Persistent Rubella Virus Production in Embryonic Rabbit Chondrocyte Cell Cultures (37465)

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Since the original report of the isolation of rubella virus in 1962 by Parkman, Buescher and Artensten (1) in primary African green monkey culture (AGMK) and Weller and Neva (2) in primary human amnion tissue, various cell culture systems have been tested for the propagation of this virus (3). Rubella virus gives a cytopathic effect (CPE) in several established cell lines such as RK 13 (4) GMK, AH-1 (5) and LLC-RK (6). In most cases, the virus had to be adapted by serial passage before CPE was observed.

Several of these cell cultures have been shown to be chronically infected. In addition, some cell cultures, obtained from rubella infected human fetuses or tissue from animals infected *in utero,* yielded virus when grown *in vitro* (7). Several primary human embryonic tissues (8) and/or continuous lines (9) inoculated with rubella virus also have been shown to be carrier cultures.

In a recent report, London *et al.* (10) observed that after inoculation on the second day of gestation of pregnant rabbits with rubella, the highest concentration of virus was found in fetal cartilage. These investigators have also shown rubella virus will grow under *in vitro* conditions in sternum derived chondrocyte cells for short periods of time (London *et al.*, unpublished observation). The purpose of this report is to describe the long-term *in vitro* growth of embryonic rabbit chondrocyte cultures and the establishment of persistent rubella infection in these cells.

These investigations were initiated to develop diploid cell cultures in which the effects of persistent rubella virus infection on chromosomes could be studied.

Materials and Methods. Cell cultures. Primary chondrocyte cultures from rabbit embryos of 28 days gestation were initiated from the tip of the sternum. Small pieces of tissue (1–2 mm) were placed on teabag paper on a 2 cm^2 elevated stainless steel grid (11) in sealed 60 mm petri dishes, containing 10 ml of Eagle's minimum essential medium (EMEM) (GIBCO powder) supplemented with 10% fetal calf serum and penicillin and streptomycin at a final concentration of 100 units and 100 μg/ml, respectively. One half of the medium was changed every other day until a monolayer developed on the surface of the glass petri dish which usually required 10 to 12 days. A 0.25% trypsin solution was used to harvest the cells which were transferred to 4 oz prescription bottles (Brockway) and grown in the same medium. Control and rubella virus infected cultures were subcultured (1:4 split ratio) once a week and fed twice a week. Both control and virus infected cell cultures were monitored periodically for mycoplasma contamination and all were negative.

Virus strain. Using the methods previously reported (12), rubella virus was isolated in first passage AGMK and stored at —70°. The virus content of the harvest was determined to be $10^{3.54}$ $TCID_{50}$/ml by interference assay (12). Rubella virus (1 ml) was inoculated into the 26th and the 42nd passage of embryonic rabbit chondrocyte cultures, which were 24 hr old, to initiate the series. Samples from the supernatant fluids were removed for virus assay at 7 days and thereafter periodically from the control and the rubella

virus inoculated cultures.

Chromosome preparations. Colcemid at a final concentration of 0.1 μg/ml was added to the cultures about 40 hr after passage for a period of 2 hr. Slides of metaphase plates were prepared according to the flame dried method of Moorhead and Nowell (15).

Fixation of slides for fluorescent antibody. Cells from control and virus infected cultures were seeded on opposite sides of the 2 chambered slides (LAB-TEK Products, Westmont, IL). On Day 8 after seeding, each chamber was fixed with a chilled mixture of 95% methanol and 5% distilled water. The slides were then washed two times with phosphate-buffered saline (pH 7.2) and one time with distilled water. The slides were then air dried and stored at 4° until time of use.

Fluorescent antibody technique. The indirect fluorescent (FA) procedure was done according to the method of Smith and Thiel (16).

Interferon assay. Samples of the supernatant fluids from the rubella virus cultures at passages 26, 37 and 104 were tested for the presence of interferon (IF). Interferon assays were performed by a cytopathic effect-inhibition method in duplicate secondary cultures of newborn rabbit kidney cells, using 200 $TCID_{50}$ vesicular stomatitis virus (VSV) (Indiana strain). The technique differed from a published procedure (17) only in that cultures were scored for cytopathic effect (CPE) 24 hr after infection.

Results. Virus titers. The results of assay for the presence of rubella virus in the media from infected cell cultures are given in Table I. In the first experiment using cultures inoculated initially at P-26, assays were carried out at 0, 13th, 25th, 48th and 78th subculture. In the second experiment, initiated at P-42, samples were obtained at the 0, 14th, and 34th subculture. Rubella virus titers ranged between $10^{3.4}$ to $10^{5.4}$ $TCID_{50}$/ml in both studies. Media taken from the control cultures at the same passage levels showed no evidence of the presence of rubella virus.

Morphological transformation. The control cultures have been subcultured 109 passages over a period of 18 mo. During this time greater than 75% of the cells appeared epithelial-like. In contrast, the persistent virus infected cultures became predominantly fibroblastic-like after the initial infection and have maintained this morphology for the duration of the passage series. (Fig. 1). In addition to cell morphological type, the control cells were very uniform in shape and arrangement while the virus infected cells were disorganized. The most striking difference between the two cultures were seen at higher magnification (Fig. 1). The nucleoli in the cells from control cultures were usually round and fairly uniform in size. The number of nucleoli varied from 1 to 3 although an occasional cell would contain up to 6. These latter cells were probably tetraploids. However, in the virus infected cultures, the nucleoli were very irregular in shape and in many cases appeared to be continuous. These were from 6 to 10 nucleoli in most cells.

Chromosomal characterization. Analyses of

TABLE I. Persistent Production of Rubella Virus by Rabbit Embryonic Chondrocyte Cultures.

Culture no.	Passage when inoculated	Passage after inoculation	Days after inoculation	Virus titer[a]/ml
p-26-2	26	0	8	5.4
		13	87	3.75
		25	175	4.45
		48	286	4.2
		78	489	4.49
p-42-2	42	0	10	4.75
		14	98	5.45
		34	209	3.45

[a] $TCID_{50}$ interference doses, average of duplicate cultures.

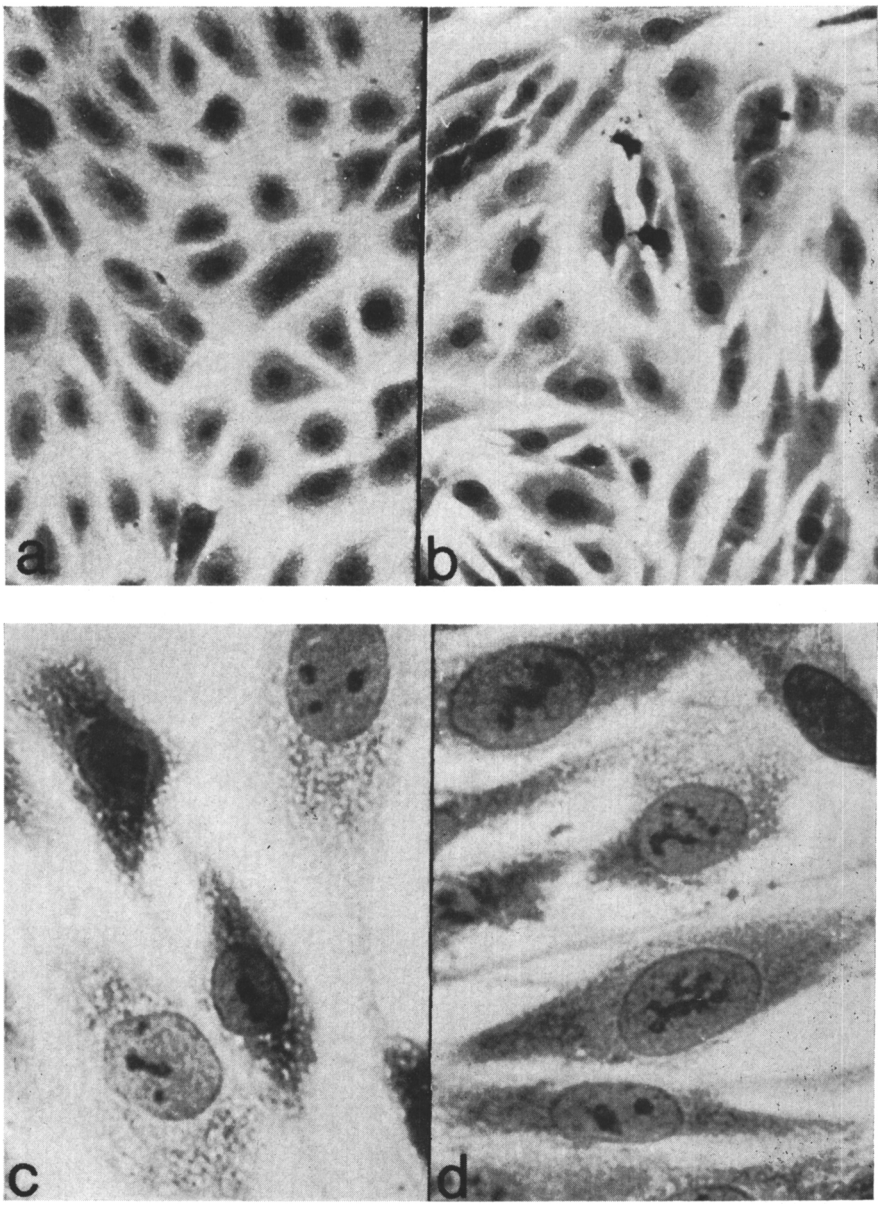

FIG. 1. Embryonic diploid rabbit chondrocyte cell cultures at passage 39. May-Greeenwald-Giemsa stain. (a.) Epithelial-like cell type seen in the uninfected control cultures. 100×. (b.) Fibroblastic-like cell type seen in the rubella virus infected cultures. 100×. (c.) Uninfected control cultures. Note the circular, orderly appearance of the nucleoli. 400×. (d.) Rubella virus infected culture. Note the long, irregular shaped nucleoli. 400×.

metaphase chromosomes and arrangement of representative karyotypes showed that the cells from the control and the chronically infected cultures were predominantly diploid to passage 53. After passage 53, a hyperdiploid stemline developed in the control cultures. Analyses of representative metaphase plates from these control cells have a chromosome count of 45, showed the presence of one additional small submetacentric chromo-

some. In contrast, the virus infected cells remained predominantly diploid until passage 94, forty passages after the control cells became hyperdiploid. The detailed analyses of the cytogenetic studies of cells from the control and the persistent rubella virus infected cultures are in preparation [for preliminary report, see Earley and Smith (14)].

FA reaction. Rubella antigen was seen as a halo around the nuclear membrane in the virus infected cells (Fig. 2). Assays were performed at passages 47 and 109, which were the 21st and 83rd subcultures after virus inoculation. Approximately 50% of the cells were infected.

Interferon assay. Interferon could not be demonstrated in the virus infected cells at dilutions of 1:4 which was the highest dilution tested. However, Hallum and Stanwick (18) found titers of IF greater than $10^{2.0}$ units/ml in these virus infected cultures using the more sensitive plaque reduction IF assay.

Discussion. Results have been reported which demonstrate the persistent infection of rubella virus in rabbit embryonic chondrocyte cells.

The titers of $10^{4.5}$ are similar to those reported by Horta-Barbosa and Warren (3) for the production of rubella virus in several different cell cultures.

The finding of London *et al.* (10) that rubella virus in the rabbit fetus is concentrated in cartilage tissue containing chondrocytes and the report here of chronic production of rubella virus in long-term chondrocyte cultures *in vitro* suggest that one of the target cells of rubella virus is the chondrocyte.

This is the first reported observation that a diploid epithelial-like morphological cell type was altered to a fibroblastic-like cell type by the presence of rubella virus in the culture.

The metabolic effects of this strain of rubella virus on these cells are being studied. Chromosomal analysis of the control and virus infected cell cultures have been reported elsewhere (14). The long-term effects of the various attenuated viruses used for vaccines should be investigated further in these cells.

Summary. Cell cultures were established from embryonic rabbit sternum. Persistent virus infected lines were established by inoculation of rubella virus at passages 26 and 42 and have produced rubella virus at titers of 10^4 or higher for 78 subcultures. The presence of virus in these cultures changed the epithelial-like morphology seen in the control

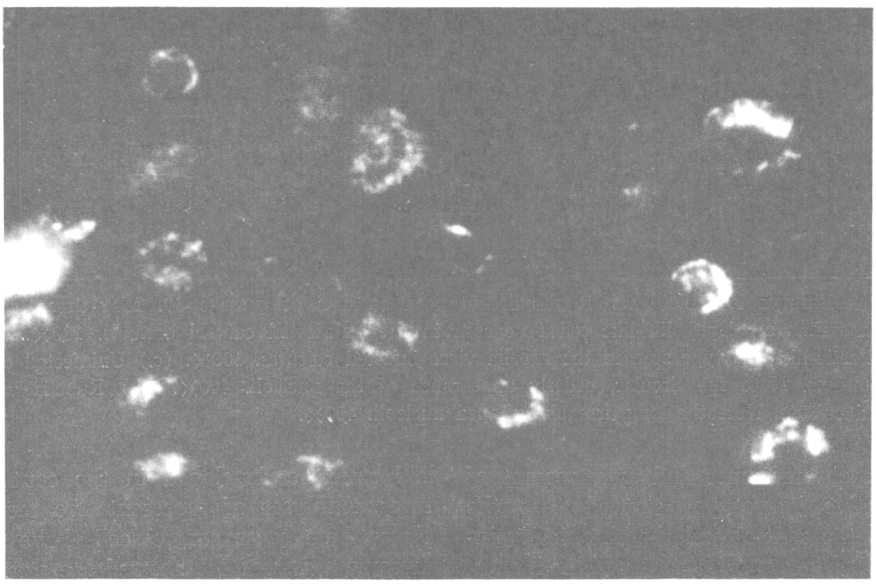

FIG. 2. Fluorescent antibody reaction of virus infected cells at passage 109.

cells to fibroblastic-like.

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