rise to a negative inotropic action, loss of intramuscular Na and an increased uptake of K. (3) Employing the isolated heart preparation, addition of 0.5 μg/ml of 9-a FF to the failing heart results in a partial recovery. Conversely, 10 μg/ml added to the 'normal' heart brings about failure quite rapidly. (4) A histological correlation of the reported results is shown. (5) The mechanism of action and significance are discussed.

1. Tanz, R. D., Clark, G. M., and Whitehead, R. W., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 167.

2. Cattell, McK., and Gold, H., *J. Pharm. and Exp. Therap.*, 1938, v62, 116.

3. Anderson, F. F., and Craver, B. N., *ibid.*, 1948, v93, 135.

4. Leitch, J. L., *Manual for the Anderson-Craver Perfusion Apparatus*, Metro Industries, New York.

5. Zwemer, R. L., Lowenstein, B. E., and Pines, K. L., *Endocrinol.*, 1940, v27, 945.

6. Abrams, W. B. and Harris, T. N., *Am. Heart J.*, 1951, v42, 876.

7. Rubin B. and Salter, W. T., *J. Pharm. and Exp. Therap.*, 1952, v105, 443.

8. Hajdu, S. and Szent-Gyorgyi, A., *Am. J. Physiol.*, 1952, v168, 159.

9. Hoffman, G., *Arch. Exp. Path. u. Pharm.*, 1954, v222, 224.

10. Booker, W. M., *et al.*, *J. Pharm. and Exp. Therap.*, 1956, v116, 8.

11. Emele, J. F., and Bonnycastle, D. D., *Am. J. Physiol.*, 1956, v185, 103.

12. Fenn, W. O., and Cobb, D. M., *ibid.*, 1936, v115, 345.

13. Fenn, W. O., *Physiol. Revs.*, 1940, v20, 377.

14. Barron, E. S. G., *Modern Trends in Physiology and Biochemistry*, Acad. Press, N. Y., 1952.

15. McDowall, R. J. S., Munro, A. F., and Zayat, A. F., *J. Physiol.*, 1955, v130, 615.

16. Gertler, M. M., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 629.

Received October 3, 1956. P.S.E.B.M., 1957, v94.

A Cytopathogenic Agent Isolated from Naval Recruits with Mild Respiratory Illnesses.* (22915)

W. PELON,† W. J. MOGABGAB,† I. A. PHILLIPS AND W. E. PIERCE
(Introduced by Laurence W. Roth)

Virology Division, Naval Medical Research Unit No. 4, Great Lakes, Ill. and Division of Infectious Disease, Department of Medicine, Tulane University School of Medicine, New Orleans, La.

A cytopathogenic agent has been isolated in monkey kidney cultures from nasopharyngeal washings obtained from Naval recruits with mild respiratory illnesses(1). In this paper, certain characteristics of the isolate are described which allow preliminary classification and identification. The relation of infection to clinical illness in 2 recruit companies and evidence suggestive of widespread infection in other populations are also presented.

Methods. Cultures were prepared with 0.25% trypsin (Difco, 1:250) dispersed kidney cells from adult Rhesus monkeys at a concentration of 500,000 cells per ml. These were incubated at 35°C in a stationary position in nutrient medium consisting of 90% mixture 199(2) (Difco Laboratories) or bovine amniotic fluid (Cudahy), 5% inactivated horse serum (56°C for 30 minutes), 5% bovine embryo extract(3), penicillin, 200 units and streptomycin, 200 μg per ml. The same media or mixture 199 alone plus antibiotics was used when the cultures were ready for inoculation. Monkey testis, cornea, human embryo kidney and chick embryo cultures were propagated similarly. HeLa, KB

* From Research Project, Bureau of Medicine and Surgery, Navy Dept., Washington, D.C. The opinions expressed herein are those of the authors and cannot be construed as indicating endorsement or approval by the Navy Dept. or of Naval Service at large. This work was also supported in part by Abbott Laboratories grant.

† Present address: Department of Medicine, Tulane University School of Medicine.

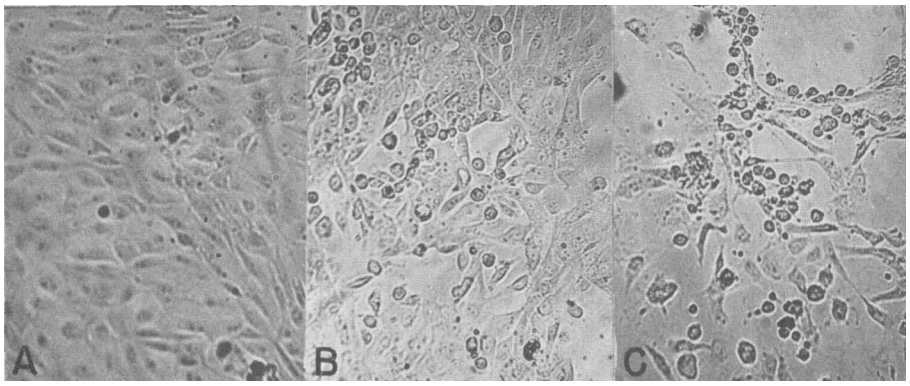

FIG. 1. Cytopathogenic effects of 2060 in monkey kidney cultures. (A) Normal, (B) 5 days after inoculation of 10^3 $TCID_{50}$, (C) 10 days after infection (55×).

(4), and human conjunctival cells(5) were cultivated in Scherer's maintenance solution (MS)(6) (Microbiological Associates), containing 40% pooled human serum, washed and maintained for inoculation with Scherer's MS containing 7.5% chicken serum and 25% tryptose phosphate broth(7). Infectivity ($TCID_{50}$) was determined by inoculating 0.1 ml of 10 fold dilutions of the agent into groups of 4 monkey kidney cultures. Readings were usually made at 10 days on the basis of cytopathogenic effects. Neutralization was done with inactivated sera (56°C for 30 min.) which were held with the agent at 4°C for 30 minutes prior to inoculation of cultures. Readings were made at the end of 10 days and titers determined on the basis of inhibition of cytopathogenic effects. Tissue culture fluids were not changed after inoculation of the agent-serum admixture.

Results. Primary isolation. Cytopathogenic effects were observed upon second monkey kidney culture passage of nasal washing No. 2060 obtained from a recruit with a mild respiratory illness in October 1954. A probable relation to infection by the agent in this individual was demonstrated by increase in neutralization titer from less than 8 in the acute serum to 56 in the convalescent serum. Isolation was repeated in kidney cultures from a different monkey inoculated with another aliquot from the same specimen. In both instances, characteristic cellular changes occurred 8 days after inoculation of the second group of cultures. Serial passage of the isolate was carried out in monkey kidney cultures.

Cytopathogenic effects. The isolate produced characteristic changes in monkey kidney cultures, manifested first by enlargement and rounding of epithelial cells, fine cytoplasmic granularity and indistinct nuclei (Fig. 1b). Involved cells later became pyknotic and fragmented (Fig. 1c). Cytopathogenic changes were not found prior to 3 days after inoculation of undiluted material. After 12 passages of the agent, complete destruction of epithelial cells occurred only after a period of 10-14 days. Many of the disrupted cells remained attached while others became loosened from the walls of the culture tube. Intranuclear or cytoplasmic inclusion bodies were not found in Giemsa stained cells. Cells treated early in the course of infection by means of the indirect fluorescent antibody technic of Weller and Coons(8), revealed an accumulation of fluorescing granules in the cytoplasm adjacent to the nucleus.‡ Staining of the entire cell occurred as infection progressed.

Classification. The relation of 2060 to known viruses was first investigated by the effects in various tissue cultures and animal hosts. The original nasal washing as well as the 12th monkey kidney passage with a $TCID_{50}$ of $10^{7.0}$ were used in separate experiments. Several blind passages were also

‡ Fluorescein isocyanate conjugated antihuman gamma globulin rabbit serum was kindly supplied by Dr. A. H. Coons.

made. Cytopathogenic effects were found in cultures of monkey testis and human embryo kidney, but not in monkey cornea, chick embryo, HeLa, KB or human conjunctival cells. Inoculation of the yolk sac, chorioallantoic membrane, amniotic or allantoic sacs of 6- to 10-day embryonated eggs did not produce visible lesions, deaths or hemagglutinins. Swiss mice less than one day or 3 weeks old were not affected by serial intracerebral, intranasal, or intraperitoneal passages. Effects were not seen in rabbits inoculated into the cornea, peritoneal cavity, or intravenously.

Antisera utilized to further exclude some viral and rickettsial agents included poliomyelitis, types 1, 2, and 3 (simian); adenoviruses, types 1 through 7 (rabbit); Coxsackie A, types 7 and 9, B, types 1 through 4 (mouse); influenza A, B and C (rooster); herpes simplex (guinea pig); distemper (canine), rubella (human), mumps (human), psittacosis (pigeon), and *Rickettsia burneti* (bovine). Pooled normal Rhesus monkey sera were also employed. Neither neutralization nor delay in appearance of cytopathogenic effect occurred with any of the antisera when tested against 10^4 $TCID_{50}$ of 2060.

Convalescent sera from the recruit from whom 2060 was isolated did not demonstrate rises in neutralizing antibody titer to the adenoviruses, types 1 through 7. Additional indirect evidence against relationship to the adenovirus or Coxsackie viruses was obtained by means of the complement-fixation test. Rises in titer of complement-fixing antibody against adenoviruses, types 4 and 7 or Coxsackie A, types 7 and 9, and Coxsackie B, types 3 and 4, were not found. All testing was controlled with known positive sera.

Inoculation of tissue culture fluids containing 2060 into bacteriological media, *i.e.*, thioglycollate broth, fresh beef heart infusion broth with 20% horse serum, incubated both aerobically and anaerobically, beef heart infusion agar enriched with 10% sheep erythrocytes and 10% horse serum, incubated aerobically and under 10% carbon dioxide, failed to yield any indication of bacterial or mycotic growth after 14 days at 37°C. Further evidence against the bacterial nature of the isolate was shown by the lack of effect of various antibiotic or chemotherapeutic compounds, including penicillin, dihydrostreptomycin, chloramphenicol, sodium sulfathiazole, sulfisoxazole diethanolamine and chlortetracycline, on the cytopathogenic effect of 2060 in monkey kidney cultures. Filterability of 2060 was demonstrated by passage of the isolate through a sintered glass filter (Corning, UF, pore diameter 0.9-1.4 μ).

Characteristics. After exposure of 10^4 $TCID_{50}$ to 70 or 95% ethanol, 1-800 para-tertiary - octyl - phenoxy - ethoxy - dimethylbenzyl ammonium chloride monohydrate and 5% phenol for 24 hours at 4°C, cytopathogenic effects were observed in monkey kidney cultures inoculated with a 10^{-1} dilution of the treated material. Controls without 2060 did not reveal specific effects, while those with the agent alone demonstrated typical changes at the same time. Similar treatment with 20% diethyl ether resulted in a delay in cytopathogenic effects. Even a pH range of 0.9 to 10.8, adjusted with HCl or NaOH, for 30 minutes at 4°C did not abolish the effects of 10^7 $TCID_{50}$; thus it appears that the agent was quite stable. As yet, a complement-fixing antigen has not been demonstrated despite repeated attempts with human and rabbit antisera and variations in amount of complement, hemolysin and time and temperature of fixation. Several methods were employed to induce agglutination of human type O, fowl, Rhesus monkey, sheep or rabbit erythrocytes. These included incubation at 4, 20 and 37°C, variation in pH from 5.3 to 10.1, dialysis of antigen and suspension of erythrocytes in 0.25M dextrose solution, dialysis and suspension in unbuffered 0.85% saline, heating the antigen at 70°C for 10 minutes, heating of the antigen followed by centrifugation at 12,000 x G for 30 minutes, and employment of erythrocytes treated with 0.1% trypsin for one hour. Hemagglutination with 2060 was demonstrated in titers of 64 to 128 with trypsin-treated human type O, fowl and monkey erythrocytes. Various proteins, including gelatin, inhibited this reaction, thus making it unsuitable for measurement of serum antibody.

Growth characteristics. Variations in the rates of increase of cytopathogenic effects depended upon the amount of the infecting agent (Fig. 2). A period as long as 2 to 3 weeks was necessary for complete destruction of cultures after inoculation with a minimal infecting dose. Because of the slow rate of growth, 10^3 or 10^4 $TCID_{50}$ as determined by cytopathogenic effects on the 10th day was used in neutralization tests to avoid non-specific changes found in older cultures. The fluid phase of the tissue culture also affected the development of cellular changes. There was a greater degree and more rapid appearance of cytopathogenic effects when mixture 199 was used without horse serum and bovine embryo extract. Similar cytopathology was obtained with bovine amniotic fluid medium, but such changes were not so pronounced when Scherer's MS, Eagle's(9) or 0.5% lactalbumin enzymatic hydrolysate media were used.

That 2060 was an infectious, propagable agent is suggested by an infectivity titer of 10^{-7} after the 13th passage of 0.1 ml aliquots in monkey kidney cultures containing 1.0 ml of fluid medium. Effects of an agent at this titer after such dilution are extremely unlikely in the absence of growth. Since the identity of early and late passage material was determined serologically, it appears that infectivity increments occurred in serial cultures.

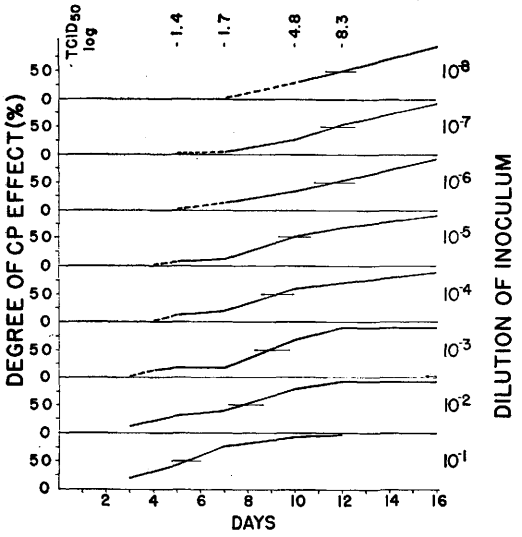

FIG. 2. Growth curves of logarithmic dilutions of 2060 based upon cytopathogenic effects in groups of four monkey kidney cultures. $TCID_{50}$ titers calculated from No. of positive cultures are shown at the time of each increment.

TABLE I. Incidence of Neutralization Titer Increments* Associated with Respiratory Illnesses in Two Recruit Companies.

Type of illness†	Company A (Oct.-Dec. 1954) No. of cases	Company A (Oct.-Dec. 1954) No. with titer rise	Company B (Mar.-May 1955) No. of cases	Company B (Mar.-May 1955) No. with titer rise
Cold	31	10	16	6
Diffuse	17	5	10	1
Pharyngitis	7	1	11	1
Chest	3	1	0	0

* 4-fold or greater increase in titer when tested with $10^{3.0}$ or $10^{4.0}$ $TCID_{50}$ of 2060 TC_{18}.

† Cold—coryza, afebrile; diffuse—sore throat, cough, coryza, with or without fever; pharyngitis —sore throat with dysphagia, with or without fever or tender cervical lymph nodes; chest—tracheitis, bronchitis, pneumonia, etc.

Neutralization by human sera. Increments in titers of neutralization were demonstrated in human sera following respiratory illnesses as shown in Table I. These were observed in the recruit company from which 2060 was isolated and, also, in a second company studied during the spring of 1955. In all cases in which a similar type agent was isolated from nasal washings, at least an 8-fold increase in neutralization titer could be demonstrated within one month. The illnesses associated with titer increments were usually mild and of short duration, lasting from 2 to 5 days (to be published). Although these results indicated antigenic stimulation by the agent, probably due to infection, relation to illness may have been circumstantial. However, isolations as well as antibody rises occurred at times which suggested an etiological association. Further evidence of the extent of infection by 2060 was shown by neutralization titers of 1:32 in two lots of pooled human gamma globulin and by the demonstration of neutralization with sera obtained from children hospitalized in Chicago during the fall of 1955. From Fig. 3, it can be seen that despite irregularities in sampling, there was a tendency for higher titers to occur in sera of older children.

Discussion. That 2060 should be considered a virus is indicated by the results of

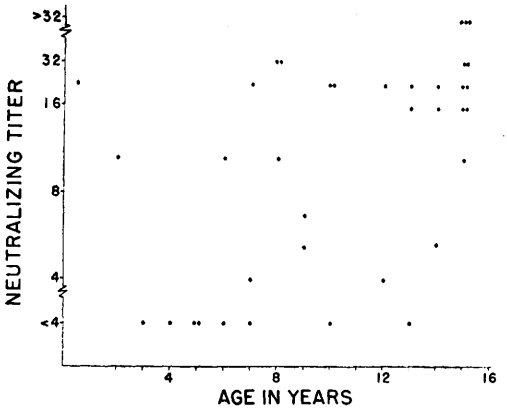

FIG. 3. Neutralization titers to 2060 in sera of children hospitalized in Chicago for a variety of medical and surgical illnesses.

these studies. Resistance to antibacterial agents, filterability, lack of growth on bacteriological media and absence of demonstrable organisms by microscopic examination of cells from tissue culture all support this contention. The lack of effect in suckling or adult mice, chick embryos, and HeLa cell cultures is characteristic of an unusual agent. When the slow rate of growth and type of cytopathogenic effect are included, the evidence favors classification of 2060 as a newly described virus or an atypical form of a known agent.

The lack of appearance of cytopathogenic effects prior to a second passage as well as increase in degree upon subsequent passages suggests adaptation to growth in monkey kidney cultures. Inhibitory substances in the nutrient medium in such cultures or in the nasal secretions, or minute quantities of virus in the original inoculum may also account for this observation. It is also of interest that certain synthetic maintenance solutions were not suitable for demonstration of cytopathogenic effects. Further studies are in progress to determine the significance of this observation and whether growth occurred in the absence of cytopathology.

That this was probably not a simian virus was indicated by lack of neutralization by at least 6 different monkey sera, complement-fixation with pooled monkey sera, or agglutination of monkey erythrocytes at 4°C as has been reported with the majority of the simian agents(10).

Infection of humans probably occurred since increases in neutralizing capacity of sera were frequently found. A total of 13 additional cytopathogenic agents was obtained from nasal washings in the recruit company from which 2060 was isolated. Of these, 5 were neutralized by anti-2060 rabbit serum. Only one recruit from whom a 2060-type agent was isolated appeared asymptomatic. Serological evidence of infection in this man could not be determined because of unavailability of convalescent serum. An index of a widespread distribution was shown by neutralization with children's sera obtained in a different locality and year and by neutralization with human gamma globulin. Although at present the relationship of this agent to respiratory illness is only presumptive, artificial transmission of the disease to man as well as repeated isolations at the onset of respiratory illnesses, associated with serological evidence of infection will provide such information.

Summary. Several agents producing the same type of cytopathology have been isolated in monkey kidney cultures from men of a recruit company in training at Great Lakes in the fall of 1954. These isolates were obtained at the onset of mild respiratory infections and rises in neutralization titers to the prototype, 2060, followed each episode. Other increments in neutralizing antibody occurred in this and another company studied during the spring of 1955. Frequency of infection by 2060 was demonstrated by titers of neutralization found in children's sera from another locale and in pooled human gamma globulin. Characteristics of the isolate indicate that it is a relatively stable virus propagable in cultures of monkey kidney and testis and human embryo kidney with a slow rate of growth. Evidence has been presented which suggests that this agent may not have been previously described. The possibilities of etiological relationship to mild respiratory illnesses have also been discussed.

1. Pelon, W., and Mogabgab, W. J., *Bact. Proc.*, 1956, 67.

2. Morgan, J. F., Morton, H. J., and Parker, R. C.,

J. Cell. and Comp. Physiol., 1950, v36, 411.
3. Robbins, F. C., Weller, T. H., and Enders, J. F., *J. Immunol.*, 1952, v69, 673.
4. Eagle, H. A., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 362.
5. Chang, R. S., *ibid.*, 1954, v87, 440.
6. Scherer, W. F., *Am. J. Path.*, 1953, v29, 113.
7. Ginsberg, H. S., Gold, E., Jordan, Jr., W. S., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 66.
8. Weller, T. H., and Coons, A. H., *ibid.*, 1954, v86, 789.
9. Eagle, H. A., *J. Exp. Med.*, 1955, v102, 595.
10. Hull, R. N., Minner, J. R., and Smith, J. W., *Am. J. Hyg.*, 1956, v63, 204.

Received October 8, 1956. P.S.E.B.M., 1957, v94.

Relation Between Degree of Fibrinogen Proteolysis by Plasmin and Fibrinolytic Paracoagulation.*† (22916)

S. SZUCHET AND M. DERECHIN.‡ (Introduced by R. Honorato)
Laboratory of Chemistry, Dental School, University of Chile.

Fibrinolytic paracoagulation, or clotting of lysed fibrinogen or fibrin(1,2), may be obtained only during a period which depends on experimental conditions. Previous works suggested a relation between degree of fibrinogen proteolysis and fibrinolytic paracoagulation which is here studied.

Materials and methods. Bovin fibrinogen (Fraction I from Armour Laboratories) was purified according to Laki(3) yielding preparations with 90-96% of total protein clottable by thrombin; chloroform activated plasmin was prepared by the method described by Loomis(4,5); Parke Davis thrombin with 5 mg/ml (approximately 100 u/ml) and 2M sodium sulphate (reagent grade) were used to effect paracoagulation, and phosphates buffer pH 7.6 to control pH. Reaction mixture: 1.5 ml of buffer and 7.5 ml of plasmin were added to 30 ml of fibrinogen solution and the mixture incubated at 40°C. Standard conditions throughout were: fibrinogen 5.150 g/l, pH 7.6 and ionic strength 0.2. Varying plasmin concentrations were employed in different reaction mixtures to reach final values of 0.60, 0.40 and 0.20 mg/ml respectively. Measurements of clotting time, paracoagulation test and non-protein nitrogen were made on aliquots withdrawn at suitable intervals. Determination of clotting time was carried out as follows: 0.9 ml of reaction mixture were transferred to test tube and 0.1 ml of thrombin added. The tube was shaken lightly and placed in water bath. The stop watch was clicked as thrombin was blown in and the time required for formation of a clot was recorded. When the mixture became unclottable paracoagulation was obtained with 0.5 ml of sodium sulphate forcibly blown into test tube, 1 minute after addition of thrombin. Paracoagulation was repeated until a negative test was obtained (extinction) and the time required for extinction was measured from initial addition of plasmin. To determine acid soluble split products, 4 ml aliquots of reaction mixture were discharged into equal amount of 10% trichloroacetic acid. The mixture was thoroughly shaken, allowed to stand 20 minutes and then filtered. Micro Kjeldahl digestions were carried out on 5 ml of filtrate using the catalyst mixture recommended by Chibnal, Rees and Williams(6) and N estimations were made according to Ma and Zuazaga(7). Acid soluble material at zero time was calculated from micro Kjeldahl estimations made on fibrinogen and plasmin specimens prior to their mixture. At least 4 curves for each plasmin concentration were carried out. Results are summarized in Fig. 1.

Results. As expected, the time required for the mixture to become unclottable by

* Presented at meeting of Soc. Biol. Santiago de Chile, July 10, 1956.

† We are indebted to Dr. J. B. Lesh from Armour Laboratories for supplying bovin fibrinogen.

‡ Research fellows of University of Chile (1955-1957).