Proteins of the Group B Arbovirus Kunjin

E. G. WESTAWAY¹ AND BEVERLEY M. REEDMAN

Queensland Institute of Medical Research, Herston, Brisbane, Australia

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Purified Kunjin virus was disrupted with sodium dodecyl sulfate, urea, and mercaptoethanol or acetic acid. Electrophoresis on 7.5% polyacrylamide gel separated four proteins of high (120,000), intermediate (65,000) and low (18,000 and 13,000) molecular weights. A "core" particle was obtained by degradation of the virion with deoxycholate at 0°C; it contained the viral ribonucleic acid and the two small structural proteins. The "envelope" material released from around the core was identified with the most prominent (intermediate) protein seen in electropherograms of virion proteins. In addition to the structural proteins, at least three additional proteins (specified by virus infection) were found in the cytoplasm. The cytoplasmic proteins associated with Kunjin virus differed in their electrophoretic profile from those associated with infection by the related Murray Valley encephalitis virus.

Characterization of group A arbovirus proteins by polyacrylamide gel electrophoresis indicates that there are only two, possibly three, structural proteins in the virion and from 5 (with Semliki Forest virus) to 14 (with Sindbis virus) proteins induced in the cytoplasm of infected cells (2, 6, 7, 13, 15, 16, 21). Group B arboviruses are smaller and comprise a larger, more complex group of viruses (1, 3). We are interested in making comparisons within group B as part of a continuing study of the antigenic and biochemical characterization of the group.

Examination by polyacrylamide gel electrophoresis of the structural and cytoplasmic proteins specified during infection by the group B arbovirus Kunjin indicate that they differ from those observed by others with group A arboviruses.

MATERIALS AND METHODS

Cells. Cultures of PS cells (a stable line of porcine kidney cells) and of VERO cells (an African green monkey kidney cell line) were grown to confluency as described previously (19, 20). Monolayers of both cell lines contained approximately 2.5×10^6 cells in 60-mm plastic petri dishes and 8×10^6 cells in 100-mm dishes.

Viruses. The sources of the cloned strain of Kunjin virus and of Murray Valley encephalitis (MVE) virus strain MRM66 have been described (4, 19).

Infection of cells. Monolayers were infected with 10 to 30 plaque forming units (PFU) per cell, and 1.5 hr later were fed with Eagle's Minimum Essential

Medium containing 0.2% bovine albumin. During radioisotopic labeling, the content of unlabeled amino acids was reduced to 1/10.

Concentration of infected culture fluids. Fluids were harvested 27 hr or later after infection, and 60% of infectivity was recovered by precipitation with polyethylene glycol (E. G. Westaway and B. M. Reedman, unpublished data). Briefly, fluids were centrifuged at $10,000 \times g$ for 10 min; polyethylene glycol was added to supernatant fluids at a final concentration of 5 to 8%. After 1 hr at 4°C, the precipitated virus was pelleted at $10,000 \times g$ for 30 min, and then was suspended in less than 2 ml of 0.1 M NaCl in 0.012 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.2% bovine albumin.

Sucrose density gradients. Concentrated virus suspensions were applied to gradients of 5 to 25% (w/v) sucrose prepared in the virus suspension buffer for the Spinco SW25.1 rotor. Sedimentation for 3 hr at 4°C at 25,000 rev/min (14) produced a single, sharp peak of infectivity, radioactivity, and viral haemagglutinin with virus grown in PS or in VERO cells. Sedimentation values for subviral particles were estimated by the method of Martin and Ames (12), using 74S ribosomes from PS cells and 19S rabbit antibody to group A arboviruses as markers. Fractions were collected, and the trichloroacetic acid-insoluble radioactivity in each fraction was counted in a liquid scintillation spectrometer.

Protein synthesis in infected cells. PS cell monolayers were treated with 3 µg of actinomycin D/ml in unlabeled medium for 1 to 2 hr at 23 hr or later after initiation of infection; they were drained and re-fed for 5 hr with similar medium that included 1 µg per ml for ¹⁴C-amino acid or ³H-leucine and ³H-tyrosine.

Preparation of cell extracts. Infected and control uninfected cultures, labeled as described above, were washed twice quickly with unlabeled medium and were dissolved by adding 1% sodium dodecyl

¹ Present address: Department of Microbiology, Monash University Medical School, Alfred Hospital, Prahran, Melbourne, Australia.

sulfate (SDS; 16), using 0.6 ml for 60-mm and 1 ml for 100-mm petri dishes.

Polyacrylamide gel electrophoresis. Proteins of the virion and of cells dissolved in SDS were analyzed by electrophoresis by a method based on the procedures of Summers et al. (18) and Strauss et al. (16). Labeled preparations were brought to the following concentrations: 1% SDS, 2% 2-mercaptoethanol, and 0.5 M urea. The mixture was incubated at 37 C for 1 hr in an atmosphere of nitrogen and then dialyzed for 5 hr at room temperature against 0.005 M sodium phosphate buffer (pH 7.2), 0.1% SDS, 0.01% ethylenediaminetetraacetic acid (EDTA), 0.5 M urea, and 10% sucrose. Samples greater than 300 μ liters were reduced in volume immediately before electrophoresis by covering the dialysis tube with Aquacide II (Calbiochem, Los Angeles, Calif.).

Electrophoresis was performed in glass tubes (0.6 by 12 cm) in vertical 10-cm gel columns comprising 7.5% acrylamide, 0.27% *N,N*'-bis-methylene acrylamide, 0.1% SDS, 0.5 M urea, and 0.1 M sodium phosphate, pH 7.2. Gels were photopolymerized under a layer of water, in a perspex water bath at below room temperature. Catalysts in the gel were 0.05% (v/v) *N,N,N*'-tetramethyl ethylene diamine and 0.0005% riboflavin. Buffer in the electrode vessels was 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS and 0.01% EDTA. Procedures for electrophoresis, fractionation of gels, and radioactivity determinations have been described (18). For doubly labeled samples, adjustment of tritium counts for spillover of carbon-14 counts into the tritium window was based on measurements made with the same quenching materials labeled with carbon-14 only.

Molecular weight determination. Mobilities of bovine albumin (monomer and dimer) and of trypsin after electrophoresis in parallel gels were compared to obtain the molecular weights of the virus-induced proteins. Each result quoted is the mean of several determinations.

Reagents. 3 H-L-leucine (1 c/mmole), 3 H-L-tyrosine (43 c/mmole), 14 C-amino acid mixtures (52 mc/milliatom of carbon), and 5- 3 H-uridine (27.6 c/mmole) were purchased from the Radiochemical Centre, Amersham, Bucks., England. Actinomycin D was supplied by Merck, Sharp, and Dohme (Aust) Pty. Ltd., Brisbane. Polyethylene glycol (molecular weight 6,000) was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

RESULTS

Proteins in the virion of Kunjin virus. The polypeptides obtained by reduction of Kunjin virus purified by sedimentation as discussed below for Fig. 2a were separated on 7.5% polyacrylamide gel. Four virus proteins were distinguished (Fig. 1). Treatment with 10% acetic acid (18) instead of mercaptoethanol also yielded the same four protein peaks after electrophoresis.

Identification of a ribonucleic acid (RNA)-protein "core" particle in the virion. Kunjin virus was labeled in RNA and protein during growth in PS cells, concentrated, and then purified by

FIG. 1. Polyacrylamide gel electrophoresis of the structural proteins of Kunjin virus. Purified virus, labeled with 3 H-leucine and 3 H-tyrosine, was treated with 1% SDS, 0.5 M urea and 2% 2-mercaptoethanol at 37 C for 1 hr, dialyzed for 5 hr, and subjected to electrophoresis on a 10-cm-long 7.5% acrylamide gel column. Electrophoresis was for 18 hr at 1.6 v/cm at room temperature. Migration is from left to right.

sedimentation (Fig. 2a). Most of the virus recovered was obtained in fraction 8. From fraction 8, 0.5 ml was diluted to reduce the sucrose concentration to 5%, and then was incubated with 0.2% deoxycholate. The treated material was applied to a 5 to 20% sucrose gradient for rate zonal centrifugation (Fig. 2b). Most of the 3 H-RNA remained associated with some 14 C-protein in a particle with a sedimentation coefficient of 120S, whereas most of the 14 C-protein was released to appear near the top of the gradient in a peak at 10 to 20S. Thus, deoxycholate treatment appears to split the virion producing an RNA-protein "core" particle and protein "envelope" material of much lower *S* value, in similar fashion to that observed with group A arboviruses (13, 16). A small amount of free viral RNA appeared to have been released also, sedimenting slightly faster than the envelope protein.

Characterization by electrophoresis of proteins in viral subparticles. Separate pools were made of fractions in the core peak and in the envelope peak of Fig. 2b. The proteins in the pools were reduced and subjected to electrophoresis in separate gels under identical conditions (Fig. 3). The envelope material was found to contain only the most prominent protein in the virion (compare peak 2, Fig. 1), whereas the core peak appeared to comprise the two smaller proteins found in the virion (compare peaks 3 and 4, Fig. 1). The corresponding peaks in Fig. 1 did not migrate as far because a lower voltage was employed for the Fig. 1 experiment. The molecular

FIG. 2. Sedimentation to purify Kunjin virus and to isolate an RNA-protein "core" particle after deoxycholate treatment of the purified virus. At 24 hr after infection, 12 60-mm petri dish cultures of PS cells were pulsed for 2 hr with 3 μ g of actinomycin/ml and then labeled for 26 hr with 30 μ Ci of 3 H-uridine and 6 μ Ci of 14 C-amino acids per plate in the presence of 0.03 μ g of actinomycin/ml. Virus was concentrated from culture fluids and purified by sedimentation through gradient "a" for 3 hr at 4°C at 25,000 rev/min in an SW 25.1 rotor. A 0.5 ml amount of the purified virus peak (fraction 8) was diluted to 2 ml in 0.2% deoxycholate (final) at 0°C for 5 min, and a 120S core particle was isolated by sedimentation in gradient "b." Arrows indicate the position of a 74S ribosomal marker from PS cells and of 19S rabbit antibody. Sedimentation is from right to left. ○, 3 H-uridine-labeled RNA; ●, 14 C-amino acid-labeled protein.

weights determined by reference to marker proteins were 65,000 for the envelope protein peak 2 and approximately 18,000 and 13,000 for peaks 3 and 4, respectively.

The large but minor protein (molecular weight 120,000) found in the virion (peak 1 in Fig. 1) was not identified in the products of deoxycholate treatment. However, its low concentration, indicated by the low incorporation of radioisotope, renders recognition difficult. If it was released by deoxycholate, it may have sedimented faster than the major envelope protein, and hence would not have been included in the fractions selected for electrophoresis.

Synthesis of virus-specific proteins in PS cells. Kunjin-infected cultures were labeled with 3 H-leucine and 3 H-tyrosine, and the polypeptides of the SDS-dissolved cytoplasm were separated on 7.5% polyacrylamide gel (Fig. 4). In addition to the four proteins noted in the virion (compare Fig. 1), at least an additional three virus-induced proteins were observed (peaks A, B and C in Fig. 4). Their approximate molecular weights are 86,000 (A), 53,000 (B) and 24,000 (C). The same electrophoretic profile was obtained from cells labeled as late as 48 hr after infection, using either 3 H-amino acids as described above or a 14 C-amino acid mixture. Peak B was more clearly separated

on a 20-cm, 5% gel but loss of resolution then occurred for the smaller proteins. A small peak was sometimes evident between peaks A and 2. Some minor peaks migrating more slowly than

FIG. 3. Electrophoresis of viral proteins associated with the nucleoprotein "core" and "envelope" material. Separate pools were made of fractions 10-14 in the 120S core peak and of fractions 28-30 in the major 14 C-protein peak (10 to 20S) of Fig. 2b. Both pools were treated and were subjected to electrophoresis in separate gels under identical conditions as in Fig. 1, but using 2 v/cm for 16 hr. Background counts in the carbon-14 window of the spectrometer were 11 counts/min.

FIG. 4. Electrophoresis of virus-specific proteins induced in PS cells infected with Kunjin virus. Cells were labeled with 3 H-leucine and 3 H-tyrosine at 23 hr after infection. A 100- μ l amount of SDS-dissolved cytoplasm was treated and subjected to electrophoresis as in Fig. 1, using 2 v/cm for 16 hr. Symbols: ●, infected; ○, uninfected cytoplasm.

peak 1 were seen also in uninfected control preparations. In some gels, the magnitude of peak A was much reduced without apparently affecting the relative proportions of the other peaks (Fig. 5). The cause of this variation is not understood.

Comparison of virion proteins with cytoplasmic proteins using double labeling (Fig. 5) showed that peak 1 in cytoplasm was considerably enriched compared to peak 1 in the virion, whereas peaks 3 and 4 were less prominent in the cytoplasm (see later Fig. 6, 7).

Variations in degradation of proteins prior to electrophoresis. Alternative methods of degrading Kunjin proteins were employed to verify the electrophoretic profiles obtained earlier. ^3H -labeled proteins of purified virions and virus-induced cytoplasmic proteins labeled with carbon-14 were co-electrophoresed on 7.5% gels after the following treatment: (i) heating at 100 C for 1 min in 2% SDS and 1% mercaptoethanol followed by dialysis for 5 hr (11); (ii) heating at 80 C for 5 min in 2% SDS, 1% mercaptoethanol, and 0.05 M Na_2CO_3 , pH 10.6, with no subsequent dialysis (5); or (iii) heating at 37 C for 15 min in 1% SDS, 0.01% EDTA, and either 0.2 or 2% dithiothreitol (DTT or Cleland's Reagent) without subsequent dialysis. Except for some variation in the magnitude of peak A, the electrophoretic profiles for both virion and infected cytoplasm remained virtually unchanged.

Virus-specific proteins produced in VERO cells. Protein synthesis in Kunjin-infected VERO cells (Fig. 6) was found to be similar to that obtained

FIG. 5. Comparison by electrophoresis of virus-induced proteins in infected cells with structural proteins of the virion. SDS-treated cytoplasm from infected PS cells with ^3H -protein labeled as in Fig. 4 was mixed with purified Kunjin virus labeled with ^{14}C -amino acids. Subsequent treatment and electrophoresis were as in Fig. 1, using 2 v/cm for 16 hr.

FIG. 6. Electrophoresis of virus-specific proteins induced in VERO cells infected with Kunjin virus. Infected cells were labeled at 24 hr with ^3H -leucine and ^3H -tyrosine, and uninfected cells were labeled with ^{14}C -amino acids. Samples of dissolved cytoplasm were mixed and treated as in Fig. 1, and the proteins were co-electrophoresed for 16 hr at 2 v/cm. Symbols: ●, infected ^{14}C -cytoplasm; ○, uninfected ^3H -cytoplasm.

FIG. 7. Co-electrophoresis of proteins induced by Kunjin virus at 44 hr with proteins induced by MVE virus at 48 hr. Amounts (50 μl) of dissolved cytoplasm from both the infected PS cell cultures were mixed, treated, and electrophoresed as in Fig. 5. Symbols: ●, MVE-infected ^{14}C -cytoplasm; ○, Kunjin-infected ^3H -cytoplasm.

in PS cells (compare Fig. 4), although the presence of peak B was not clearly established.

Proteins induced in PS cells by MVE virus. ^3H -proteins from cells infected with MVE virus were mixed with ^{14}C -proteins induced by Kunjin virus and the reduced proteins were co-electrophoresed (Fig. 7). The profile of proteins associated with MVE virus clearly differs from that observed with Kunjin virus in this and in other gels.

DISCUSSION

It has been shown that although the virions of both group A and group B arboviruses share a common structural feature, namely, the release by

deoxycholate treatment of envelope protein and a nucleoprotein core, the proteins associated with each group clearly differ. The major proteins of the envelope and core of Kunjin virus have molecular weights of 65,000 and 18,000, respectively; the two corresponding proteins of group A arboviruses have molecular weights in the range of 50,000 to 53,000 and 30,000 to 35,000 (7, 15). In addition, Kunjin virus has an additional smaller protein associated with the nucleocapsid and a large protein of molecular weight about 120,000 which may be associated with the envelope. This latter protein appears to be considerably larger than the slowly migrating core "protein 3" of Semliki Forest virus (6); however, both proteins are comparatively enriched in cytoplasm and each occurs as a minor structural component. These comparisons appear to provide evidence that serological differences between the arbovirus groups are associated with structurally different proteins in the virion. Furthermore, protein C in cytoplasmic extracts migrates between the major envelope and core peaks; there is no protein peak in the corresponding region of electropherograms of cytoplasm infected with group A arboviruses (6, 7, 15).

The total of virus-specific proteins produced in cells infected with poliovirus or Sindbis virus exceeds the total expected to be encoded in the respective viral genomes (10, 15), and in such situations early precursor proteins appear to be cleaved during virus synthesis (8, 9, 15, 17). The total of polypeptide chain which could be translated from a group B arbovirus messenger RNA may be in the vicinity of 330,000 daltons, based on the molecular weight estimation of 3.3×10^6 daltons for RNA of (group B) dengue type 2 virus (14). Although the total of Kunjin-specific polypeptides (at least 380,000) could be within the information content of the viral genome, equations are easily formulated whereby all other structural polypeptides could theoretically be derived almost exactly by cleavage from protein 1, which accumulates in cytoplasm, either directly (protein $1 \rightarrow 2 + 3 + 4 + C$) or via intermediates (protein $A \rightarrow 2 + C$; protein $B \rightarrow 3 + 4 + C$).

Because group B arboviruses exhibit extensive serological cross-reactions (3, 19), indicating very similar antigenic (hence protein) structure, the difference in electrophoretic profiles between cytoplasmic proteins associated with viruses so closely related as Kunjin and MVE was unexpected. Analyses of purified virus preparations are required to determine whether recognizable differences occur among structural proteins of different group B arboviruses.

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