Comparison of Immunofluorescence and Immunoperoxidase Staining for Identification of Rubella Virus Isolates

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To explore possible advantages which immunoperoxidase (IP) staining might have over immunofluorescence (IF) staining for identifying rubella virus isolates, direct comparative studies were done on the same coded clinical materials using the same rubella immune rabbit serum as the primary antiserum in both systems. The rubella immune rabbit serum and conjugated anti-rabbit immune globulins could be used more dilute in the IP system than in the IF system. Both IP and IF staining detected rubella antigen in all specimens which were positive by interference. IP staining also detected low levels of rubella antigen in a few additional specimens which had originally been positive for rubella virus, but which on retesting were negative by interference and IF staining. With second-cell-culture-passage material, IP and IF staining showed comparable specificity, and the few specimens which reacted nonspecifically generally did so in both systems. Cell cultures inoculated directly with urine specimens showed greater nonspecificity by IP than by IF, but this activity could be abolished by pretreatment with sodium azide and peroxide; other methods tried for inactivating endogenous peroxidase activity destroyed rubella antigen as well. The intensity of staining for positive specimens was comparable in the two systems. However, more antigen was demonstrable in both systems when BHK-21 cells were inoculated as a cell suspension and then permitted to grow into monolayers than when the same specimens were inoculated into preformed monolayers. IP staining was considered to be a highly satisfactory alternative to IF staining for identification of rubella virus isolates.

Much has been reported in recent years on the use of immunoperoxidase (IP) staining for virus identification, and various advantages which IP staining might possess over immunofluorescence (IF) staining have been indicated (1, 2, 7). However, few direct comparative studies have been done with IP and IF staining on the same materials. The present study was conducted to explore possible advantages which IP staining might have over IF staining for identifying rubella virus isolates.

Identification of rubella virus field strains presents special problems to the viral diagnostic laboratory. The virus replicates slowly and on initial isolation produces only low levels of infectious virus and viral antigen in host cell cultures. Low-passage isolates do not consistently produce a cytopathic effect, even in cell lines in which laboratory-adapted strains of rubella virus produce an extensive and clear-cut cytopathic effect. Viral isolates are generally detected in cell cultures by their ability to interfere with the replication of a challenge enterovirus. Once an interfering agent has been demon-

strated, its specific identification as rubella virus can be expedited greatly by IF staining (14), and this method has been used routinely in our laboratory for a number of years.

The comparative studies described herein were designed to compare the sensitivity, specificity, and facility of IP and IF staining for identifying rubella virus strains recovered in cell culture systems, to compare the extent of infection and amount of rubella antigen produced in cell cultures infected by two different methods, and to explore the possibility of more rapid virus identification at the first-cell-culture-passage level or directly in clinical materials.

MATERIALS AND METHODS

Materials examined by IP and IF staining. First-passage BS-C-1 or RK-13 cell culture materials previously shown to be positive or negative for rubella virus were tested under code in the identification schemes. These materials had been stored at -70°C for 2 to 12 months. Some original clinical materials (urine specimens or fetal tissues) that had been found positive or negative for rubella virus in routine testing were also examined under code. Specimens were pre-

pared for virus isolation attempts by our standard procedures (10, 14).

Cell cultures. Tube cultures of the BS-C-1 line of grivet monkey kidney for use in interference tests were prepared as described elsewhere (10).

For detecting rubella virus by IP and IF staining, materials suspected of containing virus were inoculated into the BHK-21 line of baby hamster kidney cells, since greater amounts of rubella antigen are produced in this cell type than in most other cell lines (11, 12). The BHK-21 cells were grown on 10% fetal bovine serum and 90% Eagle minimal essential medium prepared in Hanks balanced salt solution. The maintenance medium used when BHK-21 cells were inoculated with virus was 5% fetal bovine serum and 95% fortified (2× the standard concentrations of vitamins and amino acids) Eagle minimal essential medium. BHK-21 cells for IP and IF staining were grown in Lab-Tek eight-chamber slides (Miles Laboratories, Naperville, Ill.). Preformed monolayer cultures were prepared by inoculating each chamber with 15,000 BHK-21 cells in 0.4 ml of growth medium and incubating in a CO₂ incubator at 36°C for 48 h.

Infection of BHK-21 cells. Cell culture material or clinical specimens to be examined for rubella virus by IP and IF staining were inoculated onto BHK-21 cells in parallel by two procedures. For one procedure, preformed cell monolayers in Lab-Tek chambers under 0.4 ml of maintenance medium were inoculated with 0.05 ml of specimen, using two sets of four chambers each, one set for IP staining and one for IF staining. Inoculated cultures were incubated at 36°C in a CO₂ incubator for 72 h. For the other procedure, 0.5 ml of the same inoculum was mixed with 0.2 ml of a trypsin-dispersed cell suspension containing 1×10^6 BHK-21 cells per ml and 3.3 ml of maintenance medium; 0.4 ml of this mixture was then planted into each of eight Lab-Tek chambers, and incubation was conducted in a CO₂ incubator at 36°C for 72 h, during which time the cells grew into a confluent monolayer.

At the same time specimens were inoculated into BHK-21 cells for IP and IF staining, they were also inoculated in a volume of 0.2 ml into tube cultures of BS-C-1 cells to be tested for interference, and thus to confirm whether the materials still contained viable rubella virus. Interference tests were conducted after 7 days of incubation at 36°C by adding approximately 100 mean tissue culture infective doses of echovirus type 11 to the inoculated cultures and observing after an additional 3 days of incubation for inhibition of the cytopathic effect of the challenge virus.

Rubella immune rabbit serum. Antiserum to rubella virus (RV strain) was produced by immunization of rabbits with virus propagated in a rabbit serum-adapted RK-13 cell line. The immunizing antigen was prepared in cells grown and maintained on rabbit serum to circumvent the production of anti-host antibodies that might mask specific antibodies to rubella virus. Animals received five weekly intravenous injections of 1 ml of clarified, infected-cell culture fluid and were bled 2 weeks after the last immunizing injection. The same rubella antiserum was used as the primary serum in both IP and IF staining.

Conjugated anti-rabbit immune globulins. Fluorescein-conjugated and peroxidase-conjugated anti-

rabbit immune globulins produced in goats were both obtained from the same commercial source (Miles Laboratories, Inc., Elkhart, Ind.).

Indirect IF staining. After removal of the cell culture chambers, slides were washed in three changes of 0.01 M phosphate-buffered saline, pH 7.2 (PBS), and then fixed, without drying, in two changes (10 min each) of cold acetone. After drying at room temperature, slides were stored at -70°C until they were stained. Immune and normal rabbit sera were inactivated at 56°C for 30 min, and dilutions were prepared in a 20% suspension of normal beef brain in PBS (14); the use of beef brain in the diluent reduced nonspecific staining and overstaining. A suitable working dilution (1:50) of the immune rabbit serum, determined by preliminary block titrations (see below), was added in a volume of 0.1 ml to two cell cultures in each set of four; normal rabbit serum, 1:50, was added to the third, and PBS to the fourth. Incubation was conducted at 36°C for 20 min in a humidified atmosphere, and the slides were then washed in two changes of PBS (10 min each) and in distilled water for two 1-min rinses. After drying at 37°C or room temperature, 0.1 ml of an optimal (1:50) dilution of the fluorescein conjugate, prepared in 20% beef brain in PBS, was added to each cell culture. Incubation was conducted for 20 min at 36°C in a moist atmosphere, and the slides were washed as described above, drained, and mounted in Elvanol medium, pH 8.5, (5) and then examined under ultraviolet illumination as described previously (8). Specific staining of rubella virus-infected cells appeared as bright-green fluorescence in the cytoplasm. Reactions were graded as ± when one or two cells in the culture showed antigen in the cytoplasm, + when a few single or foci of infected cells were demonstrable, ++ when 25% of the cells showed positive staining, +++ when 50 to 75% of the cells were positive, and ++++ when >75% of the cells showed staining.

Indirect IP staining. A parallel set of inoculated-cell cultures was fixed, rinsed, treated with immune rabbit serum (diluted 1:75 or 1:100), normal rabbit serum, or PBS, then incubated and washed exactly as described above for IF staining. After drying, each cell culture was treated with 0.1 ml of the optimal dilution (1:150) of peroxidase-labeled anti-rabbit immune globulins. Again, the conjugate was diluted in 20% beef brain in PBS. The slides were incubated at 36°C in a humidified atmosphere for 20 min, and then washed by two 10-min rinses in PBS and two 1-min rinses in distilled water. Aminoethyl carbazole substrate (4, 6) was prepared by dissolving 2 mg of 3-amino-9-ethyl-carbazole (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in 0.5 ml of dimethylformamide, adding 9.5 ml of 50 mM acetate buffer, pH 5.0, filtering through no. 41 ashless filter paper, and, immediately before use, adding 1 drop of 3% H₂O₂. Without drying, the slides were placed in a 150-mm glass petri dish and covered with 75 ml of the substrate; incubation was conducted for 10 min at room temperature. The slides were then rinsed in distilled water, mounted in Elvanol medium (5), and observed with an ordinary light microscope. Reaction product at the site of rubella antigen-antibody complexes appeared as a red precipitate in the cytoplasm of infected cells. Reactions were graded as for IF staining.

Inactivation of endogenous peroxidase activity. Several procedures were used in efforts to inactivate endogenous peroxidase activity of certain specimens, which was evidenced by IP activity in cell cultures treated with negative serum or with conjugate alone, without destroying specific rubella antigen. Methods utilizing methanol containing nitroferrocyanate and acetic acid (15) or ethanol with HCl (16) destroyed specific antigen. However, a method employing sodium azide and hydrogen peroxide (17) effectively removed endogenous peroxidase without adversely affecting rubella antigen. For this procedure, acetone-fixed specimens were treated for 0.5 h at room temperature with 0.001 M sodium azide dissolved in 0.05 M tris(hydroxymethyl)aminomethane buffer at pH 7.6 containing 0.01% H₂O₂. After three 5-min rinses in PBS and a 1-min rinse in distilled water, the slides were air dried and then stained by the IP method as described above.

Preliminary standardization of test systems. Before beginning comparative studies on identification of rubella virus isolates, preliminary studies were performed to determine the optimal concentrations of immune reagents to use in the two test systems and the appropriate time at which to examine inoculated-cell cultures by IP and IF staining.

BHK-21 cell monolayers in eight-chamber slides were inoculated with the RV laboratory strain of rubella virus at a ratio of ≥ 1 infectious virus dose per cell. After 72 h of incubation at 36°C, when more than 90% of the cells contained antigen demonstrable by IF staining, the cell cultures, together with uninfected-cell cultures, were used for block titrations of varying dilutions of intermediate rubella immune rabbit serum against varying dilutions of the fluorescein-labeled and peroxidase-labeled anti-rabbit immune globulins. The optimal dilution of immune rabbit serum selected was one fourfold step more concentrated than the end point dilution, and the optimal dilution of conjugate was one that gave maximum staining of rubella virus-infected cells and no background or nonspecific staining. The optimal dilution of immune rabbit serum for use in IF staining was determined to be 1:50 and that for use in IP staining was 1:75 or 1:100. The optimal dilution of fluorescein-labeled goat anti-rabbit immune globulins was 1:50 and that of the peroxidase-labeled anti-rabbit immune globulins was 1:150.

To determine the appropriate time at which to examine inoculated cultures for rubella antigen by IP and IF staining, BHK-21 monolayers in eight-chamber slides were infected with graded amounts of the RV laboratory strain of rubella virus, including dilutions past the infectivity end point. At 24-h intervals, cultures infected with each virus dose were examined by IP and IF staining with optimal dilutions of rubella immune rabbit serum and the conjugated anti-rabbit immune globulins. At 72 h, positive staining could be demonstrated by both methods through the lowest concentrations of infectious virus. Based upon these findings, cells inoculated with test materials were examined after 72 h of incubation.

RESULTS

Comparative sensitivity of IP and IF staining for detection and identification of

rubella virus isolates. In developing IF procedures for identification of rubella virus isolates several years ago (14), it was noted that epithelial cells, mucus, or microbial agents present in clinical specimens frequently caused nonspecific fluorescence in cell cultures inoculated with these materials. To reduce this nonspecific activity, and also to increase levels of antigen for staining, we routinely perform two cell culture passages and examine the second-passage material by IF.

For the present studies, first-passage harvests from BS-C-1 or RK-13 cells, which had been inoculated with clinical materials and were subsequently shown to be positive or negative for rubella virus by subpassage and testing by interference and fluorescent-antibody staining (14) were inoculated into BHK-21 cells by the two procedures described above. Duplicate sets of cultures were then examined by IP and IF staining. Specimens were examined under different code numbers for IP and IF staining to avoid bias in reading the results.

Results obtained in testing 50 specimens by interference and by IP and IF staining on BHK-21 cells inoculated in suspension and grown into monolayers are summarized in Table 1. These specimens represented 25 isolation attempts made in BS-C-1 cells and 25 made in RK-13 cells. On initial testing months earlier, 22 of the specimens had given a positive interference reaction and rubella virus was identified by IF staining. On retesting, only 16 of the specimens gave a positive interference reaction; all of these were positive by both IP and IF staining, and an additional three specimens that had initially been positive by interference gave weak positive reactions only by IP staining.

Table 2 compares directly the reactions obtained in the IP system and in the IF system, and it is noteworthy that most of the nonspecific reactivity was seen with the same specimens in both systems. Four of the specimens were nonspecific in both systems, whereas one was nonspecific only by IP staining, and one was nonspecific only by IF staining.

The intensity of positive staining reactions was not markedly different in the two systems, but with a few specimens IP staining revealed slightly greater numbers of antigen-positive cells than were detected by IF staining (cf., Table 3).

Amount of rubella antigen produced in cell cultures inoculated by two different procedures. In both IP and IF systems, the BHK-21 cells infected in suspension and then permitted to grow into monolayers showed more staining than did cells infected as preformed monolayers. Table 3 compares the intensity of the reactions seen in each type of culture. In a

TABLE 1. *Comparative sensitivity of interference, IP staining and IF staining for detecting rubella virus isolates*

Interference results	No. of specimens	IP results			IF results		
		Positive	Negative	NS ^a	Positive	Negative	NS
Positive	16	16	0	0	16	0	0
Negative	34	3 ^b	26	5	0	29	5
Totals	50	19	26	5	16	29	5

^a NS, Nonspecific reaction.^b Weak positive reactions in specimens originally positive by interference.TABLE 2. *Comparative sensitivity and specificity of IP and IF staining for detecting rubella virus isolates*

IP results	No. of specimens	IF results		
		Positive	Negative	Nonspecific
Positive	19	16	3	0
Negative	26	0	25	1
Nonspecific	5	0	1	4

TABLE 3. *Intensity of positive IP and IF reactions in cultures infected as a cell suspension and as a preformed monolayer*

Specimen no.	IP reactions of cultures inoculated in:		IF reactions of cultures inoculated in:	
	Suspension	Mono-layer	Suspension	Mono-layer
6	±	±	0	0
9	+++	+	+++	+
10	+++	+	+++	+
15	++	±	++	±
20	++++	++	++++	++
21	+	±	++	+
25	+	+	+	+
30	+	+	+	0
31	+++	++	++	+
32	++	++	+	+
35	+++	+++	+++	+++
40	+	0	0	0
41	+	+	+	+
42	+++	++	+++	++
43	+	0	++	+
46	++++	++	+++	+
47	++	+	+	0
49	+++	++	+++	++
50	+	0	0	0

few instances specimens showing negative or minimal reactions in cultures inoculated as monolayers gave clearly positive reactions in cultures inoculated as cell suspensions. Figures 1 and 2 compare the extent of infection demonstrated by IP and IF staining in cultures inoculated with the same specimen by the two different procedures.

Efforts to use IP and IF staining for earlier identification of rubella virus isolates.

To explore the possibility of using IP staining for identification of rubella virus isolates at the first-cell-culture-passage level, clinical materials previously found to be positive or negative for rubella virus were inoculated into BHK-21 cells by the methods described in Materials and Methods, and after 72 h of incubation the cultures were examined by IP and IF staining. The clinical materials consisted of four urine specimens, three of which had originally been positive for rubella virus, and six fetal tissues, three of which had been positive on original testing. As shown in Table 4, only one urine and one tissue specimen gave a positive interference reaction on retesting. The positive urine specimen showed nonspecific reactivity in the IP system, as did two negative specimens; however, treatment with azide-peroxide inactivated the endogenous peroxidase activity and permitted the demonstration of one positive and two negative reactions. The single tissue specimen giving a positive interference reaction was also positive by both IP and IF, and the tissues showed no nonspecific activity.

Tissue homogenates and slip smears from the six tissue specimens were acetone fixed and examined directly by IP and IF staining. The specimens were treated with azide-peroxide to inactivate endogenous peroxidase activity before IP staining. The homogenate of the tissue that was positive by interference showed a few granules of fluorescent material of questionable specificity in the IF system and a negative reaction in the IP system. The slip smear on this same specimen gave a weak, questionable reaction in the IP system and a negative IF reaction. No other positive and no nonspecific reactions were seen with these specimens.

DISCUSSION

A preliminary report by Gerna (3) based upon the use of laboratory strains of rubella virus, together with a few field strains, indicated the feasibility of using IP staining with antisera of human origin to detect rubella antigen in infected-cell cultures. Since human sera contain

FIG. 1. Comparison of the IP staining reaction in BHK-21 cells infected with a rubella-positive specimen in (A) preformed monolayers and (B) cells infected in suspension and grown into a monolayer.

antibodies against a variety of human viruses, they are not generally recommended for use in virus identification. The present studies have shown that rubella immune rabbit serum can be employed as a specific reagent for use in identifying rubella virus strains by IP staining. However, to be suitable for use in either IP or IF staining, the rabbit antisera must be produced in such a way as to be free from antibodies to host cell components. We have found that a rubella rabbit antiserum from one commercial

source (Flow Laboratories R 860145) was unsatisfactory for identification of rubella virus by IP and IF staining because of high levels of anti-host cell reactivity.

Our comparative testing on the same materials showed IP staining to be a highly satisfactory alternative to IF staining for identification of rubella virus isolates and one which might be particularly useful in laboratories without expertise and equipment for IF procedures. Although IP staining did not show markedly improved

FIG. 2. Comparison of the IF staining reaction in BHK-21 cells infected with a rubella-positive specimen in (A) preformed monolayers and (B) cells infected in suspension and grown into a monolayer.

TABLE 4. Identification of rubella virus isolates at the first-cell-culture-passage level by IP and IF staining

Type of specimen	No. tested	Interference results	No. of specimens	IP results			IF results		
				Positive	Negative	NS ^a	Positive	Negative	NS ^a
Urine	4	Positive	1	1 ^b	0	1	1	0	0
		Negative	3	0	3 ^c	2	0	3	0
Tissue	6	Positive	1	1	0	0	1	0	0
		Negative	5	0	5	0	0	5	0

^a NS, Nonspecific reaction.
^b Specimen originally nonspecific, but gave a specific positive reaction after sodium azide-peroxide treatment.
^c Two specimens originally nonspecific, but gave negative reactions after sodium azide-peroxide treatment.

sensitivity over IF staining in terms of earlier detection of positive cultures or greater intensity of staining reactions, several findings did suggest a slightly greater sensitivity of IP staining over that of IF staining. The most notable was the fact that IP detected low levels of antigen in a few specimens that were no longer positive by interference and were negative by IF staining. In a few cases more positive cells were seen in cultures stained by IP than in parallel cultures stained by IF. Also, rubella immune serum and the labeled anti-rabbit conjugate could be used more dilute in the IP than in the IF system. Greater experience in testing rubella-positive materials by both methods will be required to conclusively establish any marked advantages of IP over IF in terms of increased sensitivity.

With small amounts of viral antigen, the contrast of positive staining might be expected to be more sharp in the IF than in the IP system. However, the red reaction product produced from the aminoethyl carbazole substrate gave excellent contrast in the IP system. Whether this contrast would be equally good in smears, rather than monolayers, of infected cells is uncertain. A major advantage to the use of aminoethyl carbazole substrate in the IP system is that, unlike the benzidine derivatives, it is not classified as a potential carcinogen.

With second-cell-culture-passage materials, IP and IF staining showed comparable specificity, and the few specimens which gave nonspecific reactions generally did so in both systems. However, first-passage-cell-culture material inoculated with urine specimens showed more nonspecific reactivity in the IP than in the IF system. This was apparently due to endogenous peroxidase activity in the inocula, and nonspecificity could be overcome by treatment of the inoculated cells with sodium azide and peroxide before IP staining.

With the use of azide-peroxide treatment, it appears that IP staining might be made feasible for identification of rubella virus isolates at the first-cell-culture-passage level, and additional studies have suggested that sensitivity might be enhanced by incubating the inoculated cultures for 5 days before IP staining is performed.

BHK-21 cells were particularly well suited to use for identification of rubella virus isolates by IP and IF staining, since detectable amounts of antigen were produced relatively rapidly, and no problems with nonspecific staining or background reactivity were seen. Gerna (3) encountered problems with nonspecific staining, particularly when the direct IP method was used, in Vero cell cultures, another of the more sensitive cell lines for propagation of rubella virus.

An important finding in these studies was that cells inoculated in suspension with viral isolates and then permitted to grow into monolayers produced greater amounts of rubella antigen than did cells infected as intact monolayers and that the use of the cell suspension inoculation technique could increase the sensitivity of IP and IF assays for detection and identification of rubella virus isolates. Previous studies in this laboratory showed that infection of BHK-21 cells in suspension with rubella virus before planting also resulted in greater yields of hemagglutinating and complement-fixing antigen than were obtained by infecting monolayer cultures (13). It is well recognized that rubella virus passes from parent to daughter cell during cellular division (9), and it appears likely that in monolayer cultures in which cells are contact inhibited there is less opportunity for cell-to-cell spread of virus via cellular division than exists in cultures in which cells infected in suspension are then permitted to grow into a monolayer.

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