rubella rash has nothing of the blotchy appearance of a well-developed measles eruption, while the brilliant phenomenon of "staining" so characteristic of measles is unlikely to occur in rubella unless the rash is of exceptional severity.

Table IV shows the incidence of physical signs in 114 patients with rubella seen in 1962.

TABLE IV.—*Incidence of Physical Signs in 114 Patients With Rubella (1962)*

	No. of Patients	
Injection of tonsillo-pharyngeal area		
Without discomfort	80	94%
With "	27	
Absent	7	
Glandular enlargement		
Posterior cervical and suboccipital	78	97%
" " only	16	
Generalized	17	
Absent	3	
Suffusion of eyes		
Present	90	85%
Severe with photophobia	7	
Absent	17	
Palatal petechiae		
Present	19 (17%)	
Absent	95	

Laboratory Aids

The white blood cell count is the only laboratory investigation that has hitherto had any practical value in the diagnosis of the disease. This is characteristically leucopenic at onset, with Türk cells appearing in considerable numbers. Hynes (1940) and Hillenbrand (1956) agree as regards these features, but the former found leucocytosis at later stages, while the latter described persistence of the original picture. Further, although Hynes found that Türk cells reached a maximum on the fourth day and disappeared by the tenth day, Hillenbrand observed their persistence up to as long as nine months from the onset. In this series leucopenia was present in 63% of cases and the Paul–Bunnell test was negative in all patients with generalized lymphadenopathy.

Recent work by Plotkin *et al.* (1963) shows that identification of the virus during the first three days of the disease is feasible and that the demonstration of neutralizing antibody may provide a valuable diagnostic test.

Complications

Most patients make an uneventful recovery and may be regarded as non-infectious after the sixth day following the appearance of the rash. Transient arthralgia is the commonest complication but may be the precursor of frank arthritis. Involvement of the central nervous system occurs more often than is generally believed (Pampiglione *et al.*, 1963). But since rubella is not a notifiable disease an accurate estimate of the incidence of encephalitis in each year proved impossible. The condition would appear to be spontaneously reversible, although Miller *et al.* (1956), reviewing 80 cases in the literature, estimated a mortality of 20% in clinically manifest encephalitis. Respiratory complications *per se* are rarely encountered; when they occur they are associated with involvement of the central nervous system. The use of corticosteroids and artificially assisted respiration may have contributed to the recent considerable fall in mortality.

Summary

Four hundred cases of rubella, or suspected rubella, admitted to two infectious diseases units from 1955–62 have been reviewed.

Even in epidemic periods, accurate diagnosis, so essential in the first 12 weeks of pregnancy, may prove difficult. Stricter attention to the clinical pattern would improve the standard of diagnosis. Confusion mostly arises with measles, scarlet fever, infectious mononucleosis, and toxic erythemata of drug origin.

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LABORATORY STUDIES ON RUBELLA AND THE RUBELLA SYNDROME

BY

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In the past few months several reports have appeared from North America of the isolation of viral agents from cases of rubella. With one exception (Weller and Neva, 1962) the isolations were made from throat washings, and in a few instances from the blood and urine, by inoculation of monkey-kidney cell cultures prepared from the African green monkey, *Cercopithecus aethiops* (Parkman *et al.*, 1962; Sever *et al.*, 1962; Sigurdardottir *et al.*, 1963). Unlike other exanthematic viruses, such as measles, varicella, and certain strains of E.C.H.O. virus, the agents from rubella patients produced no cytopathic changes in tissue culture, and evidence of virus multiplication could be demonstrated indirectly only by means of the interference phenomenon, a method similar to that employed by Tyrrell *et al.* (1960) in studies on the common cold. Monkey-kidney cultures inoculated with rubella material were superinfected with another virus, such as E.C.H.O. 11 or Coxsackie A 9, that was known to produce a cytopathic effect. The rubella-positive cultures resisted the challenge inoculation, in contrast to the control cultures, which were completely destroyed. McCarthy *et al.* (1963) have recently described cytopathic changes in a continuous line of rabbit-kidney cells inoculated with rubella virus.

Compared with measles, rubella or German measles is a mild disease and would be of little importance in human

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medicine were it not for the effect that the virus has on the human foetus. It is now well established that infection with rubella in the first three months of gestation may give rise to congenital abnormalities (Gregg, 1941 ; Lundström, 1962). Affected children typically have a combination of congenital defects which are collectively called the " rubella syndrome ": cataracts, deafness, microcephaly, mental retardation, and congenital heart disease. The rubella syndrome thus represents the result of an early intrauterine infection. From the preventive aspect there is a clear case for studying this disease, and because of the difficulties in differentiating rubella from other viral exanthemata on clinical grounds (Young and Ramsay, 1963) any laboratory methods of diagnosis would be helpful. From the immunological point of view rubella is of special interest because of the possibility that introduction of rubella antigen early in foetal development would create an immunologically tolerant state which would prevent the individual from responding to extrauterine rubella infection in the usual way. With the isolation of the virus, research into rubella can now be undertaken, and in this report we record the results of virus isolation from patients with rubella and of serological studies of cases of rubella and the rubella syndrome.

Material and Methods

Clinical Material

Patients thought to be suffering from rubella were seen either at the Hospital for Sick Children, Great Ormond Street, at the Infectious Diseases Department of the Royal Free Hospital now at Coppetts Wood Hospital, or in their own homes by the helpful co-operation of the Epidemic Observation Unit of the College of Practitioners. The clinical diagnosis of rubella was based on the following points: a rash typical in character and distribution, and occipital or post-auricular adenopathy. A low white-blood-cell count, the presence of Türk cells in the film, and a history of contact with rubella provided additional confirmatory evidence.

Material for virus isolation, which was collected as soon as the patient was seen, consisted of a swab from the posterior pharynx, 5–10 ml. of clotted blood, and in a few cases a specimen of urine. Swabs were placed into virus culture medium 199 containing 200 units of penicillin and 200 μg. of streptomycin per ml. Material for virus isolation was so far as possible inoculated within a few hours of collection, otherwise it was frozen and stored in dry ice.

Serum Specimens.—(1) A convalescent serum specimen was collected two to three weeks after the onset of the rash from as many patients as possible. (2) Cord blood sera were kindly supplied by the Obstetric Department, University College Hospital. (3) A single specimen of blood was collected from patients with the rubella syndrome. A child was regarded as having the rubella syndrome if there was a definite maternal history of an illness diagnosed as rubella in the first trimester of pregnancy and if the child was born with one or more of the following stigmata: cataracts, deafness, microcephaly, mental retardation, and congenital heart disease.

Cell Cultures

Primary monkey-kidney cell cultures from the African green vervet monkey were used. Trypsinized cells were kindly supplied by Dr. A. J. Beale, Glaxo Laboratories Ltd., in the form of a packed cell suspension. This was dispersed into tubes, approximately 75,000 cell aggregates per tube. The growth medium consisted of medium 199 with 4% inactivated calf serum. Tubes were incubated for four days at 37° C. and then the cultures were transferred to maintenance medium preparatory to inoculation. The maintenance medium consisted of medium 199, 1% inactivated calf serum, 0.088% sodium bicarbonate, and additional antibiotics. At this stage tubes were placed on a roller drum.

A continuous line of vervet-monkey-kidney cells obtained through the courtesy of Glaxo Laboratories was used in the 30th and 40th passage. Growth medium consisted of medium 199, 0.088% bicarbonate, and 8% calf serum. The maintenance medium contained 1% calf serum. A human embryo-lung-cell strain was developed from foetal lung tissue using the technique of Hayflick and Moorhead (1961). This cell strain was grown in Eagles medium with 10% calf serum and was maintained on Eagles medium 199 with 1% calf serum.

Techniques for Isolation and Characterizations of Virus

Urine specimens were first lightly centrifuged to remove debris and were then adjusted to neutral *p*H. Acute-phase blood samples were treated as follows. The serum was stored at −20° C. and the blood clot was lysed by the addition of 5 ml. of distilled water. After shaking, the lysate was separated from the debris by pipette.

Throat washings, urines, and blood lysates were each inoculated into six vervet-cell tubes, each tube receiving 0.1 ml. Inoculated tubes were placed on roller drums in the incubator at 35° C. and observed at regular intervals for cytopathic effect (C.P.E.). The maintenance medium was changed only if the *p*H became markedly acid. Between 7 and 10 days after inoculation half the tubes were superinfected with 500 $TCID_{50}$ (50% tissue culture infective doses) of E.C.H.O. 11 virus. The tubes were examined two days later for cytopathic effect as it was found that with this dose of virus the control cells were completely destroyed in this time. Tissue culture fluids for harvest and passage were first frozen and thawed and then stored in dry ice.

The same methods were used for inoculation of continuous vervet-kidney or human-embryo-lung cells. Some of the specimens were also inoculated into litters of newborn mice, each mouse receiving 0.05 ml. by the intraperitoneal and intracerebral routes. Sensitivity of the virus to ether was determined by shaking 10 ml. of tissue culture fluid with an equal volume of cold ether. The mixture was vigorously shaken for three periods of 20 minutes followed by overnight removal of the ether by vacuum in the refrigerator. An aliquot of virus fluid was shaken with medium 199 and was left overnight *in vacuo*. This provided the control.

The size of the virus was estimated by filtration through gradocol membranes.

Neutralization Test

All sera were inactivated at 56° C. for 30 minutes. Equal volumes of virus and serum dilution (usually 0.3 ml. each) were incubated in a water-bath at 37° C. for one hour, following which 0.1-ml. aliquots were inoculated direct into four to six vervet cell cultures. The virus titre was calculated so as to give 25 $TCIntD_{50}$ (50% tissue culture interfering doses) of rubella virus per 0.1 ml. of the final mixture. As control for serum toxicity and for the presence of antibodies to E.C.H.O. 11 virus, each serum was inoculated without virus into two sets of vervet-cell cultures, incubated in the same way, and superinfected with E.C.H.O. 11. None of the sera tested inhibited the E.C.H.O. 11 virus. Other controls on the neutralization test included a virus control in which the virus was

incubated with medium 199, and two negative control sera, one a guinea-pig serum, the other being obtained from a 10-months'-old infant who had not had clinical rubella or been exposed to it. After the cultures had been incubated at 35° C. for seven days, 500 $TCID_{50}$ of E.C.H.O. 11 virus was added to half the tubes. The test was read finally two days later. In these tests the presence of cytopathic changes from E.C.H.O. 11 virus indicated a lack of interference as a result of neutralization of rubella virus by specific antibody; conversely, the absence of cytopathic changes indicated that rubella virus had multiplied owing to the absence of rubella antibody.

Most of the neutralization tests were done with the WP reference strain, which was isolated in the United States and obtained through the kindness of Dr. Maurice Hilleman, of Merck Laboratories, West Point, Pa. This strain was passaged several times in vervet cells in our laboratory before use in the tests.

Results

Isolation of Virus

Throat swabs from 19 patients were tested in vervet cells, as summarized in Table I. All but 4 of the 19 swabs were inoculated within several days of collection and in many cases on the same day. Interference with the subsequent growth of E.C.H.O. 11 virus was demonstrated in 13 instances, in 11 out of the 15 that were inoculated promptly, and from two further specimens that had been stored for several months. It is noteworthy that an "interfering agent" was recovered from all six patients from whom

TABLE I.—*Results of Attempts to Isolate Interfering Agents in Primary Vervet-kidney Cells from Patients with Rubella*

Ref. No. of Patient	Sex	Age in Years	Rash Consistent with Rubella	Lymph-adenopathy	Total W.B.C. c.mm.	% Türk Cells	Definite Contact with Rubella	Day of Rash on which Specimen Taken	Results of Isolation: Throat	Blood	Urine
VL/63/39	F	18	+	+	4,600	4		1	+	−	−
VL/63 43	F	19	+	+	3,600	±†		1	+	−	−
VL/62 29	F	21	+	+	2,400	6	+	1	+	−	−
VL/63 179	F	14	+	+	N.T.	N.T.	+	1	+		
VL/63 188	M	19	+	+	N.T.	6	+	1	+		
VL/63 195	F	18	+	+	3,100	0		1	+	+	
VL/63/99	M	12	+	+	N.T.	N.T.	+	2	−		
VL/63/172	F	25	+	+	3,400	0		2	+	−	−
VL/63/174	F	19	+	+	3,200	0	+	2	+	−	
VL/63 192	M	19	+	+	5,100	3		2	−		
VL/63/212	F	11									
		12	+	−	N.T.	5	+	2	−		
VL/63/82	F	17	+	+	7,300	0		3	+		
VL/63 190	M	23	+	+	4,300	6	+	3	+		
VL/63 206	F	18	+	+	4,600	0		3	+	−	
VL/63 189	M	20	+	+	N.T.	2	+	6	−		
VL/63/130*	M	13	+	+	3,500	0		2	−		
VL/63/115*	F	21	+	+	2,300	0	+	2	+		
VL/63/113*	M	13	+	+	N.T.	N.T.		3	−		
VL/63/83*	F	32	+		4,700	±†		3	+		

N.T. = Not tested. * Specimens stored for two to three months before test. † Occasional Türk cells in film.

throat swabs were taken on the first day of the rash. Interference was also demonstrated with one out of seven blood samples obtained from patients while the rash was still present. Four urines were tested, but all were negative. With one or two exceptions these results have been confirmed by passage in vervet cell. The 19 patients from whom these specimens were derived all appeared to be typical cases of rubella (Table I), as determined by the criteria given under Materials and Methods.

Characterization of Virus

None of the specimens produced any visible cytopathic changes in primary vervet-kidney-tissue cultures over a two-weeks period of observation. The interfering agents, however, have now been passaged up to four times in vervet cells. Titres after passage have varied between $10^{2.0}$ to $10^{4.7}$ 50% interfering doses. After passage, interference could be demonstrated in continuous vervet kidney cells, but comparative titrations showed them to be at least a hundredfold less sensitive than primary cells derived from the same source. No interference could be demonstrated in human embryo lung cells or in rhesus or cynomolgus monkey kidney cells.

The MAR strain was selected as a typical local strain for further characterization. This agent produced no changes in newborn mice. Treatment with ether reduced the interfering activity of the virus from $10^{4.7}$ to less than 10^{1}. Filtration through a membrane with pores of 540 mμ did not inhibit the interfering activity, whereas after filtration through a 130-mμ filter it was completely abolished.

These viruses appeared to be highly sensitive to changes in temperature. One hour's incubation at 37° C. reduced the titre by one log. In a −20° C. deep-freeze cabinet, which was sometimes opened and shut several times a day, many of our tissue-culture specimens lost all activity, as measured by interference, in a matter of weeks.

Neutralization Tests

Table II gives the results of simultaneous titration of five pairs of acute and convalescent sera; from three of these

TABLE II.—*Results of Neutralization Tests on Acute and Convalescent Sera from Patients with Rubella Reciprocals of Neutralization Titres*

Ref. No. of Patient	Sex	Age	Typical Rubella Rash	Lymph-adenopathy	Contact with Rubella	Agent Isolated from Throat	Virus Strain used in Test	Acute Serum: Days After Onset of Rash	Acute Serum: Titre	Convalescent Serum: Days After Onset of Rash	Convalescent Serum: Titre
VL/63/39	F	18	+	+		+	MAR	1	<4	19	16
MAR.							WP*	1	<2	19	>16
									<2	43	64
VL/63/29	F	21	+	+	+	+	DAC	1	<16	15	>32
DAC.							MAR	1	<4	15	>16
							WP	1	<16	15	>32
VL/63/43	F	19	+	+		+	MAR	2	<2	21	>16
JON											
VL/63/160	M	11	+		+	N.T.	WP	2	<4	24	16
MOR.											
VL/63/176	F	47	+	+	+	N.T.	WP	3	>4	24	64
DOR											

*W_6^P. = West Point strain kindly supplied by Dr. Maurice R. Hilleman.

patients isolations were made from throat washings. All five pairs of sera showed a significant increase of titre against either the reference strain or the homologous strain. It should be noted that the increase of titre against one strain was reflected by an increase against another. In particular, MAR and DAC strains appear to be identical to the WP reference strain of the rubella virus. In addition 16 specimens of cord blood and six adult sera were tested for rubella antibody. Eighteen of these showed neutralizing antibodies with titres ranging from 1/4 to 1/1024. In four cases no activity was detected at a 1/4 dilution. These figures and the previous ones from cases of clinical rubella (Table II) suggest that the antibody response to rubella and the distribution of antibody level in the population are similar to those found in other viral infections.

Sera from 11 children with rubella syndromes presumably infected *in utero* by rubella virus were titrated for rubella-neutralizing antibody (Table III). Antibody was demonstrated in eight cases, titres ranging from 1/4 to 1/256. In the remaining three cases no neutralization could be detected at a 1/4 dilution of serum. It is probable that the antibody in the youngest child was due to residual

maternal antibody, as he was only 2 months old when the serum was collected. These results have been confirmed by repeat titrations on the negative sera and on the paired sera from cases of clinical rubella. Provided sufficient controls were included in the tests, it was possible to distinguish between sera in which no neutralizing activity could be demonstrated at 1/4 dilution from those in which there was activity at low dilutions of 1/4 or 1/8.

TABLE III.—*Neutralizing Antibody Titres to Rubella Virus*

Serum	Age in Years*	In Patients with Rubella Syndrome (Reciprocals of Neutralization Titres)		Neutralizing Titres
		Maternal Rubella in Month of Pregnancy	Congenital Defects	
VL/63/85	2/12	1st	Cat., PDA	64
VL/63/182	5/12	2nd	Microcephaly	<4
VL/63/97	11/12	1st	Deafness, cat., VSD	4
VL/63/87	1	1st	Micro., cat., deafness, PDA	64
VL/63/91	19/12	2nd	Cat., Fallot	<4
VL/63/171	2	2nd	Cat., PDA	16
VL/63/119	3	2nd	Cat.	<4
VL/63/164	4	2nd	Deafness, cat., Fallot	64
VL/62/275	5	3rd	Deafness, VSD	4
VL/63/103	10	1st	Cat., pulm. stenosis	256
VL/63/110	10	2nd	Deafness, PDA	256

* Age at time of collection of serum. Cat. = Cataracts. PDA = Patent ductus arteriosus. VSD = Ventricular septal defect. Fallot = Tetralogy of Fallot.

Discussion

In confirmation of recent reports of isolation of viruses from cases of rubella we have found that viruses interfering with the cytopathic effect of other viruses can be demonstrated with some regularity in throat swabs obtained on the first two days of the rash. Only primary vervet-kidney-cell cultures seem suitable to show this effect. These findings support the evidence obtained from earlier studies in human volunteers by Anderson (1949) and Krugman *et al.* (1953) that throat washings from rubella cases are infectious during the first 24 hours after the onset of the rash. The viruses isolated in this study to date are similar to those reported previously in that they are relatively large in size (>130), are sensitive to ether, and have no cytopathogenicity so far as is known for monkey-kidney-cell cultures.

Serological diagnosis of rubella infection was also possible using the conventional neutralization test with varying dilutions of serum. The data with human sera (Table II) suggests that antibody develops slowly in view of the length of the incubation period of rubella, which may be as long as 18 days (Krugman *et al.*, 1953), and that the viruses isolated in this series are similar antigenically to at least one North American strain. The recent report by McCarthy *et al.* (1963) that the virus has been adapted to growth in a continuous line of rabbit-kidney cells offers an alternative approach for studying rubella virus and the assay of antibody.

Apart from the academic delineation of the minor exanthemata, the fact that it is now possible to diagnose rubella infection by virus isolation from throat swabs and by serology is of practical importance in relation to rubella in pregnancy. Virological assistance could be valuable when a rubelliform rash occurs without known exposure to rubella. Moreover, as rubella can occur without a rash (Krugman *et al.*, 1953) it is now possible to determine whether in fact infection has taken place. The same applies to the laboratory diagnosis of encephalitis following a rubelliform rash. One serum has been tested from a child who developed encephalitis six days after "rubella," but no antibody could be detected in the specimen collected two months after the onset of the illness.

Children born with the rubella syndrome are of great interest immunologically. Logically, one could suppose three possible outcomes for a foetus sustaining an intrauterine rubella infection. The child could be born immune, it could be born incapable of making rubella antibodies, or it could be born without antibodies but develop them later in the course of a post-natal infection. Our preliminary data on 11 children with the rubella syndrome suggest that maternal antibodies are transmitted to the foetus but do not necessarily persist. Three children aged 5 months to 3 years had no rubella antibodies at a 1/4 dilution. On the other hand, most of the children did have rubella antibodies; but without serial studies we cannot tell whether these were acquired post-natally or not. It is conceded that we are measuring only humoral antibody; that it may not be possible by these tests to detect very low titres of antibody—that is to say, titres of less than 1/4; and there is no proof that the maternal illness was in all cases rubella. These are questions which it will be easier to answer with more experience of laboratory tests for rubella. Nevertheless, we have no evidence this far that children with the rubella syndrome differ immunologically from other children and no evidence of immunological tolerance. In this connexion it is interesting that these children do not seem to suffer fulminating infection with rubella.

Summary

The isolation is reported of viruses from typical cases of rubella which have similar properties to those recently reported from North America. The isolation rate from throat washings was significantly higher from specimens collected on the last day of the rash than on the second or third days. A rise in neutralizing antibody titre was demonstrated in convalescent sera. Sera from 11 children with the "rubella syndrome" were tested and no evidence of immunological abnormality was obtained.

A great many people have helped us in this study, and to all of them we are most grateful, especially to the patients who volunteered so willingly. We are particularly grateful to Dr. G. I. Watson and his colleagues in the Epidemic Observation Unit of the College of General Practitioners, and to the members of the consultant staff at the Hospital for Sick Children who have allowed us to investigate patients under their care, and also to Mrs. P. G. Freeman, of the Rubella Children's Association. Finally, our thanks are due to Mr. R. S. Lees, F.I.M.L.T., and the laboratory staff of the Virus Unit, the Hospital for Sick Children, Great Ormond Street, without whose help and technical skill this work could not have been undertaken.

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