respectively) by the standard method, although the probable errors ranged from 3.2% to 6.1% for the individual assays by the former method compared to 0.7% to 1.2% for those made by the latter method. The sample weights required per assay (each including triplicate determinations at each of 4 or 5 sample levels) were: casein 45 μg, gelatin 18 μg, and silk fibroin 142 μg. One hundred times these amounts were required for each assay by the standard microbiological method.

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Recovery of New Agent from Patients with Acute Respiratory Illness. (20825)

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An epidemic of acute respiratory illness occurred among the service personnel at Fort Leonard Wood, Missouri during the winter of 1952-1953(1). Influenza A′ virus was of etiologic importance in a majority of the cases during the mid-portion of the epidemic but not among the cases at the beginning or end of the outbreak. Approximately 20% of the non-influenzal patients presented clinical and X ray findings which were consistent with the diagnosis of primary atypical pneumonia (PAP) and many had acute respiratory disease (ARD)(2-5). However, in individual instances, it was difficult if not impossible to establish the etiology until the results of laboratory tests became available.

The present report is concerned with the isolation of a microbial agent, presumably viral in nature, which appears to have been of etiologic importance in the disease suffered by certain of the non-influenzal cases in the epidemic.

Materials and methods. Tissue culture. Tube and bottle cultures of HeLa cells† (human epidermoid carcinoma) were prepared by the general method of Scherer, Syverton, and Gey(6) except that tryptic digestion was not employed. The bottle (32 oz prescription size) and tube (16 x 150 mm) cultures were fed with nutrient fluid consisting of pooled human serum, 25 parts; chick embryo extract, 7 parts (2 parts 50% chick embryo extract and 5 parts 50% chick embryo extract ultrafiltrate); Hanks balanced salt solution, 68 parts, plus sufficient penicillin and streptomycin to give a final concentration of 50 units of each per ml. In order to remove the human serum contained in the nutrient fluid, the contents of the bottles and tubes were washed 3 times, immediately before use, with 20 or 1.0 ml, respectively, of 5% chicken serum in Hanks-Simms solution containing antibiotics. Finally, 30 ml (bottles) or 0.6 ml (tubes) of maintenance solution (10 parts pooled chicken serum; 5 parts 50% chick embryo extract ultrafiltrate; 85 parts Hanks balanced salt solution and antibiotics) were added to the

* The technical assistance of Vincent V. Hamparian, Sgt., U. S. A. is gratefully acknowledged.

† Bottle and tube cultures of HeLa cells and the nutrient and maintenance solutions were obtained from Microbiological Associates, Inc., Bethesda, Md.

washed cultures. At time of use, the bottle cultures contained about 6-10 million cells and were 3-5 days old; the tube cultures contained about 60-80 thousand cells and were 1-3 days old. The bottles and tubes were kept tightly stoppered prior to and following inoculation with the material under study and were incubated at 36°C in a stationary position.

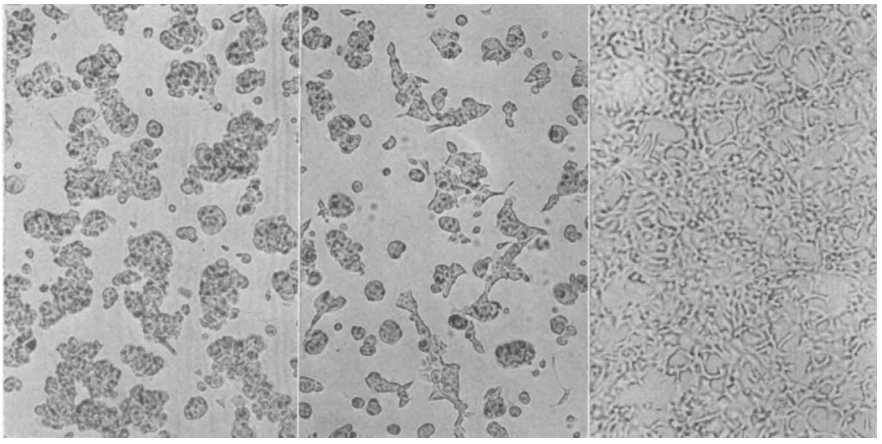
Isolation of strains of respiratory illness (RI) agents. Throat washings (collected in veal infusion broth and stored frozen at −70°C) from a patient with PAP (case 67) in the epidemic were centrifuged briefly to remove gross particles and then inoculated into roller tube cultures of adult human tracheal epithelium prepared by the general method of Enders(7). A cytopathogenic agent (RI-67) was recovered in first passage in such tissue and this organism was readily propagated in HeLa cells following 3 additional passages in tracheal cultures. All further isolations were attempted by direct inoculation into the HeLa culture medium.

For subsequent isolation attempts, the throat washings from 29 patients in the epidemic collected within the first 2 days of illness were inoculated in 0.1 ml amount into the HeLa cell culture tubes. If no cytopathogenic effect was observed within 3 days, the tissues and fluid were ground in a mortar and passed to new tubes. Cultures failing to show typical degeneration in 2 or 3 passages were considered negative and were discarded. Uninoculated control tubes were always included for comparison. Four cytopathogenic agents (in addition to the agent from patient 67 which was re-isolated directly in HeLa culture) were recovered from 2 patients with PAP and from 2 others with ARD. These agents appeared essentially identical biologically except for a minor antigenic heterogeneity detectable in neutralization tests with sera from patients convalescent from the disease. In this report, only the results of studies with the RI-67 agent are presented.

Serum neutralization test. 1. Seed virus. Flask cultures inoculated with 5.0 ml of passage virus titering 10^{-1} or 10^{-2} usually showed complete degeneration after 48 hours incubation. The cells and fluid were harvested at this time, homogenized in a Waring Blendor and the centrifuged supernate (2000 rpm for 10 minutes) which represented the seed virus was preserved at −70°C in glass-sealed ampoules. This material, titrated in HeLa cell cultures, caused significant degeneration within 2 days when diluted 10^{-1} or 10^{-2} (0.1 ml inoculum dose). *2. Neutralization procedure.* To serial 4-fold dilutions of patient's serum (inactivated at 56°C for 30 minutes) in 0.15 ml volume were added 0.3 ml amounts of seed virus diluted to contain about 10 cytopathogenic doses (strong or complete degeneration). Following incubation at 36°C for 30 minutes, pairs of tubes were inoculated with 0.15 ml amounts of each mixture. The cultures were observed for 2 or 3 days (until complete degeneration was noted in tubes in which antibody was absent) and the neutralization titer of the serum was considered to be the highest initial dilution of the serum which completely inhibited cell degeneration. Uninoculated control tubes and virus titrations were always included in the tests. Acute and convalescent sera from the same patient were tested simultaneously.

Complement-fixation (CF) test. The seed virus preparations described above were satisfactory complement-fixing antigens; uninoculated cultures, treated in the same manner, provided control antigen. The CF procedure was essentially the same as routinely employed in this laboratory for Q fever(8). The patient's serum was inactivated at 56°C for 30 minutes and 0.25 ml amounts of serial 2-fold dilutions of the serum, covering the range from 1:5-1:160, were allowed to react with 2 exact units of guinea pig complement in 0.5 ml volume and 2 units of viral antigen in 0.25 ml amount or 0.25 ml of normal control antigen in the same dilution. After incubation at 37°C for 1 hour, 0.5 ml of the hemolytic system, which consisted of a mixture of 0.25 ml of 3% sheep cell suspension and 0.25 ml of physiological saline solution containing 3 units of sheep cell amboceptor, were added and the tests were read after final incubation at 37°C for 30 minutes. The titer of the serum was considered to be the highest initial dilution of serum causing 3+ or greater fixation of complement.

Miscellaneous tests. The methods for influenza virus isolation and for the diagnostic



Complete degeneration Moderate degeneration No degeneration (control)

FIG. 1. Cytopathogenic effect of RI-67 agent for HeLa cells 2 days after inoculation, wet preparation, X70.

hemagglutination-inhibition test with cholera filtrate treated sera have already been described(9). Tests for Q fever were performed as described by Robbins *et al.*(8) and the CF test for psittacosis-lymphogranuloma venereum group infection using phenol-enhanced antigen(10) was conducted by a modification of the same general method. Cold agglutination tests were performed by the standardized procedure used in this laboratory(11) and the *Streptococcus MG* agglutination technic of Thomas *et al.*(12) was employed.

Results. Properties of the RI-67 agent. Cytopathology. Inoculation of the RI-67 agent, obtained from throat washings of patient 67, into HeLa culture tubes usually produced visible degenerative changes in cells in 24 hours and complete destruction in 48 hours. Fig. 1 shows the appearance of uninoculated culture cells and of culture cells showing moderate and complete degeneration viewed in the wet preparation. In the course of degeneration the cells lose their processes, become globoid and aggregate loosely. This is followed by accumulation of compact masses of darkly granular cells of decreased size and spherical shape. The cell surfaces may be irregular and covered with small globoid or vermiform extrusions.

Infected cells grown on coverslips, when fixed briefly with Zenker's-acetic solution and stained by the haematoxylin-eosin or Giemsa method, revealed rounding and clumping and formation of extrusions from the cell surface similar to that observed in wet preparations of the same material. In addition, intense basophilia of the nucleus and cytoplasm was observed and nuclear degeneration characterized by margination of chromatin, increased granularity, pyknosis or reticulum formation with apparent vacuolation was present. Occasionally, uninoculated cells which presented a darkened cytoplasm and appeared to be spontaneously degenerated, showed extrusions from the cell surface similar to those observed in the infected cells. No cytoplasmic or intranuclear inclusions similar to those seen in certain viral infections were observed in cells infected with RI-67 agent.

Attempt to cultivate RI-67 agent in animals and eggs. RI-67 tissue culture seed titering 10^{-2} was inoculated into 1-day-old baby mice and 5-day-old baby hamsters by the intranasal, intraperitoneal and intracerebral routes. In addition, 12-14 g mice were injected intraperitoneally and intracerebrally, and young adult guinea pigs were inoculated intraperitoneally. None of the animals inoculated intraperitoneally or intracerebrally developed signs of overt illness within 21 days following inoculation and the baby mice and hamsters inoculated intranasally did not show lesions in their thoracic or abdominal viscera when sacrificed on the fifth or eleventh day following inoculation. Passage of the agent to 8-10 g mice by the intranasal route produced no

TABLE I. Result of Neutralization and Complement-Fixation Tests with RI-67 Agent and Sera from Individual PAP, ARD and Influenza Cases, Fort Leonard Wood Epidemic.

Patient	Clinical diagnosis	Serum: Date	Serum: Day dis.	Neutralization test result: Serum dilution 1:2	1:8	1:32	1:128	1:512	Titer	C.F. titer
#67 (RI-67)	PAP	1/ 5/53	2	4+*	4+	4+			<1:2	<1:5 †
		1/17/53	14							1:5
		2/ 2/53	30	0	0	0	2+	4+	1:32	1:40
#37	PAP	1/ 3/53	3	4+	4+	4+	4+	4+	<1:2	<1:5
		1/13/53	13	0	0	3+	4+	4+	1:8	1:40
		2/ 2/53	33	0	0	0	0	3+	1:128	1:80
#34	ARD	1/ 3/53	2	4+	4+	4+			<1:2	<1:5
		1/13/53	12							1:40
		2/ 4/53	34	0	0	0	0	3+	1:128	1:80
#13	Influenza A'	12/18/52	1	0	2+	4+			1:2	<1:5
		1/ 9/53	22	0	2+	4+			1:2	<1:5

* Numbers indicate degree of tissue degeneration. 4+, complete; 3+, strong; 2+, moderate; 1+, slight degeneration. The table shows the result obtained in a single tube of the pair; the duplicate tubes in the series showed essentially identical findings. Control tests with serum alone did not show degeneration.

† None of the sera was anticomplementary and none reacted with normal control antigen.

changes which could not also be found in uninoculated mice from the same colony.

Six-day embryonated eggs inoculated via the yolk sac and 11-day eggs injected into the allantoic or amniotic cavity with the RI-67 agent showed no evidence of pathological effects following incubation at 35°C for 3 days. The allantoic and amniotic fluids as well as 20% suspensions of yolk sac and chick embryo harvested from these eggs failed to cause specific degeneration on inoculation into HeLa cultures. Furthermore, none of the allantoic or amniotic fluids agglutinated chicken or human "O" red blood cells when incubated together at 20° or 4°C.

Attempt to cultivate RI-67 agent on artificial media. HeLa culture preparation of RI-67 agent which titered 10^{-2} in HeLa tubes was inoculated on blood agar, 20% ascitic fluid agar, 10% rabbit serum-veal broth and Brewer's thioglycolate medium and incubated aerobically and anaerobically at 36°C. None of the cultures showed growth in 9 days and the ascitic agar failed to show pleuropneumonia organisms when examined‡ by the agar stain method of Dienes(13).

Serological reactions with RI-67 agent and specimens from Fort Leonard Wood cases. The results of neutralization and complement-fixation tests performed with sera from representative patients in the epidemic is shown in Table I. It is seen that the neutralizing antibody titer for the RI-67 agent rose from <1:2 to as high as 1:128 in the 3 illustrative cases of PAP and ARD while sera from the influenza patient failed to show such increase. The complement-fixation titers similarly rose from <1:5 to 1:40 or 1:80 in the patients with PAP and ARD but not with influenza.

In the group of cases listed in Tables I and II, the maximum neutralizing antibody levels against the RI-67 agent were usually attained after the 21st-30th day of the disease while maximum levels of CF antibody were generally reached by the end of the second week (case No. 67 from which the agent was isolated was the only exception found to date).

Table II summarizes the results of neutralization and complement-fixation tests performed with the sera from a representative group of patients in the epidemic and the RI-67 agent. It is seen that 12 of 14 persons with PAP or ARD showed positive findings in neutralization tests for RI-67 agent and 13/14 were positive by CF; none of the influenza cases was positive.

The cases which showed positive results with RI-67 agent gave negative findings in tests for influenza A, B and C, and for cold and *Streptococcus MG* agglutinins. Likewise, none of the sera tested showed CF antibodies

‡ We are indebted to Dr. R. G. Wittler for these examinations.

TABLE II. Serological Findings in Representative Group of Cases from the Fort Leonard Wood Epidemic.

Diagnosis	Total No. of cases	No. cases showing positive* findings: RI-67 agent Neut.	RI-67 agent C.F.	Influenza HI A' FW-1-50	Influenza HI B-Lee	Influenza HI C-1233	Cold agglut.	Strept. MG
PAP	7	6(32X)†	7(16X)†	0	0	0	0‡	0‡
ARD	7	6(16X)	6(8X)	0	0	0	0	0
Influenza	5	0(0X)	0(0X)	5	0	0	0	0

* A positive result was a 4-fold or greater increase in antibody titer.

† Numbers in parentheses are the fold-increase (geometric mean) in serum titer of the paired specimens in the group.

‡ None of the *Streptococcus MG* agglutination titers of individual specimens exceeded 1:8. The cold agglutination titers were all <1:4.

for Q fever or psittacosis-lymphogranuloma venereum group antigen.§

Serologic reaction of RI-67 agent with sera from other human diseases and from hyperimmunized animals. Neutralization tests performed with RI-67 agent and paired sera from proved cases (showing a 4-fold or greater increase in antibody titer to the responsible agent) of human influenza A′ (1 case in addition to those shown in Table II) influenza B (1 case), psittacosis (2 cases), pigeon ornithosis (1 case), Q fever (2 cases) all failed to show a rise in antibody titer for RI-67. Similar negative results were also obtained with the paired sera‖ from 3 patients with common cold and from 4 cases of primary atypical pneumonia (Cleveland, Ohio 1948 and 1953) which showed significant increase in cold or *Streptococcus MG* agglutinin. Appropriate specimens from serologically proved cases of LCM aseptic meningitis (1 case), mumps meningitis (2 cases), herpes meningitis (2 cases) and from clinical cases of infectious mononucleosis and varicella (1 each) also failed to show increase in neutralizing antibody for RI-67 agent in the convalescent specimen.

Despite this failure to obtain an antibody rise in these paired specimens, almost half (10/22) were found to have a detectable level of RI-67 neutralizing antibody which was present at a constant level (1:2-1:32) in both sera.

Further evidence for lack of relationship of RI-67 to a number of viral and rickettsial agents was obtained in tests with hyperimmune animal sera. Sera against influenza A′ (Strain A-FLW-1-52, chicken), influenza B (Motulsky-1952, chicken), influenza C (1233, chicken), ornithosis (MP-F97, chicken), Q fever (Nine Mile, guinea pig), LCM (rabbit), herpes (Laski, rabbit) and Newcastle Disease virus¶ (chicken) failed to neutralize RI-67 in a serum dilution of 1:2 or greater.

Discussion. It appears that the RI-67 virus recovered from a case of primary atypical pneumonia in the epidemic at Fort Leonard Wood is unrelated to the agents which cause Q fever, psittacosis and influenza. The possible relationship of RI-67 to any of the various infectious agents recovered during the past decade from animals, eggs, or tissue cultures inoculated with materials from cases of the common cold(5,14,15) or primary atypical pneumonia(5,14) remains to be determined.

The consistent occurrence of increase in antibody titer against RI-67 in convalescent sera from patients with PAP and ARD in the epidemic suggests that the new agent may be of etiological significance at least in certain outbreaks of these diseases.

Summary. A microbial agent, presumably a virus, was recovered from throat washings from a patient with primary atypical pneumonia in an epidemic of acute respiratory illness which occurred at Fort Leonard Wood during the winter of 1952-1953. This agent multiplies in human cell tissue cultures producing obvious cytopathogenic changes but not in common laboratory hosts. The patient whose throat washings yielded the virus de-

§ These tests were performed by A. Bankhead and O. W. Dandridge.

‖ We are indebted to Dr. W. S. Jordan, Jr. for these specimens.

¶ This was kindly furnished by the Bureau of Animal Industry, U. S. Dept. of Agriculture.

veloped specific neutralizing and complement-fixing antibodies for the agent. Other patients in the epidemic with primary atypical pneumonia (PAP) or undifferentiated acute respiratory disease (ARD) also developed antibodies for the agent but those cases with proved influenza A' did not. A portion of the population maintains an antibody level against the new agent suggesting a rather general experience with it.

Confirmatory experiments. Certain of the basic observations presented in this report have been repeated by one of the authors (M.R.H.) in the laboratory of Dr. John H. Dingle at Western Reserve University. The RI-67 agent was taken to Dr. Dingle's laboratory and the cytopathogenic effect for HeLa cells was demonstrated in cultures prepared at Western Reserve University as well as in cultures taken from this laboratory. Neutralization tests with known positive and negative paired sera were also carried out using the cultures and reagents from the 2 sources and essentially identical results were obtained.

Addendum. Since the present manuscript was submitted for publication, Rowe et al. (PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 570) *reported the recovery of an agent cytopathogenic for HeLa cells from cultures of human adenoid tissue. Tests performed in our laboratory and that of Dr. Huebner gave presumptive evidence of an immunological relationship between the RI-67 and the adenoid degenerative agent.*

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The Oxidation of Mannitol.* (20826)

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The value of mannitol, an isomer of sorbitol, as an adjunct to the diabetic diet has recently been reviewed by Olmsted(1). It was concluded that mannitol could not be of much importance as an adjunct to the diabetic diet, since 80% or more of the mannitol administered is excreted in the urine. These conclusions were apparently based on the data obtained by IV administration(2-6). Mannitol has also been known to form significant amounts of liver glycogen after oral administration(2,7), and under these conditions it is unlikely that mannitol can be excreted in the amounts indicated above. However, no posi-

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