Studies of Rubella Virus

Shunzo KONISHI and Kiyoaki NOHARA

Department of Pediatrics
(Chief: Prof. S. Konishi)
Yamaguchi University School of Medicine
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Although a fairly long time passed following the supposition that rubella was a viral disease,[1] the isolation and identification of rubella virus was difficult.

In 1962 Parkman et al.[2] first reported the isolation of rubella virus using African green monkey kidney tissue culture by the indirect method using interference phenomenon with ECHO virus. At the same time Weller et al.[3] reported the isolation of rubella virus by the direct method using primary human amnion tissue culture.

An unusually extensive epidemic of rubella throughout the United States in 1964 provided a stimulus for the study of rubella virus.

This paper presents virological and seroimmunological studies of rubella patients who had the disease during a rather large epidemic in Yamaguchi in 1966-1967, and, also studies of tissue culture cells used for the isolation of rubella virus.

MATERIALS AND METHODS

1. Clinical Materials

A fairly large epidemic of rubella was experienced in Yamaguchi districts during 1966-1967. Of patients who clinically showed rubella symptoms, 127 cases were investigated virologically and seroimmunologically.

2. Methods

(1) Specimens

(a) Throat swabs and stools were used for the isolation of the rubella virus.

(b) Sera, both in the acute and convalescent periods, were used for the determination of antibody titers.

(2) Cell cultures

(a) African green monkey (Cercopithecus aethiops) kidney cells (GMK). Monkey kidney was treated as described by Parkman, and cell cultures were used on the 4th or 8th day after inoculation when they grew full sheets.

(b) Primary rabbit embryo cells (RE).

White rabbits, New Zealand species, were opened by laparotomy between the 17th and the 21st day of conception and fetuses free of bacilli were removed. Skin and muscle layers of fetuses were cut down in size to about

5 mm square, suspended in 400-500 ml of PBSA solution with penicillin 400 u/ml and streptomycin 400 μg/ml with trypsin solution added, digested by rotation in a centrifuge at 500-600 r.p.m. for 30 minutes at 37°C, and supernatant fluids were spun in a centrifuge at 800 r.p.m. for 6 minutes at 4°C. After the sediments were washed with LE medium supplemented with 5 % calf serum, they were suspended in the same solution and were filtered through two sterilized gauzes. They were controlled with LE medium supplemented with 5 % calf serum as the number of cells was $25-40\times10^4$/ml, and were inoculated to tubes 1 ml respectively. Tubes continued to be cultivated at 37°C for 4 or 5 days, and were used for studies of viruses when cells grew full sheets.

(3) Cell culture media

(a) Media for GMK cells. (i) Growth medium: LE medium supplemented with 5 % inactivated calf serum, penicillin 100 u/ml and streptomycin 100 μg/ml. (ii) Maintenance medium: LE medium supplemented with 2 % inactivated calf serum, penicillin 100 u/ml and streptomycin 100 μg/ml.

(b) Media for RE cells. (i) Growth medium: LE medium supplemented with 5 % inactivated calf serum, penicillin 100 u/ml and streptomycin 100 μg/ml. (ii) Maintenance medium: No. 199 medium supplemented with 2 % inactivated calf serum, penicillin 100 u/ml and streptomycin 100 μg/ml.

(4) Rubella virus and antiserum

(a) RV-Y strain (Rubella virus Yamaguchi); isolated by the authors in the epidemic of rubella in 1966.

(b) M-33 strain; adapted for GMK cells and supplied from the National Institute of Health of Japan.

(c) M-33 antiserum; supplied from the Natiomal Institute of Health of Japan.

(5) Isolation of virus

(a) GMK cells-ECHO-11 virus system.

GMK cell monolayers; maintenance medium was changed to growth medium on the day prior to use; were washed twice with PBSA solution just before inoculation of specimens; 4 tubes of GMK monolayer sheets were inoculated with 0.2 ml of the specimen, remained still for an hour at room temperature for absorption, then washed twice with PBSA solution, 1 ml of maintenance medium was added, then cultured on roller drums in the incubator at 37°C for 8 days and observed daily for cytopathic effect. Media were changed if pH became markedly acid. Two of the four tubes inoculated with specimens were challenged with ECHO-11 virus 100 TCD_{50}/ml on the 9th day of culture, and the cytopathic changes on both challenge group and non-challenge group were observed. As the tubes which did not show cytopathic changes were considered as having interference phenomenon between ECHO-11 virus and agent in

specimen and the tubes which showed cytopathic changes were considered as not having interference phenomenon, it was decided that virus was positive on the former and negative on the latter.

(b) RE cells system.

The method of inoculation of specimens was the same as for GMK cells-ECHO-11 virus system. RE cell monolayers; maintenance medium was changed to growth medium on the day prior to use, were washed twice with PBSA solution just before inoculation of specimens; 4 tubes of RE monolayer sheets were inoculated with 0.2 ml of specimen respectively, remained still for an hour at room temperature for absorption, again washed twice with PBSA solution, 1 ml of maintenance medium No. 199 was added, then cultured on roller drums in the incubator at 37°C for 2 weeks, and observed daily for cytopathic changes. It was considered that viruses were present in tubes which showed cytopathic changes.

(6) Identification of virus

The same volume of rubella antiserum, 20 unit/0.1 ml, and 10-fold diluted suspension of isolated virus were mixed and the identification was carried out by the same method as for the neutralization test.

(7) Neutralization test

Rubella virus was diluted with LE solution supplemented with 10 % pooled normal rabbit serum (PNRS) so as to give 100 InD_{50} of rubella virus (M-33 strain, $10^{-3.7}$ InD_{50}) per 0.1 ml as the final mixture. All sera of patients both in the acute and convalescent periods were inactivated at 56°C for 30 minutes and diluted serially 2-fold with PNRS. Virus suspension and sera, 0.4 ml respectively, were mixed, remained still for 2 hours at room temperature, and 0.2 ml of mixture for every dilution was put into tubes of GMK cell monolayers, remained still again for an hour at room temperature, added with 1.0 ml of LE solution supplemented with 2 % inactivated calf serum, and cultivated on drums in the incubator at 37°C for 5 days. Every tube was challenged with ECHO-11 virus, 100 TCD_{50}/ml, on the 6th day and the cytopathic changes were observed daily thereafter.

RESULTS

1. Virological Studies on Primary Rabbit Embryo Cells

(1) Cytopathic changes of primary rabbit embryo (RE) cells with rubella virus:

Cytopathic changes of RE cells were observed on the 6th or 7th day with M-33 strain and on the 9th or 10th day with RV-Y strain after inoculation with rubella virus, as shown in Figure 1-3. Figure 1 shows uninoculated normal RE cells whichl are densely packed, elongated, spindle shaped with a fibroblastic

appearance. Figure 2 shows RE cells on the 8th day after inoculation with rubella virus M-33 strain. Characteristic elongated, spindle shape is being lost and becomming more round and cytoplasmic threads are becomming more obvious. Figure 3 shows RE cells on the 14th day after inoculation with the same strain. There is a generally loose appearance but with concentration in some parts; cells are more degenerated, and detaching from the surface of the tubes.

(2) Growth of rubella virus on RE cells:

Growth of rubella viruses, M-33 strain and RV-Y strain on RE cells by the direct method was investigated and compared with growth on GMK cells by the indirect method using interference phenomenon, and the results were as shown in Table 1. The titer of rubella virus on the 5th, 7th and 10th day after inoculation with M-33 strain on RE cells was somewhat lower than on GMK cells showing 1.5, 2.0 and 3.5 on the former and 2.7, 3.5 and 4.0 on the latter respectively, but they showed nearly equal titer, 3.5, on the 15th day. The titer with RV-Y strain was, by comparison, somewhat higher on RE cells than on GMK cells showing 3.5, 4.3 and 4.3 on the former and 3.0, 3.7 and 4.0 on the latter. This showed that RE cells were not inferior to GMK cells for the growth of rubella virus.

Table 1. Rubella Virus Titer on GMK and RE Cells (Negative Log)

Strain	Cell System	Titer of Rubella Virus per 0.1 ml			
		5th day	7th day	10th day	15th day
M-33 (N. I. H. J.)	GMK	2.7	3.5	4.0	3.5
	RE	1.5	2.0	3.5	3.5
RV-Y (Yamaguchi)	GMK	3.0	3.7	4.0	4.0
	RE	3.5	4.3	4.3	4.3

(3) Susceptibility of RE cells to viruses:

The susceptibility of RE cells to various viruses was investigated with results as shown in Table 2. They had sensitivity to rubella virus and also herpes simplex virus, but not to Polioviruses, Coxsackie viruses, ECHO viruses, Adenoviruses and measles virus.

2. Virological and Seroimmunological Studies on Patients with Rubella

(1) Isolation of rubella virus

(a) One hundred and twenty-seven rubella patients were investigated in order to isolate the rubella virus. By the indirect method using GMK cells, 31

Table 2. Susceptibility of RE Cells to Various Viruses

Enterovirus		
Polio	I (−)	
Polio	II (−)	
Polio	III (−)	
Coxsackie	(−)	A : 7, 9, 21
		B : 1, 2, 3, 4, 5, 6
ECHO	(−)	1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 17, 19, 20, 26, 27
Adenovirus	(−)	1, 3, 12
Measles Virus	(−)	
Herpes Simplex Virus	(+)	
Rubella Virus	(+)	

strains were isolated at the first passage, 13 strains at the second passage and 2 strains at the third passage making a total of 48 strains (37.8 %). By the direct method using RE cells from the same patients, 30 strains were isolated at the first passage, 16 at the second passage and none at the third passage making a total of 46 strains (36.2 %). All of these strains were identified as rubella virus using rubella immune rabbit serum.

Table 3. Isolation of Rubella Virus With GMK and RE Cells

Cell System	No. of Cases Tested	No. of Cases Isolated	Passage Level at Which Virus Was Isolated		
			1st passage	2nd passage	3rd passage
GMK	127	48 (37.8 %)	33	13	2
RE	127	46 (36.2 %)	30	16	0

(b) Of 6 patients with rubella encephalitis, 2 agents were isolated from throat swabs and 1 agent from cerebrospinal fluids using GMK cells. All of these agents were identified as rubella virus using rubella immune rabbit serum as shown in Table 4.

Table 4. Findings of Rubella Encephalitis

Case Number	1	2	3	4	5	6
Age	11	9	13	11	12	11
Sex	M	F	F	F	M	F
Clinical Symptoms:						
Temperature (C)	36.6	37.0	39.0	38.0	38.0	39.1
Headache	(+)	(+)	(+)	(+)	(+)	(+)
Nausea	(+)	(+)	(+)	(+)	(+)	(+)
Vomiting	(+)	(+)	(+)	(+)	(+)	(+)
Coma	(−)	(−)	(+)	(+)	(−)	(+)
Convulsion	(−)	(−)	(+)	(+)	(−)	(+)
Dist. of Sensation	(−)	(−)	(−)	(−)	(+)	(−)
P. S. R.	(+)	↑	(+)	↓	↓	↑
A. S. R.	(+)	↑	(+)	↓	↓	↑
Kernig's sign	(−)	(−)	(−)	(+)	(±)	(−)
Stiffneck	(−)	(−)	(−)	(+)	(±)	(−)
Babinski's sign	(−)	(−)	(−)	(−)	(±)	(−)
W. B. C.	5,700	7,500	7,200	14,900	9,200	14,600
Cerebrospinal Fluid:						
Cells	$^{80}/_{3}$	$^{88}/_{3}$	$^{355}/_{3}$	$^{430}/_{3}$	$^{256}/_{3}$	$^{263}/_{3}$
Protein (mg/dl)	34	35	108	81	57	162
Sugar (mg/dl)	99	64	73	100	108	100
Pandy's Reaction	(+)	(+)	(+)	(+)	(+)	(+)
Virus Isolation:						
Blood	not tested	(−)	(−)	not tested	(−)	(−)
Throat Swab	(−)	(−)	(−)	(−)	(+)	(+)
Cerebrospinal Fluid	(−)	(−)	(−)	(−)	(−)	(+)

(c) From the fetus, after it was aborted artificially, of a mother who had clinical rubella at an earlier stage of pregnancy with the rubella virus being isolated from throat swabs of the mother, interference factors using the indirect method with GMK cells and cytopathic factors using the direct method with

RE cells were respectively isolated, as shown in Table 5. These factors were all identified as rubella virus using rubella immune rabbit serum.

Table 5. Findings of Case of Woman with Rubella during Pregnancy and in the Fetus

Age : 39 years old

Mother contracted rubella during the first month of pregnancy

Clinical Findings :

Temperature 37.5°C
Typical Rubella Exanthema (+)
Swelling of Lymph Nodes (+)
Angina (+)
Conjunctivitis (−)
Chest and Abdomen ; normal
W. B. C. 3,100

Virological Studies :

Virus Isolation ; Throat Swab (+)
Blood (−)
Feces (−)
Fetus (+)

Neutralization Antibody Titer ;
Acute Phase < 4×
Convalescent phase 256×

(2) Neutralization test

(a) Neutralizing antibody titers were measured on 62 pair of sera of rubella patients and rising of titer above 4-fold was observed in 54 cases (87.0 %), 2-fold in 2 cases, 4-fold in 11 cases, 8-fold in 13 cases, 16-fold in 11 cases, 32-fold in 3 cases, 64-fold in 9 cases, and 128-fold in 7 cases, as shown in Table 6.

(b) In the antibody response of 126 pregnant women in Yamaguchi districts (shown in Table 7), the titer was under 4-fold in 19 cases (15.1 %), and above 4-fold in 107 cases (84.9 %).

Table 6. Antibody Response of Rubella Patients

No. of Cases	Indicated Fold Increase							
	0	2	4	8	16	32	64	128
62	6	2	11	13	11	3	9	7
Total	8 (13.0%)		54 (87.0 %)					

Table 7. Antibody Response of Pregnant Women

No. of Cases	Antibody Titer		
	$< 4\times$	$\geqq 4\times$	$\geqq 8\times$
126	19 (15.1 %)	107 (84.9 %)	86 (68.2 %)

DISCUSSION

In 1938 Hiro and Tasaka[1] confirmed the agent of rubella as filtrable observing that children who were inoculated subcutaneously with filtration of throat swabs of rubella patients through Berkefeld-W or Seitz-EK filters showed clinical symptoms of rubella soon afterwards. In 1953 Krugman[4] demonstrated the presence of rubella virus in the blood of persons after they had been inoculated with blood taken from patients in the acute period of rubella. But the virus was not easy to isolate and identify because unlike other exanthematic viruses, such as measles, varicella and certain strains of ECHO viruses, the agent from rubella patients produced no cytopathic changes on tissue cultures, and the multiplication of the virus could be demonstrated only indirectly by means of interference phenomenon, a method similar to that employed by Tyrell[5] in studies of the common cold, and, therefore, the study of rubella virus was suspended for a fairly long time. In 1962, Parkman et al.[2] and Weller et al.[3] using different methods succeeded in the isolation of rubella virus at almost the same time. In 1963 and 1964 a severe epidemic of rubella in the United States provided the stimulus for progress in studies of rubella not only epidemiologically, clinically, and teratologically but also virologically and seroimmunologically.

At present, there are two methods, indirect and direct, for the isolation of rubella virus.

In the indirect method, which was first described by Parkman et al.,[2] GMK cells are inoculated with the agent and then later challenged by ECHO-11 virus 100 $TCID_{50}$/ml in order to recognize the multiplication of rubella virus using the interference phenomenon between rubella virus and the challenge virus. The decision as to whether or not rubella virus multiplies on GMK cells is done as follows: if cytopathic changes do not appear on the GMK cells, rubella virus grows and the interference phenomenon is carried out between rubella virus and challenge virus, but, on the contrary, if cytopathic changes appear on the GMK cells, rubella virus is not growing and the interference phenomenon is not carried out. As challenge viruses, not only ECHO-11,[6]~[23] Coxsackie

A-9 viruses[24)~34)] but also Polio, Influenza A, Parainfluenza, Mumps and SV-40 viruses have been reported to show the interference phenomenon with rubella virus.[35)36)] Sever et al.[7)] reported that Coxsackie A-9 virus was superior to ECHO-11 virus as a challenge virus, because the interferenoe phenomenon was more distinct. But, as the authors did not find any difference between ECHO-11 virus and Coxsackie A-9 virus for interference phenomenon with rubella virus, we usually used GMK cells-ECHO-11 virus system. Anyhow, the indirect method using interference phenomenon is a complicated procedure involving the process of challenge, volume of challenging viruses and incomplete interference.

The direct method was first described in 1962 by Weller et al.[3)] who used cytopathic changes on primary human amnion cells as the direct index for the multiplication of rubella virus. Subsequently, primary rabbit kidney cell,[37)] rabbit kidney cell line (GLR K13),[37)] African green monkey kidney cell line (GMK, AH-1),[38)] rabbit cornea cell line (SIRC)[39)] and BHK 21/W12 cell[49)] were reported as being used directly for the multiplication of rubella virus. These cells, however, were not entirely satisfactory for ordinary use, because they needed a relatively long period for the appearance of cytopathic changes and the cytopathic changes themselves were sometimes obscure. Futhermore, it was rather troublesome to get these cells and to maintain them in a satisfactory condition.

The authors have confirmed RE cells as showing cytopathic changes directly with rubella virus as Reddick et al.[40)] reported. The authors partially modified Reddick's method by not using whole rabbit embryo tissue but using only skin and muscle tissues to avoid mixing with many erythrocytes and various tissue cells on dispersion with trypsin. Also, cell concentration for planting was adjusted with growth medium to $30\text{-}40\times10^4$/ml instead of 100×10^4/ml to make thin and clear monlayer sheets. When several media, No. 199 medium supplemented with 13 % horse serum, No. 199 medium supplemented with 5 % horse serum, LE medium supplemented with 5 % horse serum and LE medium supplemented with 5 % calf serum, were compared as growth media suitable for condition of cells and the multiplication of virus, the same results were obtained as Reddick[40)] reported with LE medium supplemented with 5 % calf serum. When several media, No. 199 and LE medium supplemented with various percentage of horse serum or calf serum, were compared as maintenance media suitable for appearance of cytopathic changes and lesions of cells, it was found that both media supplemented with about 2 % serum were suitable, but both media over 5% serum markedly prohibited the appearance of cytopathic changes, the same as Günalp[38)] reported about GMK, AH-1 cells. On the other hand, LE medium was inclined to lower the pH of the medium resulting in marked lesion of cells, as Günalp[38)] reported. Therefore, No. 199 medium supplemented with 2 % calf serum was used routinely, as it did not lower the pH or result

in lesion of cells.

Cytopathic effect on RE cells appeared on the 9th or 10th day after inoculation with GMK adapted rubella virus M-33 strain. The cytopathic effect of the newly isolated rubella virus, RV-Y strain, appeared on the 6th or 7th day after inoculation. Reddick[40] reported that newly isolated rubella virus AE and LB strains showed cytopathic effect on the 6th day and the other cell adapted viruses, Bell and M-33 strain, showed cytopathic effect on RE cells on the 7th day when the strain was passed ten times serially on RE cells, similar to the report by Günalp et al.[38] of the appearance of cytopathic effect comming earlier when passage of virus was repeated.

RE cells inoculated with rubella virus showed distinct differences from uninoculated normal cells. As described before, cytopathic changes were characterized by cells loosing their fibroblastic, elongated, spindle shape, becomming more round, the cytoplasmic threads becomming more obvious, and finally becomming more condensed, and detaching from the surface, the same as Reddick[40] reported.

Rubella viruses were iosolated from throat swabs,[2)7)11)41)42)] blood,[34)37)41)] stools,[4)~44)] urine,[11)12)24)43)] cerebrospinal fluid,[24)26)43)45)] lymph nodes,[37)] lens,[11)12)] iris,[46)] liver biopsy,[11)24)26)] bone marrow,[43)45)] cornea,[11)12)] necropsy materials,[12)24)26)33)] fetuses,[12)23)33)] amniotic fluids,[12)33)] etc. Of these, viruses are isolated most easily from throat swabs. Krugman[42)] reported that the percentage of virus isolation from rubella patients depended upon the time materials were obtained, and that rubella virus was isolated more often from throat swabs than from blood or stools until about one week after eruption. Frankel[47)] also reported on the isolation rate, reporting that the earlier the swabs were obtained the higher the rate of isolation. Frankel[47)] reported a high rate of 83 percent for rubella virus isolated from throat swabs obtained within 12 hours after eruption but the rate dropped to 44 percent for swabs obtained 13 to 24 hours after eruption.

The authors isolated rubella viruses from throat swabs of 127 rubella patients as follows: by the indirect method using GMK cells, 31 strains were isolated at the first passage, 13 strains at the second passage and 2 strains at the third passage making a total of 48 strains (37.8 %), and by the direct method using RE cells from the same patients, 30 strains were isolated at the first passage, 16 at the second passage and none at the third passage making a total of 46 strains (36.2 %). These results show no significant difference between the indirect method using GMK cells and the direct method using RE cells concerning with the isolation of rubella virus. Multiplication of rubella virus on both cells, GMK and RE, was almost the same although the titers of M-33 strain of rubella virus on the 5th, 7th and 10th day after inoculation on RE cells were slightly lower than on GMK cells, but they were nealy equal on the

15th day; the titers of RV-Y strain on RE cells were slightly higher at all stage than on GMK cells. The neutralization test of rubella virus by the W.H.O. method showed the same titer on both GMK and RE cells.

Thus, primary rabbit embryo cells are not inferior to African green monkey kidney cells-ECHO-11 virus systems, which at present have been generally used for the study of rubella virus. The primary rabbit embryo cells are highly susceptible to rubella virus, have the capacity of multiplication, are easy to obtain, cultivate and maintain, very simple to use, and can be observed directly for cytopathic changes.

Since Selzer[17] in 1963 isolated rubella virus from the aborted fetus of a mother who suffered from rubella, many reports[48] about the isolation of rubella virus from fetuses have been published. The authors virologically and serologically confirmed rubella infection of a pregnant woman, and isolated rubella virus from fetus which was aborted artificially on the 8th week after conception because of fear of a congenital rubella syndrome.

SUMMARY

During the rubella epidemic of 1966-1967 in Yamaguchi, one hundred and twenty-seven patints with rubella were studied virologically and seroimmunologically, and the following results were obtained:

(1) Concerning the isolation of rubella virus, the direct method using primary rabbit embryo cells was investigated and compared with the indirect method using African green monkey kidney cells and it was found that RE cells were almost the same as GMK cells for susceptibility to rubella virus, that RE cells showed simple cytopathic changes which could be observed directly, and they were very simple to obtain and to manage so that they are well suited for studies of rubella virus.

(2) From 127 patients, 48 strains (37.2 %) of rubella virus were isolated by the indirect method using GMK cells and 46 strains (36.2 %) were isolated by the direct method using RE cells.

(3) From 6 patients with rubella encephalitis, two strains of rubella virus from throat swabs and one strain from cerebrospinal fluid were isolated.

(4) From a fetus, after artificial abortion, of a mother who had had rubella at an early stage of pregnancy, rubella virus was isolated suggesting the possibility of a congenital rubella syndrome.

(5) Fifty-four cases (87.0 %) out of 62 cases of rubella patients, pair sera were obtained, showed neutralizing antibody titer rising more than 4-fold.

(6) The antibody response of 126 cases of pregnant women to rubella virus was examined and found to be under 4-fold in 19 cases (15.1 %) and above 4-fold in 107 cases (84.9 %).

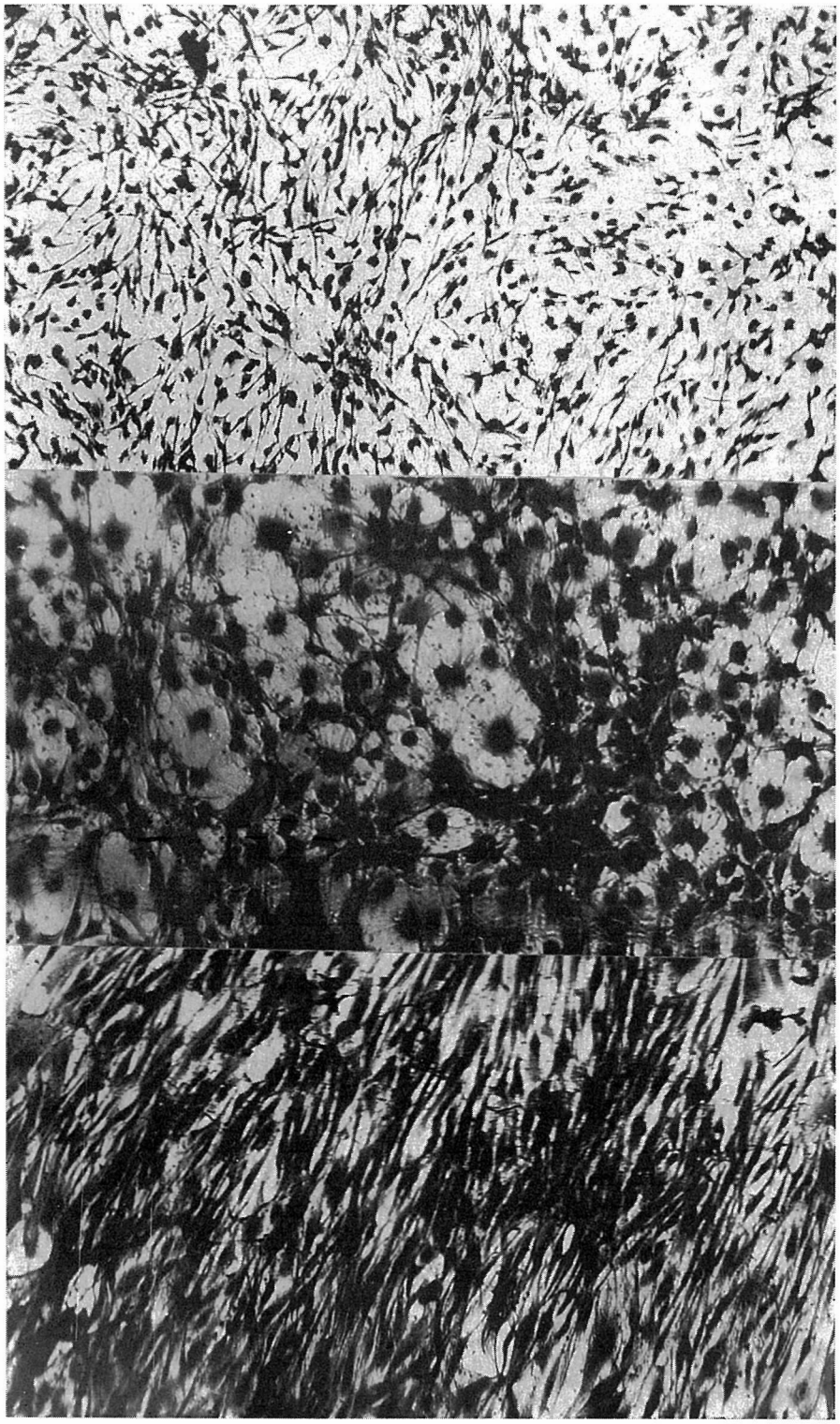

Fig. 1. Uninoculated normal RE cells.

Fig. 2. RE cells on the 8th day of inoculation of rubella virus, M-33 strain.

Fig. 3. RE cells on the 14th day of inoculation of rubella virus, M-33 strain.

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