whereas neither urokinase nor plasminogen did so alone.

1. Neurath, H., in: Advances in Protein Chemistry, C. B. Anfinsen, M. L. Anson, K. Bailey, J. T. Edsall, eds., Academic Press, New York, 1957, v12, 319.

2. Ablondi, F. B., Hagan, J. J., in: The Enymzes, P. D. Boyer, H. Lardy, K. Myrback, eds., Academic Press, New York, 1960, 175.

3. Sherry, S., Alkjaersig, N., Fletcher, A. P., Am. J. Physiol., 1965, v209, 577.

4. ———, J. Lab. Clin. Med., 1964, v64, 145.

5. Kline, D. L., Fishman, J. B., J. Biol. Chem., 1961, v236, 3232.

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Chronic Rubella Virus Infection in the Ferret *(Mustela putorius fero)* Puppy. (32200)

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Recent advances in the knowledge of the natural history of rubella virus infections have shown that intrauterine infection of rubella virus in the first trimester of pregnancy frequently results in a series of characteristic malformations. These inclube abnormalities of the eyes and heart, deafness, and mental retardation. In addition, children born with congenital rubella have been found to remain infected beyond the newborn period. Many also have thrombocytopenia, neumonitis, hepatosplenomegaly, and radiolucent long bone lesions. It would be very helpful to have experimental animal models for the study of congenital and acquired rubella. A suitable animal study system could be of particular value for evaluation of rubella vaccines and studies of the pathogenesis of chronic rubella infection.

Schiff *et al*(1) and Cusumano *et al*(2) have reported that acute limited infection could be induced in adult ferrets. Other investigators(3,4,5) have reported similar results in experimentally infected monkeys. Oxford and Schild(5) have also shown that rubella virus could propagate in the golden hamster.

The present report is from a study initiated to determine the experimental susceptibility of ferret puppies to experimental infection with rubella virus. The results presented indicate that prolonged infection can be established by inoculation of these animals during neonatal life and that the infection persists despite significant levels of antibody to rubella virus.

Materials and methods. The RV strain (7) rubella virus grown in primary African green monkey kidney (AGMK) tissue culture was used. The virus had been passed serially 14 times in primary AGMK tissue culture cells obtained from commercial sources.* Control tissue culture fluid free of virus had the same number of serial tissue culture passages in AGMK tissue culture. The virus and control tissue culture fluids were tested and determined to be free of detectable contaminating viruses, bacteria, fungi, and mycoplasma. Rubella virus was identified by neutralization tests employing paired human sera from confirmed cases of rubella virus infection. The infectivity titer of the virus was 4.5 $TCID_{50}$/ml (log_{10}).

Virus isolation. 0.2 ml of each suspension was inoculated into each of 5 primary AGMK tissue culture tubes. After incubation at 37°C for 24 hours the maintenance medium in each tube was replaced with fresh medium and the tubes were reincubated at 37°C. Eight to ten days later, 3 tubes were challenged with 100 $TCID_{50}$ of Coxsackie A-9 virus. The tubes were examined microscopically 4 days later for cytopathic effects of Coxsackie virus. Two weeks post-inocu-

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lation infected tissue culture tubes which were not challenged with Coxsackie A-9 virus were passaged into fresh primary AGMK tissue culture tubes. Four serial passages of each organ suspension or specimen were made in this manner and discarded.

Assay of isolated interfering agents. Serial 10-fold dilutions of the original 10% (W/V) organ suspension from which an interfering agent was isolated were made in Hanks' BSS and 0.2 ml/dilution inoculated into each of 5 primary AGMK tissue culture tubes. The tubes were incubated, changed, challenged, and serially passed 4 times and discarded as described in the preceding paragraph. The 50% tissue culture infective dose ($TCID_{50}$) of each tissue suspension was calculated by the method of Reed and Muench (8).

Identification of interfering agents. To determine whether each of the isolated interfering agents from tissue suspensions and specimens was rubella virus, neutralization tests(7) were carried out on all isolates using acute and convalescent human sera from confirmed rubella virus infections. In some instances non-immune and immune sera from monkeys inoculated with rubella virus infected tissue culture fluids were also used. Briefly, tests were performed by mixing equal volumes of half $\log_{10}$ dilutions of 10% infected tissue suspensions and heat inactivated (56°C for 30 minutes) serum. Mixtures were left at room temperature for one hour before inoculation into primary AGMK tissue culture. They were challenged with Coxsackie A-9 virus 8-10 days later and observed for CPE 4 days after challenge. Other details of this method have been described (7).

Serum neutralization tests. Sera from uninoculated and inoculated puppies were obtained at 4 months and one year post-infection. These were tested for the presence of antibodies to rubella virus by the neutralization method described previously(7). Sera obtained at one year post-inoculation were tested in block neutralization tests(9) in AGMK tissue culture tubes using serially diluted sera and half $\log_{10}$ dilutions of RV strain rubella virus. All sera were inactivated at 56°C for 30 minutes before use.

Complement fixation (CF) test. Sera from ferret puppies one year after infection were also tested for the presence of complement fixing antibodies. The sera were inactivated at 56°C before testing. The microtechnique CF test(10) was used. Each serum was tested against 4 units of antigen obtained from a commercial source.*

Animal inoculation. Gravid ferrets (*Mustela putorius fero*) from a commercial source† were obtained on the 30th day of gestation. They had been vaccinated against canine distemper virus. All were found to be negative for rubella neutralizing antibodies. Thirty-six 24-48 hour old puppies were inoculated by the intranasal (IN), 36 by the intracranial (IC), 36 by the subcutaneous (SC), and 25 by the intraperitoneal (IP) routes. The inoculum (0.03 ml) contained 1000 $TCID_{50}$ of the rubella virus. Thirty-one ferret puppies served as controls; 20 were inoculated SC with non-infectious tissue culture fluid and 11 were not inoculated at all. The diet of the animals (mothers and later weanling puppies) consisted of fresh horse meat, fresh beef liver, dried Brewer's yeast, pulverized chick egg shell or limestone, cod liver oil and homogenized pasteurized milk, and was the same during the course of these studies.

Virus recovery. On post-inoculation days 4, 6, and 8, and at 4 weeks, 6 weeks, 8 weeks, and 3 months post-inoculation, 4 puppies in each of the above groups, as well as 2-3 control animals, were sacrificed for study. The hearts, livers, spleens, lungs, brains, eyes, spinal cords, blood and urine of each group of animals were aseptically removed and pooled. Ten per cent (W/v) homogenate suspensions of each organ pool were prepared in Hanks' BSS with antibiotics. The suspensions were centrifuged at 4°C at 2500 rpm in a PR-2 International centrifuge for 15 minutes, and the supernatant collected and stored at —90°C.

Results. These studies were originally initiated with a total of 164 puppies. The results presented here do not, however, represent data obtained from all of these animals.

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TABLE I. Rubella Virus Isolations from Pools of Organs from Ferret Puppies Inoculated at 1-2 Days of Age with 1000 $TCID_{50}$ Rubella Virus.

Inoculum route	Specimen	Time post inoculation						
		4 days	6 days	8 days	4 wk	6 wk	8 wk	12 wk
IN	Heart	+	+	+	+	0	0	0
	Liver	+	+	+	+	0	0	0
	Spleen	+	+	+	+	0	0	0
	Lung	+	+	+	+	0	0	0
	Brain	+	+	+	+	0	0	0
	Eye	NT	+	NT	+	+	0	0
	S. cord	NT	+	NT	+	+	0	0
	Blood	+	+	+	0	0	0	0
	Urine	NT	NT	NT	0	0	0	0
IP	Heart	+	+	+	NT	NT	NT	NT
	Liver	+	+	+	NT	NT	NT	NT
	Spleen	+	+	+	NT	NT	NT	NT
	Lung	+	+	+	NT	NT	NT	NT
	Brain	+	+	+	NT	NT	NT	NT
	Eye	NT	NT	NT	NT	NT	NT	NT
	S. cord	NT	NT	NT	NT	NT	NT	NT
	Blood	+	+	0	NT	NT	NT	NT
	Urine	NT	NT	NT	NT	NT	NT	NT
SC	Heart	+	+	+	+	0	0	0
	Liver	+	+	+	0	0	0	0
	Spleen	+	+	+	+	+	0	0
	Lung	+	+	+	+	0	0	0
	Brain	+	+	+	+	+	0	0
	Eye	NT	+	NT	+	+	+	0
	S. cord	NT	+	NT	+	+	0	0
	Blood	NT	+	0	0	0	0	0
	Urine	NT	+	+	+	0	0	0
IC	Heart	+	+	+	+	0	0	0
	Liver	+	+	+	0	0	0	0
	Spleen	+	+	+	+	0	0	0
	Lung	+	+	+	+	+	0	0
	Brain	+	+	+	+	+	+	0
	Eye	NT	+	NT	+	+	+	0
	S. cord	NT	+	NT	+	+	NT	+
	Blood	+	+	0	0	0	0	0
	Urine	NT	+	+	0	0	0	0

+ = Positive for virus. 0 = Negative for virus. NT = Not tested.

Some of the animals were not sacrificed until one year after infection, while others were cannibalized by their mothers in the early stage of the study, and some died from miscellaneous causes. During the course of these studies none of the puppies showed any apparent signs of illness, nor was there any death among the puppies which could be directly attributable to rubella virus infection.

Table I shows the results of virus isolations and the duration of virus persistence in organs of infected ferret puppies. Rubella virus was isolated from most of the organs obtained on days 4, 6, and 8 post-infection. Thereafter, virus isolation from the organs varied with fewer organs yielding virus with time. Intracranially infected puppies showed the longest persistence of virus in the tissue, especially the brain, the eye (animals with eye abnormailities observed 6 weeks after infection were tested separately and were not included in the Table, see below), and spinal cord. Subcutaneously infected puppies also had persistence of virus for 8 weeks in the eye. Intranasally infected puppies showed viremia lasting through day 8 post-infection, while puppies inoculated by SC and IC routes showed viremia only through day 6 post-inoculation. Viruria persisted up to 4 weeks in SC infected animals, while it lasted only through 8 days in IC animals. It was not possible to test for viruria in other animals because of difficulties in obtaining

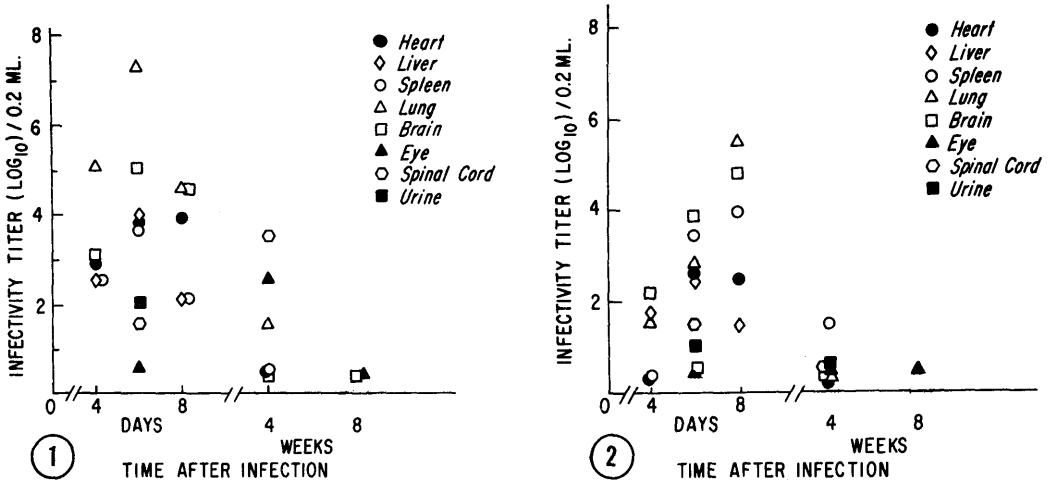

FIG. 1. Titers of rubella virus in organs of ferret puppies inoculated intracranially at 1-2 days of age.

FIG. 2. Titers of rubella virus in organs of ferret puppies inoculated subcutaneously at 1-2 days of age.

urine specimens from the bladders of these puppies; this was possible, however, as the animals grew. Virus was not isolated from organs of pupies inoculated with non-infectious tissue culture fluid. All virus isolates were confirmed by neutralization with specific antiserum.

Of 36 puppies inoculated by the IC route, 3 developed corneal cloudiness and microblepharon (in 2 animals these observations were bilateral) at 6 weeks post-inoculation; these animals were sacrificed a week later. Virus was recovered from all of the eyes of puppies inoculated by the IC, SC, and IN routes, but only these 3 animals showed gross eye abnormalities. Cultures for bacteria and fungi were negative.

The results of virus titrations on the 10% suspensions of various organs and of urine specimens from animals inoculated by the SC and IC routes and sacrificed at various intervals are shown in Fig. 1 and 2.

Animals inoculated by the IC route (Fig. 1) showed the highest virus infectivity titer 6 days after infection. The concentration of virus in the lung at that time was 30,000 times greater than in the inoculum ($10^{7.5}$ *vs* 10^{3}). Somewhat lower concentrations of virus were found in the brain, liver, spleen, and heart. Virus content of the organs and urine specimens decreased gradually, and in some no detectable virus could be found by the 8th week after inoculation. Despite the apparent differences, virus was still present in most of the organs 4 weeks after infection, with the highest level (3.5 $\log_{10}$) occurring in the spinal cord. Only the eye and the brain showed the presence of virus at 8 weeks; virus infectivity titers in these organs were less than 1 $\log_{10}$.

Puppies inoculated by the SC route (Fig. 2) showed results similar to the IC inoculated puppies in virus levels and virus shedding. However, the highest level of virus from these puppies was found 8 days after infection. In the lungs of these animals, virus titer was about 100 times greater than the inoculum. The decrease in virus content in organs from these animals was more rapid than in the IC inoculated puppies. At 4 weeks the highest titer was in the spleen, while the spinal cord, eye, urine, heart, lung, and brain showed low virus content. As in the IC inoculated animals, 8 weeks after infection virus was present only in the eye.

The levels of rubella virus in the blood at various times were not determined; nevertheless viremia was present in several instances for 6 to 8 days post-inoculation. The titers of virus in the organs could have been influenced by circulating virus in the blood in the early stages of the infection. Persis-

TABLE II. Neutralizing and Complement-Fixing Antibodies in Ferret Puppies One Year After Inoculation with Rubella Virus.*

Routes of inoculation	Antibody titers†	
	CF	Neut.
IN	0	0
	0	4
	0	0
IP	0	256
	0	256
SC	0	4
	0	4
	0	64
	0	16
	0	4
	0	4
	4	64
	0	16
IC	4	16
	0	256
	4	64
	256	256
	64	256

* Inoculated at 1-2 days of age.
† Reciprocals of dilutions.
IN, intranasal; SC, subcutaneous; IC, intracranial; IP, intraperitoneal.

tent infection, however, was not a reflection of viremia since no virus isolation from blood was made after the eighth day.

The results of complement fixation and neutralization tests carried out with sera obtained from surviving puppies inoculated by various routes one year earlier are shown in Table II. Of 3 surviving animals inoculated by the IN route, all had no detectable complement fixing antibody, while one showed low level of neutralizing antibody. Of the 2 survivals in the IP inoculated animals, neither had any detectable level of CF antibody, while each showed a neutralizing antibody titer of 1:256. Only one of 8 surviving animals inoculated by the SC route had a low titer of 1:4 of complement fixing antibody; however, all 8 had neutralizing antibodies. Among the 5 surviving animals inoculated by the IC route, one had no detectable CF antibody, 2 had titers of 1:4 each, one had a titer of 1:64, while one had a titer of 1:256. All had neutralizing antibody titers of 1:16 or higher.

Discussion. Infection with rubella virus was readily produced in one- to two-day-old ferret puppies. The fact that, regardless of routes of inoculation, every organ tested from these infected animals yielded virus indicated that the infection was widely distributed. The presence of virus in the blood for 6 to 8 days post-infection was probably a contributing factor to the wide dissemination of virus and to high virus levels in the organs. Persistent infection was also demonstrated, and in certain routes of inoculation this lasted for 2-3 months post-infection. Persistent infection was not influenced by any virus in the blood, since viremia lasted at the most 8 days post-infection. A comparison of these data with those obtained for adult ferrets indicated that suckling ferret puppies were more susceptible to rubella virus infection than adult ferrets(1,2) and that the duration of infection was longer than that seen in adult ferrets in which virus shedding was limited to the 14th through the 21st day. The ease with which suckling ferrets were infected with rubella virus should make the ferret puppy a convenient tool for laboratory study.

The corneal cloudiness and microblepharon observed in 3 of 36 puppies inoculated by the IC route suggested that the prolonged infection with rubella in these animals might be associated with tissue damage. These findings, however, occurred only in a small proportion of animals in which positive infection of the eyes was documented. Furthermore, while there was no evidence of bacterial, fungal or nutritional factors which could have produced these defects, these and other possible factors could not be completely excluded.

The antibody response in several puppies suggested that response may be related to chronic infection. Complement fixing antibody in many viral infections of humans and animals tends to fall quickly below detectable levels in a very short time. This was not observed, however, in ferret puppies inoculated by the IC route where 4 of 5 surviving animals still responded with significant complement fixing antibody levels one year post-infection. This finding paralleled the observed persistence or development of CF antibody in some children with congenital rubella(11,12).

The data presented here suggest that the most active persistent infection occurred when the virus was inoculated into the central nervous system. This was supported by the high virus titers noted and longer period of shedding of virus, which lasted through 8-12 weeks post-infection. Furthermore, significant complement-fixing antibody levels persisted in the sera of 4 out of 5 of these puppies one year after virus inoculation, suggesting that the prolonged active infection demonstrated in this group might have persisted even beyond the 12-week post-infection stage.

These findings in ferret puppies are similar to those observed in human infants with congenital rubella. Phillips *et al*(13) and Monif and Sever(14) isolated virus from the spinal fluid and brain up to the 132nd day of life of these infants. Phillips *et al*(13) found that the frequency of virus isolation from the CNS of congenital rubella infants was higher than from any other source; the virus isolations were accomplished as late as 100 days after birth. It is doubtful that the newborn ferret rubella system can be considered sufficiently analogous to the human rubella infection *in utero* to be useful as a model for the latter. On the other hand, the newborn ferret system may well provide a useful *in vivo* test system for vaccine and drug effectiveness and for studies of the pathogenesis of rubella virus.

Summary. The inoculation of newborn ferrets with rubella virus by various routes resulted in chronic infection. Specific complement fixing antibody and neutralizing antibody persisted for at least one year after inoculation. Of 36 puppies inoculated by the IC route, 3 showed corneal cloudiness with microblepharon at 6 weeks post-infection. The IC inoculated puppies had the highest titer of virus in most of the organs examined; these animals also showed longer virus shedding, higher CF and neutralizing antibodies. There was no death or evidence of illness which could be attributable to the inoculation of rubella virus by any route. These findings are similar in many respects to those observed in congenital rubella in human infants. The suckling ferret provides a useful model for the study of the prevention and pathogenesis of rubella.

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1. Schiff, G. M., Sever, J. L., Huebner, R. J., Trans. Fed. Clin. Res., 1964, v12, 245.

2. Cusumano, C. L., Sever, J. L., Schiff, G. M., Huebner, R. J., Clin. Res., 1965, v13, 41.

3. Sever, J. L., Meier, G. W., Windle, W. F., Schiff, G. M., Monif, G. R., Fabiyi, A., J. Infect. Dis., 1966, v116, 21.

4. Phillips, P. E., Parkman, P. D., Kirchstein, R. L., Meyer, H. M., Fed. Proc., 1965, v24, 570.

5. Heggie, A. D., Robbins, F. C., Proc. Soc. Exp. Biol. & Med., 1963, v114, 750.

6. Oxford, J. S., Schild, G. C., Virology, 1966, v28, 780.

7. Sever, J. L., Schiff, G. M., Traub, R. B., J. Amer. Med. Assn., 1962, v182, 663.

8. Reed, J. L., Muench, H., Am. J. Hyg., 1938, v27, 493.

9. Fabiyi, A., Wenner, H. A., J. Immunol., 1965, v94, 257.

10. Sever, J. L., Huebner, R. J., Castellano, G. A., Sarma, P. S., Fabiyi, A., Schiff, G. M., Cusumano, C. L., Science, 1965, v148, 385.

11. Sever, J. L., Huebner, R. J., Fabiyi, A., Monif, G. R. G., Castellano, G., Cusumano, C. L., Traub, R. G., Ley, A. C., Gilkeson, M. R., Roberts, J. M., Proc. Soc. Exp. Biol. & Med., 1966, v122, 513.

12. Monif, G. R. G., Hardy, J. H., Sever, J. L., Pediatrics, 1967, v39, 289.

13. Phillips, C. A., Melnick, J. L., Yow, M. D., Bayatpour, M., Burkhardt, M., J. Amer. Med. Assn., 1965, v193, 1027.

14. Monif, G. R. G., Sever, J. L., Neurology, 1966, v16, 111.

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