BIKEN JOURNAL Vol. 11, 235-241, 1968

STUDIES ON LIVE RUBELLA VACCINE

I. PROPAGATION OF RUBELLA VIRUS IN DEVELOPING CHICK EMBRYOS AND ITS LABORATORY CHARACTERIZATION

YOSHIOMI OKUNO, YOSHIICHI MINEKAWA, NORIMOTO SUZUKI and MICHIAKI TAKAHASHI

Department of Virology, Research Institute for Microbial Diseases, Osaka University, Yamada-Kami, Suita, Osaka

(Received September 21, 1968)

SUMMARY A newly isolated strain of rubella virus was serially cultivated for at least 11 passages in the amniotic cavity of developing chick embryos. The strain adapted to hen eggs differs in sensitivity to temperature from the original strain which has only been passaged several times in GMK (primary African green monkey kidney) cells.

After one passage in HK (primary human embryonic kidney) cells, the strain was inoculated into cynomolgus monkeys at the 6th and 11th passage levels and at both levels it produced high NT (neutralization) and HI (hemagglutination-inhibition) antibody responses without causing any apparent symptoms, although at both passage levels a little virus was recovered from the throat of some monkeys.

INTRODUCTION

Although rubella had been regarded as a mild exanthematous viral disease (HIRO *et al.*, 1938) of children, much attention has been payed to its specific teratogenic character since the discovery by Gregg (GREGG, 1941) that it caused the so-called congenital rubella syndrome.

But it was not until 1962 that its etiological agent was first isolated (WELLER *et al.*, 1962 and PARKMAN *et al.*, 1962) using an interference method (OKUNO *et al.*, 1956). Thereafter, studies on biological characteristics of the virus have rapidly progressed and development of a safe and effective vaccine has been generally desired and intensively investigated (PLOTKIN *et al.*, 1965, PARKMAN *et al.*, 1966, MEYER *et al.*, 1966, HILLEMAN *et al.*, 1966, MEYER *et al.*, 1968 and DU PAN *et al.*, 1968), especially after the extensive epidemic in U.S.A. in 1964 (SEVER *et al.*, 1965) and the birth of many congenitally malformed children (RUDOLPH *et al.*, 1965).

But appropriate tissue culture cells for its serial passage for attenuation cannot be easily found because its host range is relatively limited. We have attempted to adapt newly isolated strains to growth in developing chick embryos.

MATERIALS AND METHODS

1. *Virus and cells*

a) Newly isolated rubella virus

In the spring of 1966 when a relatively large scale

epidemic occurred in the Osaka district 3 interfering agents could be isolated from the throat swab specimens of patients clinically diagnosed as having rubella using the interference method in GMK cells. These agents were identified as rubella virus (RV) by the neutralization test with anti-Baylor strain hyperimmune rabbit serum.

For inoculation into developing chick embryos the newly isolated strains were used at the 6th to 19th passage levels in GMK cells at 36°C. GMK cells were used for serial passages of the strains at the second and later transfer levels to avoid contamination with simian viruses.

b) Rubella virus Baylor strain

This was supplied by Dr. K. Kawakami, Department of Pediatrics of Osaka Medical College, and passaged at 36°C in BHK21/WI2 cells.[1]

c) ECHO virus type 11 (ECHO-11)

The seed virus was supplied by Dr. T. Yamada, Virus Laboratory of Osaka Public Health Institute, and passaged and titrated in GMK cells at 36°C.

2. *Rubella virus titration*

This was carried out by the interference method (PARKMAN *et al.*, 1962) in GMK cells. One hundred TCD_{50} (50% tissue culture infectious dosis) of ECHO-11 per tube were challenged about 10 days after inoculation. The virus titer was expressed as the 50% interference dosis (InD_{50}) per 0.1 ml.

3. *Cultivation of RV in developing chick embryos*

a) Cultivation in the amniotic cavity

Fertilized domestic white Leghorn eggs of 7–8 days old were used for rubella virus cultivation. They were inoculated with 0.1 ml of virus material and incubated at 32°C for 7 days. Then the amnion was extracted and each membrane was suspended in 3 ml of PBS(-) (phosphate buffered saline without divalent cations) containing 0.1% w/v gelatine (final dilution) and homogenized at 18,000 rpm for 3 min. After centrifugation at 3,000 rpm for 15 min. at 4°C the supernatant was used for the next passage in fertile eggs.

b) Cultivation on chorioallantoic membrane

The modified method of Burnet (SCOTT, 1956) was used. The inoculum, the incubation conditions and the method for harvesting were the same as those described above.

4. *Vaccine preparation*

Two substrains of the newly isolated Matsuura strain were inoculated into HK cells after 13 serial passages in GMK cells, 2 passages in developing chick embryos, one passage in GMK cells and again 4 and 9 passages, respectively, in developing chick embryos (Fig. 3). After about 10 days incubation at 35°C, they were harvested by 3 cycles of freezing and thawing in medium 199 containing 0.1% gelatin as a stabilizer, 50 γ/ml of Kanamycin, and 50 γ/ml of Streptomycin but no serum. After centrifugation at 3,000 rpm for 30 min. at 4°C, the supernatants were used as the two vaccine strains (designated as ME6 and ME11) at total passage levels in developing chick embryos of 6 and 11. Their virus titers were 2.75 and 2.0 log InD_{50}/0.1 ml, respectively.

The purpose of passage of the vaccine strains in HK cells was to eliminate possible contaminating RIF (resistance inducing factor) (RUBIN, 1960). The COFAL (complement fixation of avian leucosis) test (HUEBNER *et al.*, 1964) became negative after passage in HK cells.

5. *Neutralization test (NT)*

Volumes of 0.4 ml of serial 2-fold dilutions of serum were mixed with an equal volume of 100 InD_{50}/0.1 ml of Baylor strain and allowed to react at 36°C for 1 hour. Then 0.2 ml of each mixture was inoculated into 2 tubes of GMK cells and allowed to be adsorbed to the cells at 36°C for 1 hour. After addition of maintenance medium incubation was continued at 36°C. On the 6th day, the supernatant fluid was replaced by fresh medium containing 100 TCD_{50} of ECHO-11 for challenge. Three or 4 days later when the CPE (cytopathic effect) caused by ECHO-11 virus in the control cells was complete, interference was evaluated and the tube without interference was judged as neutralization positive.

6. *Hemagglutination-inhibition test (HI)*

This was performed according to the modified method of STEWART *et al.* (1967) as shown in Fig. 1. Red blood cells (rbc) of the Japanese quail were used in the hemagglutination test (HA).

7. *Temperature sensitivity test of RV*

Vaccine strain ME6, Baylor strain adapted to BHK cells and Matsuura strain at the 4th passage in GMK cells (M4) were inoculated into samples of the same batch of GMK cells. After 10 days incubation at 36°C they were harvested by 3 cycles of freezing and thawing in serum-free medium 199 and the supernatants were centrifuged at 3,000 rpm

for 15 min. at 4°C and put into small test tubes. They were used as samples of seed virus for testing thermostability at 50°C.

8. *Cynomolgus monkey inoculation experiments*

a) Inoculation

First the monkeys were tested to check that they had no antibody for rubella. Then 4 monkeys were inoculated with vaccine strain ME6 and about 2 weeks later another 4 monkeys were inoculated with ME11. Inoculation was done by injection with a single dose of 0.5 ml into the femoral muscle, and a daily check was made of the general status and body temperature of the animals.

b) Antibody response after inoculation

Blood was taken from the femoral vein on appropriate days after inoculation, and the HI and NT antibody titers in the sera were measured.

c) Virus recovery test

Throat swab specimens were taken from the monkeys from the 7th to 16th days after inoculation and treated by usual methods for virus isolation using GMK cells. The material which showed no interference with ECHO-11 during 3 passages in GMK cells was judged as showing negative recovery.

RESULTS

1. *Propagation in developing chick embryos*

As a preliminary study, one newly isolated Kashihara strain at the 6th GMK cell passage was inoculated into the amniotic cavity of a 7 days old developing chick embryo. After 7 days incubation the amnion was homogenized and found to have a slight interference dosis in GMK cells.

Next the RV yields from various parts of developing chick embryos incubated at various temperatures were investigated. It was likely that almost all the viruses were associated with the amnion and did not appear in the amniotic fluid. Cultivation was better at 30°C and 32°C than at 35°C, but the death rate of eggs after inoculation was higher at 30°C.

Fig. 2 represents the growth curve of RV in the chick amnion which reached a peak after 6 days incubation. Exp. 1 was performed by inoculating RV into eggs of the same age (7 days old) and harvesting the amnions on different days, and Exp. 2 by inoculating RV into eggs of different ages (7 to 10 days old) and harvesting on the same day (from 10 to 16 days old eggs). The two experiments show similar curves and there is no difference in the sensitivity of eggs of different ages to RV.

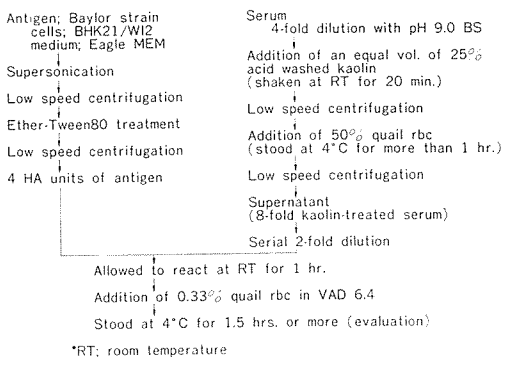
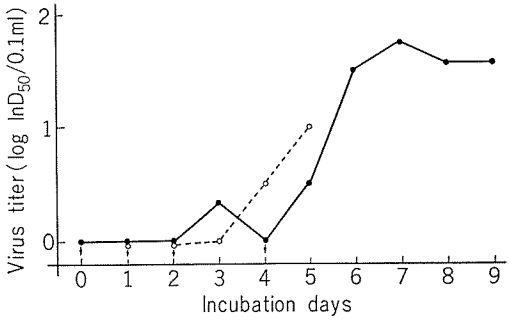


FIGURE 1

The virus titers given in Fig. 2 are those at the 1st passage level in developing chick embryos. They gradually decreased through the 2nd and 3rd passages and disappeared completely during the 4th passage. The same results were obtained with a different strain, Matsuura, and substrains at different passage levels in GMK cells.



FUGURE 2 Growth of Rubella Virus in Chick Amnion at 30°C

The Kashihara strain was used at the 6th GMK cell passage. (Virus titer: 2.83 log InD_{50}/0.1 ml) Each point shows the mean value of about 10 pooled amnions.

○------○ Exp. 1

●——● Exp. 2

The chick embryo adapted strain could be obtained by the passage method shown in Fig. 3. The Matsuura strain at the 13th passage in GMK cells was cultivated once in GMK cells after 2 passages in developing chick embryos. In the subsequent serial passages in chick amnion, the virus titer was maintained constant. Virus titers varied from 0.5 to 2.67 log InD_{50}/0.1 ml throughout these passages. The interfering agent was confirmed at intervals of 2 or 3 passages to be RV by NT with hyperimmune rabbit serum.

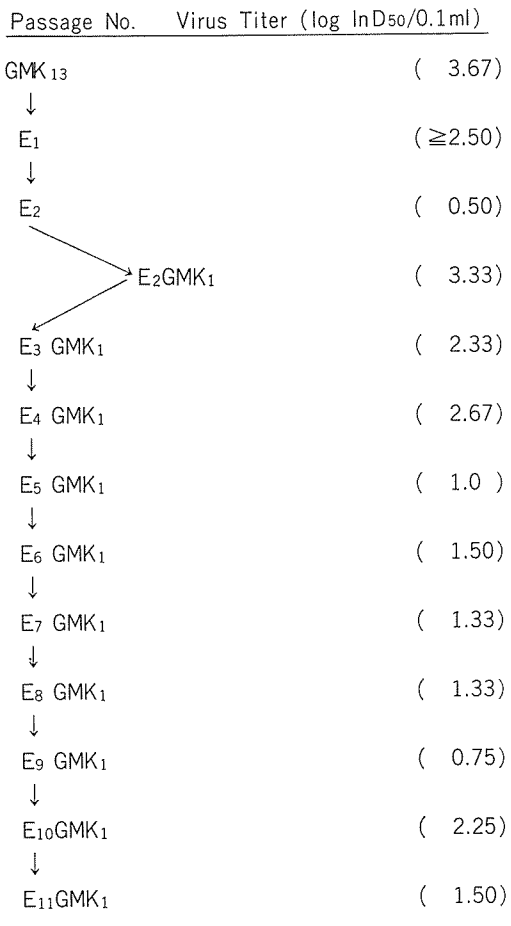


FIGURE 3 Serial Passage of RV Matsuura Strain in Developing Chick Embryos at 32°C

2. *Temperature sensitivity of ME6*

Two experiments were performed to investigate the temperature sensitivity of the vaccine strain ME6. As controls Baylor strain adapted to BHK cells and M4 which seemed comparatively near to the wild type strain were used. The results of the thermostability test at 50°C are shown in Fig. 4. Contrary to expectation, the Baylor strain was the most sensitive followed by ME6. These two strains were obviously different from M4. The second experiment was on growth rate in GMK cells at various temperatures. No clear differences between the three strains were seen. The Baylor strain seems to be able to multiply at a relatively wide range of temperatures.

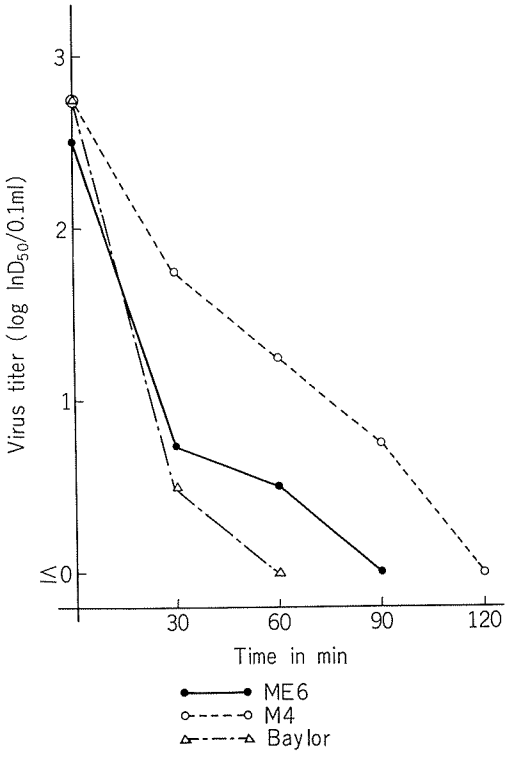


FIGURE 4 Thermostability Test at 50°C

3. *Inoculation of vaccine strains into cynomolgus monkeys*

It was confirmed by the HI method that the

monkeys purchased 2 weeks previously had no antibody for rubella. The general conditions of the monkeys were observed daily after inoculation, but they showed no particular signs such as exanthema, enanthema, or catarrh. No specific febrile curve could be obtained by rectal thermal examination. However, they showed good antibody responses to both ME6 and ME11 (Figs. 5 and 6). In the group inoculated with ME6 both the HI and NT titers were found to have risen on the 11th day after inoculation, and in the group inoculated with ME11 one monkey showed an HI antibody responses on the 10th day and all of the monkeys showed both HI and NT responses on the 13th day.

Both antibody titers were maximal on the 21st day, then decreased rather abruptly to the 28th day, and subsequently remained steady until at least the 8th week. There were almost no differences between the responses of HI and NT. In the group inoculated with ME6 the antibody titers began to rise earlier and reached higher levels but decreased more rapidly than in the group inoculated with ME11. There was no difference in the titers in the 7th to 8th week.

The virus recovery from the monkeys is summarized in Table 1. Some virus was secreted in the throat on the 8th day in 2 monkeys inoculated with ME6 and on the 10th day in one inoculated with ME11. The reisolation materials became interference positive from the 1st passage on in the ME6 group and from 2nd passage on in the ME11 group.

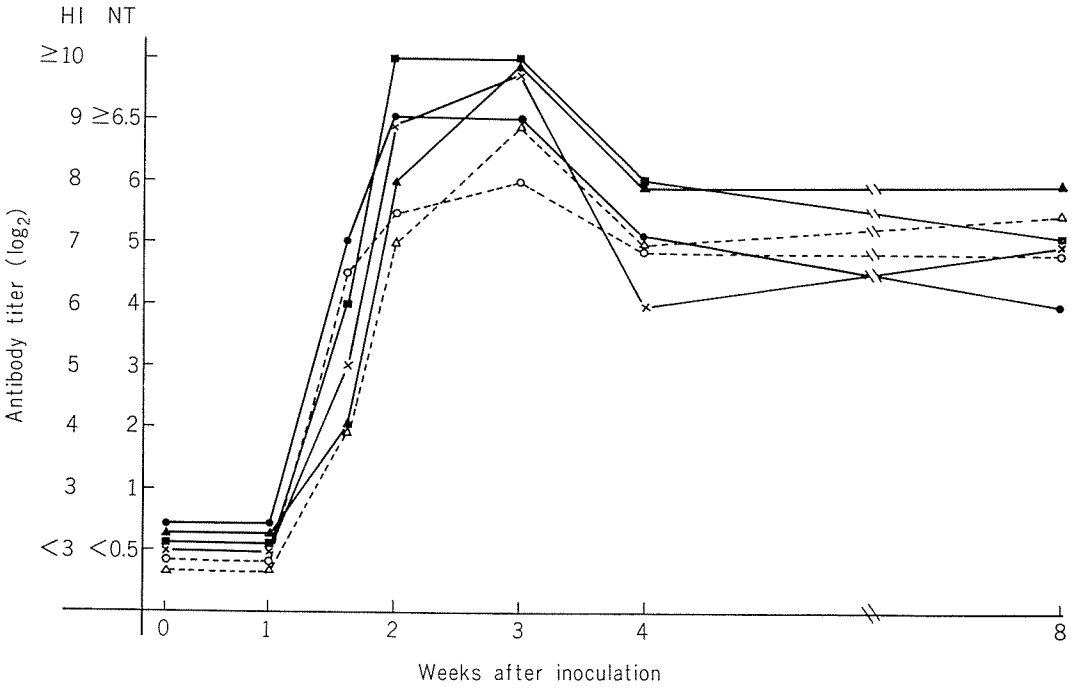


FIGURE 5 HI and NT Antibody Responses in Cynomolgus Monkeys Inoculated with Vaccine Strain ME6.
●——● No 1, HI ○------○ No 1, NT
▲——▲ No 2, HI △------△ No 2, NT
■——■ No 3, HI
×——× No 4, HI

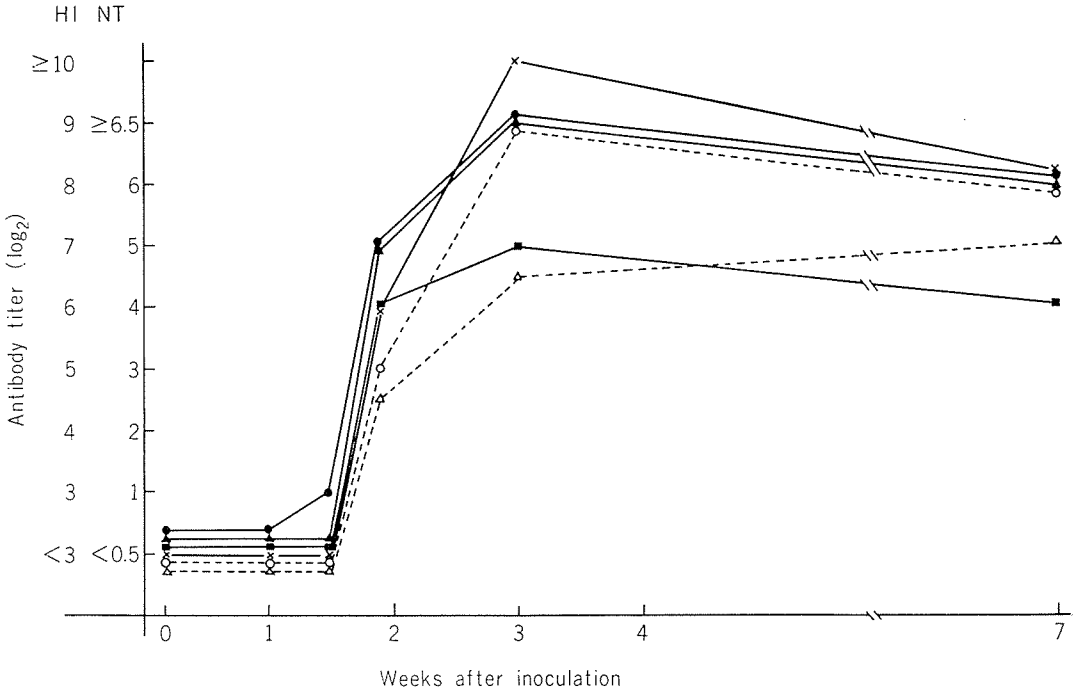


FIGURE 6 HI and NT Antibody Responses in Cynomolgus Monkeys Inoculated with Vaccine Strain MEII.
●——● No 5, HI ○------○ No 5, NT
▲——▲ No 6, HI △------△ No 6, NT
■——■ No 7, HI
×——× No 8, HI

TABLE 1 *Virus recovery from cynomolgus monkeys inoculated with RV vaccine strains*

Vaccine	Monkey No.	Days after Inoculation										
		7	8	9	10	11	12	13	14	15	16	17
ME 6	1	NE	−	−	−	−	NE	−	−	−	−	NE
	2	NE	+	−	−	−	NE	−	−	−	−	NE
	3	NE	−	−	−	−	NE	−	−	−	−	NE
	4	NE	+	−	−	−	NE	−	−	−	−	NE
ME 11	5	−	−	−	−	NE	−	−	−	−	−	−
	6	−	−	−	−	NE	−	−	−	−	−	−
	7	−	−	−	−	NE	−	−	−	−	−	−
	8	−	−	−	+	NE	−	−	−	−	−	−

NE : not examined + : recovery positive − : recovery negative

DISCUSSION

There have been no reports on successful cultivation of RV in developing chick embryos. FUCCILLO *et al.* (1967) reported that some strains of RV could multiply in chick embryo tissue culture cells but not in chicken eggs and showed only occasional growth. We also observed that plain passage without dilution of various substrains was unsuccessful and their titers were lost completely after the 3rd or 4th passage. But it was possible to adapt RV to developing chick embryos by one passage in GMK cells after two passages in chick embryos (Fig. 3). It seems probable that the affinity of the substrains for developing chick embryos were intensified in GMK cells. The reason why a low temperature is better initially is unknown. But after the 6th passage in chick embryos (i.e. ME6), the optimum temperature changed giving 1.25 log InD_{50}/0.1 ml at 30°C and 2.0 log InD_{50}/0.1 ml at 35°C, and the difference in the titers at the two temperatures became less than originally.

The localization of RV in developing chick embryos has not yet been investigated.

It is also not known what kinds of association there are between the difference in temperature sensitivity in vitro (Fig. 4) and the virulence in vivo, namely the grade of attenuation.

When monkeys were inoculated with 0.5 ml of either ME6 or ME11 at the virus titers of 2.75 and 2.0 log InD_{50}/0.1 ml, respectively, good antibody responses were evoked. The relation between the 50% interference dosis in vitro and the 50% infectious dosis of monkeys should be examined. The antibody titers of the monkeys to both ME6 and ME11 (Figs. 5 and 6) were highest in the 3rd week after inoculation, then decreased rapidly for 1 week, and subsequently remained at quite high levels. The rapid decrease was clearer in the group inoculated with ME6 than in that inoculated with ME11, but there were almost no differences in the titers in the two groups after about 2 months. This may have some relation with the decrease in IgM (immunoglobulin M) antibodies. This should be examined by another method, together with the trends of antibody titers after re-inoculation.

As with other live vaccines, the occurrence of virus shedding from the vaccinee is not desirable, especially in the case of live rubella vaccine, when there is a possibility of contact infection of pregnant women. To test this possibility with rubella virus, recovery tests were made on throat swabs of the monkeys (Table 1). The results show that there is some virus excretion from both groups and the possibility of contact infection. However, in the group infected with ME11 at a higher passage there was a tendency toward a delay in the day of onset of virus shedding and in the GMK cell passage number at which virus recovery was detected. Therefore these problems will be overcome by further passage in developing chick embryos.

ACKNOWLEDGEMENTS

We wish to express our thanks to Dr. Y. Kurose of the Department of Pediatrics of Amagasaki Hospital and to Dr. M. Tamura, a medical practitioner in Osaka city for supply of materials from patients with rubella.

Thanks are also due to Dr. T. Kan of the Kannonji Institute of the Research Foundation for Microbial Diseases of Osaka University for conducting the RIF tests.

REFERENCES

DU PAN, R. M., HUYGELEN, C., PEETERMANS, J. and PRINZIE, A. (1968). Clinical trials with a live attenuated rubella virus vaccine. *Amer. J. Dis. Child.* **115**, 658–662.

FUCCILLO, D. A., GITNICK, G. L., TRAUB, R., WONG, K., SEVER, J. L. and HUEBNER, R. J. (1967). Cultivation of rubella virus in avian tissue cultures and embryonated eggs. *Proc. Soc. Exp. Biol. Med.* **125**, 1015–1018.

GREGG, N. M. (1941). Congenital cataract following German measles in the mother. *Trans. Ophthal. Soc. Aust.* (BMA). **3**, 35–46.

HILLEMAN, M. R. (1966). Advances in control of viral infections by nonspecific measures and by vaccines with special reference to live mumps and rubella virus vaccines. *Clin. Pharmacol. Ther.* **7**, 752–762.

HIRO, Y. and TASAKA, S. (1938). Die Röteln sind eine Viruskrankheit. *Monatsschr. Kinderh.* **76**, 328–332.

HUEBNER, R. J., ARMSTRONG, D., OKUYAN, M., SARMA, P. S. and TURNER, H. C. (1964). Specific complement-fixing viral antigens in hamster and guinea pig tumors induced by the Schmidt-Ruppin strain of avian sarcoma. *Proc. Nat. Acad. Sci.* **51**, 742–750

MEYER, H. M., PARKMAN, P. D. and PANOS, P. C. (1966). Attenuated rubella virus. II. Production of an experimental live-virus vaccine and clinical trial. *New Engl. J. Med.* **275**, 575–580.

MEYER, H. M., PARKMAN, P. D., HOBBINS, T. E. and ENNIS, F. A. (1968). Clinical studies with experimental live rubella virus vaccine (Strain HPV-77). *Amer. J. Dis. Child.* **115**, 648–654.

OKUNO, Y., AOYAMA, A., TSUJIMOTO, N., KUSUMOTO, K., HOTTA, T., SUGAI, T., ISHII, S., EBARA, S. and FUKAE, S. (1956). Diagnosis of virus propagation by the interference method. *Med. J. Osaka Univ.* **7**, 257–261.

PARKMAN, P. D., BUESCHER, E. L. and ARTENSTEIN, M. S. (1962). Recovery of rubella virus from army recruits. *Proc. Soc. Exp. Biol. Med.* **111**, 225–230.

PARKMAN, P. D., MEYER, H. M., KIRSCHSTEIN, R. L. and HOPPS, H. E. (1966). Attenuated rubella virus. I. Development and laboratory characterization. *New. Engl. J. Med.* **275**, 569–574.

PLOTKIN, S. A., CORNFELD, D. and INGALLS, T. H. (1965). Studies of immunization with living rubella virus. *Amer. J. Dis Child.* 110, 381–389.

RUBIN, H. (1960). A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. *Proc. Nat. Acad. Sci.* **46**, 1105–1119.

RUDOLPH, A. J., YOW, M. D., PHILLIPS, C. A., DESMOND, M. M., BLATTNER, R. J. and MELNICK, J. L. (1965). Transplacental rubella infection in newly born infants. *J. Amer. Med. Ass.* **191**, 843–845.

SCOTT, T. F. McN. (1956). Diagnostic procedures for virus and rickettsial diseases. Amer. Public Health Assoc. New York.

SEVER, J. L., NELSON, K. B. and GILKESON, M. R. (1965). Rubella epidemic, 1964; Effect on 6,000 pregnancies. *Amer. J. Dis. Child.* **110**, 395–407.

STEWART, G. L., PARKMAN, P. D., HOPPS, H. E., DOUGLAS, R. D., HAMILTON, J. P. and MEYER, H. M. (1967). Rubella-virus hemagglutination-inhibition test. *New Engl. J. Med.* **276**, 554–557.

WELLER, T. H. and NEVA, F. A. (1962). Propagation in tissue culture of cytopathic agents from patients with rubella-like illness. *Proc. Soc. Exp. Biol. Med.* **111**, 215–225.