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ON THE RELATIONSHIP BETWEEN BLUETONGUE, AFRICAN HORSESICKNESS AND REOVIRUSES: HYBRIDIZATION STUDIES

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ABSTRACT

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The double-stranded ribonucleic acid from bluetongue virus (BTV), African horsesickness virus (AHSV) and reovirus has been tested for hybridization with messenger RNA derived from BTV and reovirus-infected cells. No relationship was found between reovirus and BTV or AHSV, but a small amount of hybridization between BTV and AHSV did occur.

INTRODUCTION

Recent studies have revealed striking similarities in the structure and chemical composition of bluetongue and African horsesickness viruses. Both viruses contain double-stranded ribonucleic acid (ds-RNA) as their genetic material, which can, after isolation, be fractionated into at least ten components of similar but not identical size distribution (Verwoerd, 1969; Oellermann, Els & Erasmus, 1970). Electron microscopic studies showed that negatively stained particles of bluetongue virus (BTV) and African horsesickness virus (AHSV) are indistinguishable. Both possess protein capsids consisting of a single layer of 32 capsomeres arranged in regular icosahedral symmetry to form a particle 55 nm in diameter (Els & Verwoerd, 1969; Oellermann *et al.*, 1970). These similarities obviously indicate a close relationship between BTV and AHSV. This is further borne out by similarities in physical, chemical and biological properties, for example their pH-sensitivity, stability towards organic solvents and transmission by Culicoides species. They are at present mainly distinguished on serological grounds and because of their different host specificities.

In genome structure both viruses closely resemble the reoviruses and a number of other ds-RNA-containing viruses of non-mammalian origin. The possible relationship between these viruses and their classification in a group called Diplornaviruses has been discussed elsewhere (Verwoerd, 1970). The main differences between BTV and AHSV on the one hand and the reoviruses on the other are found in the morphology of the capsids, reovirus particles being 75 nm in diameter and possessing double-layered capsids with 92 capsomeres.

In the experiments reported in this paper, an attempt was made by means of the hybridization technique, to obtain additional information on the mutual relationships between these viruses.

MATERIALS AND METHODS

Virus

Bluetongue virus strains were kindly provided by Dr. P. G. Howell and the African horsesickness virus by Dr. B. J. Erasmus, both of this Institute. Serotypes are indicated numerically and wild type or vaccine strains by the postscript V (virulent) or A (attenuated). Methods for the production of virus in BHK-cell cultures, and for its titration and purification have been described previously (Howell, Verwoerd & Oellermann, 1967; Verwoerd, 1969).

Phenol-extraction

The total RNA-content of infected cells was isolated by means of a slight variation of the method described by Scherrer & Darnell (1962). Cells were ruptured in a Dounce homogenizer after allowing them to swell for 30 minutes in 0.002 M Tris buffer. The nuclei were removed by centrifugation at 2000 *g* for 10 minutes and the pH of the cytoplasmic fraction adjusted to 5.2 by the addition of 10 per cent by volume of a 0.1 M acetate buffer containing 0.01 M EDTA. Sodium dodecyl sulphate (SDS) was added to 1 per cent and the solution deproteinized by two extractions with freshly distilled, water-saturated phenol at 60°C. Excess phenol was removed with ether and the RNA precipitated overnight at -4°C with two volumes of ethanol and collected by centrifugation at 30,000 *g* for 30 minutes. The precipitate was dissolved in a small volume of STE buffer (0.1 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH 6.9).

RNA from purified virus was isolated in the same way, except that the phenol extraction was carried out at pH 6.9 and room temperature.

MAK-chromatography

Columns of methylated-albumin-kieselguhr (MAK) were prepared according to the simplified procedure described by Osawa & Sibatani (1967). Ten grams of Supercel was boiled in 50 ml of 0.1 M NaCl buffered with 0.002 M Tris-HCl to pH 6.7. After cooling off, 2.5 mg of methylated albumin prepared according to Mandell & Hershey (1960) was added with careful stirring, and a column of 15 × 1.8 cm poured. A thin protective layer of 1 g of boiled Supercel was poured on top of the column. Elution of the sample was carried out with 300 ml of a NaCl-Tris-buffer at pH 6.7, using a linear concentration gradient between 0.2 M and 1.2 M.

Isolation of double-stranded RNA

Total RNA was isolated from cells harvested 18 hours after infection with bluetongue virus, by means of the phenol extraction procedure described above. Single-stranded RNA (ss-RNA), including most of

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the cellular contaminants, was removed by precipitation in 1 M LiCl for 20 hours at 4°C, followed by centrifugation at 30,000 *g* for 5 minutes. The supernatant, containing the double-stranded RNA, transfer RNA (tRNA) and sometimes small amounts of deoxyribonucleic acid (DNA), was dialysed against STE for 1 to 2 hours and finally fractionated on a MAK-column as described above. Double-stranded RNA eluted at 0.69-0.70 M NaCl. The relevant fractions were combined and the RNA precipitated with two volumes of ethanol at -20°C overnight. The precipitate was dissolved in 0.3 M STE and stored at -20°C until used.

Isolation of ^{14}C-labelled single-stranded virus-specific RNA

Virus-specific messenger RNA was obtained by first isolating polyribosomes from the cytoplasm of BTV-infected cells and then isolating the RNA from the polyribosomes (Prevec, Watanabe, Gauntt & Graham, 1968). Monolayers of cells were inoculated with BT-virus at the usual input multiplicity of 20 in the presence of 0.1 μg/ml actinomycin D. Two hours after inoculation the cells were removed from the glass surface by trypsinization and suspended at a concentration of 2×10^6 cells/ml in growth medium containing 0.5 μg actinomycin D/ml and ^{14}C-uridine at a concentration of 0.2 μCi/ml. Ten hours after infection actidione was added to a concentration of 10 μg/ml and 30 minutes later the cells were harvested by means of centrifugation and stored at -20°C.

For the isolation of polyribosomes the cells were thawed in STM buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.015 M $MgCl_2$, pH 7.4). After 15 min at 4°C Brij-58 was added to 0.5 per cent, the cells ruptured in a Dounce homogenizer and the nuclei removed by centrifugation at 2,000 *g* for 10 minutes. After adding deoxycholate (DOC) to 0.5 per cent, the supernatant was layered over 15 to 30 per cent sucrose gradients in STM buffer and centrifuged for 110 min at 24,000 rpm and 4°C. Gradients were fractionated in an Isco fractionator, recording the absorbance at 260 nm. The fractions following the sharp RNA peak and representing the polyribosomes were combined and the polyribosomes sedimented by centrifugation at 20,000 rpm overnight. The pellet was resuspended in STE and deproteinized by phenol extraction at room temperature. After precipitation with two volumes of ethanol, the RNA was finally dissolved in a small volume of STE buffer.

Thermal denaturation

Double-strandedness in ds-RNA samples was confirmed by following the hyperchromicity at 260 nm of a solution in SCC (0.15 M NaCl, 0.015 M Na-citrate) on heating in the heating cell assembly of a DK-2A spectrophotometer.

Denaturation of double-stranded RNA

For the denaturation of ds-RNA at a low temperature the method introduced by Katz & Penman (1966) was followed. To each sample, dissolved in 0.3 M STE, 10 volumes of dimethylsulphoxide (DMSO) were added and denaturation carried out at 37°C for 30 minutes. Denatured RNA was precipitated with five volumes of ethanol for 18 hours, collected by centrifuging for 30 minutes at 20,000 rpm and again dissolved in 0.3 M STE. Equally good results were obtained in hybridization experiments when denaturation was carried out by heating the mixture for 3 min at 105°C before transferring to 72.5°C for hybridization.

Hybridization

The methods used for hybridization are essentially the same as those described by Watanabe & Graham (1967) for reovirus. Single-stranded messenger-RNA (mRNA) and denatured ds-RNA, both dissolved in 0.3 M STE, were mixed in tightly stoppered glass tubes in proportions indicated in the different experiments. They were then immersed in a waterbath at 72.5°C for the various intervals indicated, after which they were removed and cooled to room temperature. STE buffer was added to bring the volume to 1 ml. Pancreatic ribonuclease (RNase) was then added to a final concentration of 4μg/ml and the samples incubated at 37°C for 30 minutes. RNase digestion was stopped by cooling to 4°C and precipitating the undigested RNA with 5 per cent trichloroacetic acid (TCA), adding 0.1 ml of a 2 per cent yeast RNA as carrier. After standing for at least 1 hour at 4°C, the precipitate was collected by filtration on Millipore filters. After washing the filters with 5 per cent TCA and 5 per cent acetic acid they were dried, immersed in 5 ml of a toluene scintillator solution (1 g PPO + 0.3 mg dimethyl-POPOP in 1 l of toluene) and the radio-activity determined in a Packard Tricarb scintillation counter. The RNase-resistant acid-precipitable counts represent the fraction of the labelled single-stranded RNA hybridized with the unlabelled viral ds-RNA.

Results

Isolation of double-stranded RNA by MAK-column chromatography

Provided that all ribosomal RNA has been selectively removed from the sample by salt-precipitation and that little DNA is present, an excellent separation of double-stranded RNA from contaminating nucleotides, tRNA and DNA can be achieved. In Fig. 1 a typical elution pattern can be seen. Three main peaks were eluted. A sharp band moving with the front contained non-precipitable radio-activity in the case of labelled material and probably represents free nucleotides and RNA-fragments. The second peak, eluting at about 0.36 to 0.40 M NaCl, is single-stranded according to its RNase-sensitivity and is presumed to be tRNA. Double-stranded RNA elutes at 0.69 to 0.70 M in a characteristic triple peak. Separation of reovirus RNA into three fractions on a MAK-column has been reported (Watanabe & Graham, 1967). Occasionally a small RNA peak of unknown identity was found at 0.5 M. This material did not hybridize with homologous single-stranded RNA and was therefore not further investigated.

Thermal denaturation

In order to confirm the double-strandedness of the ds-RNA preparations used for hybridization, melting curves were plotted for the three main samples. These are shown in Fig. 2. Characteristic profiles, almost indistinguishable from one another, were obtained, with Tm values in SSC of 96°C. The absence

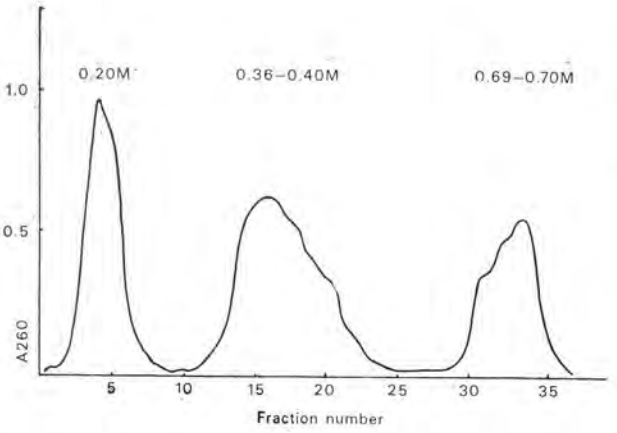

FIG. 1.—Isolation of double-stranded BTV–RNA by chromatography on a MAK-column. The molarities where the peaks are eluted are shown numerically.

of hyperchromicity at lower temperatures and a total increase in absorbance of 25 to 30 per cent are indications of the high purity of the preparations concerned.

Effect of ds-RNA concentration

Preliminary experiments indicated that the effectiveness of hybridization diminishes rapidly below a certain RNA concentration. To determine the optimal relative concentrations of the two RNA components, a series of hybridizations was carried out using a constant amount of single-stranded mRNA from BTV-10V-infected cells, and varying amounts of denatured homologous ds-RNA from BTV-10V. Experimental conditions are given in the legend to Fig. 3 in which the results are summarized.

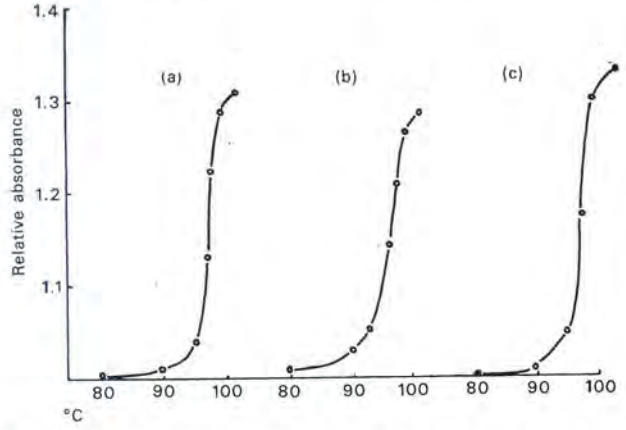

FIG. 2.—Thermal denaturation curves of the double-stranded RNA isolated from (*a*) BTV, (*b*) AHSV and (*c*) reovirus.

Rate of renaturation

In order to determine the minimal time of incubation necessary for maximum hybridization, a series of identical hybridization mixtures was set up and incubated at 72.5°C for various time intervals. The results, summarized in Fig. 4, indicated maximal hybridization after incubation for 1 hour and even a slight decline after longer periods, as judged by the amount of RNase-resistant, acid-precipitable counts obtained.

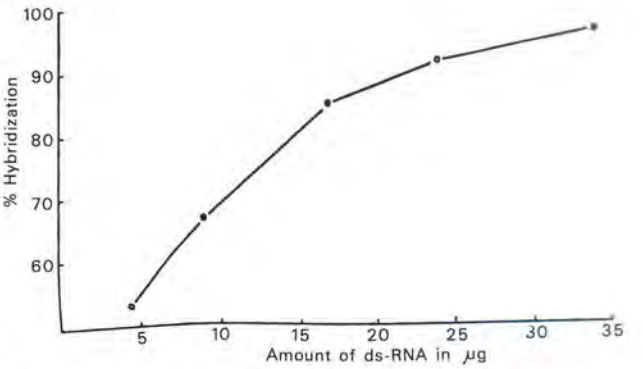

FIG. 3.—The influence of double-stranded RNA concentration on the amount of hybridization (indicated as RNase-resistant acid-precipitable counts) found between BTV-dsRNA and homologous single-stranded mRNA.

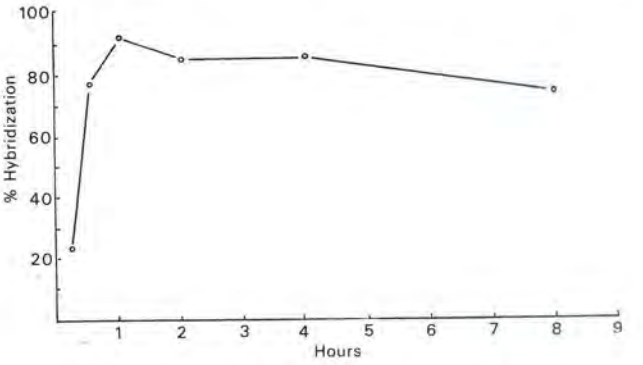

FIG. 4.—The rate of renaturation of BTV–dsRNA and homologous single-stranded mRNA at 72.5°C. The amount of renaturation is shown as RNase–resistant acid-precipitable counts.

Hybridization

^{14}C-labelled ss-RNA samples prepared from BTV-10V and reovirus type 1-infected cells were used for cross-hybridization with denatured ds-RNA from BTV-10V, reovirus 1, AHSV-3V, BTV-10A and BTV-4V. Ribosomal RNA isolated from BHK cells was included as a negative control. A single-stranded RNA sample, in which both ds-RNA addition and RNase treatment were omitted, served as a positive control. A similar one treated with RNase was used as a check on the enzyme activity. Standard conditions used for hybridization were incubation for 1 hour at 72.5°C. The hybridization mixture consisted of 0.05 ml ss-RNA containing 500 cpm, and 50 μg of ds-RNA, both in 0.3 M STE. RNase-treatment, precipitation with TCA and determination of radioactivity were done as described above.

No difference was found between the virulent (V) and attenuated (A) strains of BTV serotype 10. Considerable hybridization (64 per cent) between the different serotypes could be demonstrated, but very little homology exists between BTV and AHSV. No relationship at all could be demonstrated between

TABLE 1. — *Hybridization of BTV, AHSV and Reovirus*

ds-RNA	BTV-10V ss-RNA	Reovirus 1 ss-RNA
BTV-10V	100[a]	0
BTV-10A	100	0
BTV-4V	64	0
AHSV-3V	4	0
Reovirus 1	1	100
BHK-control	0	0

a. These figures represent the percentage of the radioactivity in the positive control recovered from each sample and are averaged from the data obtained in three separate experiments

reovirus and any of the other viruses. Complete renaturation of the denatured reovirus RNA with its own messenger RNA, however, proved its ability to hybridize with a suitable ss-RNA.

DISCUSSION

The ultimate criterion for the demonstration of relationships between organisms would be a direct comparison of the base sequences of their nucleic acids, representing as it does the encoded genetic information. As this is not yet technically feasible, however, one has to resort to methods reflecting differences or similarities in base sequences. Such a method is the hybridization technique.

Hybridization in this sense depends on the characteristic ability of double-stranded nucleic acid molecules to denature, i.e. separate into component strands on heating, and of the denatured strands to renature with homologous strands under suitable conditions. Renaturation of strands from different organisms is termed hybridization, and the degree of hybridization is regarded as a direct indication of homology, i.e. of relationship.

Hybridization has been extensively used in studies on deoxyribonucleic acid (DNA), either to investigate mutual relationships between DNA-containing organisms or to demonstrate the homology between messenger RNA and a part of the DNA genome from which it is derived. It has not yet found wide application in virology, least of all in the case of the RNA viruses, most of which contain single-stranded RNA. Only since the discovery of the formation of a double-stranded replicative form of the viral RNA in the infected cells, has it indeed become feasible to conduct hybridization studies with these viruses. A recent application has been the investigation of homologies between different poliovirus serotypes and strains (Young, Hoyer & Martin, 1968). These authors found 73 per cent hybridization between the Brunhilde and Maloney strains of Type 1 poliovirus and 25 to 34 per cent between Types 1, 2 and 3.

The double-stranded RNA-containing viruses are eminently suitable for hybridization studies for two reasons. In the first place, double-stranded viral RNA can easily be obtained in a pure form from purified virus. Secondly, a single-stranded copy of one of these strands, presumably with a messenger function, is produced in the infected cell in large excess. This mRNA does not renature with itself (Shatkin & Rada, 1967). It is therefore possible to isolate single-stranded RNA, specific for one virus and labelled with radio-isotope, and to test it for hybridization with unlabelled double-stranded RNA derived from another.

The fact that the unusual ds-RNA genomes of BTV, AHSV and reovirus are almost indistinguishable as far as their physico-chemical characteristics are concerned, naturally led to considerable speculation on the degree of relationship between them. It was even considered possible that they could represent one virus enclosed in different capsids. The methods used to study the nucleic acids, however, reflected only the basic architecture of the genome. Obviously a method of comparison that would reflect the finer detail of base sequences would be of greater value in the determination of possible relationships.

The results obtained with the hybridization technique and reported in this paper, provide information of this nature. It indicates that the similarities in genome structure are indeed superficial in nature, and that little homology exists between the genomes of the different viruses. No relationship at all was found between reovirus and either BTV or AHSV. A small and rather variable amount of hybridization between BTV and AHSV indicates the possibility of some degree of relationship, but much less than that existing between two serotypes of bluetongue virus.

SUMMARY

Hybridization techniques have been used to investigate the relationship between the double-stranded RNA-containing bluetongue, African horsesickness and reoviruses. Under experimental conditions where complete auto-hybridization of both bluetongue and reoviruses were obtained, no hybridization between reovirus and the others was found. Only a slight and rather variable amount of hybridization between bluetongue and African horsesickness viruses could be found, compared to the extensive hybridization between two bluetongue virus serotypes.

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