Comparison of Fluorescent Antibody Induced by Rubella Virus in Vaccinee and Convalescent Individuals

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Specific rubella antibody detectable by indirect immunofluorescence developed in response to immunization with attenuated rubella vaccine, HPV-77, DK-12. Fluorescent antibody (FA) was found when vaccinee sera were reacted with antigens synthesized in three different acutely infected continuous cell lines: BHK-21, LLCMK-2, and RK-13. FA titers were high, and they correlated with antibody titers obtained by hemagglutination-inhibition tests. Levels of FA in vaccinated individuals were slightly lower than those found in persons recovered from natural rubella infections. Rubella FA persists a long time in convalescent individuals and appears to be maintained for at least 19 months in vaccinees.

The need for an immunological marker that would differentiate between the antibody response induced by attenuated and wild rubella virus infections became obvious during the course of clinical trials on rubella virus vaccines. It has been reported that serological differentiation is possible by immunofluorescence (IF; references 2, 12). Because of the epidemiological significance of such a marker, we decided to evaluate critically the rubella IF system. Representative sera were selected from among more than 14,000 vaccinees tested in 1969 field trials of HPV-77, DK-12 rubella virus vaccine. This vaccine is derived from the HPV-77 rubella virus strain of Parkman and Meyer (13, 14) passed 12 additional times in canine renal cell cultures.

Our results indicated that fluorescent-antibody (FA) developed as a result of immunization with this rubella virus vaccine as has been reported for individuals recovered from natural infections (1, 3, 5, 9). A comparison of FA titers found in vaccinee and convalescent sera was made by using acutely infected BHK-21, LLCMK-2, and RK-13 cells. FA and hemagglutination-inhibition (HI) tests were done on 20 convalescent individuals, 102 vaccinated with HPV-77, DK-12 (manufactured by Philips-Roxane, St. Joseph, Mo.) and 14 inoculated with Cendehill (manufactured by Smith, Kline & French, Philadelphia, Pa.) vaccines.

MATERIALS AND METHODS

Preparation of stock viral strains. Rubella virus strain HPV-77 was obtained from P. D. Parkman of the Division of Biologics Standards, National Institutes of Health, Bethesda, Md., and strain M-33 was from E. L. Buescher, Walter Reed Army Research Institute, Washington, D.C. Both strains were adapted to grow in BHK-21, LLCMK-2, and RK-13 cell lines. Each adapted viral strain was then used to infect four 28-oz bottles of homologous cell cultures. Infected BHK-21 cultures were harvested on day 3, LLCMK-2 on day 10, and RK-13 on day 8 postinoculation. Cultures were frozen and thawed once, pooled, clarified, divided into portions, and stored at −70 C. Virus seed lots were titered in homologous cells by counting the number of fluorescing cells produced in a one-step growth cycle by 1.0 ml of virus and were expressed as cell-infecting units (CIU/ml; reference 18). The titers and passage histories for each stock seed are as follows: HPV-77, AGMK4a (primary African green monkey kidney), BHK-21a, 10⁶.0 CIU/ml; M-33 AGMK5a, BHK-21a, 10⁶.9 CIU/ml; HPV-77 AGMK3a, LLCMK-2a, 10⁵.8 CIU/ml; M-33 AGMK5a, LLCMK-2a, 10⁵.7 CIU/ml; HPV-77, AGMK5a, RK-13a, 10⁵.2 CIU/ml; M-33, AGMK5a, RK-13a, 10⁵.3 CIU/ml.

Cell cultivation and infection. A suspension of BHK-21 cells was obtained from Microbiological

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Associates, Bethesda, Md., in April 1966. This culture was free from pleuropneumonia-like organisms (PPLO). It was propagated in bottle cultures and passed twice weekly. Trypsinized cell suspensions containing a total of 450,000 cells in 3 ml of F-12 medium (Grand Island Biological Co., Grand Island, N.Y.), enriched with 10% fetal calf serum (FC), were planted in each section of quadrant petri dishes containing cover slips (22 mm square) and were incubated at 37 C for 3 days. All cover slip cultures were incubated under an atmosphere of 5% CO\textsubscript{2} in air. Medium was removed by suction, and 0.2 ml of either HPV-77 or M-33 stock virus suspension was placed on the cover slip cultures at a ratio of 1 CIU to five to six cells. Virus was adsorbed for 3 hr at 37 C. Cultures were then covered with 3 ml of maintenance medium (F-12 with 5% FC) and incubated at 35 C. BHK-21 cells were fixed 26 to 30 hr postinfection.

The LLCMK-2 cell line was obtained from the American Type Culture Collection (CCL 7.1) in July 1969 and was free from fungi, bacteria, or PPLO organisms. It was grown in Eagle's minimal essential medium (Grand Island Biological Co.) supplemented with 10% FC. Cells were maintained on the same medium with the serum content reduced to 5%. Cover slip cultures were seeded with 3 ml of a trypsinated suspension containing a total of 300,000 cells. Four to 5 days of incubation was necessary for the development of confluent monolayers. Cells were infected with 0.2 ml of stock virus in a ratio of 1 CIU to four to five cells, adsorbed for 3 hr at 37 C, covered with 3 ml of maintenance medium, and incubated at 35 C. Cell cultures were fixed 4 days after inoculation with virus.

The RK-13 cell line was obtained in 1964 from Hope E. Hoppes, Division of Biologies Standards, National Institutes of Health. Cells are free from bacteria, fungi, and PPLO organisms. Monolayer cultures were trypsinated and suspended in synthetic mixture 199 (Grand Island Biological Co.) enriched with 10% FC so that each milliliter contained 80,000 cells. A 3-ml amount of this suspension was inoculated into each quadrant and incubated for 3 days at 37 C. Cells were infected with 0.2 ml of RK-13 grown stock virus in a ratio of 1 CIU to seven to eight cells. Maintenance medium consisted of synthetic mixture 199 with 5% FC. Cultures were fixed 4 days postinfection.

**Fixation and storage of viral antigens.** When viral antigens had reached maximum intracellular concentration, as determined by pretreatments with each virus-cell system, maintenance medium was removed and the cover slips were washed three times with 0.01 M phosphate-buffered saline (PBS), pH 7.2. They were immersed in chilled absolute ethanol for 5 to 10 min and stored in fresh ethanol at -70 C. Fixed infected cells could be held for as long as 3 weeks without noticeable deterioration of the fluorescent properties of the viral antigen. Each set of infected cells was pretreated with two positive rubella sera with known FA titers before it was used for assay of unknown sera.

**Sera.** Prevaccination blood samples were drawn on each subject the same day that a single dose of HPV-77, DK-12 (Rubologen) was injected. Most of the postimmunization sera used in this study were drawn at 6 to 8 weeks. Sera were stored at -20 C until assayed.

Rubella convalescent bloods were obtained from laboratory personnel and other adults. Some of these sera had been stored frozen for as long as 6 years.

All sera were examined under code, except for the two negative and two positive controls included in each experiment. Convalescent and pre- and post-vaccinee sera were examined on noninfected as well as rubella-infected cells.

**IF technique.** The indirect IF procedure was used in this study. Fixed, dry cover slip cultures were cut into four or six pieces, placed into each section of quadrant petri dishes, covered with diluted serum, and incubated at 37 C for 1 hr. Serum was removed, and cover slips were washed with three changes of PBS and overlaid with goat antihuman gamma globulin labeled with fluorescein isothiocyanate (Biquest, Division of Beckton, Dickinson & Co., Cockeysville, Md., or Progressive Laboratories, sold through Roboz Surgical Instrument Co., Washington, D.C.). This tagged globulin was absorbed once with bovine liver powder by incubating overnight at 4 C and then spun at 35,000 rev/min for 2 hr. The cleared conjugate was diluted 1:25 with PBS for use. Cover slips were reincubated for 1 hr, washed as before, and counterstained with a 1:60 aqueous dilution of Ponta Chrome Blue Black 2F or a 1:40 aqueous solution of Flazo Orange (Nutritional Biochemicals Corp., Cleveland, Ohio) for 30 to 60 sec (8). Cover slips were washed twice, mounted in 90% buffered glycerine (pH 7.2), and examined. The intensity of fluorescence was scored by assigning values from 1 to 4+. FA titers are expressed as the reciprocal of the highest serum dilution producing a 2+ fluorescence.

**Microscopy and photography.** Stained cover slip cultures were examined with a Zeiss fluorescent microscope equipped with a dark-field condenser. An Osram HBO 200 mercury vapor bulb provided illumination. A BG-12 exciter filter was used in combination with barrier filter no. 47 or no. 50. Photographs were made with a 35-mm Zeiss camera. Eastman Kodak high-speed daylight Ektochrome film was used, and exposures varied from 1 to 3 min.

**HI test.** A modification of the rubella HI test of Stewart et al. (17) was used. The significant changes were: (i) the diluent was 0.01 M tris(hydroxymethyl)-aminomethane (Fisher Scientific Co.) in 0.9% NaCl solution, pH 7.3 to 7.4, containing 0.05 M CaCl\textsubscript{2} and 0.05 M MgCl\textsubscript{2}; (ii) the serum was heated at 56 C for 0.5 hr after adsorption with kaolin and red blood cells from 1-day-old chicks; (iii) the serum-antigen mixture was incubated at room temperature for 1 hr. HI titers are expressed as the reciprocal of the highest serum dilution showing inhibition of the virus-red cell agglutination.

**RESULTS**

**FA reaction.** Rubella antigen developed in
the cytoplasm of infected cells. In general, antigen fluoresced diffusely early in its development, but discrete granules formed about the time maximum levels of intracellular viral antigen had been synthesized. BHK-21 cells attained optimal concentration of intracellular antigen within 26 to 30 hr postinfection, but RK-13 and LLCMK-2 cells required about 96 hr. Figure 1 illustrates typical intracytoplasmic fluorescence when rubella-infected cells were examined with a convalescent and a vaccinee serum. This fluorescence was indistinguishable in any of the three cell systems. No fluorescence occurred when these sera were tested on uninfected cells. Sera from susceptible individuals (HI titer less than 1:4) failed to produce fluorescence on both infected and uninfected cells.

Serum that was examined before freezing or after one or two freeze-thaw cycles revealed optimum titers by IF. After more than four freeze-thaw cycles, the FA titers of sera decreased. HI titers were not affected in this manner. It appeared that one or more serum components deteriorated upon frequent freeze-thaw cycles and that this loss reduced the level of detectable FA. Since complement is the most likely unstable component of serum lost or reduced by this treatment, it was decided to test this by taking two sera containing moderate to high levels of FA and put them through five freeze-thaw cycles. After this treatment, the FA titers dropped fourfold, but when lyophilized guinea pig complement (Bi-oquest, Division of Beckton, Dickinson & Co.) was added nearly all of the FA titer was restored. Although effort was made to minimize the number of freeze-thaw cycles when the IF procedure was planned, under practical circumstances this was not always possible. All sera were tested without the addition of complement.

**IF reactions with HPV-77 and M-33 antigens.** Early in this study, both HPV-77- and M-33-induced antigens were used for the examination of convalescent and some vaccinee sera. There were no differences noted in the pattern of intracytoplasmic fluorescence produced by either virus grown in any of the cells. Furthermore, the FA titers obtained with a given serum and cell system were essentially the same with both antigens. Representative results are given in Table 1. Because of these findings, the bulk of the assays on vaccinee sera were performed on HPV-77 antigen, since this high-passage virus was the strain used in the preparation of the vaccine under study.

FA titers were highly reproducible. Most sera were examined at least twice with the same antigen and cell line. Only on occasion did these titers vary by one- to twofold dilution. Susceptible individuals did not have detectable FA or HI titers before immunization.

**FA titers in three cell lines.** In general, the same convalescent or vaccinee serum revealed different titers of FA, depending upon which of the three cell lines synthesized the viral antigen. The highest FA titers were obtained when viral antigen was grown in BHK-21 cells. These cells also yielded the greatest amount of infectious virus, a finding also reported by other workers (5, 7, 10). FA titers were lowest when rubella-infected RK-13 cells were employed and were intermediate on LLCMK-2 cells.

A comparison of the range and geometric mean titers (GMT) of FA obtained on all three cell systems for 20 convalescent and 37 vaccinees is shown on Table 2. For the purpose of comparison, the range and GMT obtained by HI tests for the same sera are included. Irrespective of the cell system employed, FA end points were lower for vaccinees than for convalescents. Immune status determined by HI tests also demonstrates this quantitative difference between individuals infected with wild virus and those inoculated with attenuated rubella vaccine. In all instances, if rubella antibody was present by HI determination, it was also demonstrable by IF.

**Development of immune response in vaccinees.** In our study, the immune response was first detectable 3 weeks after vaccination. Five sera taken 2 weeks after vaccination did not contain detectable amounts of antibody as determined by either serological procedure. The development of rubella antibody after immunization with HPV-77, DK-12 vaccine is shown on Fig. 2, in which the FA and HI GMT of 81 sera are plotted. FA and HI antibody were detectable at the same time and increased at the same rate. Antibody reached peak levels in 3 months, and it was maintained for at least the period studied, i.e., 19 months postvaccination. Note that the FA titer was about one-half the HI titer at each time interval after vaccination when the FA test was performed in BHK-21 cells.

Fourteen individuals inoculated with the Cendehill attenuated rubella vaccine were examined in our laboratory by IF and HI procedures. Vaccinees showing seroconversion did so by both test methods.

**DISCUSSION**

We felt the need for a serological test system to differentiate antibody response stimulated by attenuated and by wild virus infections. It
has been reported that the complement fixation (CF) and IF procedures would distinguish these immune responses. Several investigators reported that CF antibody rarely developed in vaccinees (11, 15), and others (2, 12) claimed that FA is not induced. More recent studies (6, 16) indicate that CF antibody can be found in vaccinee sera providing a sensitive test system is used. Schmidt and Lennette (16) reported that, by using their sensitive IF procedure, 17

FIG. 1. Cytoplasmic fluorescence in rubella-infected BHK-21 cells by (A) convalescent and (B) vaccinee sera; in infected LLCMK-2 cells by (C) convalescent and (D) vaccinee sera; in infected RK-13 cells by (E) convalescent and (F) vaccinee sera. ×320.
of their 22 vaccinated subjects developed significant FA titers. On the other hand, Brown and O’Leary (4) maintain that a serological marker of attenuation exists with their test system. Our own studies involving three different cell systems indicate that FA develops in response to infection induced by both attenuated and wild strains of rubella virus. Substantial levels of FA developed within 4 to 12 weeks in 102 recipients of HPV-77, DK-12 attenuated vaccine, nearly all of whom were school children. Individuals immunized with Cendehill vaccine responded similarly. Therefore, in our experience it is not possible to use this procedure to distinguish attenuated from wild virus-induced antibody.

The concentration of FA in a sample of immune serum was the same when it was examined with either HPV-77 or M-33 antigens if synthesized by the same cell type. However, the titers varied when viral antigens were propagated in different cell lines. The mechanism that controls this variation is unknown at this time. Infected BHK-21 cells developed antigen most rapidly and FA titers were highest. Thus, this cell system is the one of choice.

FA and HI end points were lower in vaccinees than in convalescent subjects. FA GMT of vaccinees were about one-half as high as

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### Table 1. FA titers obtained with HPV-77 and M-33 viral antigens synthesized by three different cells

| Sera        | HI  | BHK-21 HPV-77 | BHK-21 M-33 | LLCMK-2 HPV-77 | LLCMK-2 M-33 | RK-13 HPV-77 | RK-13 M-33 |
|-------------|-----|---------------|-------------|----------------|--------------|--------------|------------|
| Convalescent|     |               |             |                |              |              |            |
| S.P., unknown* | 512 | 512           | 512         | 256            | 256          | 128          | 128        |
| G.C., unknown  | 1,024 | 512          | 512         | 256            | 256          | 128          | 128        |
| A.S., unknown   | 256  | 64           | 64          | 64             | 64           | 64           | 64         |
| D.H., 1 month  | 2,048 | 512          | 512         | 256            | 256          | 128          | 128        |
| M.H., unknown   | 256  | 64           | 64          | 64             | 64           | 32           | 32         |
| S.W., 21 days  | 512  | 256          | 128         | 128            | 128          | 128          | 128        |
| I.N., 29 days  | 1,024 | 512          | 256         | 256            | 256          | 256          | 256        |
| Vaccinee      |     |              |             |                |              |              |            |
| B.R., 5 wk* | 64   | 32           | 32          | 32             | 32           | 16           | 16         |
| B.R., 10 wk  | 128  | 256          | 256         | 256            | 256          | 64           | 64         |
| B.R., 15 wk  | 128  | 256          | 256         | 256            | 256          | 64           | 64         |
| B.R., 25 wk  | 128  | 256          | 256         | 256            | 256          | 128          | 128        |
| 416, 3 wk    | 32   | 16           | 16          | 16             | 16           | 8            | 8          |
| 229, 10 wk   | 512  | 128          | 128         | 128            | 128          | 128          | 128        |
| 1920, 5 wk   | 512  | 512          | 512         | 64             | 64           | 64           | 64         |
| R132, 12 wk  | 1,024 | 512          | 512         | 256            | 256          | 256          | 256        |
| Negative     |     |              |             |                |              |              |            |
| E.M.        | <4   | <8           | <8          | <8             | <8           | <8           | <8         |
| B.R.        | <4   | <8           | <8          | <8             | <8           | <8           | <8         |

* Time after immunization or infection.

### Table 2. Comparison of convalescent and vaccinee HI and FA titers

| Subject | Test   | Titer | Rubella-infected cells |
|---------|--------|-------|------------------------|
|         |        |       | BHK-21 | LLCMK-2 | RK-13 |
| Convalescent (20)* | FA | Range | 64-512 | 64-256 | 32-256 |
|         | HI    | GMT*  | 284    | 142    | 100   |
| HPV-77, DK-12 vaccinee (37) | FA | Range | 8-512  | 8-256  | 8-256 |
|         | HI    | GMT   | 131    | 69     | 50    |

* Number of subjects in each group.

* Geometric mean titer.
convalescent subjects. Nevertheless, a significant amount of FA developed in response to immunization with HPV-77, DK-12 vaccine as did HI antibody. In general, most of the vaccinees' FA titers on infected BHK-21 cells were one-half the antibody level determined by HI tests. In a few instances, they were equal. Although convalescent FA titers are generally higher than vaccinee FA titers, their ranges overlap sufficiently to preclude using this criterion for differentiation.

The development of FA started with week 3 postimmunization and reached peak level within 8 to 12 weeks and remained so for 19 months. Whether the immune status of vaccinees determined by these serological procedures is of long duration, as is true with convalescent persons, or whether the duration of FA will vary from the HI antibody requires further study.

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