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A Comparative Analysis of Measles Virus RNA by Oligonucleotide Fingerprinting

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With 3 Figures

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Summary

Isolates from two cases of acute measles, one case of acute measles encephalitis and three patients with subacute sclerosing panencephalitis were compared. This comparison was based upon the electrophoretic analysis of T_1 oligonucleotides from single-stranded, full-length RNA isolated from cytoplasmic nucleocapsids. Although all viruses have oligonucleotides in common, each isolate generated a unique pattern of oligonucleotides. However, no group of oligonucleotides was observed which would allow a differentiation between viruses isolated from acute infections and those isolated from CNS diseases; indicating that probably all measles viruses differ in their nucleotide sequence, regardless of origin.

Introduction

Measles virus is an ubiquitous and highly contagious agent which infects man and also primates in close contact with man. The disease usually occurs during childhood, and normally causes a relatively mild stereotyped infection. In a few cases, a more serious complication can arise which may include acute encephalitis. In addition to this disorder another CNS disease (subacute sclerosing panencephalitis — SSPE) has been associated with measles virus infection (1—3). This association has led to many studies on the possible biochemical and antigenic differences between measles and SSPE viruses. Several reports demonstrate differences in electrophoretic mobility between the proteins of measles and SSPE viruses, especially between the P and M proteins (4—6) however the variation found between SSPE and measles isolates was of the same order as that found among the measles isolates themselves (5). Furthermore, little or no difference has been found in the antigenicity of these viruses when assayed by neutrali-

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zation, haemolysin inhibition, haemagglutination inhibition (7). Moreover, all major virus-specific polypeptides from either measles of SSPE viruses can be precipitated equally well by either homologous or heterologous rabbit sera (8). However, by using short-term immunization schedules with purified protein, antigenic differences were reported between the M protein of one SSPE isolate and one measles isolate (9). In addition, recent studies using monoclonal antibodies against measles virus haemagglutinin have demonstrated antigenic differences between several measles and SSPE isolates (10), but no characteristics, specific for SSPE isolates were observed. In addition attempts have been made to analyse the sequence homology between the RNAs of measles and SSPE viruses. YEH (11) and HALL and TER MEULEN (12) reported only 60 and 90 per cent homology respectively between RNAs from measles and SSPE isolates.

As the methods employed in these previous studies could only detect gross differences in virus-specific products, or examined one protein or a small part thereof, we have chosen in this study to analyse the oligonucleotides from a T_1 digest of the complete genome. Not only is this technique capable of detecting differences in RNA sequence throughout the genome, but is also capable of detecting differences as small as one base change. In this report, a comparative analysis between isolates from acute measles cases, a case of acute measles encephalitis and several cases of SSPE is described.

Materials and Methods

Materials

The fusion inhibitor SV 4814 was obtained from Bachem Inc. Torrance, California, U.S.A., and Actinomycin D (AMD) from SERVA, Heidelberg, F.R.G. ^{32}p orthophosphate (40 mCi/ml), Adenosine 5^{1}-^{32}p triphosphate (2000 Ci/mmol) and 5 ^{3}H Uridine (25—30 Ci/mmol) were obtained from Amersham-Buchler, Braunschweig, F.R.G. Neutral cellulose powder (Cellex N-1) was purchased from Bio-Rad and further purified by the method of SHIH and MARTIN (13). Bacterial alkaline phosphatase (BAP) was purchased from Worthington, polynucleotide kinase (from T_4 XF-1 infected E. Coli B) from P. L. Biochemicals, and T_1 ribonuclease and pancreatic ribonuclease A from Sigma. Xylene-cyanol FF(CF) and Bromophenol blue (BPB) was obtained from Chroma and Trypan red was a gift from Dr. J. J. Skehel (NIMR, Mill Hill, London).

All isolates of measles and SSPE viruses were grown on Vero cell monolayers as described (8). As the LEC isolate of SSPE virus caused extensive cell fusion which resulted in premature cell death, 10 μg/ml fusion inhibitor was added to the growth medium after the virus had been allowed to adsorb to the cell sheet. The Hallé isolate of SSPE virus was kindly given by Dr. F. B. Wild, the Mantooth isolate by Dr. Horta-Barbose, and the Braxator isolate was isolated from a case of acute measles encephalitis (14). The "CM" and "Joy" isolates from acute cases of measles were kindly given by Dr. B. Fields, Harvard Medical School, U.S.A.

The virus stocks used in these experiments had been passaged as follows. The Braxator and Mantooth isolates had been passaged 20 times in Vero cells, the LEC isolate 15 times in Vero cells and the Hallé isolate 25 times. The CM and Joy isolates were isolated in HeLa cells, passaged twice in CV_1 cells and 10 times in Vero cells.

Labelling of Cells with Radioactive Precursors

Cell monolayers were labelled with ^{32}p orthophosphate as follows. Cells (4×10^7) were infected at a m.o.i. of 1 until 20 per cent of the sheet was incorporated into syncytia (15—21 hours post infection depending on the viral isolate). The cells were then in-

cubated in phosphate-free MEM containing 1 μg/ml AMD and 50 mM HEPES pH 7.4) for 1 hour and then in 20 ml of fresh phosphate-free medium containing AMD, HEPES and 1 mCi/ml of ^{32}P-orthophate for a further 3 hours. The medium was then decanted, cells washed once in saline and pelleted by centrifugation for 5 minutes at $1000 \times g$ and 4° C.

Preparation of Nucleocapsids

The cell pellet was homogenized with a Dounce homogeniser in 20 times its volume of RSB (10 mM NaCl, 10 mM Tris pH 7.4, 1.5 mM $MgCl_2$), made 0.5 per cent with respect to NP40 and clarifed by two cycles of centrifugation at $1000 \times g$ and 4° C. The supernatent fluid was then layered onto a step gradient containing 15 and 65 per cent sucrose in TKM (150 mM KCl, 10 mM $MgCl_2$ 0.5 per cent NP40 and 10 mM Tris: pH 7.4) and centrifuged for 16 hours at $150{,}000 \times g$ and 4° C. A discreet band was clearly visible at the junction of the 15 and 65 per cent sucrose layers. This band was not visible when similar extracts were made from uninfected cells. The material at the interface of the two sucrose solution wass diluted 5× in RSB plus 0.5 per cent NP40 and centrifuged on a similar gradient at $150{,}000 \times g$ for 16 hours at 4° C. The material at the interface of the second gradient was diluted 10× in RSB plus 0.5 per cent NP40 and pelleted by centrifugation for 4 hours at $200{,}000 \times g$ and 4° C. The pellet was vigorously resuspended in extraction buffer (100 mM NaCl, 50 mM Na acetate pH 4.6, 2.5 mM EDTA) and made 1 per cent with respect to SDS. 100 μg of gradient purified 4S RNA from uninfected Vero cells was added as carrier and RNA extracted as described (15). Nucleocapsid RNA prepared by this method was assumed to be uncontaminated by messenger RNA as it did not bind to oligo dT and was unable to stimulate protein synthesis in a cell-free protein synthesizing system from rabbit reticulocytes (data not shown).

Preparation of Messenger RNA

Messenger RNA from virus-infected and mock-infected cells was extracted by phenol/SDS and oligo dT chromatography as described (15).

Purification of RNA from Intracellular Nucleocapsids by Sucrose Density Gradient Centrifugation

The final ethanolic precipitate from the RNA extraction procedure was dried, resuspended in gardient buffer (100 mM LiCl, 2.5 mM EDTA, 0.1 per cent [w/v] SDS, 10 mM Tris pH 7.4), and centrifuged for 5 hours at $200{,}000 \times g$ and 18° C on 15—30 per cent linear sucrose gradients.

Analysis of Nucleocapsid RNA Under Denaturing Conditions

RNA was pooled, precipitated with ethanol as above and the dried pellet redissolved in 30 μl of TE buffer (10 mM Tris pH 7.4, 1 mM EDTA). The RNA solution was then mixed with 100 μl Dimethyl Formamide, 50 μl Dimethyl sulphoxide, 10 μl 10 per cent (w/v) SDS and 10 μl 0.2 M EDTA. This mixture was incubated at 37° C for 5 minutes and run for 10 hours at $200{,}000 \times g$ on 15—30 per cent linear sucrose gradients containing 50 per cent Formamide.

Preparation of Single Stranded RNA by Differential Ethanol Precipitation

This was acheived by allowing the RNA to self-anneal and then separating free single strands by a modification of the method of Franklin (16). A column of purified cellulose powder (0.2 ml) was prepared in a 2 ml disposable syringe and was washed thorougly with a solution containing 35 per cent absolute ethanol and 65 per cent STE (0.15 M NaCl, 0.001 M EDTA, 0.05 M Tris/HCl pH 7.4). RNA (100 μg/ml) was hybridized in 100 μl of HB buffer (1 M NaCl, 0.023 M Tris/HCL pH 7.0,0.0015 M EDTA) by incubating at 65° C for 2 hours. The incubation mixture was then diluted with 90 μl of DB buffer (1 mM EDTA, 50 mM Tris, HCl pH 7.0)540 μl of absolute ethanol added and the mixture cooled rapidly on ice. The solution containing the RNA precipitate

was passed through the cellulose column and washed with 5 ml of a solution containing 35 per cent absolute ethanol/65 per cent STE. The column was then washed with 5 ml of a solution containing 15 per cent absolute ethanol/85 per cent STE. These fractions were pooled, precipitated at —20° with a further 3 volumes of absolute ethanol and designated "single stranded RNA". The column was finally washed with 5 ml of STE, precipitated with absolute ethanol as above and designated "double stranded" RNA.

Digestion of RNA with T_1 Nuclease and in vitro *Labelling with Adenosine 5¹- [γ- ³²p] Triphosate*

Single stranded 48S RNA from intracellular nucleocapsids was lyophilysed and dissolved in 50 µl of buffer (20 mM Tris/HCl pH 7.8). Then 2.5 µl of T_1 RNase (3.3×10^4 units/ml) and 1 µl BAP (4.5 units/ml) was added and the solution incubated for 30 minutes at 37° C. After the addition of 150 µl of water, the digest was extracted three times with an equal volume of phenol and three times with an equal volume of diethyl ether. The final aqueous phase was then lyophilysed. The dried RNA was dissolved in 70 µl of a 1 mM solution of spermidine heated, for 3 minutes at 50° C and chilled rapidly on ice. The RNA solution was incubated for 30 minutes at 37° C after the addition of 10µl of 10× kinase buffer (0.5 M Tris/HCl pH 9.5, 100 mM $MgCl_2$ 50 mM Dithiothreitol), 10 µl of [$^{-32}$p] ATP (ATP was lyophilysed and redissolved in the original volume of water immediately before use), and 10 µl of polynucleotide kinase (1 unit/µl). The reaction was stopped by the addition of 100 µl of Ammonium acetate (4 M) 20 µl SDS (10 per cent w/v) and 10 µl EDTA (0.2 M). This mixture was extracted three times with an equal volume of phenol, three times with an equal volume of ether and precipitated at —20° with 2.5 volumes of absolute alcohol after the addition of 10 µg of 4S carrier RNA.

The RNA precipitate was dried and analysed by two dimensional gel electrophoresis as described (17). Gels were dried under vacuum and exposed to X-ray film.

Results

Sedimentation Analysis of Nucleocapsid RNA

As measles virus grows poorly in tissue culture and the virion has a pleomorphic structure, the isolation of RNA from purified virus is difficult. Subsequently the yield of purified virion RNA is insufficient for adequate analysis. Therefore it was decided to use intracellular RNA from cytoplasmic nucleocapsids for this study. As RNA from such a source is known to consist of a heterogenous population (18, 19) with both positive and negative strands of various sizes (STEPHENSON and TER MEULEN, unpublished data), further purification was necessary to ensure that only full-length negative stranded 48S RNA was used in the final oligonucleotide analysis.

Initially, nucleocapsid RNA was analysed by centrifugation on aqueous sucrose gradients as described in methods. In Fig. 1a, a typical sedimentation profile is shown for nucleocapsid RNA labelled with ^{32}P *in vivo*. The majority of RNA in this sample sediments as a heterogeneously population of molecules below 30S, with only a small proportion sedimenting as an apparently homogeneous peak at about 48S. The relative proportions of 48S and heterogeneous RNA varied from isolate to isolate, but the general pattern shown in Fig. 1a was always observed. No RNA species larger than 48S were observed in nucleocapsids from infected cells. The 48S RNA was pooled, as shown in Fig. 1a precipitated with 50 µg of 4S carrier RNA and 3 volumes of absolute ethanol, and analysed on a second sucrose gradient. This sample sedimented as an apparently homogeneous species

at 48S (Fig. 1b). The sedimentation coefficient of this RNA species was indistinguishable whatever measles isolate was examined. When the 48S RNA was further analysed under denaturing conditons (Fig. 1c), heterogeneous low molecular weight RNA, with a sedimentation profile similar to that of the subgenomic nucleocapsid RNA seen in Fig. 1a, is observed. As similar RNA was not apparent of the previous aqueous gradient (Fig. 1b) it was assumed that this RNA represented subgenomic positive stranded RNA which had hybridized to full length negative stranded RNA during the extraction and purification scheme. This subgenomic RNA was present in all preparations but varied in amount relative to the full-length 48S RNA.

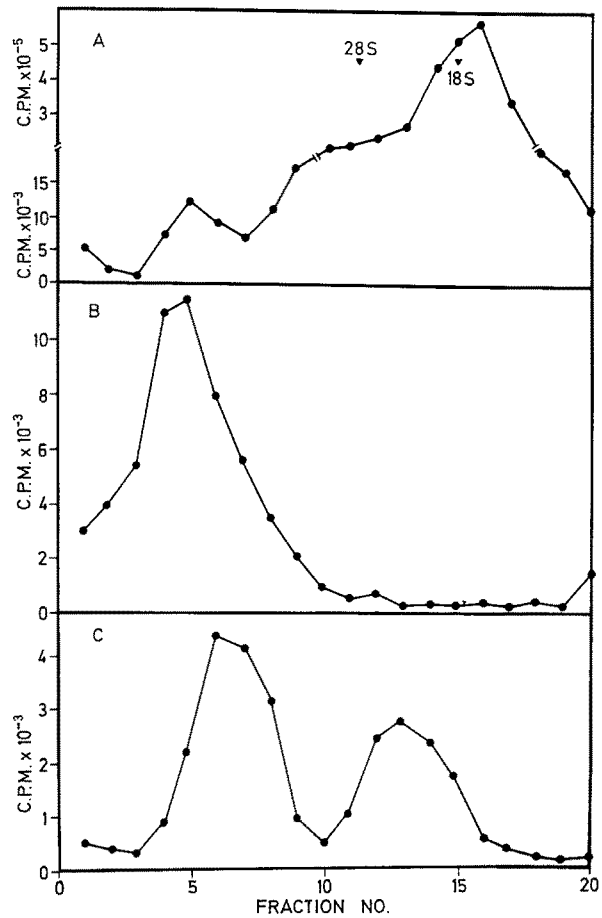

Fig. 1. Sedimentation analysis of ^{32}P-labelled RNA prepared from intracellular nucleocapsids. Sedimentation is from right to left. The position of Vero cell ribosomal RNA is shown by the arrowheads in panel A). *A* Total RNA from intracellular nucleocapsids, analysed on aqueous gradients, *B* RNA from fractions 4—6 (Panel A) analysed on a second aqueous sucrose gradient, *C* RNA from fractions 3—6 (Panel B), analysed under denaturing conditions

Purification of Single-Stranded RNA

As it was apparent that the 48S RNA species contained positive sense RNA (see previous section), and that other negative viruses, make full length positive sense strands (20) it was necessary to purify single strands of one sense only, before analysis of their T_1 oligonucleotides could be performed. If ^{32}P-labelled 48S RNA from two sequential RNA gradients is allowed to self-hybridize, and the various types of RNA separated by differential RNA precipitation, approximately 70 per cent of the radioactivity appears in the "single stranded" fraction and about 30 per cent in the "double stranded" fraction, with less than 2 per cent in the fraction containing small RNA species. This distribution of RNA species did vary from isolate to isolate, and to a lesser extent between experiments, with the highest proportion of double stranded RNA being 40 per cent of the total and the lowest level being 24 per cent. If the single stranded RNA

Table 1. *Hybridization of measles 48S RNA to cellular mRNA*

RNA	% Hybridization
48S (Snapback)	6.0 (±1.0)
48S (Self annealing)	9.5 (±1.5)
48S + uninfected cell mRNA	9.0 (±1.0)
48S + infected cell mRNA	83.0 (±3.0)

All hybridizations were performed using radioactively labelled virus (LEC) RNA and unlabelled cellular RNA as described in methods. The level of hybridization was calculated as that proportion of molecules which remained TCA precipatable after digestion at 37° C for 30 minutes with 50 μg/ml of Pancreatic Ribonuclease A and 170 Units/ml of T_1 Ribonuclease

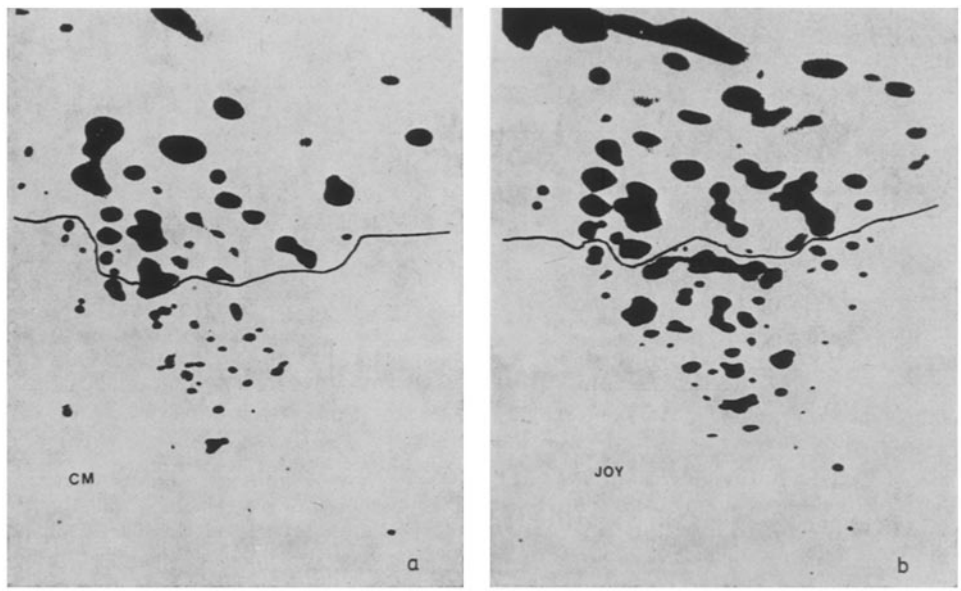

Fig. 2a, b

from such a purification scheme is hybridized to unlabelled messenger RNA from infected cells (Table 1), it can be shown that at least 85 per cent is protected from subsequent nuclease digestion and is thus assumed to be negative stranded.

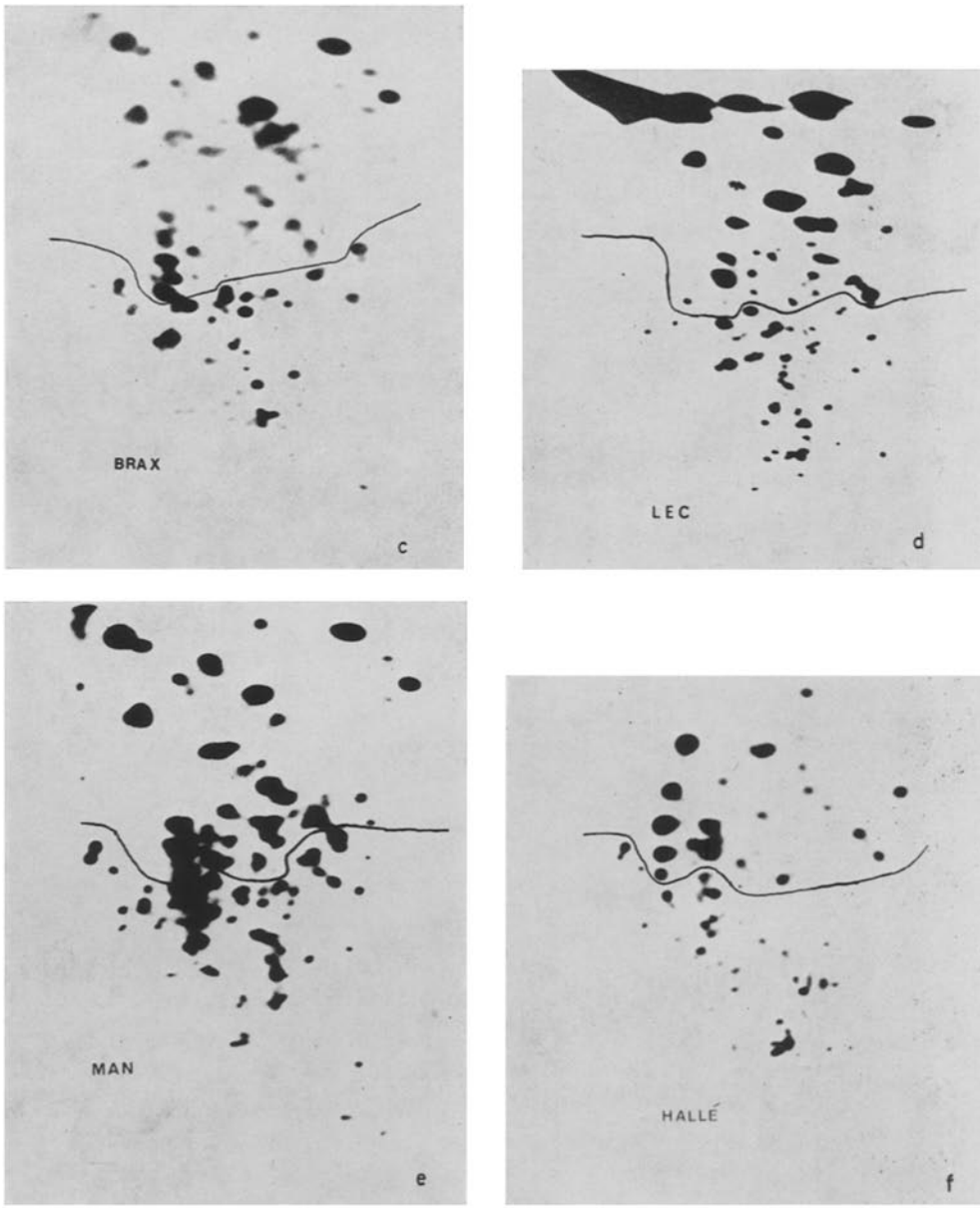

Fig. 2c—d

Fig. 2. Two dimensional electrophoresis of T_1 oligonucleotides from various strains of measles virus. Electrophoresis in the first dimension is from right to left and in the second dimension from bottom to top. The positions of the marker dyes are shown by*; *a* CM, *b* Joy, *c* Braxator, *d* LEC, *e* Mantooth, *f* Hallé

Comparative Analysis of Genomic RNA

In order to compare the genomes of various measles isolates: unlabelled, single stranded, negative sense nucleocapsid RNA was prepared from infected cell cytoplasm and purified in parallel with similar radio-labelled material as described in the previous section. This procedure was adopted as previous

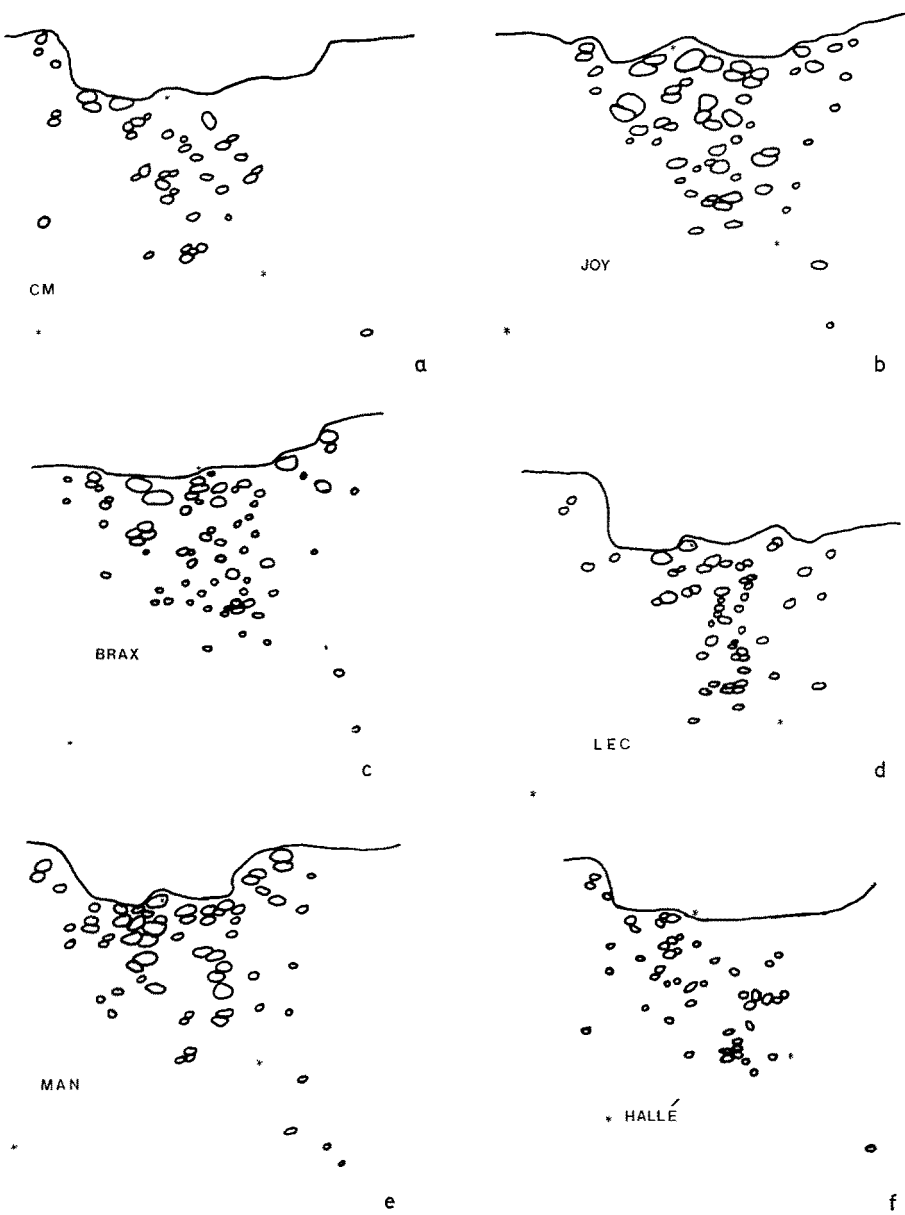

Fig. 3. Tracings of autoradiograms from Fig. 2 for comparitive analysis of virus isolates

attempts to analyse these species of RNA, labelled *in vivo*, were not successful, due to insufficient incorporation of isotope. The RNA was then digested with T_1 endonuclease, labelled with 5[1]- [γ-^{32}P] ATP and the resulting radioactive oligonucleotides analysed by two dimensional electrophoresis as described in methods. Autoradiograms of such two-dimensional gels with T_1 oligonucleotides from two acute measles isolates, one isolate of acute measles encephalitis and three SSPE isolates are shown in Fig. 2. Translucent tracings of these autoradiograms were made (Fig. 3) and these tracings were then compared.

Only the oligonucleotides beneath the line drawn in Figs. 2 and 3 were included in the analysis. It was not possible to co-electrophorese the digests of two or more isolates for this comparison, as the large number of oligonucleotides in one gel would preclude an accurate analysis of the pattern thus obtained. As seen in Fig. 2, the apparent molarity of the larger oligonucleotides is not uniform. This is assumed to be either the result of the co-migration of 2 or more oligonucleotides or an effect of the secondary structure of the oligonucleotides affecting the relative efficiency of the kinase reaction. Similar observations have been noted when the genomes of several RNA viruses were analysed by this method (21). Also the mobility and relative concentration of the smaller oligonucleotides differs in some aspects from that obtained from *in vivo* labelled RNA molecules. It is presumed that this loss of small oligonucleotides from the digest occured during the ethanol precipitation step, and is also a result of the decrease in the concentration of bisacrylamide in the second dimension of PAGE, which was introduced in order to facilitate the drying of the gels. However, these observations were judged not to affect the validity of the conclusions as identical results were obtained from duplicate or triplicate preparations from each isolate; and identical patterns were also obtained when RNAs from different passage numbers of the same isolate were compared.

Table 2. *Oligonucleotide homology amongst measles isolates*

Combination of isolates	Number of common oligonucleotides	Total specific differences[a]
All isolates	8	—
LEC, MAN, HALLE (SSPE)	0	—
BRAX, CM, JOY (measles)	0	—
HALLE only	11	16
LEC only	5	5
MAN only	10	12
BRAX only	5	5
CM only	7	11
JOY only	5	5
All isolates except HALLE	5	—
All isolates except LEC	0	—
All isolates except MAN	2	—
All isolates except BRAX	0	—
All isolates except CM	4	—
All isolates except JOY	0	—

[a] i.e. the sum of the number of oligonucleotides, specific for an isolate and the number of oligonucleotides specifically absent in an isolate

Table 2 summarizes the main differences and similarities between the various isolates examined. Thus it can be demonstrated that all isolates examined show clear differences, even though they appear virtually identical when examined by classical serology. Although 8 oligonucleotides were common to all isolates examined, none were found to be specific for SSPE isolates or for measles isolates. Similarly none were found to be characteristic for all isolates from cases of encephalitis. If the total number of characteristic changes (i.e. the sum of the oligonucleotides shown to be specific for an isolate and the oligonucleotides specifically absent in an isolate) observed in individual isolates are compared, no clear distinctions can be made between isolates from acute cases of measles acute encephalitis or SSPE. In addition, when the LEC isolate of SSPE was compared to either the Edmonston strain of measles vaccine (J. R. STEPHENSON and V. TER MEULEN, unpublished observations) or to another isolate from acute measles, WOODFOLK (22), again no clear differences were found in the oligonucleotide maps to distinguish SSPE isolates from measles isolates.

Discussion

Although many attempts have been made by morphological, antigenic and biochemical techniques to distinguish SSPE viruses from isolates derived from acute infections, no-one has been able to establish criteria specific for SSPE viruses (2, 7, 23). As previous studies have relied either on detecting gross genetic, structural or antigenic changes, we have chosen to analyse the complete genome of various viral isolates by comparing oligonucleotides generated by digestion with T_1 endonuclease. By using this technique, differences as small as one base change, occurring throughout the genome of the virus can be detected.

As it has proved difficult to obtain sufficient quantities of RNA from purified virus, intracellular nucleocapsids have been used as a source of viral RNA. However, as such material can contain significant amounts of cellular ribosomal RNA, messenger RNA and DNA, a purification scheme was devised to minimise the possibility of contamination from these cellular components. The nucleocapsids were therefore purified by centrifugation under conditions which ensured that all ribosomes, ribosomal subunits, messenger RNP, and nuclei were pelleted, while the viral nucleocapsids remained suspended at the sucrose interface. Nucleocapsid RNA prepared from such material contained no detectable amounts of ribosomal or transfer RNA when centrifugated on a sucrose gradient and scanned at $0.D_{260}$. In addition, when in vitro-labelled oligonucleotides from nucleocapsid RNA were compared to those oligonucleotides from 28S ribosomal RNA, no similarities were observed (data not shown). Thus indicating that no contaminating 28S ribosomal RNA could be detected in RNA isolated from viral nucleocapsids. Furthermore, 48S nucleocapsid RNA did not bind to oligo dT cellulose nor was it capable of stimulating protein synthesis when added to a nuclease-treated cell-free system from rabbit reticulocytes. A further complication of using infected cell material is that a significant proportion of the nucleocapsid RNA consists of subgenomic species (18, 19). These species appear to have been successfully removed by sedimentation on sucrose gradients. Also in infected cells, both positive and negative strands are necessary for the replication of paramyxoviruses (20), and can contaminate preparations of 48S RNA

from infected cells. These positive strands have been removed by allowing the RNA to self hybridize and then separating the excess single-strands by differential ethanol precipitation. RNA prepared by this technique was shown to be at least 85 per cent measles-specific negative sense RNA by hybridization to infected cell messenger RNA.

When single-stranded, full length, negative sense RNA from several measles isolates were compared by analysis of the oligonucleotides generated from a T_1 digest, a discreet family of oligonucleotides was generated from each isolate. This family of oligonucleotides was similar in number and distribution to those generated from negative-strand viruses with similar size genomes, such as VSV (24) or Spring Viraemia of Carp Virus (25). No evidence of homopolymeres, such as the poly A or poly C tracts found in positive strand viruses like poliovirus, FMDV, Coronavirus or RNA tumour viruses was found. Although all isolates appear to be virtually identical antigenically, when examined by classical serology they have only about 15 per cent or less of their specific T_1 oligonucleotides in common. Moreover, no oligonucleotides, specific for SSPE isolates or measles isolates, were observed, and concomitantly, no oligonucleotides were characteristic of encephalitic isolates. When the number of detectable specific differences in all viral isolates was compared, all isolates whether from cases of acute measles, acute measles encephalitis or SSPE show similar differences from each other.

Therefore, if we assume that all measles isolates have a common parental ancestor, viruses isolated from a chronic infection, i.e. SSPE do not appear to have mutated at a faster rate than those isolated from cases of acute measles.

However, the techniques employed in these studies cannot analyse all the potential differences in the genomes of these viruses, and such studies must await the future application of molecular cloning and cDNA sequencing to this question.

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