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SYNTHESIS OF VIRUS-SPECIFIC PROTEINS IN TICK-BORNE ENCEPHALITIS VIRUS-INFECTED PIG EMBRYO KIDNEY CELLS

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Summary. — Purified virions of tick-borne encephalitis (TBE) virus contain 3 proteins, V1, V2 and V3, with molecular weights of 8 000, 13 000 and 53 000 daltons, respectively. Seven virus-specific polypeptides were revealed in TBE-virus-infected continuous pig embryo kidney cells, namely p93—96, p79, p69, p53, p47, p16 and p13 (pN designating polypeptide with a mol wt of N × 1 000 daltons). Proteins p93—96, p69, p53, p47 and p13 corresponded by their mol wt to proteins NV5, NV4, V3 and V2 (NV1 1/2) of mosquito-borne flaviviruses. Protein p79, designated NV4 1/2, and protein p16 (the only virus-specific protein inhibited by hypertonic NaCl concentrations), had no analogues among proteins of mosquito-borne flaviviruses. The possibility of a cellular origin of protein p47 (NV3) is discussed.

Key words: *Flavivirus*; *tick-borne encephalitis virus*; *virus-specific proteins*; *elimination of cellular protein synthesis*

Introduction

As concerns protein synthesis in flavivirus-infected cells, only data concerning mosquito-borne flaviviruses have been reported (Shapiro *et al.*, 1971; Trent and Qureshi, 1971; Westaway, 1973). Identification of virus-specific proteins of flaviviruses has been difficult due to continuing cellular protein synthesis and has been based either on the exclusion of proteins identical with cellular proteins by their mobility in gel (Shapiro *et al.*, 1973; Westaway, 1973) or on the selective inhibition of cellular protein synthesis (Shapiro *et al.*, 1971; Westaway *et al.*, 1977).

These methodical approaches made it possible to identify up to 9 virus-specific proteins with molecular weights from 7 to 98 kilodaltons: NV5 — 96—98, NV4 — 69—71, V3 — 51—60, NV3 — 44—47, NVX — 31—32, NV2 1/2 — 21, NV2 — 19, NV1 1/2 — 13.5 and NV1 — 10 (Westaway, 1973; Westaway and Shew, 1977). Antigenic analysis of some of the proteins synthesised in flavivirus-infected cells showed that they possess both group-

specific and type-specific antigenic determinants (Qureshi and Trent, 1973; Eckels *et al.*, 1975).

Studies on flavivirus protein synthesis in vivo and in vitro suggest that translation of the flavivirus genome proceeds from several initiation sites (Westaway, 1977; Svitkin *et al.*, 1978), i.e. in a way basically different from the mode of translation of the majority of mRNAs in eukaryotic cells (Baltimore, 1971).

We carried out our studies on virus-specific proteins of tick-borne encephalitis (TBE) virus since no relevant data concerning tick-borne flaviviruses were available and also with the aim at obtaining information on the translation of the flavivirus genome and on the antigenic structure of virus-specific proteins of flaviviruses.

Materials and Methods

Virus. TBE virus strain Sofin, cloned three times by the plaque method, was used after it had undergone a total of 11 passages in continuous pig embryo kidney (PEK) cell cultures.

Detection of structural and nonstructural TBE virus proteins. TBE virus labelled with ^{14}C-chlorella hydrolysate (Institute of Radioisotopes, Prague) or ^{35}S-methionine (Amersham) was prepared in PEK cell cultures and purified as described (Lyapustin, 1979). Either purified virus or the protein interphase after chloroform-phenol extraction of purified virus were used as source of virion proteins. Protein from the interphase was precipitated by ethanol, washed with acetone and dissolved in electrophoresis buffer (Svitkin and Agol, 1978). To obtain in vivo labelled virus-specific proteins, in several instances we used treatment of PEK cells with cycloheximide (Serva) and hypertonic NaCl concentrations along with incubation of the cells with actinomycin D (Calbiochem). PEK cells infected with TBE virus at a multiplicity of about 1 plaque forming unit per cell were incubated in medium 199 in Earle's solution for 44 hr. Thereafter actinomycin D (5 μg/ml) was added to the medium and after 140 min the medium was changed for the same medium without amino acids but containing actinomycin D and hypertonic NaCl concentration (190 mM excess). After incubating the cells for 20 min at 37° C, ^{14}C-chlorella hydrolysate (20 μCi/ml, specific activity 1000 mCi/g) or ^{35}S-methionine (100 μCi/ml), specific activity 1200 μCi/mmole) was added to the medium. Three hours after addition of the label, the cells were lysed in electrophoresis buffer. In experiments on cycloheximide, the latter was added 30 min after actinomycin D and the cells were incubated for 30 min; thereafter the cells were washed three times with Earle's solution and treated further as described above.

Electrophoresis of the proteins in a 8–20% polyacrylamide gel in the presence of sodium dodecylsulphate, scanning of the gels and molecular weight determinations were carried out as described (Svitkin and Agol, 1978).

Protein contents of the samples were determined according to Lowry *et al.* (1951).

Results

Virion proteins

TBE virus, labelled with ^{14}C-amino acids and purified by sedimentation in a 5–20% sucrose density gradient, contained three proteins, designated V1, V2 and V3 (Fig. 1, gel 1), with mol wts of 8, 13 and 53 kilodaltons. In addition to these major proteins, we found in the preparations up to 5 minor polypeptides, probably representing proteins synthesised in infected cells which were not removed by the purification method employed. Electrophoresis of ^{35}S-methionine-labelled virion proteins revealed only V3 and V2 proteins, indicating that V1 protein is deficient in methionine (Fig. 1, gel 5).

Table 1. The effects of viral infection, treatment with hypertonic NaCl solution (190 mM excess) and cycloheximide (50 μg/ml) on total protein synthesis in PEK cells in the presence of actinomycin D (5 μg/ml)

PEK cells	Count/min per μg protein			
	Hypertonic NaCl solution			
	untreated		treated	
	Cycloheximide		Cycloheximide	
	untreated	treated	untreated	treated
Uninfected	918.9	838.6	98.9	80.5
TBE virus-infected	803.2	938.2	86.3	142.8

Virus-specific protein synthesis in TBE virus-infected PEK cells

Data on the effects of various treatments on protein synthesis in PEK cells are summarized in Table 1. The intensity of protein synthesis was somewhat higher in uninfected cells (Fig. 2, gels 1 and 2). Pretreatment of PEK cells with cycloheximide reduced protein synthesis in uninfected cells, increased it in infected cells and led to a better differentiation of the virus-specific protein bands (Fig. 2, gels 3 and 4). Treatment of the cells with hypertonic NaCl solution nearly completely inhibited cellular protein synthesis and led to a clear-cut resolution of virus-specific proteins (Table 1; Fig. 2, gels 5 and 6). The most clear picture of the virus-specific protein synthesis was obtained by treatment of the cells with hypertonic NaCl solution and cycloheximide (Table 1; Fig. 2, gels 7 and 8). This combined treatment revealed 6 virus-specific proteins: p93—96, p79, p69, p53, p47 and p13. The intensity of the p47 protein band in infected cells treated with hypertonic NaCl solution and cycloheximide was much higher than that in uninfected cells. Thus a virus origin of p47 protein can be assumed. The ratios of radioactivity in virus proteins labelled ^{35}S-methionine and ^{14}C-chlorella hydrolysate (Fig. 1, gels 2 and 4) were different, suggesting methionine deficiency of p69, p53 and p47 proteins. In addition to these proteins, also protein p16 was found in infected cells (Fig. 2, gels 1 and 3); its synthesis was inhibited by hypertonic NaCl solution (Fig. 2, gels 5 and 7).

Treatment of PEK cells with hypertonic NaCl solution not only inhibited the synthesis of cellular proteins, but also substantially reduced the synthesis of virus-specific proteins, as shown by the intensity of the radiolabel in the corresponding bands.

The V3 glycoproteid migrated somewhat more slowly (Fig. 1, gels 1 and 2) than p53 synthesised in infected PEK cells treated with hypertonic NaCl solution. Since glycosylation reduces the mobility of proteins in polyacrylamide gels (Shatkin *et al.*, 1977), it can be assumed that glycosylation of V3 is disturbed by hypertonic NaCl concentrations. The same position of p53 and V3 proteins in Fig. 1 (gels 5 and 6) was probably due to the fact that in this case the proteins reached such concentrations of the polyacrylamide gel at which further movement of proteins was limited.

Discussion

Our data on the number and molecular weights of virus-specific proteins of TBE virus differ somewhat from those of Slávik *et al.* (1973), but in view of the absence of a full report no detailed comparison is possible.

The identified structural proteins of TBE virus had a mobility characteristic of mosquito-borne flaviviruses (Westaway, 1973) as well as of TBE and Langat viruses (Shapiro *et al.*, 1972; Rosato *et al.*, 1974; Heinz and Kunz, 1977; Dzhivanyan *et al.*, 1978). The lower mobility of V1 protein of tick-borne flaviviruses observed by Shapiro *et al.* (1972) can be demonstrated only by simultaneous analysis of mosquito- and tick-borne flavivirus proteins in the same gel.

A comparison of the molecular weights of virus-specific proteins of TBE virus and of proteins synthesised in cells infected with mosquito-borne flaviviruses suggests that high molecular weight TBE virus proteins p93—96, p69 and p53 correspond to NV5, NV4 and V3 proteins of mosquito-borne flaviviruses and p47 protein to NV3 protein of mosquito-borne flaviviruses. It is possible that p47 is a virus-specific protein the identification of which is difficult because of the presence of a cellular polypeptide with a similar mobility. The synthesis of p47 in infected cells was considerably less inhibited by the combined action of hypertonic NaCl solution and cycloheximide (see Fig. 2, gels 7 and 8) than the synthesis of the corresponding polypeptide in uninfected cells. These data suggest a possible viral nature of p47. With mosquito-borne flaviviruses, peptide mapping showed that, by criteria proposed by the authors, the NV3 protein is not virus-specific (Wright *et al.*, 1977). At the same time, NV3 protein is precipitated along with other virus-specific proteins in radioimmunoprecipitation (Shapiro *et al.*, 1971). The nature of NV3 could definitely be established by comparing the peptide maps of p47 protein with those of the protein of the same molecular weight, synthesised in uninfected cells.

In the region of low molecular weight proteins, we found the p13 protein, corresponding to V2 or NV1 1/2 protein of mosquito-borne flaviviruses. For the latter it was shown that NV1 1/2 protein with a higher mobility has the same peptide composition as the V2 virion protein (Westaway and Shew, 1977; Wright and Westaway, 1977). In addition to the proteins mentioned above, virus-specific proteins p79 and p16 were found in TBE-virus infected cells. Protein p79, which could be designated NV4 1/2, does not occur in mosquito-borne flavivirus-infected cells. The synthesis of p16 protein, like that of most cellular proteins, was inhibited in the presence of hypertonic NaCl concentrations and was detected since no protein of the same mobility was found in uninfected cells. No protein analogous to p16 was found in other flaviviruses; the closest proteins NV2 and V2 differ from it by their molecular weights (19 and 13 kilodaltons, respectively).

In TBE virus-infected PEK cells we found no proteins corresponding to NV1, NV2, NV2 1/2 and NVX in mosquito-borne flaviviruses. It is not clear whether this was due to the peculiarities of the virus-cell system used, to the inhibition of NV1, NV2, NV2 1/2 and NVX protein synthesis by

hypertonic NaCl solution or the presence of cellular proteins of a similar mobility. Moreover it is possible that virus-specific proteins of tick-borne flaviviruses differ from those of mosquito-borne flaviviruses not only by a lower mobility of the structural protein V1 (Shapiro *et al.*, 1972) but also by their structure itself.

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Explanation of Figures (Plates XXIV and XXV):

Fig. 1. Structural (gels 1 and 5) and nonstructural (gels 2 and 4) proteins of TBE virus, labelled with ^{14}C-chlorella hydrolysate (gels 1 and 2) or ^{35}S-methionine (gels 4 and 5). Gel 3 — proteins of uninfected PEK cells labelled with ^{35}S-methionine. The proteins were labelled in the presence of hypertonic NaCl concentration (gels 2, 3 and 4) and after additional treatment with cycloheximide (gels 3 and 4). Gels 1 + 2 and 3 + 4 + 5 represent fragments of different slabs.

Fig. 2. Electrophoresis of ^{14}C-labelled proteins synthesised in the presence of actinomycin D (5 μg/ml) in TBE virus-infected (gels 1, 3, 5 and 7) and uninfected (gels 2, 4, 6 and 8) PEK cells. Gels 5–8 — proteins synthesised in cells treated with hypertonic NaCl solution; gels 3, 4, 7 and 8 — proteins synthesised in cells additionally treated with cycloheximide.