JOURNAL OF VIROLOGY, Nov. 1986, p. 558–563
0022-538X/86/110558-06$02.00/0

Vol. 60, No. 2

Quantitation, with a New Assay, of Theiler's Virus Capsid Protein in the Central Nervous System of Mice

EVELYNE CASH, MARIO CHAMORRO,† AND MICHEL BRAHIC*
Département de Virologie, Institut Pasteur, 75724 Paris Cedex 15, France

Received 4 May 1986/Accepted 28 July 1986

We developed a quantitative assay for antigens at the single-cell level. Tissue sections were reacted with (i) a primary antibody, (ii) a biotinylated secondary antibody, or (iii) ^{35}S-streptavidin. Binding of streptavidin to cells was quantitated by microscopic autoradiography. We showed that the number of autoradiographic grains was proportional to the amount of antigen per cell. With this assay, we studied the synthesis of Theiler's virus capsid proteins VP1, VP2, and VP3 in permissive BHK cells grown in vitro and in mouse central nervous system (CNS) cells during a persistent infection. We found that synthesis of the three capsid proteins was restricted in mouse CNS cells. Restricted virus replication could play a major role in the persistence of Theiler's virus in mouse CNS cells.

To resolve complex biological problems, e.g., development, it is often necessary to measure the expression of a specific gene at the single-cell level in tissue sections. In this context, quantitative in situ hybridization is the only way to study the synthesis of specific mRNAs (3, 10). The translation of mRNAs into proteins, on the other hand, can be studied by immunofluorescence or immunocytochemistry, techniques which offer reasonably good sensitivity but do not lend themselves easily to quantitative analysis (14).

Our study of persistent infection of mice with Theiler's virus compelled us to quantitate the synthesis of viral capsid proteins in individual central nervous system (CNS) cells. For this reason, we developed a new assay in which binding of biotinylated antibodies to cells in a tissue section is measured with radioactive streptavidin and quantitative microscopic autoradiography. This article describes this assay, as well as our results with Theiler's virus-infected animals.

Theiler's virus, a picornavirus, is the agent of a persistent infection of the mouse CNS accompanied by primary demyelination (7, 16). Both the infection and the tissue lesions predominate in the spinal cord. This slow viral infection resembles human multiple sclerosis very closely. The mechanism by which a picornavirus is able to persist in the CNS in the face of a specific immune response is one of the main question of pathogenesis in the study of this disease. We have shown that the virus infects glial cells at the site of demyelination and that viral replication is restricted in these cells (5). From these observations we proposed that persistence results from host-imposed restriction of virus replication which allows the cell to survive and, in turn, provides the virus with a shelter against immune defense mechanisms.

Recently, using quantitative in situ hybridization coupled to immunocytochemistry, we confirmed that the RNA content of infected cells was limited to a few hundred copies of viral genome per cell and that the majority (≈90%) of them did not express capsid antigens (6). A surprising result, however, was that 10% of the cells in which viral RNA replication was restricted were nevertheless immunoperoxidase positive for capsid antigens. Because immunoperoxidase is not a quantitative assay, and also because the serum that we used recognized two of the three major viral capsid proteins, it was necessary to reinvestigate this question by measuring the level of expression of individual capsid proteins in cells of infected mouse CNS. As shown below, we found that the levels of expression of VP1, VP2, and VP3 were identical and very low, similar to those observed at the very beginning of viral protein synthesis during a lytic cycle in permissive BHK cells grown in vitro. Our results demonstrated that virus capsid protein synthesis was restricted in the CNS, even in the minority of viral-RNA-containing cells which were capsid antigen positive with immunoperoxidase.

MATERIALS AND METHODS

Virus cells and animals. The DA strain of Theiler's virus was plaque purified three times on BHK cells.

SJL/J mice, 3 to 4 weeks old, were purchased from the Institut Pasteur animal facility. Mice were inoculated intracranially with 40 μl of phosphate-buffered saline (PBS) containing 10^4 PFU of Theiler's virus, strain DA.

Preparation of cells and tissue sections. Microscope slides were cleaned and treated with Denhardt medium followed by acetylation as previously described (10).

BHK-21 cells were infected at various multiplicities of infection (MOI), as indicated in Results. Cells were harvested by trypsinization, washed once in PBS, and deposited on treated slides by using a cytocentrifuge. After air drying the cells for a few minutes, we fixed them for 20 min in ice-cold PFG fixative (0.5% paraformaldehyde, 0.5% glutaraldehyde, 0.1 M phosphate buffer [pH 6.0], 1.6% glucose, 0.002% $CaCl_2$, 1% dimethyl sulfoxide). The fixative was prepared as previously described (13). After fixing, the cells were quenched by immersion for 20 min in ice-cold 0.15 M ethanolamine (pH 7.5) followed by two washes in cold PBS, dehydration (70% ethanol once, 95% ethanol twice, 100% ethanol once, xylene twice; 5 min for each step), and embedding in paraffin (twice for 10 min in Paraplast at 62°C).

Mice were perfused under anesthesia with 20 ml of PBS followed by 20 ml of cold PFG fixative (described above). Dissection of the CNS, postfixation, paraffin embedding, and sectioning were done as described previously (4).

* Corresponding author.
† Present address: Department of Microbiology, University of California, San Francisco, CA 94143.

Immune sera. The immune serum against purified virus was described previously (4). This serum binds strongly to capsid proteins VP1 and VP2 and weekly to capsid protein VP3, as shown by immunoblotting of infected BHK cell lysate.

Immune sera against purified viral proteins VP1, VP2, and VP3 were prepared as follows. Fourteen 75-cm^2 flasks of confluent BHK cells were infected at an MOI of 10 PFU per cell. The medium and cell debris were harvested after 15 h of incubation at 37°C. Triton X100 was added to a final concentration of 0.5%, and the mixture was centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was passed slowly (10 ml/h) through a column (10-ml bed volume) of anti-Theiler's virus immunoglobulins purified from the anti-whole-virion serum described above and coupled to CNBr-activated Sepharose 4B. The column was washed with several volumes of PBS–0.5 M NaCl–0.5% Triton X100, and the effluent was discarded. Bound material was eluted by boiling the Sepharose for 5 min in 13 ml of 80 mM Tris hydrochloride(pH 6.8)–3% sodium dodecyl sulfate–0.1% glycerol. After separation of the Sepharose from the eluent by centrifugation, the proteins in the eluent were precipitated with 3 volumes of methanol at −20°C for 1 h. The solution was centrifuged at 10,000 × *g* for 10 min at 4°C, and the pellet was dissolved in sample buffer (11), boiled for 3 min, and loaded on top of a preparative sodium dodecyl sulfate-acrylamide gel slab (13 by 14 by 0.15 cm). After electrophoresis, the gel was stained with Coomassie blue, destained, and soaked twice for 30 min in PBS. Three major bands were observed at positions corresponding to those of proteins VP1, VP2, and VP3. The gel bands were cut out, homogenized in 10 ml of PBS–0.1% sodium dodecyl sulfate, and the proteins were eluted overnight at room temperature with stirring. After a brief centrifugation, the supernatants were recovered, and the pellets were eluted a second time as described above. We precipitated proteins in the supernatants with methanol as described above after adding 40 μl of New Zealand White rabbit prebleed sera as carrier proteins. After centrifugation, the pellets were dissolved in 1 ml of PBS–0.1% sodium dodecyl sulfate, emulsified in 1 ml of complete Freund adjuvant, and injected into foot pads of New Zealand White rabbits. One month later, the animals were challenged intravenously with 100 μg of antigens purified as described above. Antisera were obtained 10 days later.

The specificity of each serum was checked by immunoblotting of infected BHK cell lysate. Each serum recognized exclusively its corresponding antigen and did not bind to other viral or cellular proteins.

Radioimmunocytochemistry. Radioimmunocytochemistry was performed on cytocentrifuged BHK cells or CNS paraffin sections. We removed the paraffin from the slides by dipping them twice in xylene and twice in absolute ethanol (5 min each time). Rehydration and permeabilization of the cells or tissues was obtained by dipping the slides in PBS for 5 min, PBS–0.1% Triton X100 for 4 min, and PBS twice for 5 min each time. Permeated cells and CNS sections were reacted sequentially with (i) normal goat serum (1:25 dilution in PBS), (ii) primary rabbit antiserum diluted 1:300, (iii) biotinylated anti-rabbit IgG prepared in goat (Vector Laboratories) and diluted 1:7,200, and (iv) ^{35}S-streptavidin 300 Ci/mMol (Amersham Corp.) at a final concentration of 0.5 μCi/ml. All antiserum and streptavidin dilutions were done in normal goat serum diluted 1:25 in PBS. All steps were performed at room temperature for 30 min, except incubation with ^{35}S-streptavidin, which was for 20 min. Between steps ii, iii, and iv, the slides were washed for 5 min in PBS at room temperature. After step iv, the slides were washed four times, for 5 min each time, in PBS followed by washing in a large volume of PBS overnight. We dehydrated washed slides by dipping them twice in 70% ethanol–0.3 M ammonium acetate and once in 95% ethanol–0.3 M ammonium acetate. After air drying them, we dipped the slides in NTB-2 Kodak nuclear track emulsion diluted 1:1 with 0.2 M ammonium acetate, exposed them in dark boxes for the appropriate time period, developed them in D-19 Kodak developer, fixed them in Kodak fixer, and counterstained them with Giemsa (for BHK cells) or hematoxylin (for tissue sections) as previously described (10).

RESULTS

Radioimmunocytochemistry as a quantitative assay. In this new assay, tissue sections are reacted with (i) a primary antibody specific for the antigen of interest, (ii) a biotinylated secondary antibody, and (iii) ^{35}S-labeled streptavidin, a recently introduced radiochemical. Binding of the radioactive marker is detected by autoradiography with nuclear track emulsion. Histological details can be observed simultaneously after staining. The amount of radioactivity bound to a cell is determined by counting the number of autoradiographic grains after a suitable time of exposure. As for immunocytochemistry, the concentration of the primary and secondary antibodies have to be optimized to produce a maximal signal with a minimum of background. Technical details are described in Materials and Methods, and examples are shown in Fig. 1.

We established that the technique is quantitative in the following way. BHK-21 cells were infected with Theiler's virus at MOIs of 0.25, 0.5, 1, 2, or 4 PFU per cell. The cells were harvested with trypsin 1 h after infection, a time sufficient to allow complete penetration of the inoculum. One sample of each harvest was used to extract total RNA and quantitate the average number of viral RNA genome observed per cell by using methods already published (4). This number was proportional to the MOI, and 1 PFU corresponded to 560 viral RNA molecules. Another sample of each harvest was deposited on microscope slides and carried through radioimmunocytochemistry as described in Materials and Methods. Three different primary sera were used in this experiment: anti-whole-virion, anti-VP1, and anti-VP2 sera. We processed control uninfected BHK cells in parallel to measure the background. In each case, we determined the average number of autoradiographic grains per cell and per minute of autoradiographic exposure by counting grains over 40 cells taken at random on the slide. These values were plotted against the MOI. Figure 2 shows the result of the experiment. The number of autoradiographic grains was proportional to the amount of antigen per cell for the three sera examined. Since 1 PFU corresponded to 560 viral particles, cells infected at an MOI of 0.25 PFU per cell received 140 viral particles, which explains why every cell was labeled. Cells infected at 4 PFU per cell, the highest MOI used in the experiment, received 2,240 viral particles. The absence of saturation of radioactivity binding with increasing MOI can be explained if one assumes that viral receptor densities on host cells are similar for Theiler's virus and poliovirus ($\approx 10^4$ receptors per cell) (12).

The straight lines shown in Fig. 2 were computed by linear regression. The correlation coefficients were 0.902, 0.846, and 0.933 ($P < 0.01$) for anti-whole-virion, anti-VP1, and anti-VP2 sera, respectively. The regression lines did not

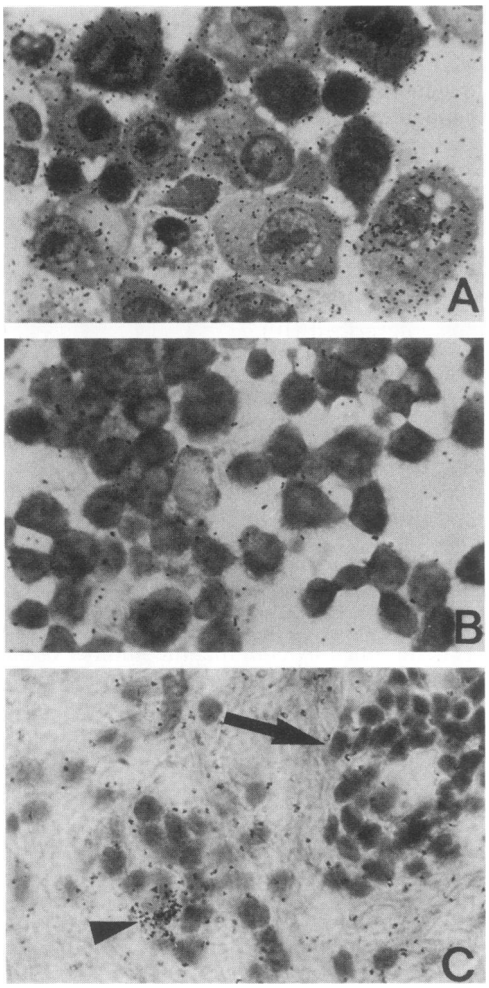

FIG. 1. (A) BHK cells were harvested 6 h after infection with 3 PFU of Theiler's virus per cell, deposited on slides, and fixed with PFG. The slides were processed for radioimmunocytochemistry as described in Materials and Methods by using anti-whole-virus serum as the primary reagent. Exposure time was 18 h. (B) Uninfected BHK cells were processed exactly as described for A. (C) An SJL/J mouse was inoculated with 10^4 PFU of Theiler's virus and sacrificed 2.5 months later by perfusion with PFG. Longitudinal paraffin sections of the spinal cord were processed for radioimmunocytochemistry with anti-VP2 primary serum. The arrow points to an inflammatory infiltrate characteristic of white matter lesions. The arrowhead indicates a cell containing VP2 capsid protein. The exposure time was 5 days.

intersect with the origin, although background, as determined by radioimmunocytochemical analysis of uninfected cells, was subtracted from each point. We believe that higher background with infected cells resulted from some cells being damaged during cytocentrifugation, thus releasing antigens on the slide. Indeed, damaged cells were routinely observed on all slides.

Two antigens (proteins VP1 and VP2) produced different coefficients of proportionality, presumably reflecting differences in the number of epitopes per protein, the affinity of different sera for their respective antigens, and the accessibility of each protein within the cells (Fig. 2). The data of Fig. 2 also give an estimate of the sensitivity of this method. The virions of picornaviruses are composed of 60 copies of each capsid protein. We could detect binding of streptavidin to cells containing 140 copies of viral RNA 1 h after infection. Therefore, we were able to detect a minimum of 8,400 copies of VP1 or VP2 per cell. This level of sensitivity is similar to that of immunocytochemistry with avidin-biotin amplification (4). It should be pointed out that this technique provides relative quantitation only, or comparisons between cells, although absolute measurements could conceivably be obtained after calibration with an enzyme-linked immunosorbent assay or a radioimmunoassay.

Quantitation of capsid protein synthesis in permissively infected BHK cells. We used radioimmunocytochemistry to study the synthesis of capsid proteins VP1, VP2, and VP3 during permissive infection of BHK cells. Cells were infected at an MOI of 3 PFU per cell with the virus suspension described above. The cells were harvested 1, 2, 3, 4, and 6 h postinfection, deposited on microscope slides, and carried through radioimmunocytochemistry by using immune sera against VP1, VP2, and VP3 proteins. Uninfected BHK cells were processed in parallel as controls. Radioactivity binding was determined by counting autoradiographic grains for

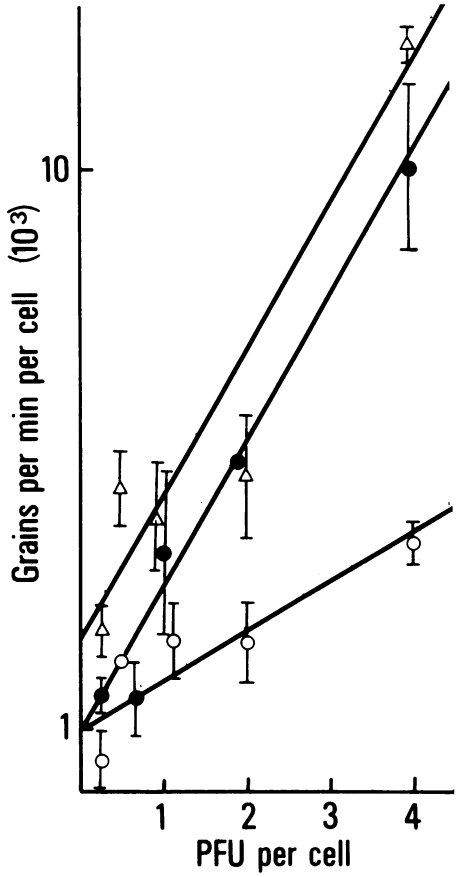

FIG. 2. Quantitation of viral capsid antigens by radioimmunocytochemistry. BHK cells were infected at the MOI indicated on the abscissa and harvested 1 h later. The cells were deposited on treated slides, fixed with PFG, and processed for radioimmunocytochemistry with: (△) anti-whole-virion, (○) anti-VP1, and (●) anti-VP2 sera. The average number of grains per cell was determined by counting grains over 40 randomly selected cells. The background, determined by processing of uninfected cells, was subtracted in each case. The points on the figure correspond to the average of three independent experiments for each MOI. The straight lines were computed by linear regression. Bars indicate standard errors.

randomly selected cells. Figure 3 shows the result of the experiment. The amount of antigen per cell decreased between 1 and 2 h postinfection, presumably reflecting the degradation of incoming capsids. Synthesis was first detected between 2 and 3 h postinfection. There was a linear threefold increase in the amount of antigen per cell between 3 and 6 h postinfection. This accumulation rate is very similar to that of viral RNA during the same period (E. Cash, unpublished data). VP1, VP2, and VP3 accumulated in infected cells at the same rate. This was expected, since picornavirus capsid proteins are translated as a polyprotein precursor. Although the average content of capsid protein per cell increased regularly with time (Fig. 3), we observed variations from cell to cell at a given time of infection (Fig. 1). We have seen this asynchronous capsid antigen synthesis in the cell population previously when using immunoperoxidase (4), and it reflects asynchronous viral RNA replication.

Quantitation of capsid protein synthesis in the CNS of persistently infected mice. The viral RNA content of infected cells is limited to a few hundred copies of genome during persistent infection of mouse CNS with Theiler's virus (6). The majority of these cells (90%) do not synthesize capsid antigens, as has been shown by coupling immunocytochemistry to in situ hybridization (6). It was important to measure the amount of capsid synthesis in the fraction of infected cells (10%) which are immunoperoxidase positive in spite of their low viral RNA content. We achieved this with radioimmunocytochemistry by comparing the amount of ^{35}S-streptavidin binding to infected cells in the CNS and to in vitro-infected BHK cells.

Six SJL/J mice, which had been inoculated intracranially with 10^4 PFU of Theiler's virus, strain DA, were sacrificed 2.5 months postinoculation and perfused with fixative. Their spinal cords were removed and embedded in paraffin, and 10 μm thick longitudinal sections of the entire spinal cord were cut and processed through radioimmunocytochemistry with anti-VP1, -VP2, and -VP3 primary sera. The slides were exposed for 5 days, developed, and systematically scanned by microscope. Autoradiographic grains were counted for every positive cell encountered. The number of positive cells per section was very close to that obtained with immunoperoxidase and varied considerably from mouse to mouse, a phenomenon already observed for viral RNA (6). Figure 4 shows the distribution of VP1, VP2, and VP3 in the population of infected cells. For the sake of clarity, the data from six mice were combined in a single histogram for each antigen. The amount of antigen per cell was expressed as the number of autoradiographic grains per cell per minute of exposure. In each case, the distribution was homogeneous, with a clear maximum. The amount of antigen corresponding to the maximum was compared with the level of antigen synthesis observed during a viral lytic cycle in BHK cells (Fig. 3). In each case it was equivalent to that observed at the very beginning of viral protein synthesis during a lytic cycle (3 h postinfection). These results demonstrated that synthesis of the three major capsid proteins was minimal in CNS cells.

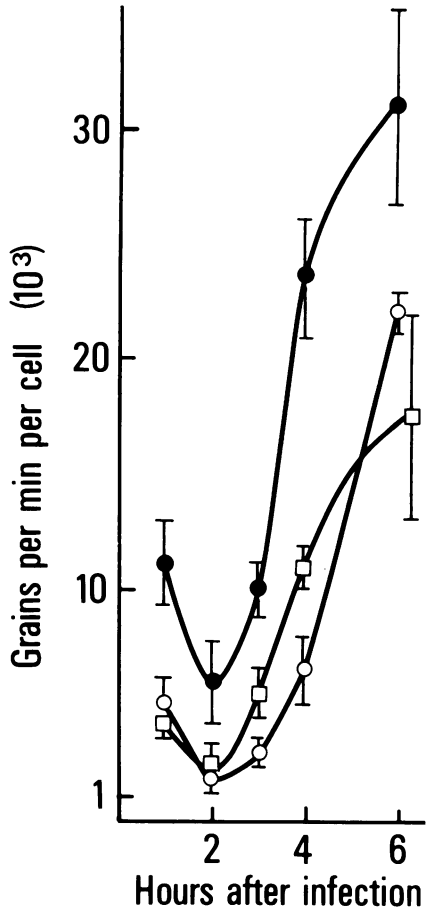

FIG. 3. Kinetics of synthesis of viral capsid proteins in permissively infected BHK cells. Cells were infected at an MOI of 3 PFU per cell and harvested at the times shown on the abscissa. The cells were processed for radioimmunocytochemistry with anti-VP1 (○), anti-VP2 (●), and anti-VP3 (□) primary antisera. The average number of grains per cell was determined by counting grains over 40 randomly selected cells. The background, determined by processing of uninfected cells, was subtracted in each case. Each point corresponds to the average of three slides processed in parallel. Bars indicate standard errors.

DISCUSSION

Several methods have been proposed to quantitate proteins in tissue sections. They consist of either computer-assisted microdensitometry of immunoperoxidase staining (1, 2, 15) or area measurement of immunoperoxidase staining with computerized image analysis (9). All require sophisticated, expensive equipment which is not routinely available. As an alternative, we propose a specific radioactive reagent and microscopic autoradiography. A similar approach has been described using ^{125}I-labeled Fab fragments or ^{125}I-labeled protein A as the radioactive reagent (8, 17). Quantitation and sensitivity were not documented in these reports. Our technique uses a biotinylated secondary antibody and ^{35}S-streptavidin. The procedure is simple and does not require image analysis equipment. We demonstrated that it is quantitative and offers good sensitivity, comparable to that of immunoperoxidase with biotin-avidin amplification.

This technique, called radioimmunocytochemistry, was used to measure the level of Theiler's virus capsid proteins in mouse CNS during the course of a persistent infection. In this disease, viral RNA replication is restricted, infected cells contain only a few hundred copies of viral genomes (6), and the majority of these cells are immunoperoxidase negative for capsid antigens. However, 10% are immunoperoxidase positive, an unexpected result since RNA replication and translation are coupled events during the lytic multiplication cycle of picornaviruses. Because immunoperoxidase

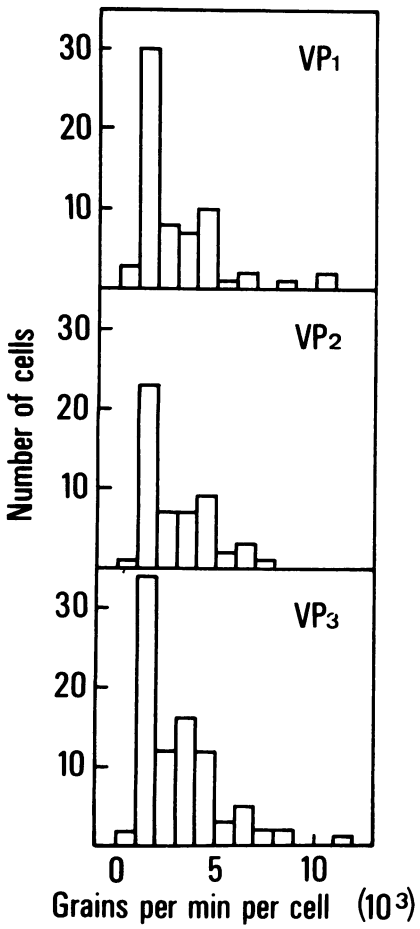

FIG. 4. Quantitation of viral capsid protein expression in the CNS of persistently infected mice. Six SJL/J mice were inoculated intracranially with 10^4 PFU of Theiler's virus and sacrificed 2.5 months later. The animals were perfused with PFG fixative. Their CNS were dissected and embedded in paraffin. Longitudinal sections of spinal cord were cut and processed through radioimmunocytochemistry with anti-VP1, anti-VP2, and anti-VP3 primary sera. Experimental conditions were identical to those described in the legend to Fig. 3. The sections were systematically scanned by microscope, and autoradiographic grains were counted for every positive cell encountered. The figure shows the distribution of radioactivity in the population of positive cells. Because the number of positive cells was small for each animal, the data from six mice were combined for each antigen. Statistical analysis produced maxima of $(2.9 \pm 0.2) \times 10^{-3}$, $(2.9 \pm 0.3) \times 10^{-3}$, and $(3.2 \pm 0.4) \times 10^{-3}$ for VP1, VP2, and VP3, respectively.

is not a quantitative technique it was difficult to interpret this result. Therefore, we used immunocytochemistry to compare the expression of capsid proteins in permissive BHK and CNS cells, based on the assumption that reagent diffusion and antigen accessibility are the same in cultured cells and tissue sections. We used thick (10 μm) CNS sections and counted grains only if they were adjacent to a clearly demarcated nucleus to minimize the sampling problem inherent to section analysis. Expression levels for VP1, VP2, and VP3 in the CNS were very small since they corresponded to those observed 2 to 3 h postinfection during a lytic cycle (Fig. 4).

In summary, our results, together with our previous findings (6), demonstrated that capsid protein synthesis was restricted in the CNS. In the majority of infected cells, it was below detection level with immunoperoxidase, and even in cells which were immunoperoxidase positive the amount of antigen was very small. Clearly, the virus replication cycle is restricted in the CNS, in term of both RNA and capsid protein synthesis.

As already discussed elsewhere (5, 6), restricted viral replication could be one of the factors allowing the survival of host cells. By providing the virus with a shelter against immune defense mechanisms, restriction may play a major role in the persistence of Theiler's virus in the CNS.

ACKNOWLEDGMENTS

We thank C. Aubert for excellent technical assistance.

This work was supported by grants from the National Multiple Sclerosis Society, the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Fondation pour la Recherche Médicale, and the Institut Pasteur Fondation.

LITERATURE CITED

1. **Benno, R. H., L. W. Tucker, T. H. Joh, and D. J. Reis.** 1982. Quantitative immunocytochemistry of tyrosine hydroxylase in rat brain. I. Development of a computer-assisted method using the peroxidase-antiperoxydase technique. Brain Res. **246:** 225–236.
2. **Benno, R. H., L. W. Tucker, T. H. Joh, and D. J. Reis.** 1982. Quantitative immunocytochemistry of tyrosine hydroxylase in rat brain. II. Variation in the amount of tyrosine hydroxylase among individual neurons of the locus coeruleus in relationship to neuronal morphology and topography. Brain Res. **246:** 237–247.
3. **Brahic, M., and A. T. Haase.** 1978. Detection of viral sequences of low reiteration frequency by in situ hybridization. Proc. Natl. Acad. Sci. USA **75:**6125–6129.
4. **Brahic, M., A. T. Haase, and E. Cash.** 1984. Simultaneous detection of viral RNA and antigens. Proc. Natl. Acad. Sci. USA **81:**5445–5448.
5. **Brahic, M., W. G. Stroop, and J. R. Baringer.** 1981. Theiler's virus persists in glial cells during demyelinating disease. Cell **26:**123–128.
6. **Cash, E., M. Chamorro, and M. Brahic.** 1985. Theiler's virus RNA and protein synthesis in the CNS of demyelinating mice. Virology **144:**290–294.
7. **Dal Canto, M. C., and H. L. Lipton.** 1975. Primary demyelination in Theiler's virus infection. An ultrastructural study. Lab. Invest. **33:**626–637.
8. **Gonatas, N. K., A. Stieler, J. Gonatas, P. Gambetti, J. C. Antoine, and S. Avrameas.** 1974. Ultrastructural autoradiographic detection of intracellular immunoglobulins with iodinated Fab fragments of antibody. J. Histochem. Cytochem. **22:**999–1009.
9. **Gross, D. S., and J. M. Rothfeld.** 1985. Quantitative immunocytochemistry of hypothalamic and pituitary hormones: validation of an automated, computerized image analysis system. J. Histochem. Cytochem. **33:**11–20.
10. **Haase, A. T., M. Brahic, L. Stowring, and H. Blum.** 1984. Detection of viral nucleic acids by in situ hybridization. Methods Virol. **7:**189–226.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–682.
12. **Lonberg-Holm, K., and L. Philipson.** 1974. Early interactions between viruses and cells, p. 1–148. *In* J. Melnick (ed.), Monographs in virology, vol. IX. S. Karger, Basel.
13. **Morris, R. G., and P. C. Barber.** 1983. Fixation of Thy-1 in nervous tissue for immunohistochemistry. J. Histochem. Cytochem. **31:**263–274.
14. **Sternberger, L. A.** 1979. Immunocytochemistry. John Wiley &

Sons, Inc., New York.
15. **Sternberger, N. H., Y. Itoyama, M. W. Kies, and H. D. Webster.** 1978. Myelin basic protein demonstrated immunocytochemically in oligodendroglia prior to myelin sheath formation. Proc. Natl. Acad. Sci. USA **75**:2521–2524.
16. **Theiler, M.** 1937. Spontaneous encephalomyelitis of mice, a new virus disease. J. Exp. Med. **55**:705–719.
17. **Wright, G. M., and C. P. Leblond.** 1980. An attempt at quantitation of procollagens I and III and collagen IV in tooth sections by exposure to the corresponding antibodies followed by ^{125}I-protein A an radioautography. J. Histochem. Cytochem. **28**:1355–1358.