JOURNAL OF VIROLOGY, Aug. 1988, p. 2859–2866
0022-538X/88/082859-08$02.00/0
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Vol. 62, No. 8

Generation of Measles Virus Defective Interfering Particles and Their Presence in a Preparation of Attenuated Live-Virus Vaccine

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Received 21 March 1988/Accepted 4 May 1988

By starting from a thrice-purified wild-type measles virus plaque, the generation of detectable subgenomic RNAs was achieved within a series of five serial infections of Vero cells. The evolution of these subgenomic RNAs was followed for seven serial passages and ended with the preparation of a highly interfering viral stock. On the other hand, the detection of discrete subgenomic RNAs was achieved during the first infection of Vero cells with at least one of three measles virus vaccine preparations tested. These subgenomic RNAs, which interfered very efficiently with the replication of the endogenous standard genomes upon vaccine infection but showed a moderate interfering activity with a standard virus stock derived by plaque purification from the vaccine preparation, resulted from the presence of defective interfering particles in the vaccine preparation. The relevance of this finding for the attenuation, stability, and potential capacity for persistent infection of such a vaccine is discussed.

The use of live-virus vaccines against measles virus is being strongly encouraged in Western countries where they have been proven safe and effective in the prevention of this disease and of its complications (35). Moreover, the eradication of measles by the widespread vaccination of children is one of the current priorities of the World Health Organization (14).

In the late 1950s, Enders et al. (13) were the first to adapt a measles virus strain to growth in chick embryos, after its carriage through 24 primary renal cell and 28 primary amnion cell passages. When subjected to further passages in chick embryos, the strain (Edmonston) proved to be attenuated and immunogenic in animals and could be successfully used in susceptible children (47). However, a high rate of fever and rash following vaccination with the Edmonston strain (49) led to the development of further-attenuated strains such as Edmonston-Enders, Edmonston-Schwartz, and Edmonston-Zagreb (ZAG). These strains resulted from subsequent passages of the Edmonston strain in chick fibroblasts (18, 48) or in dog kidney and human diploid cells successively (29). In particular, the final procedure leading to the production of the ZAG strain involved 19 successive passages in human diploid cells of undiluted suspensions and ended with a virus titer of 10^6 50% tissue culture infective doses per ml (29). The virus was plaque purified in passages 9, 11, and 13.

Experimentally, successive undiluted passages of a virus stock favor the generation of homologous defective interfering (DI) particles (17, 25, 40, 52). DI particles, by interfering with their standard counterpart, reduce the yield of fully infectious virions and can lead to persistent infections in tissue culture (19, 45) and attenuation in vivo (2, 8). DI particles, moreover, constitute a mutational force leading to genome instability in that they cause the standard (St) genome to escape interference (10, 24). DI virions are characterized by the presence of nucleocapsids of subgenomic size corresponding to deletions of the St genome (1, 25, 36, 43). Among negative-stranded RNA viruses, DI particles have been observed for vesicular stomatitis virus (9, 15, 26, 27) and rabies (30), influenza (42), parainfluenza type 3 (41), and Sendai (32) viruses. The generation of DI particles (16) and the establishment of persistent infection (44) with measles virus have also been reported.

In view of the method developed for the production of further-attenuated measles virus vaccines, we hypothesized that these vaccines might contain DI particles. To verify this assertion, we first followed in the present study the process of measles virus DI particle generation. We then analyzed three vaccine preparations for the possible presence of DI particles. The ZAG vaccine preparation was found to be contaminated by DI particles. The relevance of this finding for the attenuation of the vaccine or for possible undesired effects are discussed.

MATERIALS AND METHODS

Cells. Vero, Hel R66, MK2, and CV1 cells were routinely subcultured in minimal Eagle medium (MEM) supplemented with 5% fetal calf serum. Unless otherwise specified, we routinely used Vero cells (about 10^7 cells per petri dish) to grow the various measles virus preparations.

Viruses. Measles virus Hu2 wild-type strain (a gift from B. K. Rima, The Queen's University of Belfast, Northern Ireland) was isolated and propagated as described previously (28). A virus stock was derived from Hu2 after three serial plaque purifications. A plaque resulting from this series of plaque purifications was then amplified on 10^7 Vero cells. This plaque amplification was followed by two further passages by using as inoculum the 20-fold-diluted supernatant of the previous infection. Finally, the infected cells resulting from the second infection were collected and sonicated, and they represented the stock called PP3 (with a titer of 7×10^7 PFU/ml). This stock resulted therefore from three serial infections starting from a three-time plaque-purified virus inoculum.

Three attenuated live-virus measles vaccine strains currently available in Switzerland were used: Edmonston-Enders (Attenuvax; Merck Sharp & Dohme), ZAG (Moraten; Berna), and Edmonston-Schwartz (Rimevax; Smith-Kline).

The Edmonston-Enders strain was only available in the commercial form, i.e., as vials of lyophilized powder con-

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taining at least 1,000 50% tissue culture infective doses. Upon infection with one corresponding dose of such strain, Vero cells became detached from the culture plates after about 10 min. This detachment was due likely to a deleterious effect of a stabilizer contained in the powder. Therefore, after 10 min of infection, the cells were trypsinized and seeded on new petri dishes. The supernatant of these infected cells was harvested when extensive cytopathic effect (CPE) developed and served as the working stock for this vaccine preparation (titer, 2×10^6 PFU/ml).

The Edmonston-Schwartz and ZAG strains were provided by the manufacturers as supernatants of Vero cells infected under the conditions routinely used in the production of vaccines. The ZAG strain was supplied as a concentrated frozen culture medium with a titer of 1.2×10^7 PFU/ml and was used as such. The Edmonston-Schwartz strain was supplied as lyophilized cell supernatant containing less than 1,000 PFU/ml. Therefore, a working stock containing 1.6×10^6 PFU/ml was prepared after a first passage of this vaccine in Vero cells at a low multiplicity of infection (MOI).

Infections. Infections were performed on confluent cell monolayers grown on petri dishes. After being washed with MEM without fetal calf serum, the cells were incubated for 1 h at 32°C with the appropriate viral preparation diluted in MEM without fetal calf serum. The virus inoculum was then removed, replaced by MEM supplemented with 2% fetal calf serum, and further incubated at 32°C until the appearance of CPE.

Plaque assay. Infections were carried out as described above, with serial dilutions of viral preparations. Infected cells were then incubated under a 0.4% agarose overlayer for about a week at 32°C and finally stained with crystal violet in 20% ethanol (20) to allow plaque counting.

Nucleocapsid preparation. Measles virus nucleocapsids were isolated on cesium chloride gradients by the method of Kolakofsky (33), with the modification that 0.2% Sarkosyl (CIBA-GEIGY Corp.) was added to cell extracts and to CsCl solutions.

Electrophoresis and Northern (RNA) blot. Electrophoresis of RNA was carried out on horizontal 1% agarose gels under denaturing conditions, as described previously (39). The RNA samples were prepared after the pelleting of nucleocapsids in 10 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–1 mM EDTA (TNE) (300,000 × *g*; 4°C for 1 h), resuspension of nucleocapsids in TNE–0.5% sodium dodecyl sulfate, and dilution with 3 volumes of loading buffer (13% 3-morpholinepropanesulfonic acid, 20% formaldehyde, 67% formamide). The samples were heated for 10 min at 60°C before loading. The solubilization of nucleocapsids in 0.5% sodium dodecyl sulfate without further extraction was sufficient to dissociate genomic RNA from proteins and to allow the correct separation and hybridization of nucleic acids. RNA was transferred to nitrocellulose sheets (BA85; Schleicher & Schuell, Inc.) according to standard procedures (37). Autoradiography was performed with Kodak X-Omat films.

Radioactive probes. (i) Random genomic probe. Genomic RNA was purified from intracellular nucleocapsids isolated from PP3-infected cells by digestion of the nucleocapsid with proteinase K and extraction with phenol-chloroform. A randomly primed cDNA probe was then produced by reverse transcription of this genomic RNA in the presence of [α-^{32}P]dCTP, as described by Dowling et al. (12). Unincorporated nucleotides were then removed by chromatography on a Sephadex G-50 column, and the RNA template was destroyed by alkaline hydrolysis (38). Because measles virus nucleocapsids contain plus- and minus-strand RNAs (51), this probe (the RT probe) contained a mixture of cDNA fragments complementary to both genomic strands.

(ii) Gene-specific probes. Riboprobes specific for parts of the N, H, and L genes were prepared from plasmids pI62-N1 (6), pGEM-H, and pGEM-DI (5), respectively. In these plasmids, the measles virus-specific inserts span, starting from the genome 3′ end, the following segments of the genome (a total of 15,900 bases): pI62-N1 (N probe), bases 50 to 1740; pGEM-H (H probe), bases 7670 to 8,460; pGEM-DI (DI probe), bases 14310-14690 (5, 6; R. Cattaneo, personal communication). In our hybridization experiments, only plus-sense probes were used.

RESULTS

Generation of interfering particles from wild-type measles virus. Certain in vitro cell lines have, surprisingly, been found not to support the replication of DI particles even when they are perfectly permissive to the homologous St virus (21). To perform our study, we first had to ensure that the cell line used did, indeed, support measles virus DI particle replication. This preliminary study was also necessary because of the incomplete information available in previous reports (4, 16, 31) on the rate of measles virus DI particle generation.

Serial passages with high MOI were therefore performed by starting with a wild-type DI-free viral stock (PP3) obtained after a series of three passages following a three-time plaque purification procedure (see Materials and Methods for details). The first high-multiplicity passage presented here (which constituted, in fact, passage 4 after the plaque purification) was then followed by a series of infections for which the sonicated infected cells from the preceding passage served as the inoculum for the subsequent infection. At each passage, a sample of the yield of the preceding infection was analyzed to detect signs of the generation of DI particles. The emergence of interfering activity was indicated by decreased titers of the virus produced and by modification of the nature of the CPE i.e., slower spreading and incomplete degeneration of syncytia (Table 1). By passage 6, syncytia never reached confluence and areas of cell degeneration were progressively filled in by surrounding intact cells. At this point, a viral stock, called P6, resulting from two cycles of amplification of the virions generated at passage 5 by coinfection with the parental standard virus (PP3) was finally established.

TABLE 1. Cell-associated infectious virions produced during successive undiluted passages of a wild-type measles virus strain PP3[a]

Passage no.	Yield (PFU/ml)	Time to maximum CPE (h)
1	10^7	60
2	ND[b]	48
3	10^6	36
4	10^5	90
5	2×10^6	104
6[c]	2×10^5	144

[a] Confluent Vero cells were infected first with PP3 at a MOI of 5 (passage 1). When maximum CPE was reached, the cells were collected, suspended in 4 ml of MEM, and sonicated. A portion (100 μl) of this suspension was used for titration of the yield, and 3 ml was used undiluted to infect a new series of Vero cells.

[b] ND, Not determined.

[c] Passage 6 corresponds to the infection leading to the establishment of P6 stock (see text).

TABLE 2. Interference assay for PP3 and P6[a]

Sample	P6 inoculum (total PFU)[b]	Yield (total PFU)	
		Cell-associated virus	Virus in supernatant
A		1.2×10^8	1.1×10^7
B	4.5×10^6	3.5×10^4	1.1×10^3
C	4.5×10^5	6.4×10^5	1.2×10^5
D	4.5×10^4	9.5×10^6	1.6×10^6

[a] A series of Vero cells (10^7) were infected as indicated. By 112 h of infection, when syncytia had completely (samples A to D), partially (sample C), or minimally (sample B) degenerated, the cells were collected and separated from their supernatants by centrifugation at 2,000 × *g* for 10 min. The titers of cell-associated virus and that present in the supernatants were then determined after one cycle of freeze-thawing at −70°C.

[b] PP3 inoculum for all samples was 2×10^4 PFU.

Interfering activity of P6 viral stock. The presence of interfering activity in the P6 viral stock was confirmed by the results of the interference assay (Table 2). The P6 stock exhibited a straightforward interfering activity. At the highest P6 MOI (P6 inoculum of 4.5×10^6 PFU/ml), the infectious virus production dropped by 4 orders of magnitude during coinfections of P6 with PP3. This interfering activity in turn decreased (increase of infectious virus production by 1 order of magnitude) when the P6 inoculum was reduced.

Generation of subgenomic RNAs during serial undiluted passages. In parallel with the determination of the interfering activity generated during the series of high-multiplicity virus passages (Table 1), we characterized the viral genomic RNAs recovered from the serially infected cells and from the second amplification of passage 6 resulting in the P6 stock by Northern blot analysis (Fig. 1). This analysis showed that a subgenomic RNA with an estimated length of 700 bases, compared with the 28S and 18S ribosomal RNAs, was detectable by passage 2 (Fig. 1, lane 2). This 700-base RNA became the predominant nucleocapsid RNA species in passages 3 and 4 (Fig. 1, lanes 3 and 4). In passage 5 (Fig. 1, lane 5), however, the 700-base RNA was replaced by two new subgenomic RNAs with estimated sizes of 1,000 and 500 bases. Finally, after two series of amplification of passage 5 by coinfection with PP3, the nucleocapsids produced no longer contained the 500-base RNA but contained exclusively the 1,000-base RNA (Fig. 1, lane 6). This defective RNA appeared extremely fit for replication and interference, since it not only displaced the other subgenomic RNA but also was vastly overrepresented over the full-length (50S) genomic RNA. This high capacity for interference agreed with the results of the interference assay (Table 2).

The generation of measles virus DI particles thus paralleled that of other negative-strand viruses, such as vesicular stomatitis virus and Sendai virus (1, 22, 23, 33, 34, 46). During serial high-multiplicity passages, as well as during the evolution of a DI particle-induced persistent infection, various subgenomic RNAs arose with no preferential selection for shorter species.

As for the primary structure of the DI RNA present in P6 stock, it failed to hybridize in Northern blots with specific probes representing sequences in the NP, H, and L genes (see Materials and Methods; data not shown). It should, however, be noted that if the 1,000-base DI RNA is of the copy-back type and contains sequences from the last 1,000 bases of the genomic 5′ end, it would not be detected with our specific L-gene probe (DI probe; see Materials and Methods) which terminates 1,210 bases from the 5′ end.

Regardless of the precise molecular structure of this predominant DI RNA, it was of interest to observe that a significant amount of subgenomic interfering genomes were detected in infected cells after a series of five passages following plaque purification (i.e., two passages in the present experiment plus the three passages involved in PP3 stock preparation; see Materials and Methods).

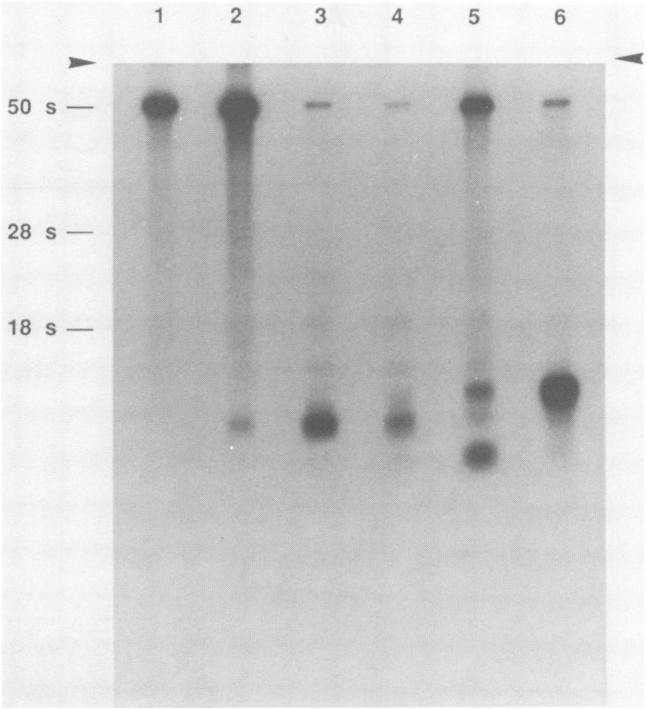

FIG. 1. Northern blot analysis of intracellular nucleocapsid RNAs produced during successive undiluted passages of the standard strain PP3. Samples (900 μl) of the viral suspensions produced during each viral passage (Table 1 and Materials and Methods) were used to infect a series of 3×10^7 Vero cells. When the CPE was extensive, cells were collected, intracellular nucleocapsids were isolated, and nucleocapsid RNA was characterized by Northern blot analysis. Lanes: 1, intracellular nucleocapsid RNA synthesized after infection with PP3; 2 to 5, nucleocapsid RNA synthesized after infections with virions produced during passages 2 to 5, respectively (Table 1); 6, nucleocapsid RNA synthesized after infection with virions produced after two cycles of coinfection of the virus produced after undiluted passage 5 and PP3. Random cDNA probe (RT) was used. The electrophoretic migration of 18S and 28S ribosomal RNAs was determined by staining with methylene blue, and the size of the full-length (50S) measles virus RNA is indicated. Arrows, Origin of the gel.

Analysis of attenuated live-virus vaccine preparations. The results presented so far showed the ability of the cell line used to support the replication of measles virus DI particles. Also, the relative ease with which the measles virus DI particles were generated upon serial passages made it extremely likely that we would detect their presence in the attenuated vaccine preparations, because, as noted above, the attenuation was achieved after numerous serial passages in various host cells. The characterization of nucleocapsid RNA present in Vero cells infected with the three vaccine preparations was therefore undertaken after infection at an MOI of about 0.1 to 1. To obtain such MOI, it is important to remember that the Edmonston-Schwartz and Edmonston-Enders strains had to be passaged first at very low MOI (about 10^{-4}) from preparations supplied by the manufacturers to obtain working stocks. In contrast, this preliminary

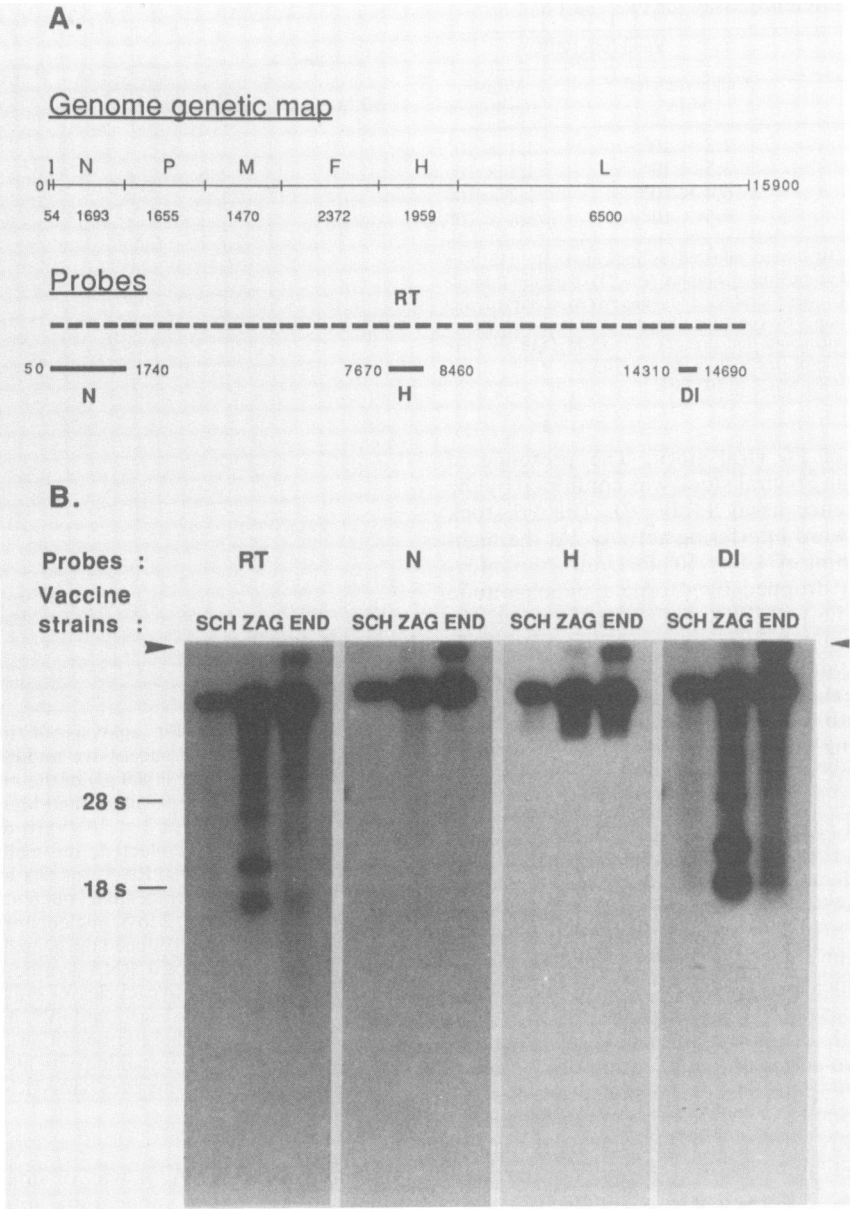

FIG. 2. Northern blot analysis of measles virus nucleocapsid RNAs purified from vaccine-infected Vero cells. (A) Description of probes used. The measles virus genomic map is drawn according to reference 5 , and the positions of the probes are indicated. RT, random cDNA genomic probe; N, H, and DI, riboprobes (see Materials and Methods). (B) Northern blot analysis. Vero cells were infected with Edmonston-Schwartz (SCH), Edmonston-Enders (END), and ZAG vaccine preparations, as indicated in Materials and Methods, and intracellular nucleocapsid RNAs were analyzed by Northern blot and hybridization with the probes described in panel A. Arrows, Origin of the gel.

passage was not required for the ZAG strain which was supplied at virus titers of sufficiently high infectivity (see Materials and Methods). After infection of Vero cells with the three vaccine strains, the isolated intracellular nucleocapsid RNAs were characterized by Northern blot analysis with the random cDNA genomic probe (RT probe), as well as riboprobes specific for various parts of the genome (Fig. 2). The results obtained differed with the three vaccine preparations and with the type of probe used. With the use of the random cDNA genomic probe, three discrete subgenomic RNAs (estimated sizes, 5,000, 2,900, and 2,000 bases) were clearly detected in the cells infected with the ZAG vaccine preparation, and a few diffuse but weak bands were present in the Edmonston-Enders sample. Under the same conditions, no RNA species except for the full-length genomic RNA could be seen in the Edmonston-Schwartz sample. No subgenomic RNAs were detected with the specific probes N and H, but the DI probe detected the same subgenomic RNAs as those detected by the RT probe in the Edmonston-Zagreb sample (Fig. 2B, probe DI, lane ZAG) and possible diffuse RNAs species in the Edmonston-Enders sample (Fig. 2B, probe DI, lane END). These data therefore convincingly demonstrated the presence of discrete subgenomic viral RNAs in cells infected with the ZAG vaccine preparation.

Structure of subgenomic RNAs present in ZAG vaccine

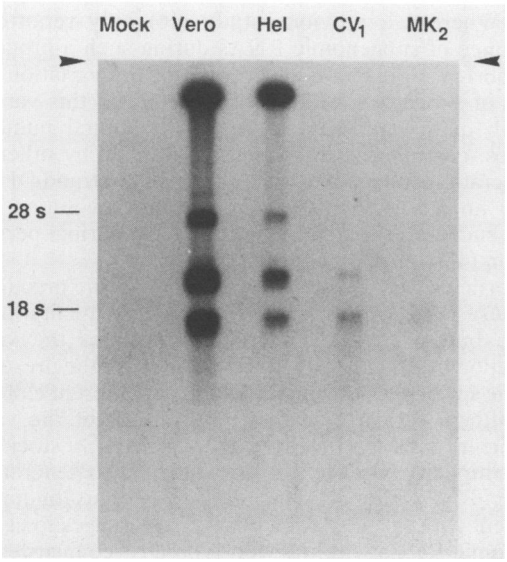

FIG. 3. Pattern of intracellular nucleocapsid RNAs recovered after infection of various cell types with the ZAG vaccine strain. Vero, Hel R66, CV1, and MK2 cells were infected with the ZAG vaccine (MOI of about 5), and the intracellular nucleocapsid RNAs were isolated and characterized by Northern blot analysis with the RT probe. Arrows, Origin of the gel.

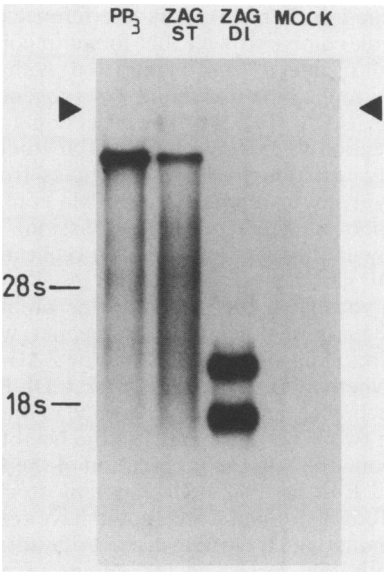

FIG. 4. Northern blot analysis of nucleocapsid RNAs isolated from cells infected with different stocks of the ZAG strain. Vero cells were infected with stocks derived from the ZAG vaccine strain either after a series of three plaque purifications (ZAG ST) or after two undiluted pasages (ZAG DI). Intracellular nucleocapsid RNAs were then characterized by Northern blot analysis with the DI riboprobe. PP_3, Intracellular nucleocapsid RNA isolated from PP3-infected cells used as a reference for a preparation free of detectable subgenomic RNAs; mock, sample isolated from uninfected cells. Arrows, Origin of the gel.

preparation. The increased ratios of the ZAG subgenomic RNAs to the genomic RNA seen with the use of the DI probe (Fig. 2B, probes DI and RT, compare the intensity of the subgenomic RNAs with that of the genomic RNA) suggested that the subgenomic RNAs were enriched for 5′-end genomic sequences. Moreover, the H and DI probes appeared to detect a smear of RNA species extending in length from the size of the full-length genome to the size separating each probe from the genomic 5′ end (Fig. 2B, probes H and DI, lanes ZAG and END). This result was again consistent with the presence of a large number of minor RNAs, all originating from the genomic 5′ end. These assertions would clearly have to be confirmed by using more-specific techniques; it was, however, noteworthy that the present gross analysis appeared to indicate that the measles virus subgenomic RNAs detected tended to retain the genomic 5′ end, an observation consistent with the most prevalent structure of the nonsegmented negative-strand subgenomic RNAs (19, 36, 43).

Independent amplification of ZAG subgenomic RNAs in different cell types. To ensure that the subgenomic RNAs were actually present in the vaccine and were not merely generated by the single passage needed to isolate nucleocapsids, four different cell types (Vero, Hel R66, CV1, and MK2) were infected at comparable MOI (MOI of 5). The rationale behind the experiment was the observation that different cell types generate nonuniform sizes of subgenomic RNAs (23). It was therefore expected that subgenomic ZAG RNAs would vary in size in the different cell types if they were generated during the first cell passage. This hypothesis was not verified in the present study (Fig. 3). Although the efficiency of virus replication in the four cell types used differed, the same three discrete subgenomic RNAs were recovered. Therefore, the subgenomic RNAs detected upon infection with the ZAG vaccine preparation were presumably present in the vaccine preparation rather than generated during Vero cell infection.

Interference ability of ZAG subgenomic RNAs. To assess the interfering capacity of the ZAG subgenomic RNAs, a viral stock (ZAG-St) was derived from the vaccine preparation after a series of three plaque purifications followed by a cycle of growth at low MOI. In addition, a ZAG-DI stock was prepared in which the subgenomic RNAs present in the preparation were amplified by two cycles of high-multiplicity infections. The characterization of nucleocapsid RNAs present in the cells infected with these two viral stocks (Fig. 4) showed that ZAG-St constituted a viral stock devoid of detectable subgenomic RNAs. In the ZAG-DI stock, however, the subgenomic RNAs or at least two of them had been amplified over the genomic RNA (compare Fig. 4, lane ZAG DI with Fig. 2B, probe DI, lane ZAG). Upon coinfection with these two viral stocks, the interference activity resulted in the drop of about 1 order of magnitude in infectious virus

TABLE 3. Interference assay for ZAG-St and ZAG-DI[a]

Sample	ZAG-DI inoculum (total PFU)[b]	Cell-associated virus yield (total PFU)
Expt 1		
A		5.3×10^7
B	4.5×10^6	7.0×10^6
C	4.5×10^5	3.6×10^7
D	4.5×10^4	6.2×10^7
Expt 2		
A		2.2×10^8
B	2.6×10^6	1.4×10^7
C	2.6×10^5	3.8×10^7
D	2.6×10^4	8.0×10^7

[a] The experiments were carried out as described in Table 2, footnote *a*, except that ZAG-St and ZAG-DI stocks were used.

[b] The ZAG-St inocula were 2×10^4 and 2.2×10^6 for experiments 1 and 2, respectively.

particle production (Table 3). This interference activity was somehow weak compared with that found in coinfections of PP3 with P6 (Table 2) and contrasted with the strong advantage in replication exhibited by subgenomic RNAs over genomic RNA (Fig. 4). The selection (achieved by plaque purification to generate the ZAG-St stock) of a virus already partly resistant to the DI particles from which it previously evolved, as reported by DePolo et al. (10), could explain this poor interference (see Discussion).

Effect of low-multiplicity infections on replication of ZAG-DI RNAs. DI particles of discrete RNA sizes were only found unequivocally in the ZAG vaccine preparation. Because the attenuation of the two other vaccines was achieved with procedures similar to that used for the ZAG vaccine, we questioned whether the failure to detect DI RNAs arose because of technical reasons. A difference in the analyses of ZAG and the two other vaccines was the need for an initial low MOI (about 10^{-4}) in the preparation of the Edmonston-Schwartz and Edmonston-Enders working stocks (see Materials and Methods). Such a step could have resulted in the loss of contaminating DI particles. To verify this hypothesis, infections with the ZAG vaccine strain were performed under conditions of increasing dilution of the inoculum, i.e., of decreasing MOI. The characterization of the intracellular nucleocapsid RNAs generated during such infections (Fig. 5) showed that decreasing the MOI resulted in a progressive loss of subgenomic RNA amplification and unequivocally demonstrated that infection at an MOI of 10^{-4} was equivalent to a plaque purification. For that reason, one could not conclude that the Edmonston-Schwartz and Edmonston-Enders vaccine preparations were free of subgenomic RNA-containing virions.

DISCUSSION

The present study constitutes the first passage-to-passage follow up of measles virus subgenomic RNA generation and amplification taking place during a series of undiluted passages. Whereas a previous study (16) only reported the appearance of subgenomic RNAs during such a process, in this report, we present a description of the variation in the pattern of subgenomic RNAs. The extent of this variation, although quite remarkable for such a limited number of passages, corroborate the observation made by others that the generation and amplification of negative-strand DI RNAs lead to an RNA population in constant evolution either during successive passages (22, 23) or during persistent infection (46).

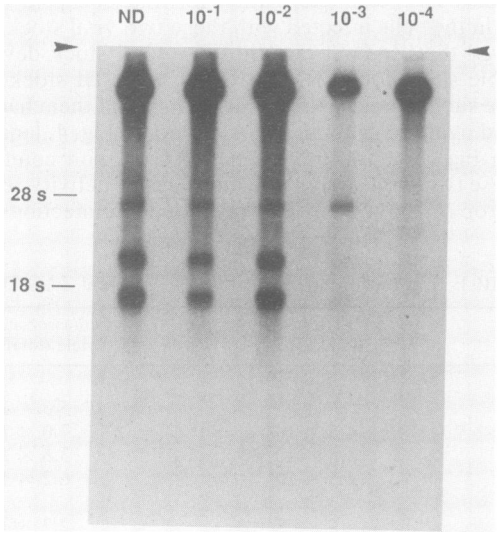

FIG. 5. Effect of decreasing MOI on recovery of intracellular subgenomic RNAs from ZAG vaccine strain-infected cells. Vero cells were infected with nondiluted (ND) ZAG vaccine strain (MOI of about 1) or with increasing dilutions of the vaccine strain as indicated. Intracellular nucleocapsid RNAs were then isolated and analyzed by Northern blot with the RT probe. Arrows, Origin of the gel.

DI particles were present in the ZAG vaccine preparation. That these DI RNAs were not generated by the first in vitro passage but resulted from contamination of the vaccine preparation by DI particles was supported by the presence of the same species of subgenomic RNAs in different cell types. Also, after a series of plaque purifications of the vaccine resulting in a DI particle-free stock (ZAG-St stock), one high-multiplicity passage was not sufficient to generate subgenomic RNAs (Fig. 4, lane ZAG ST). This finding was consistent with the need for at least five passages to generate subgenomic RNAs with the wild-type DI-free measles virus stock PP3 (Fig. 1).

The presence of DI particles in a preparation of attenuated measles virus vaccine has to be considered in view of the effects that DI particles have on the course of viral infection and on the evolution of the St virus genetic information.

Measles virus DI particles have been suspected to play a role in the pathogenesis of subacute sclerosing panencephalitis, a rare fatal disease caused by measles virus after years of latency (50); this suspicion is based on the well-documented role of negative-strand DI particles in the establishment and maintenance of in vitro viral persistence (19). However, statistical evidence indicates that the use of measles virus vaccine has contributed to a significant decrease in the incidence of subacute sclerosing panencephalitis (7). Rare cases of subacute sclerosing panencephalitis in measles virus vaccinees could in fact be explained by incomplete vaccine efficacy or by the possibility of prior exposure to measles virus (53).

On the other hand, the possible involvement of these DI particles in vaccine attenuation has to be considered as well. The role of DI particles in virus attenuation is supported by the recent observation that a DI-containing influenza virus strain was responsible for a natural epizootic in chickens. This DI-associated strain caused less severe morbidity and significantly reduced mortality than did a DI-free influenza virus strain (2, 8).

The in vivo effects of these vaccine DI particles will ultimately depend on their ability to replicate upon vaccination. Because vaccination is performed with the equivalent of about 10^3 PFU, the ability of the DI particles to replicate in vivo will depend mainly on the number of initially exposed cells (i.e., initial MOI). In the case of effective low MOI, the intracellular stability of DI nucleocapsids waiting for an incoming St nucleocapsid generated during the first cycle of in vivo replication may be a determinant. The intracellular stability of negative-strand virus DI nucleocapsids have been shown to depend on the type of virus: about 6 to 12 h for vesicular stomatitis virus (11) to 17 to 25 days for influenza virus (3). The stability of measles DI nucleocapsids will therefore have to be determined before we can speculate on the chances of their amplification upon vaccination.

Finally, the role of DI particles in the stability of the attenuated strain has to be discussed. As reported by DePolo and co-workers (10), coinfection of St and DI particles during serial passages leads to the evolution of viral gen-

omes. The selection of variant St viruses resistant to interference is constantly taking place, leading to the emergence of viruses which have accumulated mutations at least at the genomic 5′ end, as well as in the nucleocapsid protein gene. The presence of DI particles therefore constitutes a driving force leading to virus evolution. With the hypothesis that attenuation results from the selection of a particular variant, with no way to control the further evolution of such variant, the mutational driving force represented by DI particles may be undesirable.

Clearly these issues need to be studied further. They are important in many respects. If measles virus DI particles participate in attenuation, vaccine strains should be developed containing DI particles with the highest interfering activity. On the other hand, if the DI particles are not responsible for attenuation, an effort should be made to obtain DI-free vaccine preparations. This DI-free preparations could minimize the putative hazard caused by persistent infection, contribute to the stability of the attenuated strain, and on a more practical point, certainly increase the virus yield during vaccine manufacture.

ACKNOWLEDGMENTS

We thank Merck Sharp & Dohme, Smith-Kline, and Berna for supplying the vaccine preparations; Roberto Cattaneo and Martin Billeter for the kind gift of the measles virus-specific probes; Geneviève Mottet for skillful technical assistance; and Joe Curran and Daniel Kolakofsky for helpful discussions and comments.

This work was supported by grants from the Roche Research Foundation (to P.C.), Geigy-Jubilaümstiftung, and the Swiss National Foundation for Scientific Research.

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