

Biological Characterization of Clones Derived from the Edmonston Strain of Measles Virus in Comparison with Schwarz and CAM-70 Vaccine Strains

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Four virus clones were derived from the Edmonston strain of measles virus by repeated plaque purification. These clones were compared with the vaccine strains Schwarz and CAM-70 in terms of biological activities including plaque formation, hemagglutination, hemolysis and replication in Vero cells and chick embryo fibroblasts (CEF).

Two clones of intermediate plaque yielded mixed plaque populations on subcultivation whereas the other two, showing small and large plaque sizes, showed stable plaque phenotypes. The vaccine strains showed consistent homogeneous plaque populations.

All the Edmonston clones showed agglutination of monkey erythrocytes in isotonic solution while both vaccine strains hemagglutinated only in the presence of high salt concentrations.

Variation in the hemolytic activity was observed among the four clones but no hemolytic activity was detected for the vaccine virus strains.

Vaccine strains replicated efficiently both in Vero cells and CEF. All four clones showed efficient replication in Vero cells but different replication profiles in CEF. Two of them replicated efficiently, one was of intermediate efficiency and the other showed no replication in CEF.

Two of the clones showed characteristics similar to vaccine strains. One in terms of size and homogeneity of plaques, the other for a low hemolytic activity and both for the efficiency of propagation in CEF.

Key words: biological characterization - plaque variants - measles - vaccine

Measles is a viral exanthematic disease, considered as one of the most contagious infections of mankind, which still causes the death of over one million children per year, mainly in the developing world. The severity of measles is due to the immune suppression caused by the virus leading to secondary infections and also for the role in the establishment of a slow progressive disease of the nervous system, subacute sclerosing panencephalitis (SSPE). After the successful introduction of the vaccine in 1964 together with the fact that measles virus is considered monotypic and has no animal reservoir, measles was considered as potentially eradicable. Current licensed vaccines are safe and effective. They are, however, limited by the requirement for constant refrigeration and by interference of transplacentally acquired maternal antibodies (Gellin & Katz 1994). Variation exists between virus strains in monoclonal antibody reactivity (Hu et al. 1993), plaque formation (Rapp 1964, De Maeyer & Enders 1965), temperature sensitivity (Haspel et al. 1975), ability to induce

interferon (McKimm & Rapp 1977), hemagglutination (Schluederberg & Nakamura 1967), as well as in nucleotide sequence (Rota et al. 1994). However, the biological significance of this variation and the correlation with virulence and attenuation need to be established in order to evaluate the efficacy of eradication programmes. The envelope of measles virus carries two transmembrane glycoproteins which are biologically important antigens for representing the primary targets in immune response. The hemagglutinin (H) is responsible for host cell recognition through receptor binding and agglutination of red blood cells. The fusion (F) protein is involved in fusion of the viral and cell membranes, viral penetration and hemolysis (Bellini et al. 1994). In this study we compare biological activities of different strains of measles virus such as plaque formation, hemagglutination, hemolysis and replication in Vero cells and in chick embryo fibroblasts (CEF).

MATERIALS AND METHODS

Virus strains - The cell culture adapted Edmonston strain was provided by CDC, Atlanta, (Hummel et al. 1992) and passaged five times in Vero cells. This strain yielded a mixed plaque population from which were derived four clones

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by plaque purifications. The Schwarz vaccine strain was obtained from the Rouvax (Pasteur Mérieux) commercial vaccine. The vaccine strain CAM-70 was obtained from the commercial vaccine from Bio-Manguinhos. All the strains studied were passaged twice in Vero cells to prepare the seed lot.

Cells - Vero cells (Rhim et al. 1969) were obtained from the American Type Culture Collection and primary chick embryo fibroblasts (Rubin 1973) were cultured in 199 Earle's medium containing 5% foetal bovine serum and 40µg/ml gentamycin. Monolayers of Vero cells for plaque assays were prepared at a density of 2.6×10^5 cells/cm² for an area of 9.6cm²; monolayers of Vero cells for 25cm² flasks were prepared in a density of 1.2×10^4 cells/cm². Monolayers of CEF for 25cm² flasks were prepared in a density of 6×10^3 cells/cm². The cultures were kept in an incubator with 5% CO₂ in air, at 37°C.

Plaque selection and plaque assays - Plaque assays were performed according to general method of Dulbecco and Vogt (1954). The viruses were inoculated in a volume of 0.3ml/9.6cm² in 6-well plates and allowed to adsorb for 1 hr. The inoculae were then removed and replaced with 3ml of medium containing 0.6% agarose. After seven days of incubation at 37°C the cells were stained by the addition of neutral red in medium to a final concentration of 1:20,000. Plaques were counted and measured at a magnification of 12.5x. For plaque selection the dye was removed and a plug of agarose and cells was aspirated with a pasteur pipette from selected well separated plaques and inoculated into tubes with 1ml of medium containing 2×10^5 Vero cells. The cultures showing cytopathic effect were frozen and the cloning procedure repeated three to five times.

Hemagglutination - The hemagglutination tests were performed in round bottomed microtitre plates (Allison 1985). Twofold dilutions of the virus samples were prepared starting from 1:6, 1:7 and 1:8 using as diluent both PBS and 1.6 M (NH₄)₂SO₄ in PBS (Shirodaria et al. 1976). An equal volume of rhesus monkey red blood cells 1% in PBS was added. Titres were determined after incubation at 37°C for 2 hr as log₁₀ of the reciprocal of the highest dilution which produced 50% agglutination. The amount of infectious virus required to hemagglutinate red cells was calculated from plaque assay results as log₁₀ pfu/HAU.

Hemolysis - Since hemagglutination is a prerequisite for hemolysis, hemolysis tests were initiated as described above (Allison 1985). The plates were then vigorously shaken and reincubated at 37°C overnight. Controls representing 100% hemolysis were prepared by mixing equal volumes of 0.1% Triton-X-100 with controls of non-agglu-

tinated red blood cells. One hundred µl of PBS were added to all the wells and the plates centrifuged at 1000x g for 10 min. The supernatants were transferred to flat bottomed plates in a volume of 100µl. Optical densities (OD) were measured on a microplate reader at 405nm. The mean absorbance value produced by the 100% hemolysis controls and by the control RBC's was calculated. The background OD value represented by the control RBC's was subtracted from the OD values for the virus samples and the 100% hemolysis controls. The results were calculated as the log₁₀ of the dilution which gave 50% hemolysis, relative to the controls. The amount of infectious virus required to produce 50% hemolysis of red cells was determined from plaque assay results as log₁₀ pfu/HLU.

Propagation in Vero cells and CEF - The efficiency of replication of the virus strains was evaluated by inoculating five cultures of each cell type with 1ml containing 10⁶ pfu of each virus sample, at a MOI of 2.8 for CEF and 1.4 for Vero cells. The viruses were allowed to adsorb for 1 hr at 37°C. The inoculae were then removed, replaced by 10ml of fresh medium and the cultures reincubated at 37°C. The culture flasks of each virus were frozen at -70°C on days 0, 2, 4, 6 and 8 post-infection (p.i.) for Vero cells and days 0, 2, 4, 7 and 9 p.i. for CEF. After a single freeze/thaw cycle, virus samples were clarified at 2000x g for 10 min, and titrated by plaque assay.

RESULTS

The plaque phenotype produced by all the strains was observed after incubation of seven days at 37°C. The Schwarz virus produced small plaques of 0.5mm in diameter. The plaques produced by the CAM-70 strain were of intermediate size, with 2.5mm. The starting Edmonston virus contained a heterogeneous plaque population from which were derived four clones. Clone 1 was obtained after four plaque purifications and showed consistently mixed progeny of intermediate (2.5mm) and small (0.5mm) plaques. Clone 2 was also obtained after four plaque purifications and showed a stable population of small plaques (0.5mm) which were indistinguishable from those of the Schwarz strain. Clone 3 was obtained after five plaque purifications and, as with clone 1, showed a mixed progeny of intermediate and small plaques. For clones 1 and 3 the intermediate CAM-70 like plaque predominated. The plaque population produced by clone 4 was of stable, large plaques, with 7.5mm in diameter (Fig. 1). Both vaccine strains gave consistently homogeneous plaque populations (Table I).

Both of the vaccine strains hemagglutinated only in hypertonic saline while the four Edmonston clones hemagglutinated both in hypertonic and isotonic conditions (Table II).

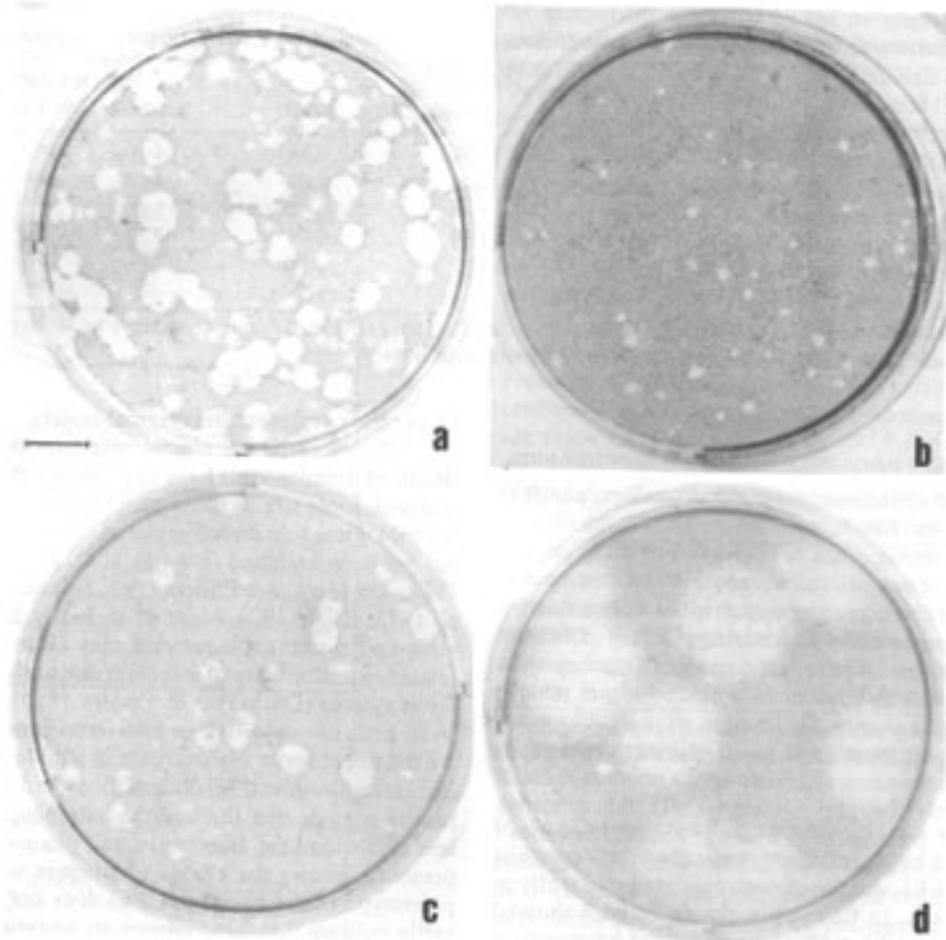


Fig. 1: plaques produced by clones derived from the Edmonston strain in Vero cells under agarose overlay, stained with neutral red. a: clone 1, b: clone 2, c: clone 3, d: clone 4 (Bar: 0.5cm) .

TABLE I

Plaque type produced in Vero cells infected with measles virus under agarose overlay

Virus	Plaque type	Diameter (mm)	Progeny
clone 1	intermediate	2.5	mixed
clone 2	small	0.5	stable
clone 3	intermediate	2.5	mixed
clone 4	large	7.5	stable
Schwarz	small	0.5	homogeneous
CAM-70	intermediate	2.5	homogeneous

The vaccine strains Schwarz and CAM-70 did not produce hemolysis, even under hypertonic conditions. The relationship between infectious and hemolytic titres is shown in Table III. The results of the hemolysis assays for the four clones are shown in Fig. 2.

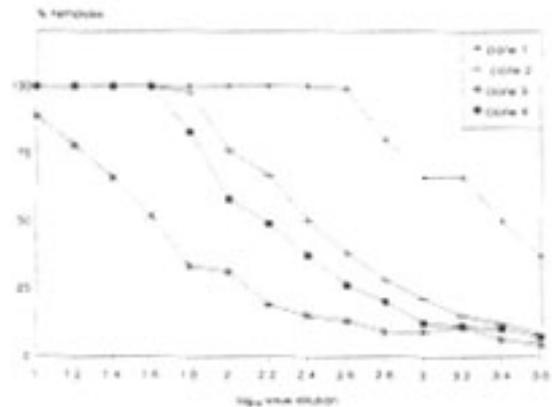


Fig. 2: relationship between dilution of the virus (\log_{10}) and percentage of hemolysis induced in a red blood cell suspension.

TABLE II
Relationship between infectious titres and hemagglutination under isotonic and hypertonic conditions

Virus	Infectious titres ^a	HA titres ^b			log ₁₀ pfu/HAU	
		isotonic	hypertonic	ratio*	isotonic	hypertonic
clone 1	9.30	5.01	5.39	0.38	4.29	3.91
clone 2	8.34	3.76	4.79	1.03	4.58	3.55
clone 3	9.22	3.58	3.95	0.37	5.64	5.27
clone 4	9.20	3.58	4.49	0.91	5.62	4.71
Schwarz	8.43	<1.60	3.81	>2.20	-	4.62
CAM-70	8.90	<1.60	3.50	>1.90	-	5.40

a: values expressed as log₁₀ pfu/ml; *b*: values expressed as log₁₀ HAU/ml; *: rate of increase under hypertonic conditions; HA - hemagglutination; HAU - hemagglutination units.

TABLE III

Relationship between infectious and hemolytic titres

Virus	Infectious titres ^a	HL titres ^b	log ₁₀ pfu/HLU
clone 1	9.30	4.72	4.58
clone 2	8.34	3.71	4.63
clone 3	9.22	3.21	6.01
clone 4	9.20	3.46	5.74
Schwarz	8.43	-	-
CAM-70	8.90	-	-

a: values expressed as log₁₀ pfu/ml; *b*: values expressed as log₁₀ HLU/ml; HL - hemolysis; HLU - hemolysis units.

The vaccine strains Schwarz and CAM-70 showed highly efficient replication in Vero cells and in CEF. All the clones replicated efficiently in Vero cells. In CEF, only clones 2 and 3 showed efficient replication; clone 1 showed intermediate replication efficiency and clone 4 showed no apparent replication (Fig. 3).

DISCUSSION

The Edmonston strain from which the clones were derived was passaged 70 times in tissue culture since its isolation. MRC-5, a human diploid cell line, was the last cell type in which it was passaged before coming to our hands, and no passages in chick cells were performed. Both vaccine strains, Schwarz and CAM-70 were attenuated through 163 and 147 passages, respectively, most of them in chick cells. The first licensed attenuated measles vaccine, Edmonston B, was derived from the Edmonston strain by additional passages in chick embryos and chick cells. The administration of this vaccine was associated with symptoms of the disease such as rash and high fever (Markowitz & Katz 1994). Since the Edmonston virus used in the present study had not been passaged in chick embryo cells, it is highly likely to be more virulent than the Edmonston B strain, but considering the number of passages, it may no

longer truly represent the original isolate.

Differences in the plaque size produced by strains of measles virus have been documented. In addition, some strains show multiple plaque sizes. No biological role directly related to plaque sizes has yet been assigned (Buynak et al. 1962, Rapp 1964, De Maeyer & Enders 1965, Schumacher et al. 1972, Gould 1974, Mann et al. 1980). In some virus-cell systems, plaque size may be inversely related to multiplicity of infection due to the interferon system (De Mayer & Enders 1965). Since Vero cells are defective in interferon production we did not observe any decrease in plaque size by increasing the infectious dose of the inoculum. Our results suggest that the intermediate plaques are less stable than the large and small plaques. The presence among the clones of plaques with the phenotype of the vaccine strains does not necessarily indicate that these viruses are attenuated.

Salt-dependent hemagglutination was first demonstrated by Schluenderberg and Nakamura (1967). Measles virus isolates showing low hemagglutination activity or salt-dependent hemagglutination have been described (Shirodaria et al. 1976, Saito et al. 1992, Shibahara et al. 1994). Salt-dependence of the Schwarz strain, as well as the isotonic hemagglutination of the Edmonston-Zagreb (Ikic et al. 1972) vaccine strain was previously demonstrated (Shirodaria et al. 1976, Allison 1985). In our study we found the Schwarz and CAM-70 vaccine strains to be salt-dependent while all of the clones also hemagglutinated under isotonic conditions. The relationship between infectivity and hemagglutination titres showed quantitative differences between the clones. The difference between the vaccine strains and the clones and between clones under isotonic and hypertonic conditions was striking. The demonstration of hemagglutination and salt-dependent agglutination in non-attenuated strains as well as in vaccine strains indicates that the type of hemagglutination cannot be considered as an exclusive attenuation marker.

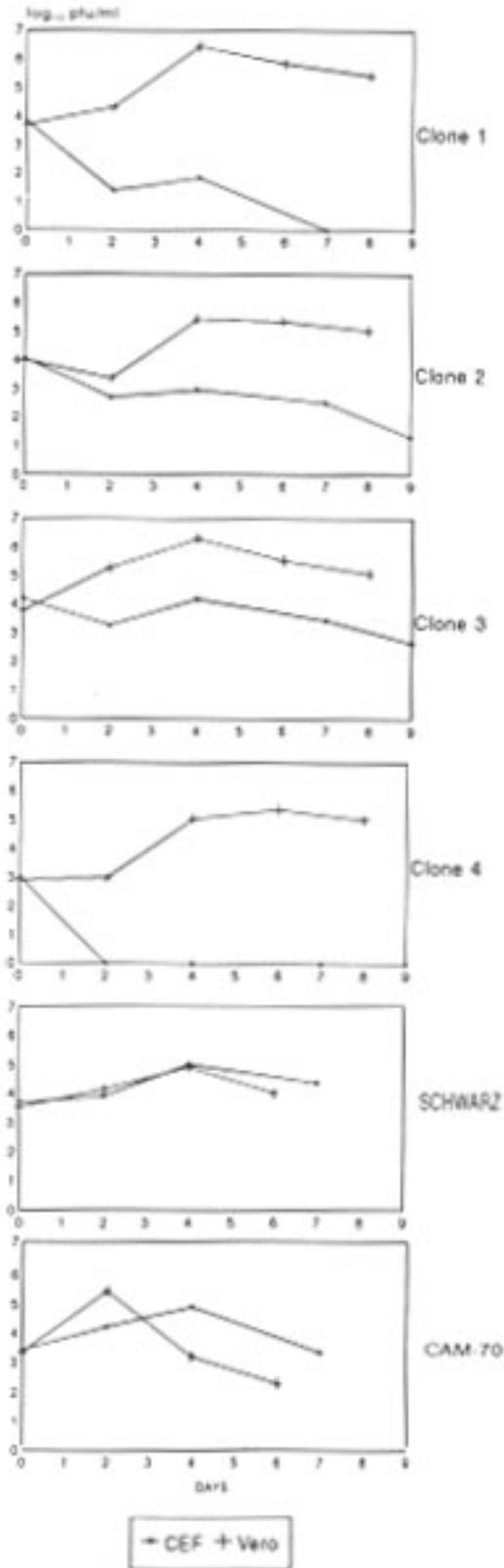


Fig. 3: viral replication in chick embryo fibroblasts and Vero cells. Relationship between infectious titre and days post-infection.

Hemolysis mediated by the measles virus fusion protein requires the participation of the hemagglutinin (Wild et al. 1991, Malvoisin & Wild 1993). The vaccine strains Schwarz and CAM-70 did not produce hemolysis even in hypertonic conditions. The relationship between infectious and hemolytic titres may prove useful for the evaluation of quantitative differences between viruses. Hemolysis by a strain of measles virus having a salt-dependent hemagglutination has been described (Breschkin et al. 1977). On the other hand, the presence of hemagglutinin and absence of hemolysis has also been demonstrated (Norrby & Falksveden 1964, Haspel et al. 1975). Fifty percent hemolysis were obtained in dilutions $10^{-3.3}$ for clone 1, $10^{-2.4}$ for clone 2, $10^{-1.6}$ for clone 3 and $10^{-2.16}$ for clone 4. Allison (1985), using the Edmonston-Zagreb strain, obtained 50% hemolysis with dilution $10^{-0.2}$. Sinitsyna et al. (1990), have suggested that the decrease in immunogenicity of overattenuated vaccines is related to a reduction in the expression of the fusion protein. It is possible, therefore, that a high number of passages and a corresponding reduction in the expression of the F protein might be reflected in the decrease or absence of hemolytic activity. Despite the apparent lower hemolytic activity of the Edmonston-Zagreb strain in relation to the clones, it was nevertheless demonstrable. This result is relevant since the Edmonston-Zagreb strain was attenuated by 108 passages, and only 20% of those were performed in chick cells. These observations strengthen the hypothesis that the number of passages, particularly in CEF, affects the expression of the F protein.

Non-egg adapted viral strains replicate poorly or not at all in chick cells (Buynak et al. 1962). Similarly, the propagation in chick embryo fibroblasts was selected as the only marker which could distinguish vaccine from virulent strains (Schumacher et al. 1972). It is not clear whether the adaptation of the virus to chick cells is mediated by the presence of variants in the virus population which are selected through an alternative attachment and penetration pathway or if it is mediated by mutations accumulated during viral passages (Dörig et al. 1993, Dunster et al. 1995). Since RNA virus genomes show high mutation frequencies, the adaptation to specific host cells can possibly be related to nucleotide changes in the RNA molecules (Holland et al. 1982). For measles virus the molecular biological changes underlying this process remain unclear.

Our results confirm the ability of the Schwarz and CAM-70 vaccine strains to propagate in CEF, and a similar ability was observed in Vero cells. All the clones showed efficient replication in Vero

cells. Based on the titres obtained for the clones grown in CEF, clones 2 and 3 showed efficient replication since both had detectable titres on day 9 p.i. Clone 1 showed intermediate replication efficiency with lower titres persisting to day 4 and no detectable virus on day 7. Clone 4 showed no replication with results compatible with virus inactivation. Two of the clones showed characteristics similar to vaccine strains: clone 2 in terms of size and homogeneity of plaques, clone 3 in terms of hemolytic activity and both for the efficiency of propagation in CEF.

In our study the fact that all the plaque isolates do not completely share the biological features of the vaccine viruses can suggest that, if present, the attenuated phenotype was represented only in a very small proportion of the population and could not be selected by plaque picks. Also, repeated passages which were not performed in the Edmonston samples, could have favored mutations responsible for attenuation.

While none of the biological variations of measles strains have yet been explained on the basis of nucleotide sequence changes, further studies on the adaptation to chick cells and comparative genome sequencing can lead us to the identification of those sequences responsible for attenuation and contribute to the improvement of current vaccines.

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