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# Cultivation of Rubella Virus in Avian Tissue Cultures and Embryonated Eggs. (32264)

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Rubella virus infection during early pregnancy frequently causes major congenital malformations. Concentrated effort by a number of laboratories is now being directed toward the development of an effective live attenuated rubella vaccine. Recently Meyer and Parkman and their associates prepared a live attenuated rubella vaccine with virus grown in primary African green monkey kidney (AGMK) tissue cultures(1,2). The vaccine produced good antibody responses; however, virus was excreted by some of the vaccinees. The possibility of simian or covert virus contamination of the AGMK cultures, especially after high serial passage, suggests that this tissue culture system may be less desirable for parenteral administration of live virus preparations. Furthermore, little data are available on the effects of parenteral administration of non-inactivated AGMK material. For this reason studies were undertaken, by our laboratory and others, to adapt rubella virus to RIF-free avian tissue culture systems and embryonated eggs. Several of these host systems have received extensive clinical and laboratory evaluation in studies of parenterally administered live yellow fever and rubeola vaccines and have been found free of known oncogenic agents.

Recently Hilleman reported on the development of a live rubella vaccine grown in duck embryo tissue culture(3,4). Preliminary studies indicated antibody response occurred in all recipients. Virus shedding was infrequent, if present. Details of the methods and conditions of cultuvation used in these studies have not been reported.

The present paper describes our experience in the serial cultivation of 9 strains of virulent or attenuated rubella virus in duck and RIF-free chick tissue cultures and embryonated eggs.

*Materials and methods.* Nine strains or different passages of virulent or attenuated rubella virus were selected for these investigations. The passage histories and temperatures of cultivation of each strain are given in Table I. The RV strain was originally obtained in a throat swab from a 6-year-old

TABLE I. Rubella Virus Seeds—Passage History.

| | |
|---|---|
| 1. | RV $AGMK_{50}$ (37°)* |
| 2. | RV $AGMK_{37}$ ($37°_{14}$, TD† $37°_{23}$) |
| 3. | RV $AGMK_{37}$ ($37°_{14}$, $35°_{23}$) |
| 4. | RV $AGMK_{33}$ (37°) |
| 5. | RV $AGMK_{29}$ ($37°_{14}$, $35°_{5}$, $32°_{10}$) |
| 6. | RV $AGMK_{18}$ (37°) |
| 7. | HPV $AGMK_{78}$ (35°) |
| 8. | Brown $AGMK_6$ (37°) |
| 9. | Friedman (Gargle) |

* AGMK, primary African green monkey kidney tissue culture. 50, 50th passage. 37°, passages made at 37°C.

† TD, terminal dilution at 15th passage.

child(5). Lower passages in AGMK tissue have been fully virulent for man(5,6). Higher passages of the RV strain may be attenuated since intranasal inoculation of volunteers with 14th passage virus did not result in infection or clinical disease. The HPV vaccine strain was received from Drs. Meyer and Parkman at the 77th passage and originated from the M-33 strain(1). The Friedman gargle and Brown strain were obtained from adults with rubella. Cultivation in tissue culture was carried out at 28°C, 32°C, 35°C, and 37°C. Stationary and rolled cultures were passed at intervals of 4, 7, 10, and 14 days. Serial passages were continued for a minimum of 10 passages.

Primary cell cultures of duck embryo and chick embryo were prepared by Flow Laboratories of Rockville, Md., and Microbiological Associates of Bethesda, Md., using the ordinary trypsinization procedure. Nine- to eleven-day-old fertilized Pekin duck eggs and white Leghorn eggs from RIF-free chicken flocks were used throughout the study. These eggs were obtained from Truslow Farms, Chestertown, Md., and SPAFAS Inc., Norwich, Conn. Growth medium was 199 containing 5% inactivated fetal calf serum and maintenance medium consisted of Basal Medium Eagle's with 1% inactivated fetal calf serum. All media contained 0.005% Neomycin and 1.0% glutamine.

Seven- to nine-day-old duck and RIF-free chicken embryonated eggs were inoculated intra-amniotically and were passed serially at 7- to 10-day intervals. These eggs were obtained from the same sources noted above and were incubated at 34°C.

Titrations for viral infectivity were carried out by the inoculation of undiluted and serial 10-fold dilutions of each passage material into AGMK tissue cultures and detection of viral interference using Coxsackie A-9 virus according to the methods previously reported (7). In each case these cultures were incubated at the same temperature as was used for the previous passage in the avian system. When titrations demonstrated the presence of an interfering agent, neutralization index tests using known positive rubella antiserum were performed to confirm the presence of rubella virus(7). The 50% tissue culture interfering doses were calculated according to the method of Reed and Muench(8).

*Results.* The infectivity titers of the 9 seeds of rubella virus through 3 passages at the various temperatures and passage intervals are summarized in Table II. All seeds studied showed some evidence of growth in either duck or chick tissue cultures through the 3rd passage for at least one of the temperatures and passage intervals. In duck embryo tissue, titers were between 1.7 and 4.2 $\log_{10}$ and in chick embryo tissue they ranged from 1.5 to 3.8 $\log_{10}$. On the basis of

TABLE II. Initial Three Passages Growth of Rubella Virus in Avian Tissue Cultures.†

| | Seed No. | First passage | Third passage | Temp (°C) | Passage interval (days) |
|---|---|---|---|---|---|
| Duck | 1 | 2.5* | 4.2 | 35 | 7 |
| | 1 | 2.4 | 2.4 | 37 | 7 |
| | 1 | 1.9 | 1.2 | 32 | 7 |
| | 1 | 2.7 | 1.4 | 35 | 7 |
| | 2 | 1.4 | 3.7 | 37 | 7 |
| | 3 | 2.4 | 4.0 | 37 | 7 |
| | 4 | <.5 | 1.7 | 37 | 7 |
| | 4 | 2.7 | 2.7 | 32 | 10 |
| | 5 | 3.5 | 2.9 | 28 | 7 |
| | 5 | 3.2 | 2.2 | 28 | 10 |
| | 8 | 1.8 | 4.0 | 35 | 7 |
| | 9 | 1.0 | 1.0 | 37 | 10 |
| Chick | 1 | <.5 | 1.7 | 37 | 7 |
| | 1 | 2.5 | 1.5 | 32 | 10 |
| | 2 | 1.9 | 2.4 | 35 | 7 |
| | 3 | 1.4 | 3.7 | 35 | 7 |
| | 5 | 1.2 | 1.2 | 28 | 7 |
| | 5 | 1.3 | 1.8 | 35 | 7 |
| | 6 | <.5 | 3.8 | 35 | 7 |
| | 7 | 1.0 | 1.4 | 35 | 7 |
| | 8 | .9 | 1.5 | 32 | 10 |

* $TCID_{50}$ $Log_{10}$/ml.

† All seeds studied at 28°, 32°, 35°, 37° with passages at 7, 10, or 14 days.

Omission of data from table indicates titer of <.5 $Log_{10}$ for seed under those conditions.

TABLE III. Growth of Rubella Virus in Avian Tissue.

| Seed No. | 1st passage | 5th passage | 7th passage | Temp (°C) | Passage interval (days) |
|---|---|---|---|---|---|
| | | Duck | | | |
| 1 | 3.2* | 4.5 | 4.0 | 32 | 10 |
| 4 | 1.5 | 1.2 | | 32 | 14 |
| 5 | 3.2 | 1.2 | | 37 | 7 |
| 6 | 1.7 | 1.7 | 1.2 | 32 | 14 |
| 8 | <.5 | 1.7 | | 32 | 4 |
| | | Chick | | | |
| 2 | <.5 | .9 | | 35 | 14 |
| 4 | 1.7 | 1.5 | | 32 | 4 |
| 5 | 1.0 | .9 | | 35 | 14 |
| 7 | 2.5 | 1.0 | | 35 | 10 |

* Infectivity titer expressed as $TCID_{50}$ $Log_{10}$/ml.

these results further passages were continued.

The growth of rubella virus through the 5th and 7th passages is summarized in Table III. In general the seeds passed in duck embryo tissue culture grew best at 32°C Of the 5 seeds demonstrating titer, 4 were from the RV strain and one was the Brown strain. Infectivity titers ranged from 1.2 to 4.5 $logs_{10}$. The highest titer was obtained with an RV seed which had 50 passages in AGMK tissue. Titers with rolled and stationary cultures were not significantly different.

Four seeds showed evidence of growth in chick embryo tissue culture at the 5th passage level. The titers were approximately one $log_{10}$/ml. Three seeds were from the RV strain and the other was HPV. There were no significant differences in titers obtained with rolled or stationary cultures.

Multiple prior passages in AGMK tissue culture did not appear to play an important part in modifying the virus for growth in avian tissue culture. RV strain with 50 passages and HPV with 78 passages in AGMK tissue showed growth through the 7th and 5th passages of duck and chick tissue, respectively. Brown strain with only 6 passages in AGMK also had infectivity at the fifth passage level in duck embryo tissue culture. However, throat gargle material containing rubella virus, inoculated directly in duck and chick tissue culture, grew only through 3 passages in the duck culture. Passage intervals of 4 to 14 days resulted in growth through the 5th passage, but in general titers were better with longer passage intervals particularly in duck.

Attempts to cultivate the various virus seeds in duck and chicken eggs by the intra-amniotic route showed occasional growth of virus in the early passages in duck eggs, but no growth in chicken eggs. One RV seed, No. 5, in duck eggs had a titer of 2.4 $log_{10}$/ml at the 4th passage. On later passages, however, virus was no longer detectable.

*Discussion.* The data presented here indicate that rubella virus will replicate in RIF-free chick embryo tissue cultures. The adaptation of rubella to avian tissue cultures, particularly to chick embryo tissue culture, is of importance to the development of a safe attenuated rubella virus vaccine for parenteral administration. While the titer and passages are low, the several seeds cultivated in this tissue demonstrate that this system is capable of supporting the growth of the virus. The successful cultivation of several rubella virus strains in duck embryo tissue culture confirms the report of Hilleman on the cultivation of the virus in this tissue (3,4). Further studies of the conditions and variables for optimal growth in chick and duck embryo tissue culture are warranted.

The lack of detectable virus in some of the later passages may be a reflection at least in part of problems we encountered with growth and maintenance of the avian tissue cultures at that time. Spotty growth of cultures was much more of a problem with chick embryo tissue than with duck tissue culture. We also noted with early passages that occasionally passages in poor tissue produced little detectable virus, while subsequent passages in healthy tissue resulted in good titers. With both tissue systems there was no difference in titers between rolled and stationary cultures. Additional studies of the other variables which may affect the growth of rubella virus in duck and chicken eggs are needed. Thus far the tissue culture approach has been more productive in our hands than has propagation in embryonated eggs.

*Summary.* The cultivation of rubella virus in avian tissue culture systems, and particu-

larly in RIF-free chick embryo tissue culture, is of importance in the development of safe attenuated rubella virus vaccines for parenteral administration. Nine virulent or attenuated rubella virus strains were propagated for at least 3 passages in RIF-free chick embryo tissue culture or duck embryo tissue culture. Several strains showed growth in duck and chick tissue through the 5th to 7th passages. Highest titers were usually obtained when passages were made at intervals of 7 or more days. Virus titers varied from 1.2 to 4.5 $TCIND_{50}$ log $_{10}$/ml in duck tissue culture at the 5th to 7th passages, and 1.0 to 1.5 $TCIND_{50}$ $log_{10}$/ml in chick tissue culture at the 5th passage level. Virus growth was demonstrated in early passages in embryonated duck eggs but no detectable growth was obtained in chick eggs. The propagation of rubella virus in RIF-free chick embryo tissue culture warrants further efforts at the development of an attenuated rubella vaccine in this tissue culture system.

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## Changes in Pituitary Prolactin Release and Hypothalamic PIF Content During the Estrous Cycle of Rats.*† (32265)

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In randomly cycling rats, considerable variability has been observed in pituitary prolactin content, pituitary prolactin release *in vitro,* and in hypothalamic prolactin inhibiting factor (PIF) content(1-3). These variations may be due to differences in secretion of the gonadal hormones during each stage of the estrous cycle. Changes during each phase of the estrous cycle of rats have been observed in pituitary LH concentration (4), plasma LH content(5) and hypothalamic content of luteinizing hormone releasing factor (LRF)(6). It was of interest therefore, to determine whether differences could be detected during each stage of the estrous cycle of rats in pituitary prolactin content, pituitary prolactin release *in vitro* and in hypothalamic PIF content.

*Materials and methods.* Mature female Sprague-Dawley rats (Spartan Research Labs., Inc., Haslett, Mich.) were housed in a temperature (75 ± 1°F) and light (14 hr/day) controlled room. Vaginal smears were taken each morning between 9-10 A.M. beginning one week after arrival of the rats. Only rats which had exhibited at least 2 regular cycles of 4 or 5 day length were used in this study. At each stage of the estrous cycle (except metestrus), the rats were killed by guillotine, and the anterior pituitaries and hypothalami were quickly removed. The posterior lobe was discarded and the anterior pituitary was weighed, frozen and stored at −20°C until assayed. The hypothalamus and median eminence were collected in chilled .1N HCl (10 hypothalami/2 ml)

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