Large-Scale Purification of Rubella Virus and Preparation of an Experimental Split Rubella Virus Vaccine

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Large-scale purification of rubella virus from tissue culture fluid and preparation of an experimental rubella subunit vaccine are described. The virus, purified isopycnically in sucrose gradients, was Tween 80-ether treated, and the product was filtered sterile. The vaccines were of two types, formalinized and nonformalinized. They were not infectious, had only a slight pyrogenicity, and caused no harmful symptoms in animals. The immune response in guinea pigs was better when the nonformalinized vaccine was used and the dosage was 2,500 hemagglutinating units per injection (0.5 ml).

“Live” attenuated virus vaccines have been developed for the prophylaxis of rubella, and large-scale vaccinations have been started in several countries. These vaccines give a good seroconversion rate and protect the vaccinee against the clinical disease. But the vaccinees may become reinfected without clinical symptoms; they then excrete the vaccine virus from the respiratory tract and may even cause the transmission of vaccine virus to a susceptible contact (12, 14, 19, 23, 27). In addition, rubella vaccine virus has been recovered from the uterine cervix and the products of conception (22, 27).

Only a few attempts to develop “killed” rubella virus vaccines have been made. Some of the preparations seemed to induce both antibody formation (3, 6) and give protection (24), but it has also been reported that one purified and concentrated killed vaccine, although it induced antibody formation, failed to protect against challenges with live virus (4, 14).

The poor protection from serological reinfec-
tion (30) and the risk of inadvertent vaccination of pregnant women with “live” rubella virus vaccine focus more interest on an effective “killed” virus vaccine that could possibly induce higher levels of rubella antibodies.

The present report describes large-scale purification of rubella virus by continuous-flow gradient centrifugation and the preparation of an experimental split rubella virus vaccine.

MATERIALS AND METHODS

Virus production. The RA 27/3 strain of rubella virus was grown in BHK-21/13S suspended cell cultures (28). The virus-containing medium was clarified by low-speed centrifugation, its pH was adjusted to 7.3 with NaHCO₃, and it was either frozen rapidly to −70°C for storage or immediately processed.

Hemagglutination (HA) and plaque assay in BHK-21/WI-2 cells and preparation of electron micrographs have been described earlier (28), as has the treatment with Tween 80 and ether (26).

Continuous-flow rotor B-XVI. Initially we had some difficulties in operating the rotor because of incomplete information from the manufacturer, Beckman Instruments, Inc., Spinc Division, Palo Alto, Calif. A major problem was the original upper seal housing, which was not designed to stand the high velocity of the rotor and its resonance vibrations. Thus, one of the primary demands was the adjustable seal housing (no. 332393).

During operation, the cooling water pressure was kept at 15 to 20 psi. The feeding pressure into the rotor varied between a suction of 7 psi and a pressure of 20 psi.

Gradient. Linear 0 to 55% (w/w) sucrose gradients were in phosphate-buffered saline (pH 7.3) containing 0.003 M ethylenediaminetetraacetic acid (EDTA; reference 28) and stabilizing protein. The gradient was exhausted with 65% (w/w) sucrose in water. The densities were determined refractometrically with an Abbé refractometer.

Virus purification and subunit vaccine production. Before purification, EDTA (0.003 M) was added and the pH was adjusted to 7.3 with NaHCO₃. The fluid was clarified by low-speed centrifugation (Sorvall, GSA-rotor, 8,000 rev/min, 15 min) and then pumped through the B-XVI rotor at a flow rate of 2,000 ml/hr at 39,000 rev/min and 4°C. After an equilibration time of 2 hr, the gradient was exhausted and collected in 20-ml fractions and the peak fractions (monitored by the HA test) were pooled.

Sucrose was removed by dialysis and lipid by
2Log HA/ml

\[ \begin{array}{c}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 \\
1.10 & 1.12 & 1.14 & 1.16 & 1.18 & 1.20 & 1.22 & 1.24 \\
\end{array} \]

Fig. 1. Sedimentation of rubella virus in sucrose gradient for 2 hr. The two lines represent the two experiments that differ most from the mean sedimentation pattern.

Tween 80-ether treatment. Thereafter, the liquid was sterilized (Millipore filter, 220 nm).

The sterile product was diluted in gradient buffer to 5,000 or 500 hemagglutination units (HAU)/ml, with a final concentration of 1.5 mg of AlPO₄ per ml to form the experimental split rubella virus vaccine. We also made an experimental vaccine which was treated for 4 days in 1:6,000 formaldehyde at 4°C before sterile filtration.

Pyrogen tests. To test the pyrogenicity, 1 ml of vaccine was injected intravenously into rabbits, and the rectal temperature was followed for 3 hr.

Immunization test. Guinea pigs were immunized by injecting 0.5 ml of vaccine three times fortnightly. Blood samples were taken at time zero, at the time of injection, and 1 week after the last injection and were tested for hemagglutination-inhibition (HI) antibodies (11).

RESULTS

Buoyant density of the virus. The virus formed a relatively sharp band in the B-XVI rotor gradients at densities ranging from 1.165 to 1.200 g/cm³ (Fig. 1). In our experiments, the average buoyant density was 1.175 to 1.180 g/cm³, which is in accordance with previous observations (18, 28) for the buoyant density of rubella virus in sucrose. Usually a 2-hr equilibration period was used. The whole purification procedure could then be accomplished in 6 hr. With longer equilibration times (Fig. 2), the virus band tended to be sharper but its position was not appreciably altered. In practice, 2 hr was sufficient and between 85 to 90% of the virus in the gradient could be collected in four to five fractions (80 to 100 ml). Similar buoyant densities were obtained when the virus was purified from suspension media or monolayer cultures of BHK-21 cells. Freezing and thawing of the virus-containing medium before purification had no detectable effect.

The peak of infectivity coincided with the HA activity peak. In a typical experiment (Fig. 3), the total input infectivity was 10¹³-¹⁴ plaque-forming units (PFU) and the main peak had a total infectivity of 10¹⁰-¹¹ PFU, which is about 60% of the input of infectious virus. The concentration factor when calculated from all peak fractions is 10, and from the top peak fraction it is about 100. The PFU/HAU ratio of the top fraction was 2Log HAU/ml.

FIG. 2. Effect of the equilibration time on the sedimentation of rubella virus in sucrose gradients. Dashed line, 2 hr; solid line, 4 hr.
PURIFICATION OF RUBELLA VIRUS

10^9.42/10^8.31 = 10^9.11, a value comparable with previous reports (28).

Effect of flow rate. The determination of the recovery rate of the virus from the gradient was often disturbed by HA inhibitors (8) in the serum-containing medium. However, purification experiments on high-virus titer lots (32 to 128 HAU/ml) indicated that with flow rates up to 2.5 liters/hr less than 10% of the virus input was not recovered from the gradient.

Infectivity titrations of virus in the input, gradient, and outflow media also indicated that with flow rates of 2 to 2.5 liters/hr about two-thirds of the input infectivity may be collected in the gradient peak and less than 10% flows through the rotor.

Properties of the experimental rubella vaccine. Electron micrographs (Fig. 4 and 5) show that preparations purified by the B-XVI rotor mainly contain typical rubella virus particles, 60 to 70 nm in diameter (28), and small amounts of amorphous material of variable size. Some of the particles were deformed or partially disintegrated.

Tween 80-ether treatment removed all virus infectivity and caused a slight increase in HA activity. In electron microscopy, no virus particles or derivatives could be identified among the stabilizing protein present in the preparation.

The rubella subunit preparation passed the usual safety tests conducted on inactivated influenza virus vaccines (tests for infectious virus negative, sterility tests negative, and lack of toxicity in growing mice). The pyrogenicity was minimal: a temperature rise of only 0.3°C was found in rabbits.

We tested the immune responses of guinea pigs to a formalinized and a nonformalinized vaccine. The doses were either 250 HAU per injection (0.5 ml) or 2,500 HAU per injection (Fig. 6 and 7).

FIG. 3. Comparison of the sedimentation of infective rubella virus and HA-active particles in sucrose gradients. Dashed line, infectivity (PFU/ml); solid line, HA activity (HAU/ml).

FIG. 4. Rubella virus particles (purified from the stabilizing protein) taken from the top of the HA activity peak from a sucrose gradient. ×100,000.

FIG. 5. Tween 80-ether-treated rubella virus preparation with stabilizing protein. ×100,000. Bar, 100 nm.
nucleic acid and core protein from the envelope are not feasible at present.

Our split rubella virus vaccine causes a relatively large increase in antibodies (nonformalized, 2,500 HAU/injection) in guinea pigs. It is of the same magnitude as the antibody increase in natural human rubella infections (19). The possible role of HI antibody quantity in the prevention of reinfection has been reported (14, 30).

The vaccines were prepared in BHK-21 cell cultures, an oncogenic hamster kidney cell line (25) not suitable for human vaccine production. In acceptable cell systems (primary cell cultures or diploid cell lines), rubella virus grows to lower titers; e.g., in WI-38 cell cultures about 10^4.5 to 10^5 PFU/ml were achieved. This was about 5 to 10 times lower than in the BHK-21/13S suspension culture system.

The altered reactivity to measles and respiratory syncytial virus infections in vaccinees after...
"killed" measles and respiratory syncytial vaccines is probably associated with virus antibody complexes or alum-containing preparations (7, 9, 10, 16, 21). The same phenomenon might be expected in the case of rubella virus vaccine. Thus, adjuvants other than AlPO₄ should be tested for subunit-type vaccines.

Purification of rubella virus or other viruses growing in low titers necessarily involves large volumes. Volume concentration of rubella with organic solvents or salts is too drastic a procedure. Also recovery of infectivity of pelleted rubella virus has been low. The present continuous-flow gradient system (1, 2, 5) seems to be a gentle means for both rapid concentration and purification in one step. In the peak fractions, a concentration factor of 30 to 40 can be obtained and the electron micrographs suggest a relatively high degree of purity.

Provided a proper cell culture system has been developed for rubella virus production in high titers, the present method should give a convenient and effective procedure for subunit vaccine production.

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LITERATURE CITED

1. Anderson, N. G. 1963. Virus isolation in the zona ultracentrifuge. Nature (London) 199:1166–1168.
2. Anderson, N. G., H. P. Barringer, J. W. Amburagey, Jr., G. B. Cline, C. E. Nunley and A. S. Berman. 1966. Continuous-flow centrifugation combined with isopycnic banding: rotors B-VIII and B-IX. Nat. Cancer Inst. Monogr. 21:199–216.
3. Beck, E. S. 1969. Review of studies with inactivated rubella virus. Amer. J. Dis. Child. 118:328–333.
4. Buynak, E. B., M. R. Hilleman, R. E. Weibel and J. Stokes. 1968. Live attenuated rubella virus vaccines prepared in duck embryo cell culture I. Development and clinical testing. J. Amer. Med. Ass. 244:103–108.
5. Cline, G. B., L. E. Nunley, and N. G. Anderson. 1966. Improved continuous flow centrifugation with banding. Nature (London) 212:487–489.
6. Frankel, J. W. 1964. Neutralizing antibody responses of guinea-pigs to inactivated rubella virus vaccine. Nature (London) 204:655–656.
7. Fulginiti, V. A., J. H. Arthur, D. S. Pearlmam, and C. H. Kempe. 1968. Altered reactivity to measles virus. Amer. J. Dis. Child. 115:671–676.
8. Furukawa, T., S. A. Plotkin, D. Sedwick, and M. L. Profeta. 1967. Hemagglutinin of rubella virus. Nature (London) 211:172–173.
9. Gardner, P. S., J. McQuillen, and S. D. M. Court. 1970. Speculation of pathogenesis in death from respiratory syncytial virus infection. Brit. Med. J. 1:327–330.
10. Gokiert, J. G., and W. E. Beamish. 1970. Altered reactivity to measles virus in previously vaccinated children. Can. Med. Ass. J. 183:724–727.
11. Halonen, P. E., J. A. Stewart, and A. D. Hall. 1967. Rubella hemagglutinin prepared in serum free suspension culture of BHK-21 cells. Annu. Med. Exp. Biol. Binn. 45:182–183.
12. Hilleman, M. R., E. B. Buynak, J. E. Whitman, R. W. Weibel and J. Stokes. 1969. Live attenuated rubella virus vaccines. Amer. J. Dis. Child. 118:166–171.
13. Horstmann, D. M., H. Liebhaber, and E. I. Kohorn. 1970. Post-partum vaccination of rubella-susceptible women. Lancet 2:1003–1006.
14. Horstmann, D. M., H. Liebhaber, G. L. LeBouvier, D. A. Rosenberg, and S. B. Halstead. 1970. Rubella: reinfection of vaccinated and naturally immune persons exposed in an epidemic. N. Engl. J. Med. 283:771–778.
15. Hovi, T., and A. Valveri. 1970. Infectivity and some physico-chemical characteristics of rubella virus ribonuclease acid. Virology 42:1–8.
16. Lancet. 1969. Vaccine against respiratory syncytial virus. Vol. 2:311.
17. Laufs, R., and R. Thomsen. 1968. Eigenschaften eines mit Tween 80 und Äthylyther behandelten Rubella-virus-Hämaggulutins. Arch. Gesamte Virusforsch. 24:164–180.
18. McCombs, R. M., and W. E. Rawls. 1968. Density gradient centrifugation of rubella virus. J. Virol. 2:49–414.
19. Meyer, H. M. Jr., P. D. Parkman, T. E. Hobbs and F. A. Ennis. 1968. Clinical studies with experimental live rubella virus vaccine (strain HPV-77). Amer. J. Dis. Child. 115:648–654.
20. Norrby, E. 1963. Hämaggulutination by measles virus. III. Identification of two different hemagglutinins. Virology 19:147–157.
21. Norrby, E., R. Lergercrantz, and S. Gard. 1965. Measles vaccination IV. Responses to different types of preparations given as a fourth dose of vaccine. Brit. Med. J. 1:813–817.
22. Phillips, C. A., J. V. S. Maek, W. A. Rogers, and H. Savel. 1970. Intratracheal rubella infection following immunization with rubella vaccine. J. Amer. Med. Ass. 213:624–625.
23. Schiff, G. M., R. Donath and T. Rotte. 1969. Experimental rubella studies. Amer. J. Dis. Child. 118:269–274.
24. Sever, J. L., G. M. Schiff and R. J. Huebner. 1963. Inactivated rubella virus vaccine. J. Lab. Clin. Med. 62:1015.
25. Stoker, M., and I. MacPherson. 1964. Syrian hamster fibroblast cell line BHK-21 and its derivatives. Nature (London) 202:1355–1356.
26. Valveri, A., K. Penttinen, P. Viäininen and C.-H. von Bonsdorff. 1970. Hemagglutination activity and morphology of influenza virus. Arch. Environ. Health 21:328–331.
27. Valveri, A., T. Vesikari, N. Oker-Blom, M. Seppläli, J. Veronelli, F. C. Robbins, and P. D. Parkman. 1969. Transmission of attenuated rubella vaccines to the human fetus. Amer. J. Dis. Child. 118:243–246.
28. Valveri, A., C.-H. von Bonsdorff, T. Vesikari, T. Hovi and P. Viäininen. 1969. Purification of rubella virus particles. J. Gen. Virol. 5:39–46.
29. Waterson, A. P., and R. Rott. 1963. The components of measles virus and their relation to rinderpest and distemper. Z. Naturforsch. 18B: 377–384.
30. Wilkins, J., J. M. Leedoom, B. Portnoy, and M. A. Salvatove. 1969. Reinfection with rubella virus despite live vaccine induced immunity. Amer. J. Dis. Child. 118:275–294.