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Measles Virus Nucleocapsids: Large-Scale Purification and Use in Radioimmunoassays

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Nucleocapsids in quantities approaching 1 mg were purified from 10^9 measles virus-infected cells. They contained one polypeptide species with a molecular weight of 59,000. Antiserum was raised in rabbits against purified nucleocapsids and used in a competitive radioimmunoassay. Because of their instability, purified nucleocapsids were not suitable for use in such an assay. Instead, partially purified nucleocapsids from HEp-2 cells persistently infected with measles virus and labeled in vitro with [^{35}S]methionine were used as the source of radioactive antigen. The radioimmunoassay thus developed measured less than 5 ng of nucleocapsids in infected cells.

Measles virus (MV) has been implicated in at least two chronic diseases of the central nervous system, subacute sclerosing panencephalitis and multiple sclerosis (for a recent review, see reference 9). The evidence for its involvement in multiple sclerosis is indirect and inconclusive; elevations of MV antibodies have been observed in multiple sclerosis patients (1, 3, 14-16), and MV-like nucleocapsids occasionally have been observed in clinical material (18, 19). A critical analysis of the possible involvement of MV requires the application of assays that are sufficiently sensitive to detect very small amounts of viral macromolecules in clinical material. Low yields of measles virions and purified viral polypeptides have made it very difficult to develop such assays; however, large numbers of viral nucleocapsids accumulate in infected cells. In this manuscript we describe a method for their purification and the development of a competitive radioimmunoassay (RIA) that detects nucleocapsids in quantities of less than 5 ng.

MATERIALS AND METHODS

Buffers and solutions. The following buffers and solutions were used: TDE [0.002 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4-0.002 M ethylenediaminetetraacetate (EDTA)-2% dimethyl sulfoxide], TE (0.002 M Tris [pH 7.4]-0.002 M EDTA), 30% Renografin (30 ml of Renografin-76 [E. R. Squibb & Sons] + 69 ml of water + 1 ml of 100× TE buffer), PBS (0.02 M sodium phosphate buffer [pH 7.2]-0.15 M NaCl), NET (0.05 M Tris [pH 7.2]-0.15 M NaCl-0.005 M EDTA), and solubilization buffer (0.1 M Tris [pH 6.8]-2% sodium dodecyl sulfate [SDS]-2% 2-mercaptoethanol-20% glycerol).

Cells and virus. Vero cells were grown in monolayers in Eagle minimal essential medium supplemented with 10% fetal calf serum. The Edmonston vaccine strain of MV was passaged in Vero cells and plaque purified three times. Viral inocula were prepared in Vero cells that were infected at a multiplicity of 0.01. After 40 h, cells were scraped into the medium, subjected to sonic treatment, and centrifuged at 1,000 × *g* for 5 min. Most of the infectious virus was in the supernatant, and this was stored in portions at −70°C. HEp-2-PI cells, a line of HEp-2 cells persistently infected with the Edmonston vaccine strain of MV (Hayes and Zweerink, submitted for publication) was grown in Joklik-modified minimal essential medium supplemented with 5% fetal calf serum.

Radiolabeling of viral ribonucleic acid and polypeptides in infected cells. To label viral ribonucleic acid in Vero cells, 2 μg of actinomycin D per ml was added to the medium at 24 h postinfection, and 2 h later [^{3}H]uridine (New England Nuclear Corp.; 40 to 50 Ci/mmol) was added to 25 μCi/ml. To label polypeptides in HEp-2-PI cells, the growth medium was replaced with Eagle minimum essential medium containing 0.45 mg of methionine per liter and 5% dialyzed fetal calf serum at 24 h after plating of cells, and 0.5 h later [^{35}S]methionine (New England Nuclear Corp.; 400 Ci/mmol) was added to 25 μCi/ml. Cells were harvested 6 h later, and washed two times with complete growth medium.

Nucleocapsid purification. Most of the nucleocapsids in MV-infected cells remain cell associated (unpublished data). Therefore, they were purified from cytoplasmic extracts prepared when more than 90% of the cells in the culture had fused (approximately 40 h postinfection). Vero cells infected with MV were grown in roller bottles and harvested by scraping them into the medium. Next they were mixed

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with 5×10^6 [^{3}H]uridine-labeled infected cells and pelleted by centrifugation at $500 \times g$ for 5 min. (The latter cells contained [^{3}H]uridine-labeled ribonucleic acid in nucleocapsid, and this facilitated their purification.) After suspension at 10^7 cells per ml in TDE buffer and Dounce homogenization, the nuclei were pelleted ($500 \times g$ for 5 min). The cytoplasmic extract was layered over 2-ml cushions of 30% Renografin-76 in TE buffer and centrifuged at 40,000 rpm for 60 min in a Beckman SW41 rotor. The pellets were resuspended by Dounce homogenization in 5 ml of TE buffer per 10^8 cells and incubated with 0.5% deoxycholate at 37°C for 5 min. Next, the material was layered over preformed 20 to 80% Renografin gradients and centrifuged for 3 h at 25,000 rpm in a Beckman SW27 rotor. Fractions of 1.2 ml each were collected, and the radioactivity in each was determined by spotting a small sample (0.05 ml) on filter paper (4). Fractions containing nucleocapsids (at approximately 60% Renografin) were diluted with an equal volume of TE buffer, layered on top of preformed cesium chloride gradients (1.2 to 1.4 g/ml in TE buffer), and centrifuged for 16 h at 20,000 rpm in an SW27 rotor. Again, fractions of 1.2 ml were collected, and the radioactivity in each was determined. Nucleocapsids banded at a density of 1.30 g/cm^2. The appropriate fractions were combined and diluted with 2 volumes of PBS, and the nucleocapsids were pelleted by centrifugation at 40,000 rpm for 90 min in an SW41 rotor.

In some instances, particulate fractions that were enriched for nucleocapsids were prepared by Dounce homogenizing HEp-2-PI cells in PBS containing 0.002 M phenylmethyl sulfonyl fluoride and 2% Triton X-100 followed by incubation at 23°C for 15 min and centrifugation through a cushion of 40% sucrose (wt/vol) in TE buffer (1.5 h at 45,000 rpm in an SW50 rotor). These preparations will be referred to as partially purified nucleocapsids.

SDS-polyacrylamide gel electrophoresis. Polypeptides were analyzed in 10% discontinuous Tris gels as described by Maizel (8). Unlabeled polypeptides were visualized by staining with Coomassie brilliant blue (20), and [^{35}S]methionine-labeled polypeptides were visualized by fluorography (2). The molecular weight of the nucleocapsid polypeptide was calculated by comparing its relative migration rate with those of reovirus polypeptides. The nucleocapsid polypeptide was quantitated by comparing its binding to Coomassie brilliant blue with that of reovirus polypeptides. The latter served as standards because their molecular weights and relative amounts in reovirions are known (20). Coomassie brilliant blue binding was determined by scanning stained gels (Quickscan, Helena Laboratories), and the areas under the peaks were integrated with a PDP-11 computer.

Antisera. Rabbits were prebled and subsequently injected at six to eight subcutaneous sites with a total of 400 to 600 μg of purified nucleocapsids in PBS mixed with an equal volume of incomplete Freund adjuvant. Animals were boosted intraperitoneally with the same amount of nucleocapsids after 1 month, and they were bled after an additional 10 days. An immunoglobulin G-rich fraction was obtained from heat-inactivated serum (30 min at 56°C) by three successive precipitations with 35% saturated $(NH_4)_2SO_4$ (5). The precipitated material was reconstituted to its original volume with PBS and stored at −70°C.

Preparation of *Staphylococcus* type A immunoadsorbent. The Cowan I strain of *Staphylococcus* type A was grown, fixed in Formalin, and heat inactivated as described by Kessler (6, 7). The bacterium was stored as a 10% (vol/vol) suspension in PBS in 2-ml portions. Before use, *Staphylococcus* type A suspensions were thawed, pelleted at $1,000 \times g$ for 10 min, and suspended for 15 min at 23°C in 2 ml of NET buffer containing 0.02% sodium azide and 0.5% Triton X-100. Cells were washed three times in NET containing 0.02% sodium azide and 0.05% Triton X-100 and resuspended to 10% (vol/vol) in the same buffer. *Staphylococcus* type A thus prepared was stored at 4°C and used within 48 h.

Immune precipitations of viral nucleocapsids. Partially purified nucleocapsids (see above) were prepared from 10^5 [^{35}S]methionine-labeled HEp-2-PI cells. They were resuspended in 100 μl of NET buffer containing 0.05% Triton X-100 and 0.002 M phenylmethyl sulfonyl fluoride and subjected to sonic treatment for 1 min in a Bronsonic-12 water bath. This preparation was preadsorbed for 15 min at 23°C with 25 μl of the 10% suspension of *Staphylococcus* type A, and the *Staphylococcus* type A was pelleted at $12,000 \times g$ for 1 min. The supernatant was removed, and portions of 10 μl were incubated at 23°C for 1 h with 20 μl of dilutions of the nucleocapsid-specific antiserum in NET buffer. Antigen-antibody complexes were precipitated by the addition of 100 μl of *Staphylococcus* type A (15 min at 23°C) followed by centrifugation at $12,000 \times g$ for 1 min. The pellet was washed three times in 1 ml of NET buffer containing 0.05% Triton X-100 and 0.002 M phenylmethyl sulfonyl fluoride. During the course of this work, we found that the addition of cytoplasmic extract from unlabeled, uninfected cells in fourfold excess (compared with labeled extract) minimized nonspecific binding of labeled polypeptides. This extract was added to the reaction mixture after the 1-h incubation with antiserum. The antigen was eluted from the *Staphylococcus* type A as described by Kessler (6) and counted in Triton-toluene (17). The counts associated with the *Staphylococcus* type A pellet were expressed as the percentage of the total trichloroacetic acid-precipitable radioactivity in the reaction mixture. For analysis of the radioactive material in the immune precipitate by SDS-polyacrylamide gel electrophoresis the antigen-antibody-*Staphylococcus* type A complex was boiled for 2 min in 0.05 ml of solubilization buffer. *Staphylococcus* type A was pelleted; the supernatant was applied directly to SDS-polyacrylamide gels; and the polypeptides were visualized by fluorography.

Competitive RIA. The dilution of anti-nucleocapsid serum which precipitated approximately 70% of the total nucleocapsids in 10 μl of the [^{35}S]methionine-labeled HEp-2-PI cell extract was determined experimentally (see above). Portions (10 μl) of this antiserum dilution were incubated for 2 h at 23°C with 20 μl of increasing concentrations of partially purified unlabeled nucleocapsids. Next, 10λ of partially purified nucleocapsids from [^{35}S]methionine-labeled HEp-2-PI cells (see above) were added, and incubation was continued for 1 h. The immune complexes were precipi-

tated as described above. The radioactivity associated with the *Staphylococcus* type A pellet was expressed as the percentage of the trichloroacetic acid-precipitable radioactivity in the reaction mixture.

RESULTS

Purity of nucleocapsids. Yields of purified nucleocapsids were between 0.5 and 1 mg per 10^9 infected Vero cells. Their purity was ascertained by analysis of the constituent polypeptides by SDS-polyacrylamide gel electrophoresis and by electron microscopy (data not shown). A single polypeptide species with a molecular weight of 59,000 was present, and negatively stained preparations showed helical structures that are typical for MV and paramyxoviruses (11, 12). They were 18 mm in diameter and of variable length; the latter may be due to breakage during sample preparation.

Development of RIA for nucleocapsids. Antiserum raised in rabbits against purified nucleocapsids formed precipitin lines with sonically treated, purified nucleocapsids in a double-diffusion immunoprecipitation test, whereas preimmune serum did not (data not shown). We attempted to develop RIA with this antiserum and purified nucleocapsids. This was not successful because nucleocapsids adhere to glass and plastic and they are extremely unstable. Storage at various temperatures in buffer containing SDS, a variety of protease inhibitors, or as lyophilized material resulted in their degradation into trichloroacetic acid-soluble material. Furthermore, attempts to radioiodinate nucleocapsids with chloramine-T, lactoperoxidase, or the Bolton-Hunter reagent were unsuccessful. Therefore, partially purified nucleocapsid preparations from [^{35}S]methionine-labeled cells were used as the source of labeled antigen, and they were prepared immediately before the assay from cells that were kept at −70°C. HEp-2-PI cells were chosen because they contain constant and high levels of nucleocapsids. Small portions of extracts from HEp-2-PI cells were mixed with increasing amounts of nucleocapsid-specific antiserum, and antigen-antibody complexes were precipitated with *Staphylococcus* type A. Figure 1A shows that 2% of total trichloroacetic acid-insoluble radioactivity was precipitated in the absence of antiserum. Addition of nucleocapsid-specific antiserum increased this to 18%. A particulate fraction was also prepared from uninfected [^{35}S]methionine-labeled HEp-2 cells. The addition of nucleocapsid antiserum to this extract and the addition of preimmune serum to HEp-2-PI extract did not increase the radioactivity of the immune precipitate over background.

To develop a competitive RIA, we employed

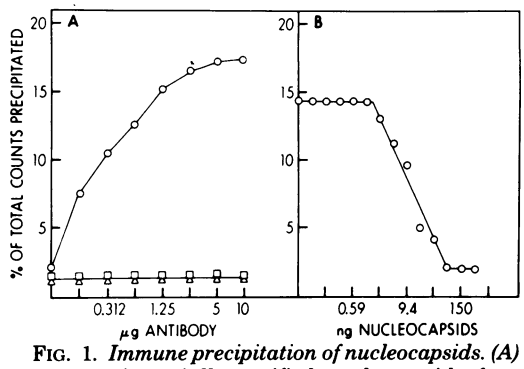

FIG. 1. *Immune precipitation of nucleocapsids. (A) Portions of partially purified nucleocapsids from [^{35}S]methionine-labeled HEp-2-PI or from a particulate fraction of [^{35}S]methionine-labeled uninfected HEp-2 cells were mixed with varying amounts of sera and precipitated with Staphylococcus type A. Symbols: ○, anti-nucleocapsid serum and HEp-2-PI extract; □, anti-nucleocapsid serum and HEp-2 extract; △, preimmune serum and HEp-2-PI extract. (B) Portions of anti-nucleocapsid serum in limiting amounts (1.25 μg/10 μl) were incubated with varying amounts of partially purified, unlabeled nucleocapsids from infected Vero cells. Next, 10-μl samples of partially purified nucleocapsids from [^{35}S]methionine-labeled HEp-2-PI cells were added. Immune complexes were precipitated with Staphylococcus type A.*

a dilution of the nucleocapsid-specific antiserum that precipitated 70% of the partially purified nucleocapsids in the [^{35}S]methionine-labeled HEp-2-PI cells. Portions of the antiserum were incubated for 2 h with various concentrations of unlabeled, partially purified nucleocapsids. (This preparation was sufficiently enriched for nucleocapsids so that they could be visualized by staining of polyacrylamide gels with Coomassie brilliant blue, thus allowing for their quantitation.) Next, partially purified [^{35}S]methionine-labeled nucleocapsids from HEp-2-PI cells were added, the incubation was continued, and antigen-antibody complexes were precipitated. Figure 1B shows that the amount of labeled material in the immune precipitate decreased with increasing amounts of cold nucleocapsids and that a 50% reduction was achieved at approximately 10 ng of nucleocapsids. The addition of particulate material from unlabeled, uninfected HEp-2 cells did not inhibit the reaction between [^{35}S]methionine-labeled nucleocapsids and anti-nucleocapsid serum.

The specificity of these reactions was investigated by analyzing immune precipitates by SDS-polyacrylamide gel electrophoresis. Figure 2A shows the labeled polypeptides in partially purified, [^{35}S]methionine-labeled HEp-2-PI extracts. The nucleocapsid polypeptide species can be observed (see arrow), but many other polypeptides were also present. Anti-nucleocapsid

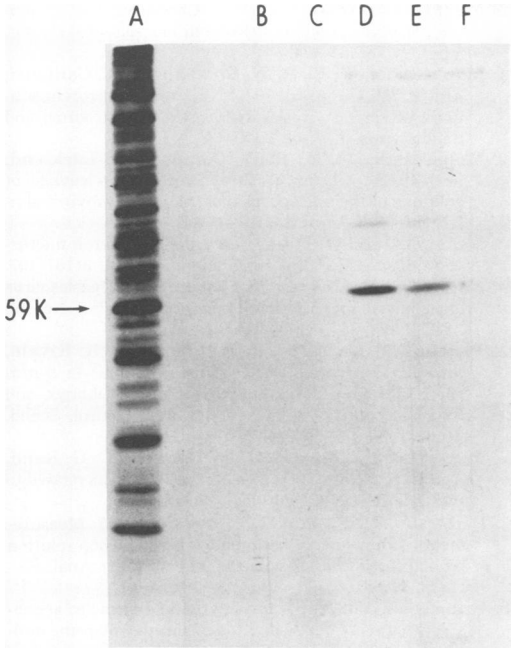

FIG. 2. *SDS-polyacrylamide gel electrophoresis of partially purified nucleocapsids and immune precipitates. Fluorograms of 10% discontinuous Tris-SDS-polyacrylamide gels: (A) 10 μl of a preparation of [^{35}S]methionine-labeled, partially purified nucleocapsids from HEp-2-PI cells. (B through F), immune precipitates of: (B) anti-nucleocapsid serum and the [^{35}S]methionine-labeled particulate fraction from uninfected HEp-2 cells; (C) preimmune serum and [^{35}S]methionine-labeled, partially purified nucleocapsids from HEp-2-PI cells; (D) anti-nucleocapsid serum and [^{35}S]methionine-labeled, partially purified nucleocapsids from HEp-2-PI cells; (E) anti-nucleocapsid serum, 10 ng of partially purified nucleocapsids, and [^{35}S]methionine-labeled, partially purified nucleocapsids from HEp-2-PI cells; (F) anti-nucleocapsid serum, 100 ng of partially purified nucleocapsids, and [^{35}S]methionine-labeled, partially purified nucleocapsids from HEp-2-PI cells.*

serum precipitated the nucleocapsid polypeptide species and two minor polypeptide species (Fig. 2D). Addition of increasing quantities of unlabeled, partially purified nucleocapsids reduced the amount of label associated with the nucleocapsid and one of the minor polypeptide species (Fig. 2E and F). The incubation of preimmune serum with partially purified, labeled HEp-2-PI nucleocapsids resulted in a precipitate without identifiable major polypeptides (Fig. 2C), as did the incubation of anti-nucleocapsid serum with labeled extract prepared from uninfected HEp-2 cells (Fig. 2B).

DISCUSSION

The purpose of this work was to purify sufficient quantities of MV nucleocapsids for the preparation of specific antiserum and to develop a sensitive assay for the detection of MV nucleocapsids in infected cells.

Nucleocapsids were purified from the cytoplasm of infected cells rather than from virions. The yield of virions in tissue culture is very low, and nucleocapsids have been observed to accumulate in the cytoplasm of infected Vero cells (13; unpublished data). Purification employed differential centrifugation and equilibrium centrifugation in Renografin and cesium chloride. We found that equilibrium banding in cesium chloride alone resulted in nucleocapsid preparations with several minor contaminating polypeptide species in addition to the major polypeptide (molecular weight, 59,000). A similar major nucleocapsid polypeptide was observed by Waters and Bussell (21) and Mountcastle and Choppin (10). The purity of the nucleocapsids and the specificity of the antiserum prepared against these nucleocapsids were clearly demonstrated in Fig. 2. Antiserum reacted with material in MV-infected cells and not with material in uninfected cells (Fig. 1A and 2B and D). The immune-precipitated material consisted of a major polypeptide that comigrated in SDS-polyacrylamide gels with the nucleocapsid polypeptide species (Fig. 2D). Two minor polypeptides were also precipitated: one with a molecular weight of 70,000 and the other of very high molecular weight. The first one of the minor species may be the polypeptide that has been observed by Mountcastle and Choppin (10) in association with nucleocapsids. The high-molecular-weight material most likely represents host material that co-precipitated with the antigen-antibody complexes.

We attempted to develop a competitive RIA by radioiodinating purified nucleocapsids. This turned out to be very difficult, partially because of their instability and their tendency to adhere to plastic and glass. Therefore, we developed an assay using partially purified nucleocapsids from cells that were labeled with [^{35}S]methionine in vitro. This assay will detect less than 5 ng of nucleocapsids. The sensitivity can be increased several-fold by raising the amount of [^{35}S]methionine in the medium and by extending the labeling period. The assay will be used to quantitate MV nucleocapsids in lytically and persistently infected cells both in vivo and in vitro. Furthermore, the analysis of clinical material from patients with multiple sclerosis will be an important step in assessing the role of MV virus in the etiology of this disease.

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