a bright greenish-yellow fluorescence. 2. The fluorescent circumoval precipitin technic demonstrated excellent diagnostic performance. Positive reactions were obtained in 30 sera from individuals with known infections. Negative reactions were obtained in 20 sera from individuals not infected with *S. mansoni* and in the sera of 25 children, heavily parasitized with intestinal helminths, but not with *S. mansoni*. 3. The sensitivity and specificity of the circumoval reaction, in addition to the advantages introduced by the fluorescent antibody technic, might make the fluorescent circumoval precipitin technic highly reliable for serological diagnosis of schistosomiasis.

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Propagation in Tissue Culture of Cytopathic Agents from Patients with Rubella-Like Illness.* (27749)

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Recognition of the association between maternal rubella and congenital anomalies of the human fetus has stimulated increasing interest in a clinical entity previously considered benign. Although a viral etiology of rubella was suggested by the demonstration of the infectiousness for human volunteers of filtered inocula(1,2), reports of transmission to monkeys(3,4) and of propagation of the agent in the embryonated hen's egg(4) or tissue culture(5) have not been confirmed. Thus, the nature of the etiologic agent remains uncertain. The lack of specific diagnostic procedures has made evaluation of rubella-like illness in the pregnant female a perplexing problem. Similarly, interpretation of reports such as the isolation of adenoviruses from individuals with rubella(6) has, of necessity, been speculative.

In October, 1960, attempts to isolate an agent from the urine of a boy with an exanthematous illness resulted in recovery of a virus characterized by unique cytopathic activity in primary human amnion cell cultures. The attendant circumstances led to investigation of an institutional outbreak of rubella at Phillips Exeter Academy, Exeter, N. H., in February, 1961. Similar viruses were recovered from the urine or blood of 2 patients; this was made possible by Dr. James T. Heyl, Medical Director, and our indebtedness to him for clinical data and for collection of sera is gratefully acknowledged. During the

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spring of 1962, Dr. Donald M. Clark of Phillips Academy in Andover, Mass. and Dr. Curtis Prout of the Harvard Health Services made cases of rubella available; to date, a fourth isolate has been obtained from a patient's urine.

While these studies were in progress, we learned of concurrent efforts at the Walter Reed Army Institute of Research (WRAIR) (7,8) to recover agents from patients with rubella using a different technical approach. Through the cooperation of E. L. Buescher, isolates were exchanged and found to be related.

This preliminary communication presents information on the newly recognized viruses recovered from patients with rubella-like illnesses, and evidence bearing on their etiologic significance. The unusual cytopathic activity (CPE) of the agents in primary human amnion cultures (PHA) has been utilized as the index of viral activity and this phenomenon is therefore emphasized.

Materials and methods. Tissue cultures.† Human amnion (PHA) obtained by Caesarean section, and chick embryo were grown as primary trypsinized cultures in 16 × 150 mm tubes with 1.5 ml medium. Human embryonic skin-muscle (SM) was cultured as explants in plasma clots in 20 × 150 mm tubes with 2.0 ml medium. Nutrient media for these cultures consisted of a mixture of 45% bovine embryonic fluids collected by the "blind" puncture method(9), 45% Hanks' balanced salt solution (BSS), 5% inactivated horse serum derived from multiple bleedings of the same animal, and 5% beef embryo extract. Phenol red, penicillin (100 U/ml) and streptomycin (100 μg/ml) were added. The medium was stored at −15°C. To initiate growth of amnion cells, the medium was modified for the first 4-6 days by including Mycostatin® (50 U/ml), and inactivated horse serum in a final concentration of 20%. Cell lines of HeLa, FL human amnion, and human liver (Chang) were obtained from Dr. R. S. Chang and propagated on modified Eagle's medium(10). Established cultures were rotated at 35°C, and medium changed at intervals of 5-7 days. After fixation with Bouin's, cells grown on cover slips or prepared by the collodion embedding technic(11) were stained with hematoxylin and eosin for histologic study.

Each experiment included multiple control cultures of the same tissue used for infected cultures and both groups received media from the same batch. For serial transfer of virus, inocula of scraped cells plus fluid, of harvested fluids only, or of supernates of fluids centrifuged at 1500 rpm for 15 minutes were used. Comparable material was passed to new control cultures. For quantitative titrations 10-fold dilutions of centrifuged fluids in BSS were inoculated in 0.2-ml amounts into a minimum of 3 PHA cultures. Infectivity titers were based on specific cytopathic changes (CPE) developing over a 28-day period and were calculated by the Reed and Muench method(12).

Serologic technics. Serum samples were stored at −15°C. The neutralization test (NT) was performed as follows: Patients' sera were heated at 56°C for 30 minutes, then diluted 1:2 by addition of unheated, pooled normal rabbit serum (PNRS). To these serum mixtures were added equal volumes of virus at 10^{0}, $10^{-1.0}$, or $10^{-2.0}$ diluted in BSS containing 10% PNRS. Equal volumes of PNRS were added to each virus control dilution. Virus control dilutions and serum-virus mixtures were held at 37°C for 2 hours; then 0.2 ml of each were inoculated into 3 PHA cultures. Thus, inactivated patients' serum at a constant final dilution of 1:4 was allowed to react with a series of virus dilutions in the presence of 25 or 30% PNRS; virus controls were exposed to 50 or 55% PNRS. In each instance the final total serum content was 55% since undiluted virus in tissue culture media contained 5% horse serum. Paired sera were tested simultaneously. The maximum virus dose was $>10^{2}$ in all but one test. Uninoculated PHA cultures and cultures inoculated with serum alone were included routinely.

† We are indebted to Dr. Duncan Reid and the staff of the Boston Lying-In Hospital for cooperation in providing tissue specimens, to Mr. John Carabitses for preparation of photomicrographs, and to Dr. Geoffrey Edsall and staff of the Massachusetts Public Health Biologic Laboratories for horse serum.

Cultures were examined for CPE without content identification by the authors at intervals of 2-3 days over a period of 18 to 28 days; *i.e.*, until titers stabilized. Titers of virus in each group were calculated at each reading by the Reed-Muench method, and neutralization indices determined.

Cases from whom agents were isolated. Strain RW. On 9/30/60, after 2 days of slight cough and painful cervical lymph nodes, RW, age 10 yrs., developed fever of 39.4°C, severe pain behind the eyes, and prominent posterior cervical and suboccipital adenopathy. Next day fever rose to 40.4°C, and he complained of severe pain in the right ankle and metatarsal joints bilaterally. On 10/2 a fine macular rash appeared on the trunk, became generalized and more papular, and presented large macules on palms of the hands and soles of the feet before clearing after 3 days. Further joint symptoms of the right shoulder and both hips appeared on 10/4. Fever and joint symptoms subsided by 10/7, but fine desquamation on finger tips and feet occurred on 10/17. The only medication during the illness was aspirin. Urine was collected on 10/3.

Strain Bell. A 16-year-old Exeter student (AB) noted "bumps" on back of the neck and under chin, and retro-orbital pain on movement of eyes. The third day, 2/25/61, a rash appeared, and he was hospitalized. Examination showed a maculopapular rash on face, trunk, and abdomen; enlarged postauricular and cervical nodes; conjunctival injection; and a temperature of 36.7°C. Blood and urine were obtained on 2/25, within 6 hours after rash was noted.

Strain Gol. A 16-year-old Exeter student (BG) became aware of enlarged lymph nodes behind the ears on 2/21/61. These persisted without other symptoms until 2/24 when a faint skin rash developed and he was hospitalized with a temperature of 37.2°C. Following day he showed a prominent and generalized rubelliform rash and bilaterally enlarged postauricular nodes. Blood and urine were obtained on 2/25, within 24-30 hours after rash appeared.

Strain Chav. Without prodromata except possible fever, a 22-year-old Harvard student (PC) noted a skin rash on 4/30/62. His temperature was 37.6°C. After 24 hours fever declined and the rubelliform rash became more prominent extending from face to hips. Bilaterally palpable postcervical and axillary lymph nodes were present. Blood and urine were obtained on 5/1, 24-30 hours after appearance of rash. *Handling of specimens.* Blood was kept at room temperature for 2-4 hours, centrifuged, and the clot ground with an approximately equal volume of BSS; after allowing large particles to settle, the suspension was used as inoculum. Urines were centrifuged at 1500-2000 rpm for 20 minutes to provide sediment as well as supernate. Cultures were inoculated with 0.1 to 0.25 ml volumes of these materials. The interval between collection and inoculation of positive specimens did not exceed 4 hours. Two of the 3 positive urine specimens were transported at wet ice temperature.

Results. (a) *Isolation of agents.* (1) *RW strain from urine.* Isolation was made in each of 2 cell culture systems without common origin. SM cultures were inoculated with urine supernatant and kept for 45 days before ground tissue plus fluid was passed to PHA cultures. CPE was suspected in the PHA cultures between days 26 and 33, and confirmed in preparations fixed on day 33. Isolation was also accomplished by inoculation of the urine into PHA cultures. These were kept for 50 days, then passed as scraped cells plus fluid to SM cultures for 46 days, followed by a 3rd passage to PHA. The first evidence of CPE, later confirmed in stained material, was observed in the 3rd passage PHA on the 21st day. Transfer of infected material has continued serially in PHA cultures, at intervals of 7 to 27 days, to the present 38th tissue culture passage for a cumulative period of cultivation of 620 days.

(2) *Bell strain from clotted blood.* Clot suspension was inoculated into PHA cultures. From these, scraped cells plus fluid were passed to PHA on day 45, even though no CPE was seen. Between days 17 and 21 of the 2nd passage, CPE appeared and was confirmed in stained preparations of the 3rd passage. Strain Bell was passed thereafter at 10- to 16-day intervals in PHA and is currently in the 36th tissue culture passage.

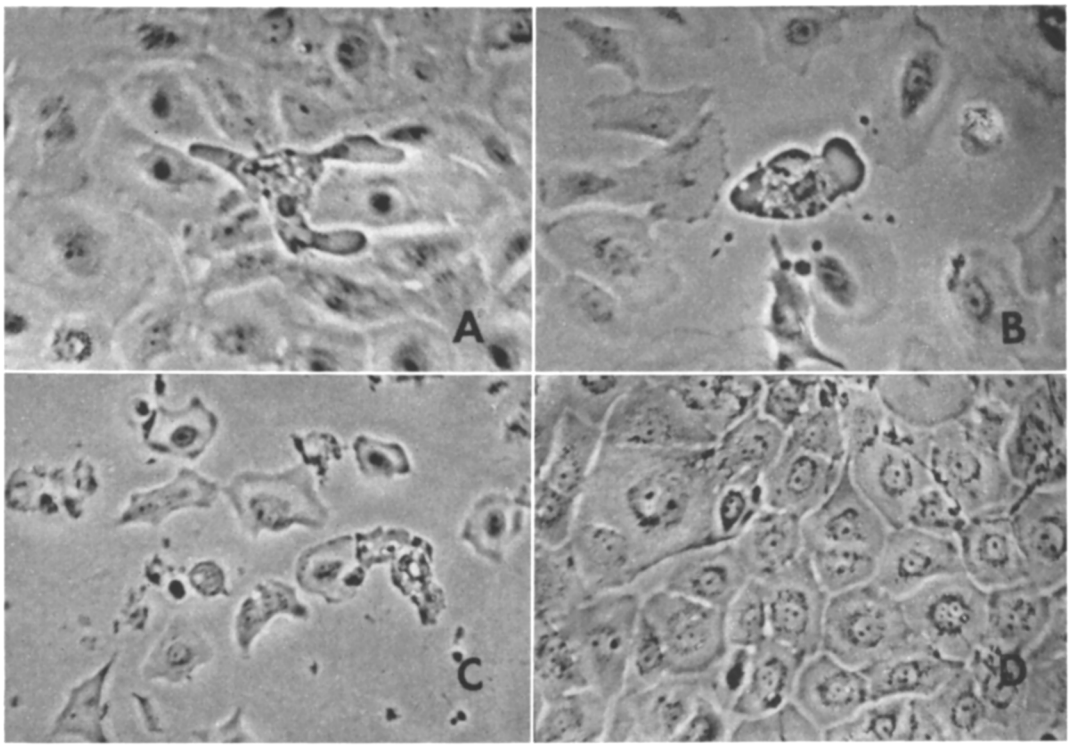

FIG. 1. Phase contrast photographs of PHA cultures illustrating CPE produced by 32nd passage RW virus. All ×225. A. Cell in central area of cell sheet showing typical amoeboid appearance with clear pseudopodia and inclusion bodies; 10th day after inoculation with 10^3 $TCID_{50}$. B. Rounded amoeboid cell, undergoing dissolution with a blunt clear pseudopodium and cytoplasmic inclusion, seen at edge of cell sheet on 22nd day as first evidence of CPE in a neutralization experiment. C. Central area of culture showing >90% specific cell destruction when photographed on 32nd day following inoculation. D. Central area of uninoculated culture from same experiment as "C" on 32nd day.

Similar handling of urine and throat garglings from Bell gave negative results.

(3) *Gol strain from urine.* PHA cultures inoculated with urine sediment showed suggestive changes on the 37th day. A 2nd passage of scraped cells plus fluid harvested on day 48 to PHA cultures resulted in specific CPE on the 24th day. CPE also was demonstrated in stained material. Subcultures of the agent were carried out in PHA at 10- to 33-day intervals to the 24th passage. Although Gol strain was isolated at the same time as strain Bell, the PHA cultures used derived from different amnions.

(4) *Chav strain from urine.* Urine supernatant and sediment from this patient produced no definite changes in PHA cultures after 38 days. A 2nd passage to PHA was made on day 41, and CPE was detected on the 19th day. This 1962 strain is currently in the 3rd tissue culture passage. (b) *Properties of the agents.* (1) *Cytopathic changes in PHA cultures.* For detection of early changes, observation with a magnification of 100× and maintenance of a confluent monolayer of thin, clear, uniformly polygonal cells is essential. Initially, scattered cells in the sheet appear refractile, become irregularly oval, or elongate, and seem less firmly attached to the glass surface. The nucleus can no longer be seen. Inclusions appear, varying from numerous minute granules to a few large, homogeneous-appearing, palely refractile bodies that approach or exceed nuclear size. At this stage affected cells frequently become amoeboid in shape (Fig. 1, A and B) with extrusion of wide and sometimes multiple pseudopodia that often terminate in a clear ectoplasmic-like tip. Amoeboid forms are fragile, having been observed on occasion

to disintegrate in 60-90 minutes. Cell destruction is accompanied by further loss of adherence, so that filamentous fragments of cytoplasm may wave in the media film. While the majority of affected cells detach, some persist as smudges of cytoplasmic and inclusion debris. In some cultures, for reasons not understood, amoeboid cells are rare; then, affected cells remain rounded and contain refractile inclusions. The number of amoeboid forms may be reduced for 1 or 2 days following a change of medium. The first evidence of CPE usually is seen at the top or margins of the cell sheet. While affected cells may show some concentration in particular areas, the process typically involves scattered individual cells. It is to be emphasized that as the process progresses, the great majority of cells remaining attached to the glass appear normal; superficial examination, therefore, will reveal only an increasingly sparse cell population. In the end stages, the surviving cells are widely separated and cellular detritus accumulates (Fig. 1, C and D). In one series of 3 passages (Gol strain) in PHA maintained on Eagle's medium, CPE appeared to diminish progressively.

With low passage strains CPE was first noted 17 to 34 days after inoculation and progressed slowly for weeks thereafter. The prepatent period changed on serial cultivation (>20th p.) to as short as 5 days, and the cell sheet frequently was estimated as being 90% destroyed after 3 to 5 weeks; with progressive destruction, a distinct pH differential was observed between media of infected and control cultures.

Study of stained preparations made at various times from numerous passage levels confirmed that at any one time there were few affected cells in comparison to the total number of cells present. Isolated involved cells, however, showed striking nuclear changes characterized by the aggregation of basophilic material into small and later large clumps. Concurrently, prominent eosinophilic bodies were seen in the cytoplasm and were also intimately associated with the basophilic masses. Cells illustrating a possible sequence of stages are depicted in Fig. 2. Typical amoeboid forms were not seen in the stained material; this was explained when it was demonstrated that these forms disappear during the fixation procedure used.

(2) *Cultivation in other cell systems.* The RW, Gol, and Bell agents each multiplied in FL amnion, Chang's liver, and HeLa cell lines. No CPE was observed in these cells, or in SM cultures, but multiplication was demonstrated by assay of harvested fluids in PHA cultures. Attempts to demonstrate growth in cultures of embryonic chick tissue were negative.

(3) *Host range.* Inoculation of RW virus (27th p., titer $10^{-2.7}$) in 0.2-ml amounts on the chorioallantoic membrane of 10-day-old embryonated eggs produced no lesions. In 2 trials the same RW virus passed 3 times at 6-7-day intervals *via* amniotic sac of 7-day eggs could not be recovered in PHA cultures.

Newborn Swiss white mice were inoculated intraperitoneally and intracerebrally with fluids harvested on the 24th day from 4th pass RW cultures; 3 passages using brain and spleen tissue were made at 6-13-day intervals in newborn mice. Starting with Bell (6th pass, titer $>10^1$) and Gol (4th pass, titer $>10^1$) material, 3 passages of brain and carcass tissue in newborn mice were made at 7-day intervals. No obvious disease developed in the mice.

Inoculation with infectious culture fluids of ordinary bacteriologic media, and of media for pleuropneumonia-like organisms, yielded no growth. RW and Bell agents produced characteristic CPE in PHA cultures when tetracycline (10 μg/ml) was added to the media.

(4) *Hemagglutination and hemadsorption.* PHA cultures infected with RW, Gol or Bell agents, or the fluids therefrom, at times when CPE was evident were tested with guinea pig, newborn chick, hen, sheep, rhesus, or human "O" red cells; these tests done at room temperature and at 4°C showed no evidence of hemadsorption or hemagglutinin activity.

(5) *Interference with cytopathic activity of Sindbis virus.* When it was learned that agents recovered from rubella interfered with CPE of ECHO 11 virus(7), test was made of possible interference by our agents with Sindbis

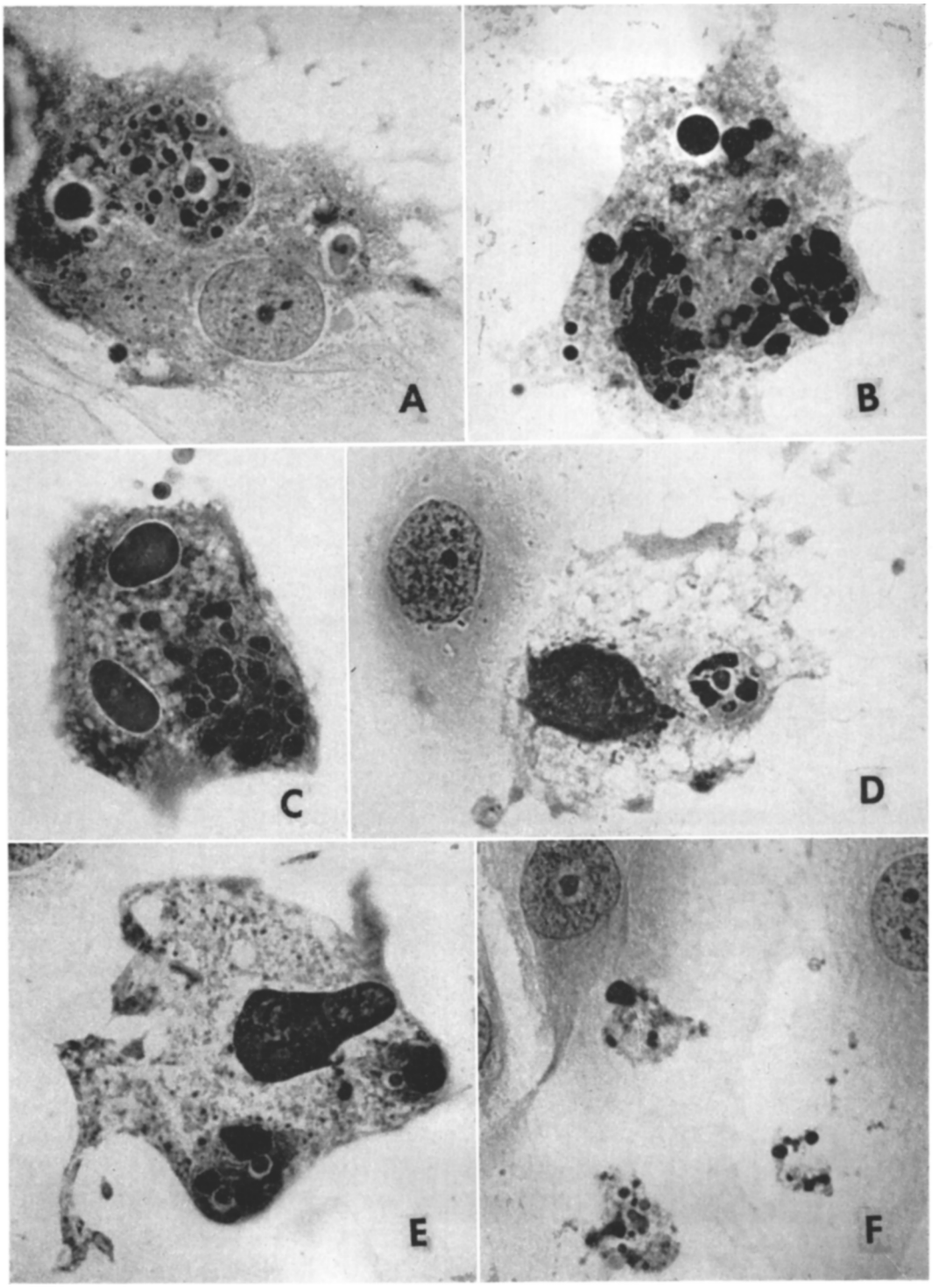

FIG. 2. Examples of specific cellular changes produced by 3 virus strains in primary human amnion cultures. Preparations fixed in Bouin's and stained H and E. ×2050. A. Normal cell with associated early stage infected cell; latter with small eosinophilic cytoplasmic bodies and

aggregation of nuclear chromatin into basophilic bodies. RW strain, 7th TC pass.; fixed day 18. B. Binucleate infected cell with prominent basophilic elongated chromatin masses and round intensely eosinophilic staining inclusions in nuclear regions as well as cytoplasm. Bell strain, 11th TC pass.; fixed day 16. C. Infected cell with prominent nuclear chromatin aggregates, and 2 large, as well as smaller, eosinophilic inclusions. Gol strain, 7th TC pass.; fixed day 33. D. An uninvolved and an infected cell; the latter undergoing dissolution, showing chromatin aggregation and containing a large, irregular eosinophilic cytoplasmic inclusion. RW strain, 31st pass., day 17. E. Infected cell with cytoplasmic filaments undergoing disintegration; large elongate cytoplasmic inclusion and 2 aggregates of basophilic material each associated with small, round eosinophilic bodies. RW strain, 3rd TC pass., day 30. F. Three aggregates of small basophilic and eosinophilic bodies, representing remnants of degenerated infected cells, with adjacent normal cells. RW strain, 7th pass., day 18.

TABLE I. Summary of Filtration Experiments.

Filter	Strain and titer of virus used	Prewash membrane with 2% proteose-peptone	Titer of virus in filtrate
450 mμ Millipore	Gol (24th p., $10^{-2.0}$)	No	$10^{-1.0}$
"	"	Yes	$10^{-2.0}$
"	RW (32nd p., $10^{-2.8}$)	"	$10^{-3.2}$
300 mμ Millipore	RW (27th p., $10^{-2.5}$)	Yes	$>10^{-1.5}$
"	RW (32nd p., $10^{-3.0}$)	"	$>10^{-1.5}$
"	Bell (34th p., $10^{-3.0}$)	"	$>10^{-1.0}$
150 mμ (SS Type A)*	Gol (24th p., $10^{-2.0}$)	No	None
100 mμ Millipore	RW (18th p., $10^{-1.0}$)	No	None
"	RW (27th p., $10^{-2.5}$)	Yes	? " †
"	Bell (34th p., $10^{-3.0}$)	"	"
50 mμ Millipore	RW (18th p., $10^{-1.0}$)	No	None

* Carl Schleicher and Schuell Co., Keene, N. H.
† 1/4 cultures positive at 10°; ? significance.

virus‡ in PHA cultures. Cultures infected with the RW and Bell strains were completely resistant to 10^3-10^4 doses of Sindbis virus. The same challenge with Sindbis virus of control cultures, as well as of cultures inoculated with limiting dilutions of RW and Bell viruses, but lacking CPE thereof, produced Sindbis CPE.

6. *Other differential properties. Membrane filtration:* Filtration experiments, summarized in Table I, indicated that the agents pass a 300 mμ Millipore® membrane prewashed with 2% proteose-peptone. At the termination of each filtration, the system was tested to verify that it would not pass *Serratia marcescens*. *Thermal stability:* As summarized in Table II, the heating of undiluted virus in tissue culture medium at 56°C resulted in significant, but incomplete, loss of infectivity. When preserved at –50°C in culture medium, the agents were quite stable, but showed more lability on storage at higher temperatures (Table III). In an experiment not tabulated, infectivity was preserved at –50°C for a period of one year. *Effect of ether and sodium desoxycholate. Ether*: Centrifuged RW 10th p. virus was mixed with an equal volume of ether, held at 5°C for 24 hours, the ether removed and the material then tested for infectivity. No virus was detected, whereas a control aliquant handled without ether had an infectivity titer of $10^{-2.5}$. Bell virus (15th p., titer $10^{-2.5}$) was similarly exposed to ether with complete loss of infectivity. *Sodium desoxycholate:* Bell virus (15th p., titer

‡ AR 339 strain received in 1957 as mouse brain virus from R. M. Taylor, and provided by T. E. Frothingham after 11 chick culture passages and one or two passages in PHA.

TABLE II. Stability of Virus in Tissue Culture Medium at 56°C.

Virus	Initial titer	Heated at 56°C; final titer
RW 10th p.	$10^{-3.0}$	120 min. = $10^{-0.0}$
		30 min. = $10^{-1.7}$
Bell 6th p.	$10^{-2.25}$	60 min. = $10^{-0.25}$
" 27th p.	$10^{-2.0}$	60 min. = $10^{-0.3}$

TABLE III. Stability of Virus in Tissue Culture Medium When Stored at Different Temperatures.

Storage temp., °C	Virus pool	Titers after varying periods of storage
−50	RW 10th p.	12 days = $10^{-2.75}$; 36 days = $10^{-2.5}$; 4 mo = $10^{-3.0}$; 5 mo = $10^{-2.5}$
−50	" 18th p.	4 days = $10^{-3.7}$; 3 mo = $10^{-3.0}$; 3½ mo = $10^{-2.5}$
−20	Bell 22nd p.*	Initial titer = $10^{-3.0}$; 17 days = $10^{-2.0}$
−20	" 28th p.*	Initial titer = $10^{-2.0}$; 28 days = $10^{-1.5}$
4	" 22nd p.*	Initial titer = $10^{-3.0}$; 10 days = $10^{-2.5}$; 32 days = $10^{-2.0}$; 63 days = $10^{-1.9}$
4	" 28th p.*	Initial titer = $10^{-2.0}$; 28 days = $10^{-1.4}$

* Bell pools made in Eagle's medium with 5% horse serum.

$10^{-3.0}$) and RW virus (10th p., titer $10^{-2.5}$) exposed one hour at 37°C to 1:1000 sodium desoxycholate in phosphate buffered saline (pH 9.0) containing 0.75% bovine albumin both showed >100-fold loss of infectivity.

(c) *Serologic studies indicating relationship to rubella-like illness:* (1) *Development of serologic procedures:* Attempts to prepare active complement-fixing antigens and to demonstrate specific antigen-antibody reactions with agar-cell culture-precipitation test(13) or fluorescent-antibody technics have been unsuccessful. Efforts, therefore, were concentrated on demonstration of neutralizing antibody. This approach was complicated by the nature of the CPE and by characteristics of the antigen-antibody reaction not yet completely elucidated. Tests of the neutralizing activity of dilutions of acute and of convalescent phase sera from rubella cases following inactivation at 56°C and combination with 100 $TCID_{50}$ of virus for varying periods at 5°C or 37°C resulted in irregular inhibition of CPE in cultures receiving the 1:4 dilution of convalescent serum. However, it was noted that 5 of 6 uninactivated sera from adults when combined in a 1:4 dilution with 100 ID_{50} of virus for one hour at 5°C gave complete suppression of CPE over a 30-day period, while 6 uninactivated sera from infants, aged 3 to 9 months, showed no significant neutralization. One of these positive adult sera (from an individual with a history of rubella) was studied; heating to 56°C/30 min almost abolished neutralizing capacity, but this was partially restored by the incorporation in the system of 10% uninactivated normal rabbit serum. An NT based on constant serum *vs* varying virus dilutions and incorporating PNRS in the system developed from these observations. This has given satisfactory results, but requires repetitive readings as CPE is usually incompletely suppressed.

(2) *Results of neutralization tests:* In Table IV are presented detailed results of representative tests. These illustrate typical but variable patterns of neutralization observed with paired sera from suspected rubella patients. In rare instances, (BG), neutralization of virus by convalescent phase serum was complete; in others (WD), neutralization was initially prominent but later decreased, while frequently (RW, WM, JP, AB) there was a low but persistent degree of neutralization. Included are findings on an undiagnosed patient without rash (EN) who showed no neutralization, and data on a sporadic case, clinically considered as rubella, whose acute and convalescent sera both neutralized virus completely (RR).

Table V summarizes the NT findings on 15 paired sera from cases of rubella; these include data on 3 patients from whom virus was recovered, as well as on all specimens from epidemic cases tested to date. In all 15 patients, antibody rises were demonstrated to one or both virus strains. The phenomenon of virus breakthrough often led to a lower final neutralization index than was observed early in the test. In contrast to the results with sera from epidemic rubella cases, tests on paired sera from cases of varicella (3 cases), measles (2 cases), infectious mononucleosis (2 cases), ECHO virus infection (2 cases), mumps (1 case) and primary herpes simplex (1 case) yielded in no instance a maximum log neutralization index exceeding 0.5.

TABLE IV. Serial Readings in 3 NTs with RW and Bell Viruses *vs* Paired Sera from Patients Clinically Suspected of Rubella.*

Virus and sera tested	Negative log virus titer on day: 10	14	16	18	20	22	24
Test 1. RW virus							
†RW — day 7	.7	1.7	2.2	2.7	2.7	2.7	
RW — day 400	1.2	1.5	1.5	1.5	1.5	1.5	
WM— day 3	<0	1.2	1.5	1.5	1.5	1.5	
WM— day 41	<0	.2	.5	.5	.5	.5	
JP — day 2	.2	1.2	1.2	1.2	1.2	1.2	
JP — day 433	<0	.2	.2	.2	.2	.2	
Test 2. Bell virus							
WD— day 1	.7	1.5	1.5	1.5	1.5	1.5	1.5
WD— day 14	<0	<0	<0	<0	.5	.7	.7
RR — day 5	<0	<0	<0	<0	<0	<0	<0
RR — day 47	<0	<0	<0	<0	<0	<0	<0
EN — day 3	1.5	2.2	2.2	2.2	2.5	2.5	2.5
EN — day 32	1.5	1.7	1.7	2.5	2.5	2.5	2.5
Test 3. Bell virus							
†AB — day 3	<0	.5	.7	.7	1.2	1.5	1.5
AB — day 27	<0	<0	.2	.2	.7	.7	.7
†BG — day 5	<0	.5	.5	1.0	1.0	1.6	1.6
BG — day 74	<0	<0	<0	<0	<0	<0	<0

* Patient EN had lymphadenopathy without rash; RR and RW were clinically suspected cases with rash, but in non-epidemic circumstances; remainder, cases occurring in epidemic situations.

† Patients from whom RW, Bell, and Gol strains were isolated.

(d) *Behavior of agents received from WRAIR:* WRAIR agents(8) M-30-T (Grivet MK p. 15) and M-33-T (Grivet MK p. 17) have been passed 3 times in PHA cells with the appearance in each of CPE resembling that produced by our agents, and confirmed by the demonstration in stained affected cells of basophilic aggregates and eosinophilic inclusions. PHA cultures infected with WRAIR strains were resistant to challenge with Sindbis virus.

Discussion. The agents described appear to be representatives of a group of viruses not previously recovered from man. That they originated from the inocula of blood or urine and not from biologic components of the culture system is indicated by the following facts. RW virus was recovered in 2 different human tissue systems; the Bell and Gol agents were isolated in different lots of PHA. Further, no agents were recovered from cultures of the same tissues simultaneously employed in other long-term experiments. Finally, it is to be noted that control cultures routinely maintained in parallel showed no specific changes.

An etiologic role of the agents is suggested by the results of the neutralization tests. Sera were examined from representative cases of rubella occurring in typical institutional epidemics; these derived from 5 different outbreaks distributed over a period of 13 years. In each of these patients, an increase in neutralizing capacity for the viruses was demonstrated. Examination of paired sera from other viral infections revealed little or no alteration in neutralizing ability. The low neutralization indices demonstrated with rubella sera may be in part the consequence of specific antibody in acute phase sera. They may also reflect the conditions of the test system, or possibly, inherent characteristics of the host response to the virus. The indicated relationship between our agents and those recovered at the WRAIR further supports the concept that these viruses are the etiologic agents of rubella.

Further refinement of the neutralization

TABLE V. Results of Neutralization Tests with RW and Bell Viruses on Paired Sera from Patients with Clinical Rubella.

Patient	Age	Days of Illness*	Log neut. index *vs* RW† Final	Log neut. index *vs* RW† Max.	Log neut. index *vs* Bell Final	Log neut. index *vs* Bell Max.
Cases from whom agents isolated:						
RW	10	7;400	1.2	1.2	not tested	
AB	16	3;27;41	1.0	1.5	.7	.7
BG	16	5;74	not tested		>1.6	>1.6
1961 Epidemic at Exeter:						
MC	17	7;76	1.0	1.1	.6	>1.6
WM	17	3;41	1.0	1.0	>1.2	>1.2
JP	16	2;433	.5	1.0	not tested	
JC	15	6;44	not tested		1.5	>1.7
1962 Epidemic at Andover:						
TE	15	1;63	.7	1.0	1.7	1.7
BN	14	1;59	1.0	1.2	1.2	1.2
WB	15	2;41	.7	1.0	.2	>1.0
Cases from previous epidemics:‡						
WD	14	1;14 (1956)	.7	.7	.7	>1.5
GT	8	1;34 (1952)	1.0	1.7	not tested	
AH	±15	1;36 (1949)	.5	1.2	not tested	
HS	±15	1;39 (1949)	not tested		1.5	>1.5
PB	±15	2;42 (1949)	not tested		1.2	>1.2

* Acute and convalescent serum collected on these days.

† Final neut. index = log titer of virus with acute serum minus log titer of virus with conval. serum at end of test. Max. neut. index = maximum difference in log titers during test before virus "breakthrough" occurred.

‡ 1956—St. Marks School, courtesy T. H. Ingalls; 1952—Wrentham, courtesy D. H. Jolly; 1949—Exeter.

procedure should result in an increased sensitivity. Although application of the interfering capacity of the rubella agent to the problem of antibody assay has not been explored in detail by us, preliminary tests suggest the use of Sindbis virus in PHA cultures for this purpose. This would minimize problems associated with pre-existing antibody to ECHO 11 in human sera and the possible presence of simian agents in a monkey kidney system.

Information on the sensitivity of PHA cultures for isolation of rubella virus is not yet available. The 4 isolates here reported were obtained during attempts to recover virus from urine or blood of 15 patients with clinical rubella since 1960. The frequency, timing, and duration of the viremia and viruria in rubella remain to be defined.

Summary. Viruses, apparently not heretofore described, have been isolated from the urine or blood of 4 patients with rubella-like illnesses. These agents were serially propagated in primary human amnion cultures and produced unique cytopathic changes characterized by the aggregation of nuclear material and the presence of inclusion bodies. Other attributes demonstrated included: filterability, failure to grow in embryonated eggs or produce disease in newborn mice, lack of hemadsorption or hemagglutination, interference with Sindbis virus, relative stability on storage, and destruction by ether. By inhibition of specific cytopathic activity, low but consistent rises in levels of neutralizing antibody were demonstrated in paired sera from cases of rubella. Moreover, patients from 5 epidemics of rubella, widely separated in time, exhibited serologic evidence of infection with the agents studied. These findings suggest that the viruses herein described may be responsible for a significant proportion of illnesses now clinically diagnosed as rubella, and, in particular, those occurring in epidemic situations.

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Recovery of Rubella Virus from Army Recruits. (27750)

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During February and March 1961, a new agent was isolated repeatedly from military recruits hospitalized with rubella at Fort Dix, N. J.(1,2). This agent, recovered from throat washings, has been found to propagate only in a limited number of cell lines. In these it fails to hemadsorb, or to produce cytopathologic effect (CPE) or sufficient antigen to react in *in vitro* serological tests. It is recognizable only by its ability to interfere with ECHO virus, Type 11 (E-11). This preliminary report describes methods used for recovery of this agent, its association with rubella, and certain characteristics which identify it presumptively as a virus.

Materials and methods. Between 7 February and 27 March 1961, 79 male recruits between 17 and 25 years of age were hospitalized with febrile exanthems at Walson Army Hospital, Fort Dix. In 20 of these a definite clinical diagnosis of rubella was made; in 21 others rubella seemed the most probable diagnosis. Twenty-five had scarlet fever, and 13 had atypical exanthematous disease. The criteria used for these diagnoses are shown in Table I. Throat washings were obtained from each patient within 24-48 hours of appearance of rash; patients gargled with 15 ml of Hanks' balanced salt solution (BSS), containing 0.4% bovine plasma albumin (BPA), and no antibiotics. Throat washings were stored at −65°C until tested. Throat cultures obtained simultaneously were plated on sheep blood agar for recovery of β hemolytic streptococci (3). Acute phase sera were collected upon admission; convalescent specimens were obtained 1-26 weeks later in all but 5 patients. All sera were stored at −20°C and were heated at 56°C for 30 minutes before use in neutralization tests. *Cell cultures,* experimental animals, and challenge virus.* Primary cultures of African Green monkey kidney (*Cercopithicus aethiops*, GMK) and Hep2 cells in continuous passage were maintained with Eagle's basal medium containing 2% chicken serum and penicillin and streptomycin in final concentrations of 100 u or μg/ml. Monolayers of human embryonic kidney (HEK) were maintained with serum free medium 199 containing the same antibiotics. Media were changed in inoculated tubes at 3-4-day intervals. Embryonated eggs, suckling mice, and adult rabbits were obtained through the Dept. of Animal Husbandry of this Institute. E-11 virus used for challenge of infected cultures was in 9th or 10th rhesus kidney passage and was received as strain Gregory in 7th passage in 1956 from Dr. A. B. Sabin. *Virus isolations.* Throat washings as collected were incubated at room temperature for 20-30 minutes with penicillin (1000 u/ml), streptomycin (1000 μg/ml) and Mycostatin® (500 u/ml). One-half ml of this mixture, without further treatment, was added to each of 2-4 tubes of HEK, GMK, and Hep2 cells. Acute serum specimens in 0.2 ml volume were similarly inoculated. Initially, duplicate cultures were incu-

* Cell cultures and media were obtained from Microbiological Associates, Bethesda, Md. and Flow Laboratories, Arlington, Va.