, both of which are susceptible to LCMV infection, but not in RIE cells, which are relatively resistant to LCMV infection (3). Furthermore, NGF as well as the supernatant from NGF-treated PC12 cells did not have any virucidal effect on LCMV infectious particles (data not shown).

We then investigated whether the decreased viral production in NGF-treated PC12 cells was correlated with reduced levels of LCMV replication and transcription. Results presented in Fig. 5 demonstrated that replication and transcription of LCMV were significantly reduced in NGF-treated PC12 cells, as determined by Northern blot hybridization. Both the genomic S RNA, a measure of replication, and the NP mRNA,

FIG. 8. NGF treatment does not affect VSV replication in PC12 cells or LCMV replication in BHK-21 cells. Untreated and NGF-treated (100 ng/ml for 72 h) PC12 and BHK-21 cells were infected with VSV and LCMV ARM 53b, respectively, at an MOI of 5 PFU per cell. At the indicated time points after infection, supernatant virus titers were determined as described in Materials and Methods. In each case, values represent averages of two independent experiments. Standard deviations (not shown) were always within 20% of the average values obtained.

a measure of transcription, were detected 12 h after infection in untreated cells, whereas in NGF-treated cells, the S segment was first detected 24 h after infection. Levels of genomic S segment and NP mRNAs were estimated to be 10- and 100-fold higher, respectively, in untreated cells than in NGF-treated PC12 cells. Untreated and NGF-treated PC12 cells showed similar levels of transcription for the housekeeping gene encoding cyclophilin.

Lack of cell proliferation is not responsible for the decreased LCMV replication in NGF-treated PC12 cells. The NGF-induced sympathetic neuron-like phenotype in PC12 cells is accompanied by a cessation of cell growth (25). To investigate whether the lack of cell proliferation was responsible for the decreased LCMV replication in NGF-treated PC12 cells, we compared levels of LCMV replication in proliferating and nonproliferating undifferentiated PC12 cells. Absence of serum prevented cell proliferation but also caused significant cell death, which reached 25% of the cell population after 48 h. To overcome possible effects on LCMV replication related to cell death, we also used treatments with two agents, forskolin (10 μ M) and insulin (1 mM), known to rescue nonproliferating PC12 cells from serum-free cell death (53). Virus titers at different time points after infection revealed differences in the kinetics and total yield of LCMV infectious particles between proliferating and nonproliferating PC12 cells, with viral titers being fivefold higher in proliferating than in nonproliferating PC12 cells (Fig. 6A). Nevertheless, levels of LCMV replication and transcription were not significantly affected in nonproliferating undifferentiated PC12 cells (Fig. 6B), a result which contrasted with the significant differences found between untreated and NGF-treated PC12 cells (compare Fig. 5 and 6B). Moreover, the small decrease in LCMV production caused by the lack of cell proliferation was not exclusive to PC12 cells. As presented in Fig. 7, similar results were found when LCMV production was examined in quiescent and exponentially growing 3T3 fibroblast cells. A two- to fivefold-decreased LCMV yield was found in quiescent 3T3 cells (Fig. 7A), but no significant differences were found in LCMV replication and

FIG. 9. Lack of selection of viral variants resistant to the NGF effect on LCMV replication in PC12 cells. Virus obtained 72 h after infection from supernatant of untreated and NGF-treated PC12 cells were used to infect untreated and NGF-treated PC12 cells at an MOI of 0.1 PFU per cell. At the indicated time points after infection, virus titers in supernatants were determined as described in Materials and Methods. Values represent averages of two independent experiments, with standard deviations (not shown) within 20% of the average values. Symbols: $\circ$, untreated PC12 cells infected with ARM 53b; $\square$, NGF-treated PC12 cells infected with ARM 53b; $\bullet$, untreated PC12 cells infected with ARM 53b; $\blacksquare$, NGF-treated PC12 cells infected with ARM 53b.

transcription between quiescent and proliferating 3T3 cells as determined by Northern blot hybridization (Fig. 7B).

NGF does not affect VSV replication in PC12 cells or LCMV replication in BHK-21 cells. To investigate whether the decreased LCMV replication in NGF-treated PC12 cells was a general phenomenon affecting other viruses, we compared VSV replication in untreated and NGF-treated PC12 cells. As shown in Fig. 8, similar kinetics and levels of VSV replication were obtained in the two cases. Similarly, we investigated the possibility that NGF was able to cause a reduction in LCMV replication through a pathway unrelated to the induction of the neuron-like phenotype in PC12 cells. Results presented in Fig. 8 indicate similar levels of LCMV replication in untreated and NGF-treated BHK-21 cells.

Lack of selection of LCMV variants with the ability to escape from NGF effects. Despite the high reduction in LCMV yield, the supernatant from NGF-treated PC12 cells still contained significant amounts of LCMV infectious particles ($\sim 10^4$ PFU/ml). We explored the possibility that NGF treatment had selected for LCMV variants with the ability to overcome the restriction of replication caused by NGF in PC12 cells. Results presented in Fig. 9 showed that this selection did not take place. LCMV recovered from supernatant of untreated or NGF-treated PC12 cells was used to infect untreated and NGF-treated PC12 cells. The two viral populations were able to replicate to similar high levels ($\sim 5 \times 10^7$ PFU/ml) in untreated PC12 cells, whereas replication of LCMV-infected untreated and NGF-treated PC12 was equally restricted ($\sim 10^4$ PFU/ml) by NGF treatment.

Return to chromaffin phenotype after withdrawal of NGF restores normal levels of LCMV replication in PC12 cells. PC12 cells differentiated into a neuron-like phenotype by exposure to NGF are able to restore a chromaffin phenotype after removal of NGF (25, 26). Under the hypothesis that the NGF-induced neuron-like phenotype was responsible for the decreased LCMV replication, it would then be expected that normal levels of LCMV replication would be restored after

FIG. 10. Return to chromaffin phenotype after withdrawal of NGF restores high levels of LCMV replication and transcription in PC12 cells. (A) Restoration of high LCMV yields after NGF withdrawal in PC12 cells. Untreated ($\circ$) and NGF-treated ($\bullet$) PC12 cells were infected with LCMV ARM 53b at an MOI of 5 PFU per cell. At 24 h after infection, NGF-treated PC12 cells were washed and infection was allowed to proceed in the absence of NGF (Δ). At the indicated time points, virus titers in supernatants were determined as described in Materials and Methods. Values represent the averages of two independent experiments with standard deviations. (B) NGF withdrawal restores high levels of LCMV replication and transcription in PC12 cells. Cells were treated as described for panel A; at the indicated times postinfection (p.i.), total cellular RNA was extracted and analyzed by Northern blot hybridization. (i) Untreated ($-$ NGF) and NGF-treated ($+$ NGF) PC12 cells at different time points after infection with LCMV ARM 53b ($+$) (uninfected controls [$-$]) were analyzed only for 72 h; (ii) samples in which NGF was removed 24 h after LCMV infection. (a) Hybridization with an LCMV NP probe; (b) hybridization to a specific probe for the housekeeping *GAPDH* gene; (c) ethidium bromide staining of the gels before transfer; (d) the same blot presented in panel i but using a three-times-longer exposure time.

NGF removal and return to the chromaffin phenotype in PC12 cells. Results presented in Fig. 10A indicated that upon NGF withdrawal 24 h after LCMV infection, the return to the chromaffin phenotype was accompanied by restoration of normal levels of LCMV replication. Thus, NGF-treated PC12 cells had titers of $\sim 5 \times 10^2$, $\sim 1 \times 10^3$, and 5×10^3 PFU/ml at 24, 48, and 72 h after LCMV infection, respectively. In

contrast, upon NGF withdrawal 24 h after infection, LCMV titers were $\sim 5 \times 10^5$ and $\sim 1 \times 10^7$ PFU/ml at 48 and 72 h after infection, respectively. Concomitant with the restoration of normal levels of LCMV infectious particle yield, restoration of LCMV replication and transcription to normal levels was also found (Fig. 10B).

DISCUSSION

We presented evidence that NGF differentiation of PC12 cells into a neuron-like phenotype leads to a severe reduction in viral yield upon LCMV infection (Fig. 1). This effect on viral production is not related to a decreased susceptibility to LCMV infection in NGF-treated PC12 cells, as demonstrated by the results of infectious center assays (Fig. 2 and 3). Thus, although 100% of NGF-treated PC12 cells scored as positive infectious centers after LCMV infection at an MOI of 5 PFU per cell, there was a 1,000-fold reduction in supernatant LCMV titers compared with untreated PC12 cells (Fig. 1 to 3). The lack of viral spread through the NGF-treated PC12 cell population after infection at low MOI (Fig. 2) likely reflects the drastic reduction in the yield of mature infectious virus particles per cell. The finding that untreated and NGF-treated PC12 displayed similar kinetics of LCMV binding (Fig. 4A) as well as similar expression levels of the putative LCMV receptor (Fig. 4B) provides further support for the conclusion that untreated and NGF-treated PC12 cells have similar susceptibilities to LCMV infection, suggesting that the reduction in viral yield is related to an intracellular block.

Despite the fact that 100% of the NGF-treated PC12 cells were harboring infectious virus after infection at an MOI of 5 PFU per cell, most of them (92%; Fig. 2) expressed low levels of the NP viral antigen, undetectable by immunofluorescence (Fig. 3). Nevertheless, a significant proportion of the NGF-treated PC12 cells (8.0%; Fig. 3) expressed normal levels of NP antigen, indicating that the reduced NP expression does not account for the 1,000-fold reduction in LCMV production found in differentiated PC12 cells. Moreover, the observation that the majority of NP-positive cells in the LCMV-infected NGF-treated PC12 cells also displayed the morphological characteristics of neuronal differentiation (Fig. 3) argues against the possibility that these NP-expressing cells represent a subpopulation of cells unresponsive to NGF. Finally, from results presented in Fig. 9, it is also unlikely that the selection of viral variants with the ability to overcome the NGF-induced restriction of LCMV replication in PC12 cells is responsible for the establishment of cells expressing high levels of NP antigen within the NGF-treated PC12 cell population.

Dependence of virus yield on the metabolic activity of the cell has been previously reported for LCMV as well as other arenaviruses (15, 48, 50, 58). Thus, the cessation of cell proliferation associated with the transdifferentiation of PC12 cells from a chromaffin to a neuronal phenotype (25, 39) may have been responsible for the reduced LCMV production in NGF-treated PC12 cells. However, only a fivefold reduction in LCMV yield was found in nonproliferating undifferentiated PC12 cells (Fig. 6A), which, although significant, does not account for the much higher differences found between undifferentiated and NGF-differentiated PC12 cells (compare Fig. 1 and 6). Levels of intracellular cyclic AMP (cAMP) vary with the state of cell growth and differentiation, being relatively high in quiescent and terminally differentiated neural cells (27, 52), and cAMP has been reported to exert a variety of effects on viral replication (21, 43, 63). In particular, it has been proposed that the shift from acute to persistent measles virus infection may depend on the intracellular level of cyclic

nucleotides (43). PC12 cells treated with forskolin, an activator of adenylate cyclase (17), showed only a moderate decrease (5- to 10-fold) in LCMV production, similar to that found in quiescent 3T3 cells (Fig. 6 and 7). These results suggest that although intracellular levels of cAMP may contribute, they are not the sole cause for the down-regulation of LCMV replication in differentiated PC12 cells. On the other hand, levels of 2'-5'-adenylate (2-5A)-dependent RNase and 2-5A synthetase, both members of the 2-5A system, are elevated in growth-arrested cells (31, 59). In addition, 2-5A-dependent RNase levels have been reported to be elevated during cell differentiation (33). Because several investigations have implicated the 2-5A system in the mechanism by which interferon inhibits the replication of picornaviruses (9, 35, 56), it is conceivable that the 2-5A system contributes to the restricted LCMV replication in NGF-treated PC12 cells. Nonetheless, after exposure to gamma interferon (100 U/ml for 24 h), BHK-21 cells had levels of 2-5A synthetase higher than that found in NGF-treated PC12 cells but only a very modest (5- to 10-fold) concomitant decrease in LCMV production (data not shown), suggesting that it is very unlikely the 2-5A system is responsible for the 1,000-fold reduction in LCMV yield found in NGF-treated PC12 cells. The well-established antiviral activity displayed by gamma interferon during LCMV infection *in vivo* (32, 36, 62) likely does reflect not only a direct effect on LCMV replication within the infected cell but also the contribution of interferon-induced immune processes (62).

Reduced titers of LCMV in tissue culture supernatant correlated with decreased levels of viral replication and transcription in NGF-treated PC12 cells, as determined by diminished levels of the S RNA and NP mRNA, respectively (Fig. 5). However, replication and transcription of LCMV in NGF-treated PC12 cells were differently affected. Thus, expression of the S RNA, although significantly reduced, was readily detected at 24 h after LCMV infection, with increased levels at 72 h postinfection (Fig. 5). In contrast, under the same experimental conditions, expression of the NP mRNA was not detected in the NGF-treated PC12 cells (Fig. 5). Very low levels of NP mRNA were detected by Northern blot hybridization at 48 h after infection by using an exposure three times longer than the one presented in Fig. 5 (data not shown).

The detailed mechanisms by which viruses are able to establish and maintain long-term persistent infections in a tissue are largely unknown, and differences occur with different virus-cell interactions (34, 42, 60). Both viral genes (11, 12, 28, 29, 49) and host cellular genes (40) which promote viral persistence by enhancing cell survival during infection by cytolytic viruses have been identified. Although LCMV replication is exquisitely noncytopathic for the infected cells, subtle functional and morphological alterations are sometimes detected (reviewed in reference 38). In several instances, the LCMV-induced disorder of a cellular differentiated function causes disturbance in the host homeostasis and leads to disease (reviewed in references 14 and 44).

Persistent LCMV infection, both *in vivo* and *in vitro*, is associated with significant reduction in the formation of infectious progeny virus and changes in the regulation of viral expression (5, 38, 47). Whether specific cellular genes may contribute to or determine the establishment and regulation of LCMV persistent infection is unknown. The observation that there is a restoration of normal levels of LCMV replication concomitant with NGF withdrawal and return to the chromaffin phenotype in PC12 cells suggests that induction or changes in expression levels of specific cellular gene products contribute to the restricted LCMV replication in differentiated PC12 cells. Interestingly, merely the induction of any given cell

differentiation program does not necessarily interfere with LCMV replication, as noted earlier during differentiation of embryonal carcinoma cells into epithelial cells (19). Moreover, the expression of neuronal markers does not necessarily cause LCMV replication to be restricted, as indicated by the high levels of LCMV replication found in the neuronal cell line OBL21 (data not shown). The action of NGF is transcription dependent (6), and NGF is known to induce or increase the transcription of many different genes (2, 24, 61), with some of them, such as the proto-oncogenes *c-fos* and *c-myc*, exhibiting a very rapid response to NGF (24). Whether and how these early NGF response genes may contribute to the LCMV-restricted replication in NGF-treated PC12 cells remains to be determined. Other NGF-induced genes such as PC4 (61), which are related to the interferon and lymphokine genes, may regulate the expression of other genes leading to a complex network of interactions, some of which interfere with the replication of LCMV.

Expression of NP antigen (51) and S RNA (22) is readily detected in the CNS of mice persistently infected with LCMV, but electron microscopic examination has failed to reveal mature viral particles budding from infected neurons within the brain parenchyma (51). Interestingly, we have also found that in mice persistently infected with LCMV, the amount of infectious virus, measured as PFU per gram of tissue, recovered from brain parenchyma, free of leptomeninges and choroid plexus, is 100-fold lower than that recovered from brain samples containing leptomeninges and choroid plexus (data not shown). Detection of NP antigen and S RNA by immunohistochemistry and in situ hybridization procedures does not necessarily reflect the rate of transcription and translation, respectively, but rather may reflect, as found with influenza virus (8), a very long half-life of the viral ribonucleoprotein (RNP) complexes. Presently, we are trying to determine the half-life of LCMV RNPs in undifferentiated and differentiated PC12 cells. A restricted productive LCMV infection of neurons together with highly stable LCMV RNPs would explain the observed slow clearance of viral material from the brain in the absence of lymphocytic infiltration into the brain parenchyma or necrosis of brain tissue (46).

Further investigation of the PC12 tissue culture paradigm, although far from representing the complexity of the interaction between LCMV and the CNS, should contribute to the dissection and clarification of the specific molecular mechanisms by which LCMV can establish a long-term persistent infection in the murine CNS, where, despite viral effects on behavioral and differentiated functions of CNS, no vital functions are compromised.

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