The Proteins of Murray Valley Encephalitis Virus

By E. G. WESTAWAY

Department of Microbiology, Monash University Medical School,
Alfred Hospital, Prahran, Melbourne 3181, Australia

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SUMMARY

Proteins specified by Murray Valley encephalitis virus were labelled during virus growth in Vero and in PS cells, and separated by polyacrylamide gel electrophoresis. The purified virus particle contains three proteins (V-1, V-2 and V-3) whereas the slow sedimenting haemagglutinin or virus sub-particle lacks the core protein V-2 but contains NV-2, a non-structural protein. Seven non-structural proteins in addition to V-2 and V-3 were identified in infected cells. Electrophoretic profiles of virus-specified proteins in both cell lines were almost identical after elimination by a double-label technique of the background of continuing host-cell protein synthesis. Glucosamine was incorporated into the envelope protein V-3 and NV-2. From 26 to 46 h post-infection in Vero cells, the proportion and amounts of virus-specified proteins remained constant and they were non-equimolar; incorporation of labelled leucine into V-2 was much greater than incorporation into NV-2, whereas in cells infected with Kunjin (a related flavivirus) this ratio of incorporation was reversed. At 21 to 25 h, the synthesis of V-2 was less prominent but there was an enhanced synthesis of NV-X. Apart from V-1, NV-1, NV-4 and NV-5, all the proteins are larger than the corresponding Kunjin virus proteins and together represent about $400 \times 10^4$ daltons of polypeptide synthesis, which is close to the maximum coding content of the flavivirus genome.

INTRODUCTION

Murray Valley encephalitis (MVE) virus is included in a major subgroup of flaviviruses (Westaway, 1966), formerly group B arboviruses. The virus particle of flaviviruses comprises three structural proteins, the largest or envelope protein being a glycoprotein (Stollar, 1969; Westaway & Reedman, 1969; Shapiro et al. 1971; Trent & Qureshi, 1971). Purified MVE virus contains 11% lipid and 7.8% RNA (Ada, Abbot & Anderson, 1962); after extraction from virus particles the RNA is infectious (Anderson & Ada, 1959) and is similar in mol. wt. ($4.2 \times 10^6$) to that of another flavivirus, Kunjin (Boulton & Westaway, 1972). The diam. of the virus particle measured in thin sections is 40 nm (Filshie & Rehacek, 1968).

At least nine proteins are specified in flavivirus-infected cells (Westaway, 1973). Electrophoretic profiles of infected cytoplasm labelled in amino acids provide poor resolution because host-cell protein synthesis is not ‘switched off’ during flavivirus infection; hence, apart from showing differences from a Kunjin virus-specified profile, we were unable to satisfactorily resolve MVE virus proteins in infected cytoplasm (Westaway & Reedman, 1969). The host cell component of such profiles can now be eliminated by a double label and
subtraction technique (Westaway, 1973), recently termed ‘difference analysis’ (Hightower &
Bratt, 1974).

In this paper the structural and non-structural proteins of MVE virus are defined, and
evidence is adduced indicating some form of translational control during flavivirus infections.

METHODS

Cells. Vero cells and PS cells (Westaway & Reedman, 1969) were grown to confluency in
medium 199 containing 10% foetal calf serum and maintained during experiments in
Eagle’s minimum essential medium containing 0.1% bovine serum albumin.

Viruses. MVE virus (strain MRM66) and Kunjin virus (strain MRM61C) pools were 10% (w/v) suspensions of infected suckling mouse brain (Westaway & Reedman, 1969).

Virus purification. MVE virus and Kunjin virus were labelled with radioactive amino acids
from 16 to 52 h during growth in Vero or PS cells, concentrated from infected cell-culture
fluids by precipitation with polyethylene glycol, and purified by velocity sedimentation
through 5 to 25% (w/v) sucrose gradients, as previously described (Westaway & Reedman,
1969).

Haemagglutination assays. The haemagglutinin content of fractions from virus purification
gradients was assayed by the microtitre method using the buffer systems of Clarke & Casals
(1958). The optimum pH for haemagglutination for both MVE and Kunjin viruses was 6.6.

Preparation of radioactively labelled cytoplasnm. Vero cell or PS cell monolayers in 60 mm
Petri dishes were infected at an input multiplicity of about 30 p.f.u./cell; control cultures were
mock infected. At 3 h prior to commencement of labelling fresh medium was supplied,
containing actinomycin D (a gift of Merck, Sharp and Dohme (Aust.) Pty Ltd, Sydney) at
3 µg/ml, and being deficient in leucine (prior to [4H]- or [14C]-leucine labelling) or in glucose
(prior to [3H]- or [14C]-glucosamine labelling). Labelled compounds (all obtained from the
Radiochemical Centre, Amersham, Bucks.) were incorporated in the medium as follows:
[4,5-3H]-leucine (22 Ci/mmol) 10 µCi/ml, [14C]-l-leucine (331 mCi/mmol) 1 µCi/ml, [6-3H]-
D-glucosamine (12.6 Ci/mmol) 25 µCi/ml and [14C]-D-glucosamine (318 mCi/mmol) 2.5
µCi/ml. Labelled cytoplasm was harvested by washing cell monolayers with saline, and
dissolving the cells in 0.6 ml 1% SDS.

Polyacrylamide gel electrophoresis. The proteins in fractions from virus purification
gradients or in labelled cytoplasm were heated at 45 °C for 30 min in 1% SDS, 1%, dithio-
threitol, 0.01% EDTA and 0.005 M-sodium phosphate, pH 7.2. The heated reduced
samples were applied directly to 8% (w/v) polyacrylamide gels and electrophoresed in SDS-
phosphate buffer as previously described (Westaway, 1973).

RESULTS

Proteins in virus particles and in subviral particles of MVE virus

Two peaks of radioactivity and haemagglutinin were resolved in a velocity sedimentation
gradient of culture fluid concentrates (Fig. 1). The major peak represents the virus particle,
containing nearly all the infectivity; the slower sedimenting peak of haemagglutinin has been
termed SHA and is associated similarly with other flaviviruses (Shapiro et al. 1971; Della-
Porta & Westaway, 1972). In comparisons with purification gradients of Kunjin virus,
similar rates of sedimentation were observed for virus particles (approx. 200S) and for each
SHA (65 to 80S).

Samples from each peak were electrophoresed on 8% SDS-phosphate polyacrylamide
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Fig. 1. Velocity sedimentation of MVE virus. Virus was labelled with [3H]-leucine during growth in Vero cells and concentrated from culture fluids by precipitation with polyethylene glycol. The concentrate in 2.4 ml was layered on a 5 to 25% (w/v) sucrose gradient and sedimented for 3 h at 24,000 rev/min in an SW27 rotor. Direction of sedimentation is represented as from right to left. SHA is the slow sedimenting haemagglutinin. ●—●, radioactivity, ct/min; ○—○, H.A.U.

The virus contains three structural proteins (V-1, V-2 and V-3). SHA also contains three proteins, V-1, V-3 and a non-structural protein NV-2 (see later). Electrophoresis of a top fraction of the purification gradient (fraction 33 of Fig. 1) revealed only the three virus particle proteins, probably arising from degraded virus particles. The absence of NV-2 in the latter material indicates that SHA is stable during the concentration and purification procedures.

Comparisons with other flavivirus proteins

Co-electrophoresis of structural proteins of MVE and Kunjin virus particles yielded similar profiles (Fig. 3a) with peaks of similar magnitude, but V-3 (envelope) and V-2 (innermost core protein) of Kunjin virus migrated more rapidly than the corresponding proteins of MVE virus. Comparisons of proteins specified in Vero cells by both flaviviruses (Fig. 3b) showed clear differences in several respects. Although the MVE-infected cells were labelled very late in infection, the proportion of virus to host-cell protein was obviously much less than the proportion in Kunjin-infected cells. Not only is the slower migration of V-2 and V-3 of MVE virus again evident, but NV-2 in MVE-infected cells also migrates more slowly, by comparison with NV-2 for Kunjin virus. However, the most striking difference is the
Fig. 2. Polyacrylamide gel electrophoresis of proteins of (a) purified MVE virus (fraction 6 in Fig. 1) and of (b) slow sedimenting haemagglutinin (fraction 24 of Fig. 1). The gel origin is at the left of figure. In (b), the proteins were identified by electrophoresis of virus particle proteins and of cytoplasmic proteins in parallel gels.

Fig. 3. Comparison by co-electrophoresis of the proteins of MVE virus (●—●) and of Kunjin virus ○—○. (a) Structural proteins of virus particles labelled with [3H]-leucine (MVE) or with mixed [14C]-amino acids (Kunjin); (b) proteins specified in Vero cells by MVE virus at 42 to 46 h post-infection and by Kunjin virus at 26 to 30 h, labelled with [3H]-leucine or [14C]-leucine respectively. The structural proteins are designated V-1 to V-3 and the non-structural proteins NV-1 to NV-5 (Westaway, 1973); an additional protein NV-X is resolved in subsequent figures. In (b), V-2 of MVE virus was identified by electrophoresis of proteins of the virus particle in parallel gels, and the reference Kunjin proteins are identified with labelled arrows.
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Fig. 4. (a) Co-electrophoresis of [3H]-leucine-labelled MVE virus-infected Vero cells at 42 to 46 h post-infection (●–●) and [14C]-leucine-labelled uninfected cells (○—○). (b) The 'net profile' of MVE virus-specified proteins (●—●) was derived by elimination of host cell proteins as follows: the [14C], ct/min in (a) were multiplied by an appropriate constant factor (here 1.9) and subtracted from the [3H], ct/min. The net or virus-specified proteins represent 31% of total protein synthesis during the labelling period. Note that NV-3, NV-X and NV-2½ were not resolved in the 'gross profile' in (a). For comparison with Fig. 3 the net profile of Kunjin virus-specified proteins (26 to 30 h) is co-plotted (○—○) in (b); labelled arrows designate the Kunjin proteins readily distinguishable from those of MVE virus.

ratio of label incorporated in V-2 and NV-2; for Kunjin virus, incorporation in NV-2 is much greater than that for V-2, whereas for MVE virus the corresponding ratio of incorporation is virtually reversed.

Elimination of host-cell protein from electrophoretic profiles

Co-electrophoresis of extracts of infected (42 to 46 h) and uninfected cells showed the extent of labelling of host-cell protein in MVE-infected Vero cells (Fig. 4a). This large contribution of host-cell protein was eliminated from gel profiles (Fig. 4b and subsequent figures) using the double label and subtraction technique applied previously to other flaviviruses (Westaway, 1973). In the net profiles so derived, virus-specified proteins are clearly defined and additional proteins are resolved. Thus, in Fig. 4b the proteins NV-3, NV-X and NV-2½ (identified previously in Kunjin-infected cells; Westaway, 1973) are now observed, and the ratio of V-2 to NV-2 is enhanced. All these proteins migrate more slowly than the corresponding Kunjin proteins. NV-1 appears more prominent than in Kunjin-infected cells. In most net profiles of MVE-infected cells (see later figures), a small shoulder or peak is evident on the leading edge of V-2; its significance is uncertain.

Note that by comparison with V-3, the incorporation of labelled leucine into V-2 is much greater in cytoplasm than in the virus particle (Fig. 2, 3) indicating that an excess of V-2 is in fact being produced.
Protein synthesis at different periods after infection

Because of the unusual NV-2/V-2 ratio observed for MVE proteins, and the need to confirm the occurrence of the smaller peaks NV-3, NV-X and NV-2½, net profiles were obtained for earlier labelling periods. At 26 to 30 h (Fig. 5b), the features observed at 42 to 46 h were confirmed, the smaller peaks being defined more clearly. At 21 to 25 h (Fig. 5a), synthesis of V-2 was somewhat reduced, but synthesis of NV-X was markedly increased. The net incorporation of label into virus proteins was 21% (21 to 25 h), 32% (26 to 30 h) and 31% (42 to 46 h). As in other flavivirus-infected cells (Westaway, 1973), there is no late 'switch-off' of host-cell protein synthesis.

The varying incorporation of label into individual virus-specified proteins at different periods indicates that some measure of translational control is effective in MVE-infected Vero cells.

Protein synthesis in PS cells

To establish that all protein peaks observed in the net profiles of infected Vero cells were indeed virus-specified and did not include induced host-cell proteins, infected and uninfected PS cells were labelled with radioactive leucine at 26 to 30 h (Fig. 6). The contribution of host-cell protein synthesis was even greater than that in Vero cells; virus proteins represented only 13% of total synthesis. However, the PS cell net profile (Fig. 6b) is almost identical to the Vero cell net profile labelled during the same period (compare Fig. 5b). In three further analyses of this PS cell material, the net profiles were reproducible. At 31 to 34 h, the net profile was similar, but incorporation of label into virus proteins represented 25% of the total incorporation.

Glycoprotein synthesis in infected cells

Proteins V-3, NV-3 and NV-2 were reported to be glycoproteins in cells infected with Japanese encephalitis virus (Shapiro, Kos & Russell, 1973a), which is closely related antigenically to MVE virus (Westaway, 1966). Infected Vero cells were labelled with [3H]-glucosamine at 20 to 26 h; V-3, NV-3 and NV-2 were clearly labelled (Fig. 7a). Another labelled peak in the usual migration position of NV-5 was largely eliminated by
Fig. 6. Electropherograms of MVE virus-specified proteins in PS cells at 26 to 30 h post-infection. 
(a) •--•, [\(^{3}H\)]-leucine-labelled infected cells; O---O, [\(^{14}C\)]-leucine-labelled uninfected cells. (b) 
Net profile of virus proteins obtained from (a) as in Fig. 4, which represents only 13% of total 
protein synthesis.

Fig. 7. Gross (O---O) and net (•--•) profiles of [\(^{3}H\)]-glucosamine-labelled infected cells. (a) 
MVE virus-infected Vero cells at 20 to 26 h post-infection; (b) MVE virus-infected PS cells at 22 to 
34 h. The net profiles were obtained from gross profiles as in Fig. 4, by subtraction of adjusted 
counts of co-electrophoresed [\(^{14}C\)]-glucosamine-labelled uninfected cells (not plotted). Preparation 
(a) only was dialysed against pH 7.2 phosphate buffers (0.1 M for 16 h, 0.005 M for 2 h) prior to 
electrophoresis. Arrows indicate the migration positions of leucine-labelled virus proteins in 
parallel gels.
co-electrophoresis and an appropriate subtraction of counts in [14C]-glucosamine-labelled uninfected cells. The subtraction technique was essential to define V-3, NV-3 and NV-2 as glycoproteins in infected PS cells (Fig. 7b) but a small residue corresponding to NV-5 persisted; its significance is uncertain.

In the net profiles in Fig. 7a a large and broad peak of glucosamine label migrated in the position corresponding to NV-1. It was partially removed by dialysis of samples prior to electrophoresis. Similar observations have been made using Kunjin virus-infected Vero cells, where such material was found associated with cell membranes (R. W. Boulton & E. G. Westaway, unpublished results), and it probably represents, at least in part, an increased synthesis of host cell glycolipid associated with the proliferation of membranes and vacuoles induced during flavivirus infection (Matsumura, Stollar & Schlesinger, 1971). In Japanese encephalitis virus-infected cells, a glucosamine-labelled peak 'not present in every experiment' also co-migrated with NV-1 (Shapiro, Kos & Russell, 1973a).

DISCUSSION

While the structural proteins of MVE are similar to those of other closely related flaviviruses, there is a striking contrast in the electrophoretic profiles of proteins specified in infected cells, especially in the relative migration and ratio of NV-2 to V-2. Although V-1, NV-5, NV-4 and NV-1 of MVE and Kunjin viruses migrate identically, all other proteins specified by MVE virus are 3 x 10⁸ to 6 x 10⁸ daltons larger. These differences are surprising for the following reasons. Kunjin, together with Japanese encephalitis and St Louis encephalitis viruses, is included in the 'MVE subgroup' of flaviviruses (Westaway, 1966) whose members all exhibit strong antigenic cross-reactions, and a great deal of biochemical similarity within the subgroup is expected. For example, the proteins specified by these three viruses show only trivial differences, if any, in electrophoretic migration (Westaway, 1973). The uniqueness of the MVE proteins suggests that during evolution MVE virus has diverged significantly from Kunjin and other members of the MVE subgroup, even though the MVE and Kunjin virus strains were isolated in the same region of Australia.

Several proteins, e.g. NV-3, NV-X, NV-2½, were defined only in subtracted or net profiles; the similarity of such profiles from Vero or PS cells established the virus-specific nature of the nine proteins identified. These could all be related to the nine similar proteins identified in other flavivirus-infected cells (Westaway, 1973). Protein NV-3 has been of questionable significance in previous reports (Westaway & Reedman, 1969; Shapiro et al., 1971; Shapiro et al., 1973a; Westaway, 1973) but it is here clearly defined in several net profiles labelled in leucine or in glucosamine in two cell lines; hence it is a real entity. Similarly, protein NV-2½ trails further (and hence separates better) from NV-2 than in Kunjin-infected cells. The proteins specified by Kunjin virus amount to approx. 368 x 10⁸ daltons (Westaway, 1973); because several corresponding proteins specified by MVE virus are 3 x 10⁹ to 6 x 10⁹ daltons larger, the total of MVE proteins comprises about 400 x 10⁹ daltons, very close to the maximum which could be coded for by a flavivirus genome of 4.2 x 10⁶ daltons (Boulton & Westaway, 1972).

As in other flavivirus infections, the structural protein V-1 is found in SHA but is not found in cytoplasm. Evidence has been advanced by Shapiro, Brandt & Russell (1972) that V-1 is possibly derived by cleavage from NV-2 during virus membrane maturation leaving SHA as a residue. However, the association of NV-2 with SHA has been variable (Stollar, 1969; Shapiro et al. 1971) and this may reflect small differences observed in the rate of sedimentation of different preparations of SHA (unpublished results); the possible role of NV-2 and SHA in maturation of the virus particle remains unresolved.
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The large accumulation of the core protein V-2 during MVE virus replication is unique compared with the results observed in four other flavivirus infections (Westaway, 1973) and is reminiscent of the accumulation of core protein in Sindbis virus-infected Vero cells (Westaway, 1973). This observation and the singular early accumulation of NV-X and its later decline in synthesis in both MVE virus- and Kunjin virus-infected cells (Fig. 5; Westaway, 1973) accentuate the non-equimolar nature of the translation products of flavivirus infections. In Kunjin virus-infected cells all these products except V-2 are stable, and no precursor-product relationships have been detected (Westaway, 1973). Recently Qureshi & Trent (1973) showed that the largest protein NV-5 (their antigen III) in St Louis encephalitis virus-infected cells contained only one antigenic determinant, which was unrelated by immunodiffusion analysis to the envelope protein (their antigen I). Furthermore, Shapiro, Kos & Russell (1973b) have shown a differential inhibition by puromycin on synthesis only of non-glycosylated non-structural proteins of Japanese encephalitis virus, indicating at least two functionally distinct modes of translation of flavivirus mRNA. The virus genome is the only single-stranded species of RNA found in flavivirus-infected cells (Stollar, Schlesinger & Stevens, 1967; Trent, Swensen & Qureshi, 1969). Obviously, the functioning of the flavivirus genome as a polycistronic messenger is more complex than that observed in alphavirus and in picornavirus infections, where post-translational cleavage occurs following translation of the polycistronic messenger (Jacobson, Asso & Baltimore, 1970; Schlesinger & Schlesinger, 1972). Further elucidation requires positive identification of the primary gene products, and definition of the relationships (if any) of the smaller to the larger virus-specified proteins.

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REFERENCES


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