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Immunization with Modified Vaccinia Virus Ankara-Based Recombinant Vaccine against Severe Acute Respiratory Syndrome Is Associated with Enhanced Hepatitis in Ferrets

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Severe acute respiratory syndrome (SARS) caused by a newly identified coronavirus (SARS-CoV) is a serious emerging human infectious disease. In this report, we immunized ferrets (*Mustela putorius furo*) with recombinant modified vaccinia virus Ankara (rMVA) expressing the SARS-CoV spike (S) protein. Immunized ferrets developed a more rapid and vigorous neutralizing antibody response than control animals after challenge with SARS-CoV; however, they also exhibited strong inflammatory responses in liver tissue. Inflammation in control animals exposed to SARS-CoV was relatively mild. Thus, our data suggest that vaccination with rMVA expressing SARS-CoV S protein is associated with enhanced hepatitis.

Severe acute respiratory syndrome (SARS) is a serious emerging human infectious disease of the 21st century. The causative agent was identified and characterized as a new member of the family *Coronaviridae*, SARS-associated coronavirus (SARS-CoV) (8, 14). The first SARS outbreak was primarily contained by the means of quarantine. As evidenced by the recently reported new cases (http://www.wpro.who.int/sars/docs/pressreleases/pr_27122003.asp and http://www.who.int/csr/don/2004_04-23/en/), it is almost certain that SARS-CoV remains a constant threat to public health. Efforts to develop SARS vaccine candidates are under way worldwide (9). Several recent reports have described studies on evaluation of SARS vaccine candidates in monkey and mouse models (1, 7, 15, 19). It has been shown that a recombinant adenovirus-based SARS vaccine candidate expressing SARS-CoV spike (S) and nucleocapsid proteins could induce strong neutralizing antibody and T-cell responses in monkeys (rhesus macaques) (7). However, the protection potential was not evaluated by challenge experiment. The neutralizing antibody response induced by recombinant modified vaccinia virus Ankara (rMVA) expressing SARS-CoV S protein (rMVA-S) has been shown to inhibit SARS-CoV replication in a mouse model (1). Since replication of SARS-CoV in mice can last only approximately 3 days postinfection, the memory immune response, which is essential for an effective prophylactic vaccine, is difficult to evaluate in mice. In this communication, we evaluated the effects of rMVA-S in ferrets.

Coronavirus S is the major antigenic protein responsible for inducing neutralizing antibody responses (4, 6), while MVA is a widely used recombinant poxvirus vector for development of safe and effective recombinant vaccines (11). We constructed rMVA-S using a standard protocol for construction of recombinant poxviruses with the recombinant vector pJS5, provided by Bernard Moss at the National Institutes of Health (2, 5). The S gene was synthesized based on a SARS-CoV Tor2 isolate (8) by reverse transcription-PCR (RT-PCR) with primer pair AGGC*GAATTC*ATGTTTATTTTCTTATTATTTCTTACTCTCACT (N terminus primer; EcoRI site in italics) and TATA*CCCGGG*TTATGTGTAATGTAATTTGACACCCTTGAGAA (C terminus primer; SmaI site in italics). Expression of the S protein was confirmed with Western blot analysis using a specific monoclonal antibody against the SARS-CoV S protein (Fig. 1).

Since it has been reported that ferrets were susceptible to SARS-CoV infection (10), we decided to use ferrets for an immunization-and-challenge study. Animal housing and manipulations were approved by the Animal Care Committee of the Canadian Science Centre for Human and Animal Health and met the Canadian Council on Animal Care guidelines. Six- to 10-week-old male (castrated) ferrets were purchased from Marshall Farm Pet Supplies (Wolcott, N.Y.). Enzyme-linked immunosorbent assay (ELISA) and neutralization tests were performed to confirm that there was no antibody cross-reactivity against SARS-CoV in all ferrets before the experiment. Ferrets were divided into three groups (see Tables 1 and 2;

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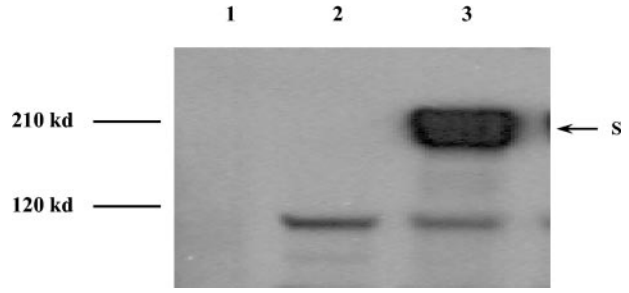

FIG. 1. Expression of SARS-CoV S proteins by rMVA-S. The S protein was detected by Western blotting with a SARS-CoV S-specific mouse monoclonal antibody (1). Lane 1, mock-infected BHK21 cell control; lane 2, lysate from MVA-infected BHK21 cells; lane 3, lysate from rMVA-S-infected BHK21 cells.

ferrets were housed individually, i.e., one ferret per cage) and were immunized with parental MVA (ferrets 1 to 3), rMVA-S (ferrets 7 to 9), or phosphate-buffered saline (PBS; ferrets 10 to 12) on day 0 with a dose of 10^8 PFU of the corresponding virus per ferret by intraperitoneal and subcutaneous routes, and a booster immunization was given on day 14 with the same regimen. Due to the lack of ferret-specific reagents for analysis of cell-mediated immune responses, only neutralizing antibody responses were monitored by microplaque reduction neutralization test. Briefly, a triplicate of serial dilutions of heat-inactivated ferret sera were incubated with 100 PFU of the SARS-CoV in a 100-μl volume for 60 min at 37°C. After incubation, the serum–SARS-CoV mixture was added to monolayers of Vero-V76 cells in a 96-well tissue culture plate. Infected Vero-V76 cells were incubated at 37°C in 5% CO_2 for 3 to 4 days with a 2% carboxymethylcellulose overlay and then fixed with 4% formaldehyde and stained with 0.5% crystal violet. Neutralization titers were determined as the reciprocal of the highest serum dilution that neutralized at least 70% of the formation of plaques on Vero-V76 cells. As shown in Table 1, neutralizing activity was detected in sera collected from all three ferrets 7 days after booster immunization with rMVA-S virus, while the titer declined to undetectable level 14 days after the booster. The serum immunoglobulin G titer determined by ELISA corresponded with the neutralization results (see online supplementary data). Moreover, the antibody response specific for the S protein in rMVA-S-vaccinated ferrets was confirmed by Western blotting.

TABLE 1. Neutralizing antibody response following rMVA-S immunization and SARS-CoV challenge

Ferret no.	Immunogen[a]	Neutralization titer on indicated day(s):									
		Postvaccination					Postchallenge				
		0	7	14[b]	21	28	3–5	7–9	13–15	20–22	27–29
1	MVA	−	−	−	−	−	−	320	320	160	320
2	MVA	−	−	−	20	−	−	160	160	640	640
3	MVA	−	−	−	−	−	40	160	320	640	640
7	rMVA-S	−	−	−	40	−	20	1,280	1,280	640	640
8	rMVA-S	−	20	−	40	−	80	1,280	640	640	1,280
9	rMVA-S	−	−	−	20	−	640	2,560	320	1,280	1,280
10	PBS	−	−	−	−	−	−	320	320	1,280	1,280
11	PBS	−	−	−	−	−	−	320	320	640	1,280
12	PBS	−	−	−	−	−	20	80	320	1,280	1,280

[a] Immunogen MVA is the parental virus of rMVA-S.
[b] The day booster immunization was given; the neutralizing antibody response was determined by microplaque reduction neutralization test, and the lowest dilution used was 1/20. −, negative.

Two weeks after the booster immunization, ferrets were challenged with 10^6 PFU of the SARS-CoV Tor2 isolate by the intranasal route in our biosafety level 4 facility. Ferrets immunized with rMVA-S developed a neutralizing antibody response as early as 3 days after SARS-CoV challenge, while neutralizing antibodies were not detected in most of the control ferrets until 7 days after SARS-CoV infection (Table 1). This shows that a typical memory immune response occurred in ferrets immunized with rMVA-S following SARS-CoV challenge. Furthermore, ferrets immunized with rMVA-S developed peak neutralizing antibody titer between 7 and 9 days after SARS-CoV challenge. In contrast, other challenged ferrets developed comparable levels of neutralizing antibodies 13 days later (Table 1). To our knowledge, this is the first report showing that immunization with a SARS vaccine candidate induced a rapid memory immune response, which is an essential feature for an effective prophylactic vaccine, following SARS-CoV challenge in an animal model.

Although no clinical signs (e.g., elevated temperature and altered behavior including feeding) were observed up to 29 days postchallenge, viral RNA was detected in pharyngeal swabs and blood samples by RT-PCR from all ferrets challenged with SARS-CoV by our previously reported protocols (17). The viral RNA could be detected in pharyngeal swabs starting from 1 day after SARS-CoV infection (Table 2). Ferret 8 (vaccinated with rMVA-S) still shed virus in the pharyngeal excretion up to 22 days postchallenge. Interestingly, viral RNA could be detected in blood specimens only at 8 days postchallenge and lasted up to 22 days postinfection. Moreover, live SARS-CoV could be isolated from pharyngeal swabs early in the infection (up to 5 days postinfection; level ranged from 4×10^3 to 1.4×10^4 PFU per pharyngeal swab, and no significant difference between rMVA-S-immunized and control ferrets was observed), while an attempt to isolate SARS-CoV from sera was not successful (data not shown). We speculate that the failure to isolate live virus from pharyngeal swabs (5 days postinfection) and sera (8 days postinfection) is related to the increased neutralizing antibody response (Table 1). Real-time RT-PCR to quantify the virus loads from the positive blood specimens (as determined by classical RT-PCR; Table 2) was also unsuccessful. The sensitivity of our real-time RT-PCR has been titrated to be 0.1 PFU per ml, while the classical RT-PCR showed sensitivity as low as 10^{-4} PFU per ml (A. Andonov and H. Weigartl, unpublished data). Thus, although SARS-CoV could enter into blood after approximately 8 days of challenge, viral load was low (lower than 0.1 PFU per ml). Nonetheless, our data indicate that immunization with rMVA-S had no significant effects on the level of SARS-CoV replication in ferrets, although a rapid, vigorous memory neutralizing antibody response occurred. In contrast, a live SARS-CoV (15), a DNA-based vaccine expressing the S protein (19), and rMVA-S (1) have been reported to induce significant protective immunity in mice following SARS-CoV challenge. The most likely cause for the difference in the protective efficacy in mice and ferrets immunized with rMVA-S is that

TABLE 2. Detection of viral RNA by RT-PCR from blood and pharyngeal swabs.

Ferret no.	Immunogen	Presence of viral RNA[a] on indicated days in:											
		Blood						Pharyngeal swabs					
		1–3	4–6	8–10	13–15	20–22	27–29	1–3	4–6	8–10	13–15	20–22	27–29
1	MVA	−	−	+	−	+	−	+	−	+	−	−	−
2	MVA	−	−	−	−	+	−	+	+	+	−	−	−
3	MVA	−	−	−	+	−	−	+	+	−	−	−	−
7	rMVA-S	−	−	+	+	−	−	+	+	+	−	−	−
8	rMVA-S	−	−	+	+	+	−	+	+	+	+	+	−
9	rMVA-S	−	−	−	+	−	−	+	+	+	−	−	−
10	PBS	−	−	+	+	−	−	+	+	−	−	−	−
11	PBS	−	−	−	+	+	−	+	+	+	+	−	−
12	PBS	−	−	−	−	−	−	+	+	+	−	−	−

[a] Due to the limited working capacity in a biosafety level 4 space, only one ferret from a group could be manipulated for blood and pharyngeal swab collection on a particular day after SARS-CoV challenge. +, RT-PCR positive; −, RT-PCR negative.

SARS-CoV exhibits different replication kinetics in these two animal systems. For example, SARS-CoV could replicate to a high titer in the first 3 days after challenge in mice, while the virus titer decreased sharply afterwards (15). Replication of SARS-CoV in ferrets, however, could last up to 22 days after infection (Table 2). Thus, further studies to elucidate the kinetics of SARS-CoV replication in ferrets should aid in understanding the difference between these two animal models for development of SARS vaccines.

Biochemical tests of blood samples and histopathological examination of various tissues were performed to investigate any pathological effects as consequences of rMVA-S vaccination and SARS-CoV challenge. The VetTest dry chemistry analyzer, with the protocol and reagents provided by the man-

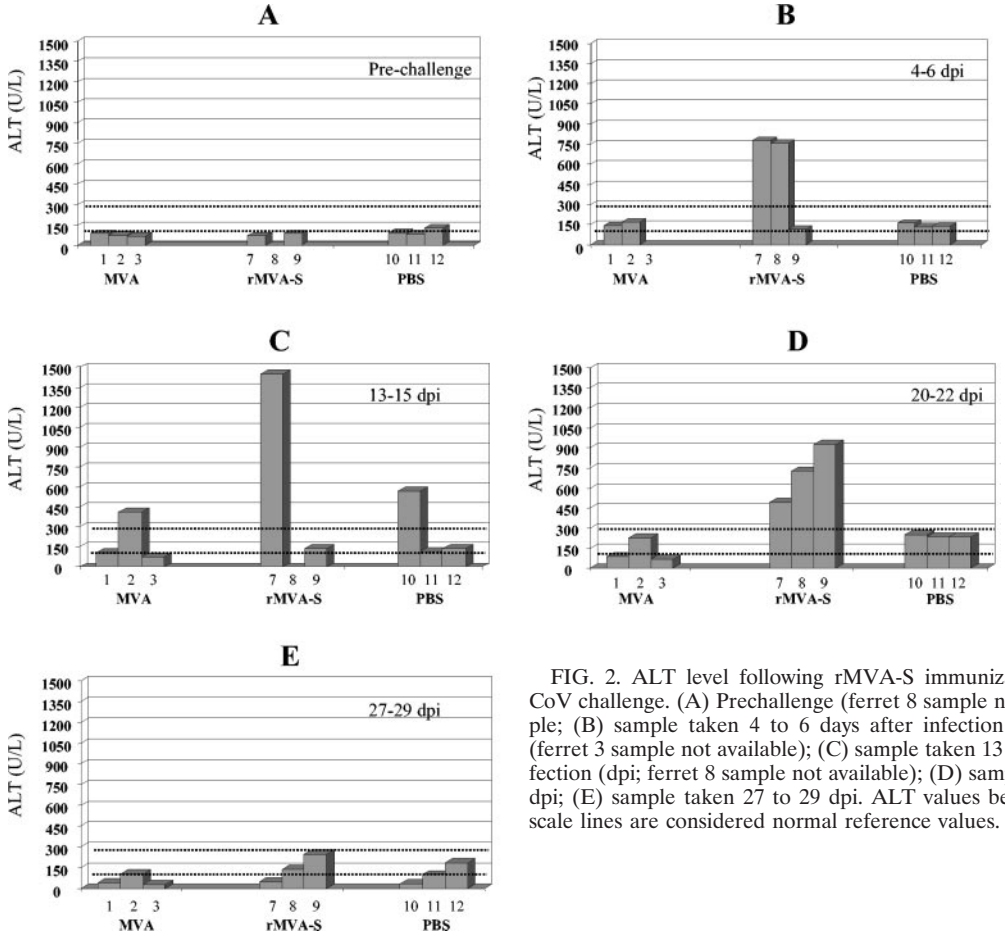

FIG. 2. ALT level following rMVA-S immunization and SARS-CoV challenge. (A) Prechallenge (ferret 8 sample not available) sample; (B) sample taken 4 to 6 days after infection with SARS-CoV (ferret 3 sample not available); (C) sample taken 13 to 15 days postinfection (dpi; ferret 8 sample not available); (D) sample taken 20 to 22 dpi; (E) sample taken 27 to 29 dpi. ALT values between the dotted scale lines are considered normal reference values.

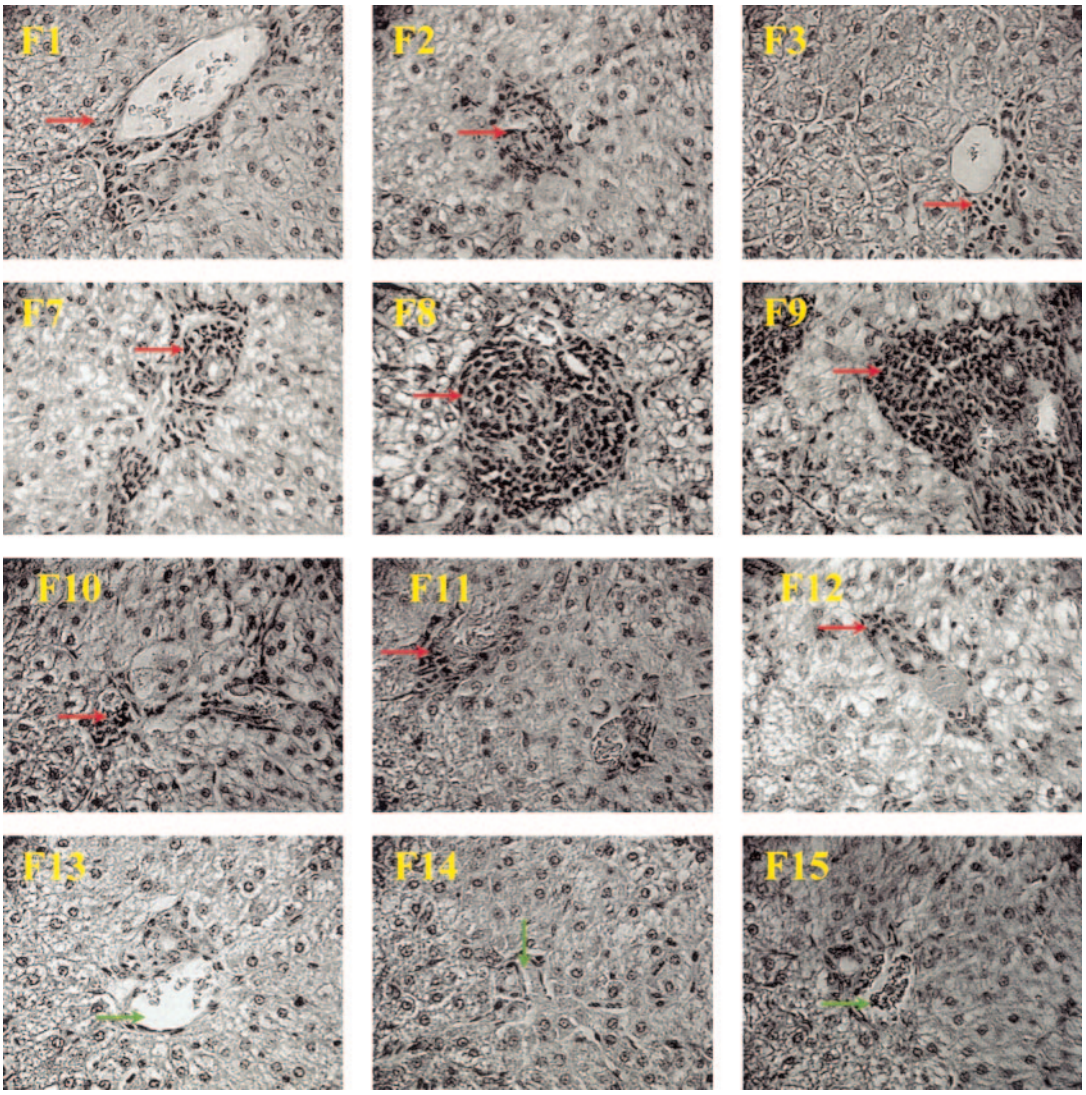

FIG. 3. Histopathology of ferret livers following immunization and SARS-CoV challenge. Representative pictures were taken at 27 to 29 days postinfection (ferrets [F] 1 to 3, 7 to 9, and 10 to 12) or no infection (naive ferrets 13, 14, and 15; exposed to neither MVA nor SARS-CoV) at ×40 magnification. Perivascular mononuclear infiltrates (red arrows) were present in all livers from ferrets infected with SARS-CoV, ranging from mild (ferrets 1, 2, 3, 10, 11, and 12) to severe (ferrets 7, 8, and 9) lesions. In addition, intralobular infiltration of mononuclear cells was extensive in livers from ferrets 7, 8, and 9. No significant liver lesions were found in naive ferrets (ferrets 13, 14, and 15, which did not receive MVA or SARS-CoV and which were used for preliminary examination of ferrets. Green arrows, vein of portal triads.

ufacturer (IDEXX Laboratories Inc. USA), was used to examine blood samples taken at various time points for the levels of alkaline phosphatase (an indicator of hepatic disease involving the biliary system), alanine aminotransferase (ALT; an indicator of hepatic parenchymal lesions), albumin (an indicator of abnormality of hepatic and renal function), creatinine (an indicator of renal disease), total bilirubin (an indicator of obstructive liver disease), total protein (an indicator of abnormality of hepatic and renal function), and urea (an indicator of renal disease). Surprisingly, ferrets vaccinated with rMVA-S demonstrated a significantly higher level of ALT after challenge with SARS-CoV than the control ferrets (Fig. 2). The elevated level of ALT was evidenced by 4 to 6 days after SARS-CoV infection and lasted until day 22. All the other parameters tested fell into the normal or slightly higher-than-normal (alkaline phosphatase) physiological range compared to the reference value (based on the recommendation from IDEXX Laboratories Inc. USA and data from 60 serum samples from healthy ferrets provided by the ferret supplier, Marshall Farm).

Histopathological examination was performed on liver sections (fixed with 10% PBS-buffered formalin, embedded in wax, and stained with hematoxylin and eosin) from ferrets sacrificed between 27 and 29 days postchallenge. It was found that ferrets immunized with rMVA-S (particularly ferret 9) developed severe periportal and panlobular mononuclear hepatitis (Fig. 3). In contrast, only mild periportal mononuclear hepatitis was observed in control ferrets receiving parental MVA or PBS. In addition, the panlobular hepatitis observed in rMVA-S-immunized ferrets was also accompanied by signs of focal necrosis of liver cells, including swelling of hepatocytes (hydropic degeneration), increased acidophilia, and hyper-

chromatic and fragmented nuclei (karyorrhexis). Thus, the histopathological finding is in line with the blood chemistry analysis. A summary of histopathological findings was included in the online supplementary materials. Note that the liver tissue specimen for pathological sectioning was collected postmortem (27 to 29 days after the challenge); by then the ALT level had already declined to (or slightly below) the normal range (Fig. 2E) and no viral RNA could be detected (Table 2). Not surprisingly, no viral antigen was found in the liver tissue by immunolabeling with the specific anti-S monoclonal antibody (data not shown). Therefore, it is likely that the liver inflammation shown in Fig. 3 does not reflect the true severity of the hepatitis associated with rMVA-S vaccination and SARS-CoV challenge and, in fact, may represent the recovering stage. Detailed pathological examination at the time when the ALT level is at the highest should be performed in future studies. Other organs were only mildly affected by SARS-CoV infection (data not shown).

It is known that neutralizing antibodies induced by the S protein of feline infectious peritonitis virus (also a coronavirus) often lead to accelerated infection by the mechanism of antibody-dependent enhancement of virus infectivity (12, 13, 16, 18). SARS-CoV has been shown to infect hepatocytes and cause hepatitis in humans (3). Here, we found that immunization with rMVA-S is associated with enhanced hepatitis in ferrets after SARS-CoV challenge. However, our present data cannot conclusively demonstrate whether or not the enhanced liver inflammation was the consequence of accelerated virus infection of livers or simply enhanced immunopathological effects on livers as a combined result from rMVA-S immunization and SARS-CoV infection. Further investigation, such as passive transfer of immune sera from vaccinated or SARS-CoV-infected ferrets to naive ferrets, which then are challenged with SARS-CoV, should aid in understanding the link between the immune responses induced by SARS-CoV antigens and enhanced liver inflammation.

In this communication, we demonstrated that vaccination with rMVA-S could induce a rapid and vigorous memory neutralizing antibody response, which is an essential feature for an effective prophylactic vaccine, in ferrets after challenge with SARS-CoV. On the other hand, our data suggest that vaccination with SARS-CoV S may lead to enhanced liver damage following SARS-CoV infection. This information is extremely important for development of safe SARS vaccines. Extra caution should be taken in proposed human trials of SARS vaccines (9) due to the potential liver damage from immunization and virus infection.

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REFERENCES

1. **Berry, J. D., S. Jones, M. A. Drebot, A. Andonov, M. Sabara, X. Y. Yuan, H. Weingartl, L. Fernando, P. Marszal, J. Gren, B. Nicolas, M. Andonova, F. Ranada, M. J. Gubbins, T. B. Ball, P. Kitching, Y. Li, A. Kabani, and F. Plummer.** 2004. Development and characterisation of neutralising monoclonal antibody to the SARS-coronavirus. J. Virol. Methods **120:**87–96.

1a. **Bisht, H., A. Roberts, L. Vogel, A. Bukreyev, P. L. Collins, B. R. Murphy, K. Subbarao, and B. Moss.** 2004. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc. Natl. Acad. Sci. USA **101:**6641–6646.

2. **Chakrabarti, S., J. R. Sisler, and B. Moss.** 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. BioTechniques **23:**1094–1097.

3. **Chau, T. N., K. C. Lee, H. Yao, T. Y. Tsang, T. C. Chow, Y. C. Yeung, K. W. Choi, Y. K. Tso, T. Lau, S. T. Lai, and C. L. Lai.** 2004. SARS-associated viral hepatitis caused by a novel coronavirus: report of three cases. Hepatology **39:**302–310.

4. **Collins, A. R., R. L. Knobler, H. Powell, and M. J. Buchmeier.** 1982. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology **119:**358–371.

5. **Falkner, F. G., and B. Moss.** 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62:**1849–1854.

6. **Fleming, J. O., S. A. Stohlman, R. C. Harmon, M. M. Lai, J. A. Frelinger, and L. P. Weiner.** 1983. Antigenic relationships of murine coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus. Virology **131:**296–307.

7. **Gao, W., A. Tamin, A. Soloff, L. D'Aiuto, E. Nwanegbo, P. D. Robbins, W. J. Bellini, S. Barratt-Boyes, and A. Gambotto.** 2003. Effects of a SARS-associated coronavirus vaccine in monkeys. Lancet **362:**1895–1896.

8. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper.** 2003. The genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.

9. **Marshall, E., and M. Enserink.** 2004. Medicine. Caution urged on SARS vaccines. Science **303:**944–946.

10. **Martina, B. E., B. L. Haagmans, T. Kuiken, R. A. Fouchier, G. F. Rimmelzwaan, G. Van Amerongen, J. S. Peiris, W. Lim, and A. D. Osterhaus.** 2003. Virology: SARS virus infection of cats and ferrets. Nature **425:**915.

11. **Moss, B.** 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc. Natl. Acad. Sci. USA **93:**11341–11348.

12. **Olsen, C. W.** 1993. A review of feline infectious peritonitis virus: molecular biology, immunopathogenesis, clinical aspects, and vaccination. Vet. Microbiol. **36:**1–37.

13. **Pedersen, N. C., and J. W. Black.** 1983. Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. Am. J. Vet. Res. **44:**229–234.

14. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.

15. **Subbarao, K., J. McAuliffe, L. Vogel, G. Fahle, S. Fischer, K. Tatti, M. Packard, W. J. Shieh, S. Zaki, and B. Murphy.** 2004. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J. Virol. **78:**3572–3577.

16. **Vennema, H., R. J. de Groot, D. A. Harbour, M. Dalderup, T. Gruffydd-Jones, M. C. Horzinek, and W. J. Spaan.** 1990. Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. J. Virol. **64:**1407–1409.

17. **Weingartl, H. M., J. Copps, M. A. Drebot, P. Marszal, G. Smith, J. Gren, M. Andova, J. Pasick, P. Kitching, and M. Czub.** 2004. Susceptibility of pigs and chickens to SARS coronavirus. Emerg. Infect. Dis. **10:**179–184.

18. **Weiss, R. C., and F. W. Scott.** 1981. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. Comp. Immunol. Microbiol. Infect. Dis. **4:**175–189.

19. **Yang, Z. Y., W. P. Kong, Y. Huang, A. Roberts, B. R. Murphy, K. Subbarao, and G. J. Nabel.** 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature **428:**561–564.

the duodenum as a blind pouch (Group I dogs).

1. Dragstedt, L. R., Oberhelman, H. A., Smith, C. A., *Ann. Surg.*, 1951, v134, 332.
2. ———, *A.M.A. Arch. Surg.*, 1951, v63, 298.
3. Koennecke, W., *Arch. f. Klin. Chir.*, 1922, v120, 537.
4. Sauvage, L. R., Schmitz, E. J., Storer, E. H., Kanar, E. A., Smith, F. R., Harkins, H. N., *Surg. Gyn. Obst.*, 1953, v96, 127.
5. Wangensteen, O. H., *J.A.M.A.*, 1952, v149, 18.
6. ———, *Gastroenterol.*, 1953, v20, 611.

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Propagation in Tissue Cultures of Cytopathogenic Agents from Patients with Measles.*† (21073)

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Numerous attempts have been made in the past to propagate the agent of measles in lower animals, in chick embryos and in tissue cultures(1-3). The results of different investigators were often at variance or directly contradictory. It has been made reasonably clear, however, that monkeys, especially *M. mulatta,* are moderately susceptible to experimental inoculation(3). Furthermore the researches of Rake, Shaffer and their collaborators have provided evidence suggesting that the agent which passed through bacteria-retaining filters could be maintained indefinitely in serial passages in the developing chick embryo(4,5). These workers(5) also confirmed the earlier observations of Plotz(6) who apparently had succeeded in growing the agent in a modified suspended cell culture of chick embryonic tissues. Egg passage in the hands of Shaffer and his coworkers(7,8) regularly appeared to alter the pathogenicity of the agent for man as indicated by the development of a mild and much modified disease following the inoculation of egg adapted materials into susceptible children. In certain cases this modified disease seemed to be followed by resistance to measles as indicated by the results of subsequent natural or artificial exposure to the virulent form of the agent(9). Since 1943 when the last of the communications by Rake and his collaborators appeared, no important progress has been made in the study of the etiology of measles. This fact may in large part be attributed to the lack of a convenient laboratory method for the demonstration of the presence of the agent which induced no recognizable changes in eggs or cultures of chick tissues. Moreover, repeated attempts by Shaffer(10) to demonstrate a serologic reaction, such as complement fixation, using materials from the infected chick embryo failed. Accordingly, the only available technics have consisted in the inoculation of man or the monkey. The former is obviously impractical as routine and the latter tedious, expensive and frequently inconclusive because of variation in individual susceptibility.

With these considerations in mind we have recently attempted to cultivate the agent of measles in cultures of human and monkey cells employing procedures applied successfully to the propagation of the poliomyelitis viruses(11-13).* In blood and throat washings of typical cases of measles agents have been demonstrated that can be maintained in serial

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TABLE I. Cytopathogenic Agents Isolated in Tissue Cultures from Throat Washings and Blood of 5 Measles Cases.

Material and No. of passages				Time of collection of materials	
T.W.*	Passages	Blood	Passages	Days after onset	Hours after rash
—†	—	+	3	5	29
+	3	+	3	3	17
+	4	+	10	2	14
+	3	ND‡	ND	4	6
+	2	+	1	?	±24

* T.W. = Throat washings.
† No virus isolated.
‡ ND = Not done.

passage in tissue cultures and which induce distinctive cytopathic changes in renal epithelial cells. A certain amount of evidence has been accumulated indicating that antibodies specific for these agents develop during the course of the disease. It is our purpose to describe here these observations in a preliminary manner. Additional evidence for the relationship of these agents to measles will be sought in future investigations.

Materials and methods. Collection of specimens. Throat washings, venous blood and feces were obtained from 7 patients as early as possible after a clinical diagnosis of measles was established. In 5 instances the time at which specimens were collected in relation to the onset of exanthem is given in the case histories described below or in Table I. When capable, patients were asked to gargle with 10-15 ml of sterile neutralized fat-free milk. Certain specimens from the throats of younger children were obtained by cotton swab previously moistened in milk. After swabbing the throat the swab was immersed in 2 ml of milk. Penicillin, 100 u/ml, and streptomycin, 50 mg/ml, were added to all throat specimens which were then centrifuged at 5450 rpm for about one hour. Supernatant fluid and sediment resuspended in a small volume of milk were used as separate inocula in different experiments in amounts varying from 0.5 ml to 3.0 ml. About 10 ml of blood immediately after withdrawal were placed in tubes containing 2 ml of 0.05% solution of heparin. As inocula for tissue cultures amounts varying from 0.5 ml to 2.0 ml of the whole blood were employed. After addition of antibiotics as described above 10% fecal suspensions were prepared by grinding the material in bovine amniotic fluid medium. The suspensions were then centrifuged at 5450 rpm for about one hour and the supernatant fluids used as inocula, in amounts varying from 0.1 ml to 3 ml. All specimens were refrigerated in water and ice or maintained in the cold at about 5°C from the time of collection until they were added to the cultures. The maximum time that lapsed between collection of specimens and inoculation was 3½ hours.

Tissue culture technics. In the initial isolation attempts roller tube cultures(11,12) of human kidney, human embryonic lung, human embryonic intestine, human uterus and rhesus monkey testis were employed. Subsequent passages of the agents isolated were later attempted in human kidney, human embryonic skin and muscle, human foreskin, human uterus, rhesus monkey kidney and embryonic chick tissue. Stationary cultures prepared according to the technic of Youngner(13) with trypsinized human and rhesus monkey kidney were later employed for isolation of agents and their passage. The culture medium consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics, and phenol red as an indicator of cell metabolism(12). Soybean trypsin inhibitor was added to this medium unless it was used for the cultivation of human and monkey kidney (11). Fluids were usually changed at intervals of 4-5 days. For histological examination the cell growth after fixation in 10% formalin was embedded in collodion, dehydrated and stained with hematoxylin and eosin.‡

‡ We are indebted to Dr. William J. Cheatham for the following account of the collodion embedding technic and for the preparation of material for histologic examination. *Collodion membrane technic.* The culture fluid is removed from the roller tube and replaced by fixative sufficient to cover the areas of outgrowth; formalin has generally been used. Fixation for one hour is adequate with the method of tissue culture used. Following fixation, dehydration is carried out with increasing concentrations of alcohol ending in ether-alcohol mixture (50 per cent of each).

Manner of passage in tissue culture. Serial passage of the various strains (Table I) was accomplished as routine by removal of the culture medium between the 4th and the 16th day after inoculation and immediate transfer of 0.1 ml to each of a number of fresh cultures. Successful passage of the agent with fluids that had been previously centrifuged at 2500 rpm to remove cellular elements has also been repeatedly demonstrated. Larger inocula (up to 1.0 ml) were often used during the initial experiments before the resistance of the agent to storage at various temperatures had been determined. *Virus neutralization and complement fixation tests.* The procedures employed are described subsequently in the text. *Description of cases from which materials were obtained.* During an outbreak of measles at a private boarding school for boys in Southboro, Mass., isolations were attempted from throat washings and stools of 4 patients and blood of 3 of the same patients. The latter are designated as Cases 1, 2 and 3. Throat washings of 2 siblings in a small epidemic of measles in Wellesley, Mass., (Cases 4 and 5) and blood from 2 cases of measles admitted to the Boston City Hospital during an epidemic in Boston (Cases 6 and 7) are under investigation at the present time. Details of these typical cases are omitted for the sake of brevity.

Case 1: D.R., age 11, was in contact with a "probable" case of measles 10 days before symptoms commenced on 1/20/54. The latter consisted of signs of an upper respiratory infection including sore throat and fever to 101°F. He soon developed conjunctivitis and a bad cough. These symptoms became aggravated and on 1/24 in the morning there was a suggestion of a rash on his face. Temperature at 4:30 p.m. was 105.5°F. Koplik's spots were noted on the buccal mucosa by the school physician the next morning. On 1/25 at 1 p.m. he was seen by TCP with findings of temperature 98° p.o., mild to moderate conjunctivitis, moderate generalized adenopathy and characteristic blotchy maculopapular rash in full bloom, extending to involve even the palms and soles. No Koplik's spots were seen at this time. Specimens of throat washings, blood and stool were collected at 1:30 p.m. on 1/25. *Case 2:* H.J., age 13, with no known contact other than Case 1, developed signs of an upper respiratory infection on 1/26/54. He complained of sore throat and cough the following day and of photophobia on 1/28. At this time the infirmary nurse noticed a questionable trace of a rash on his forehead. He was seen on 1/29 by TCP with findings of temperature 101° p.o., and maculopapular rash developing over the face, mildly over the chest and abdomen, minimally on the upper extremities and none on the lower extremities. He had moderate conjunctivitis, cough and Koplik's spots on the buccal mucosa. Specimens of throat washings, blood and stool were obtained at 11 a.m. on 1/29. *Case 3:* D.E., age 13, a close contact of both Cases 1 and 2 during their prodromal stages, experienced a gastrointestinal upset on 2/8/54 with cramps and nausea. Temperature was 99.6°F. Faint rash was first noted on his face in the late evening of 2/9, and he was seen by TCP at 12 noon on 2/10 with findings of typical rash over the face with minimal extension to the chest, abdomen and back. Koplik's spots

The latter is then replaced by a dilute solution of collodion in ether-alcohol allowing an hour or more for penetration of the collodion into the tissues. The concentration of collodion is best determined by trials in which films are removed from blank tubes. The collodion is poured from the tube, rotating the latter so that the entire inner surface is covered by a thin film. The collodion is allowed to dry partially, rotating the tube constantly. This requires 1-2 minutes and can be arrived at by experience, employing as a guide the time at which the odor of ether-alcohol is barely detectable. Cold water is then run slowly into the tube to harden the membrane. The upper edge of the tube is rimmed and the membrane carefully separated from the glass with a thin glass rod. With patience a cast of the tube including the tissue is obtained; this is cut into a flat sheet which can be stained and trimmed according to the distribution of tissue. The technic of staining with hematoxylin and eosin is used and need not be described in detail here. Alcoholic eosin has been found most satisfactory. Complete dehydration using absolute alcohol cannot be used because of the solubility of collodion. Clearing is therefore carried out from 95 per cent alcohol in oil of Origanum followed by xylol. After clearing the membrane is mounted using any satisfactory mounting media.

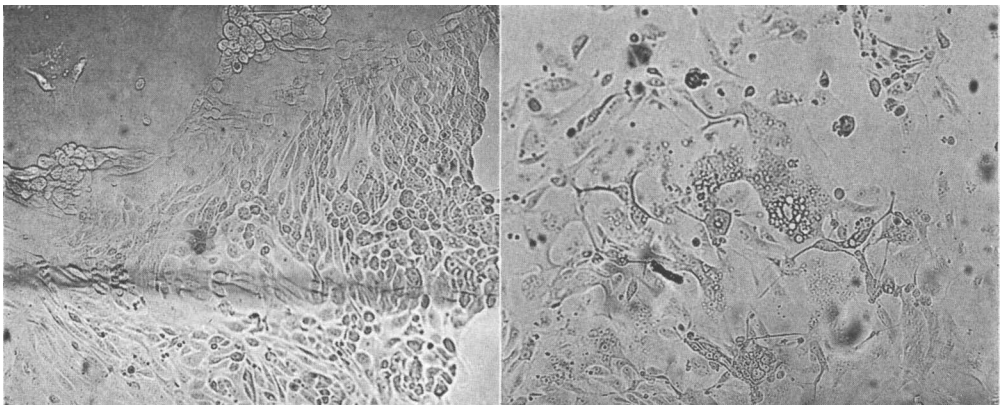

Fig. 1. Fig. 2.

FIG. 1. Outgrowth of normal human kidney cells in an uninoculated roller tube culture (×130). Control for cultures shown in Fig. 2 and 3.

FIG. 2. Area of syncytial giant cells with small cytoplasmic vacuoles. Note many nucleoli and faint nuclear outlines. 9th day after inoculation; 7th passage, agent from Case 3 blood (×130).

were present on the buccal mucosa. Cough and conjunctivitis were mild. His temperature was 102° p.o. Specimens of throat washings, blood and stool were obtained at this time. His temperature was 103.6°F with rash in full bloom in the afternoon. On the following morning his temperature was 104°F.

Experimental. Cytopathic changes induced by agents isolated from cases of measles. The first of 8 agents obtained from blood or throat washings of measles cases and exhibiting comparable properties was isolated in cultures of human kidney tissue following addition of the blood of Case 3. In each of the 3 cultures that were inoculated cytopathic changes were observed on the 7th day. Since these changes presented a characteristic appearance not heretofore associated definitely with a virus they have provided the means for the further investigation of this agent as well as others that have been recently isolated. Accordingly, here at the beginning these changes will be described in detail. Observation of *fresh preparations* under low magnification (80X) revealed within the sheet-like outgrowth of renal epithelial cells discrete areas of varying size and shape in which the cell boundaries were obliterated and the nuclei often difficult to visualize. Within these areas, which may be described as non-refractile "glassy" plaques, large and small vacuoles were often numerous lending them a foamy or lace-like quality. The number and size of the vacuoles increased as incubation was continued. On careful examination of these areas many small, slightly refractile bodies were seen that resembled nucleoli within nuclei whose outlines could often be distinguished only with difficulty. The total effect thus suggested the presence of large vacuolated giant cells. After further cultivation the extent of the areas initially present was slowly extended or was enlarged by coalescence with neighboring plaques while others developed elsewhere. In addition to the formation of vacuoles degenerative changes gradually appeared within the affected areas suggesting coagulation necrosis. At the end of three weeks most of the epithelial cells appeared to be involved, yet here and there small aggregates of normal cells remained. These seemed, however, to be composed mainly of spindle-shaped cells. Reference to Fig. 1, 2 and 3 will aid in the visualization of these changes as they are manifest in the natural state. In contrast to the appearance of the normal cell outgrowth shown in Fig. 1 the smooth confluent area of affected cells stands out clearly in Fig. 2. While a slight degree of vacuolization is evident in this figure, it is extensive in Fig. 3 especially along the margin of cell growth where it is first apt to become apparent.

The interpretation that has just been presented of the changes observed in fresh prepa-

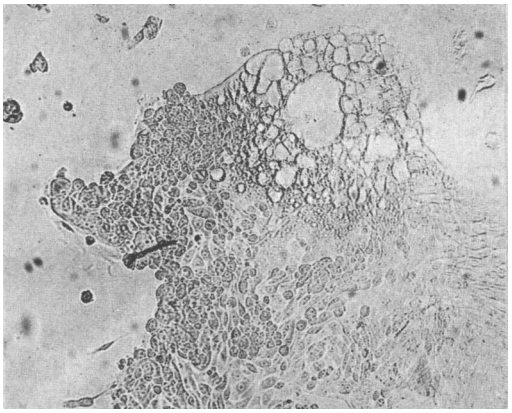

FIG. 3. Lace-like network of vacuoles at tissue margin from the same culture (×130).

rations was supported by examination of fixed and stained materials. Under these conditions the glassy areas were clearly revealed as collections of nuclei surrounded by a common cytoplasmic matrix. As many as 40 to 100 nuclei were counted in such syncytial formations. Often the limits of the encompassing cytoplasm were sharply defined thus contributing to the impression that development of true giant cells has occurred *in vitro*. Whether or not this is actually the case, the phenomenon is of much interest in view of the constant presence of giant cells in lymphoid tissues during the early stages of measles in man(14,15).

Examination of *stained materials* also revealed significant changes within the nuclei of the giant cells that were not visible in fresh preparations. These consisted in a redistribution of the chromatin which ultimately assumed a marginal position where it formed a dense ring or crescent that stained intensely with the basic dye. Concomitantly the central portion of the nucleus came to be occupied by an apparently homogeneous substance, acidophilic in character, that approximated closely to the chromatin ring. Since in these and other preparations that have been examined subsequently no clear unstained zone has been observed between the chromatin and this acidophilic mass, it cannot be asserted that the latter represents an intranuclear inclusion body of the type characteristically associated with viral infections. Nevertheless, as far as can now be determined, its presence along with the margination of the chromatin affords a useful criterion of infection for the agents under study. It should be emphasized, however, that the changes as just depicted are encountered in cultures that have been incubated for relatively prolonged periods (*e.g.* 14-21 days). When the interval between inoculation of the agent and examination of the stained cells (*e.g.* 4 days) is shorter, margination of the chromatin may be incomplete or inapparent and the acidophilic substance may only be seen in small rounded masses distributed here and there amid nuclear materials that approximate the normal arrangement. Fig. 4, 5, 6 and 7 illustrate well-developed nuclear changes and also the general similarity of the affected areas to the giant cells encountered in lesions associated with measles. Of particular interest in this latter connection are the basophilic "pseudoprotozoal" bodies that Bonenfant(16) has recently described in the mucosa and lymphoid follicles in cases of this disease. These bodies were usually surrounded by an acidophilic homogeneous substance. As described and pictured these bodies with their matrix strikingly resemble the giant cells in tissue cultures that exhibit well-developed nuclear changes.§

Some biologic properties of the agents isolated from measles. Certain of the biologic properties of the agents isolated from patients with measles have been definitely determined, others in a preliminary or tentative manner. In several instances these properties have been studied only in respect to the strain first isolated from the blood of Case 3. Since, however, the other strains, as far as they have been examined, behave in a similar manner it is probable that all of them, when thoroughly studied, will exhibit the same general characteristics.

A) *Source of virus.* As repeatedly stated, agents have been recovered from both blood and throat washings. In three cases yielding viruses from one or another of these sources

§ Since this manuscript was submitted for publication materials fixed in Bouin's fluid, employing the usual alteration of washing with 70 per cent alcohol following fixation, have been examined. Typical acidophilic intranuclear inclusion bodies were regularly seen surrounded by a clear zone or "halo."

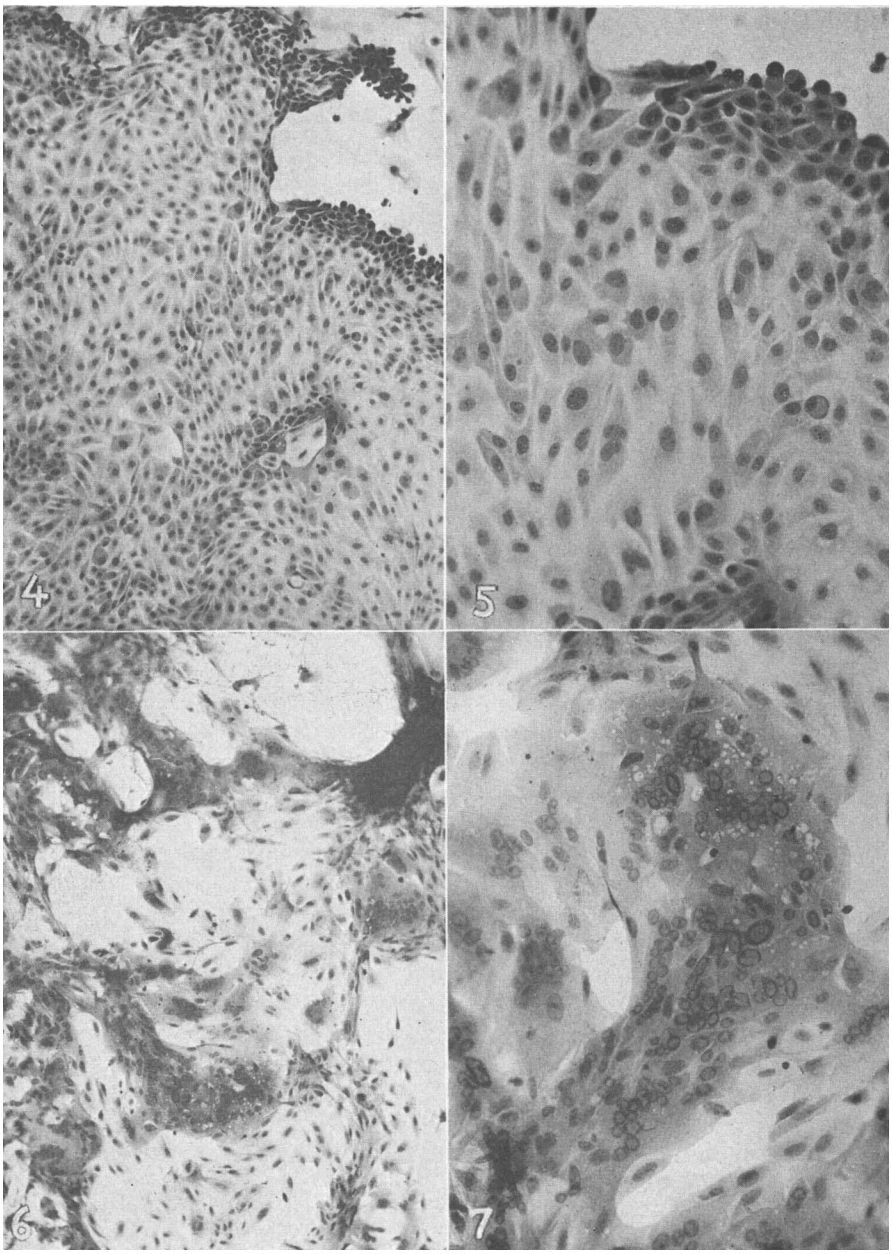

FIG. 4. Outgrowth of normal human renal cells fixed and stained with hematoxylin and eosin. Control for cultures shown in Fig. 5-7 (×110).

FIG. 5. A portion of the cell outgrowth shown in Fig. 4 more highly magnified. Hematoxylin and eosin stain (×300).

FIG. 6. Outgrowth of human renal cells showing giant cell formation and nuclear changes 20 days after inoculation with 2 ml blood from Case 3. Hematoxylin and eosin stain (×110).

FIG. 7. A portion of cell outgrowth shown in Fig. 6 more highly magnified. Hematoxylin and eosin stain (×300).

fecal suspensions have likewise been examined by the tissue culture technic. In none was evidence for the presence of an agent obtained. Further examinations of fecal specimens are necessary before it can be stated whether or not the virus is present in the intestinal excreta.

B) *Cytopathogenic range.* Monkey kidney

is the only other tissue employed that has yielded a growth of cells in which the characteristic changes described above have been definitely observed following inoculation of virus. In cultures consisting largely of monkey renal epithelial cells as prepared by Youngner's modification of Dulbecco's technic (13) cytopathic changes have been regularly observed which resemble closely those produced by these agents in human renal cells as seen in both fresh and stained preparations. These effects followed the addition of blood or throat washings from cases of measles as well as infected tissue culture fluids derived from previous passages. Monkey kidney cultures may, therefore, be applied to the study of these agents in the same manner as cultures of human kidney. In so doing, however, it must be borne in mind that cytopathic effects which superficially resemble those resulting from infection by the measles agents may possibly be induced by other viral agents present in the monkey kidney tissue (*cf.* last paragraph under G) or by unknown factors. In a few cultures of human prepucial tissue inoculated with one of the measles agents changes resembling those seen in renal cells were noted in the epithelial outgrowth about certain fragments. Additional observations, however, will be required before it can be confidently asserted that dermal epithelial cells are specifically attacked by these viruses. In a single experiment no cytopathic manifestations were seen during a period of 31 days following inoculation of infected tissue culture fluid into cultures of human embryonic skin and muscle, human uterine tissue or embryonic chick tissue. Tests for the presence of complement fixing antigen in the fluids removed from the cultures on the 31st day were negative. These serologic results suggest that growth of the virus did not occur, since, as will be shown subsequently, the antigen appears to develop regularly after several days in cultures of renal tissue infected with the virus.

C) *Failure to induce demonstrable changes in mice or chick embryos.* Two litters of suckling white mice (1-day-old) were inoculated with infective tissue culture fluid by both the intraperitoneal and intracerebral routes. The animals remained well during an observation period of 21 days. Employing the same material as inoculum 0.1 ml amounts were introduced into the amniotic sac of 7-day embryonated hen's eggs. After 7 days incubation at 36°C the amniotic fluid and membranes were harvested, ground with alundum and centrifuged at 1500 rpm. The supernatant fluid was used for a second egg passage which was carried out in the same manner. Whereas inoculation of 0.1 ml aliquots of the first egg passage material into cultures of monkey renal epithelium was followed by characteristic cytopathic changes on the 8th day, the addition of 0.5 ml of second egg passage material to such cultures failed to produce this effect. No complement fixing antigen was detected in the materials from the egg passages. Although these results suggest that the virus is not readily adapted to growth in the chick embryo, it is evident that much further investigation will be required to determine the degree of susceptibility of this host.

D) *Serial passages.* Serial passages of several of the strains have been carried out without difficulty in cultures of human or monkey renal cells. Employing 0.1 ml of the fluid phase as inoculum, 10 passages of the first strain isolated have so far been accomplished in human cells of this type. With other strains fewer passages have been completed as indicated in Table I.

E) *Assay of infectivity.* As yet only one attempt has been made to measure infectivity of virus propagated in tissue culture. In this case fluids and cells were removed from 15 cultures of human kidney tissue on the 6th day after inoculation of fluid from the 4th tissue culture passage of the agent from Case 3. These materials were pooled and the cells were ground with alundum in the presence of the fluid. After centrifugation for 15 minutes at 2500 rpm the supernatant fluid was titrated for infectivity in cultures of human kidney tissues. For this purpose 3 cultures were each inoculated with 0.1 ml of the suspension diluted by a factor of 10. The endpoint of viral activity as indicated by the highest dilution causing cytopathic changes was about $10^{-2.5}$. This low titer was somewhat unexpected in view of the fact, as will be shown here-

TABLE II. Neutralization of Cytopathogenic Effect of Virus from Measles Case 3 by Convalescent Sera from Two Other Cases of Measles.

Serum source	Serum drawn (days after rash)	Neutralizing serum dilution*
Case 1	1	<1:8
	17	1:32
2	0	<1:2
	12	1:32
	70	1:32

* Readings taken on 10th day after addition of virus.

after, that tissue culture fluids contain sufficient antigen to fix complement in the presence of convalescent measles serum. Without more experimental data, however, it cannot be assumed that maximal infectivity titers lie within this range.

F) *Neutralization of cytopathogenicity by convalescent measles sera.* That the cytopathogenic capacity of at least one strain of the agents associated with measles is inhibited by serum factors developing during the course of the disease has been demonstrated in two experiments. Employing 100 ID_{50} of the viral suspension mentioned in the previous paragraph neutralization tests were carried out in cultures of monkey renal epithelial cells. Sera taken during the acute and convalescent stages from two of the cases occurring at the boys' school were stored at −15°C and inactivated at 56°C for 30 minutes before they were diluted and used in the test. As diluent bovine amniotic fluid was employed. Dilutions of serum and virus were mixed and kept at 5°C for one hour when 0.1 ml of each mixture was added to each of three tissue cultures. In both tests the cultures were examined every day or every 2 days and the final readings were recorded on the 10th day. The results are summarized in Table II. They indicate that significant increases in substances occurred in the serum of both patients that neutralized the cytopathogenicity of the agent isolated from the blood of a third patient. In considering these results it is pertinent to recall that agents with similar characteristics have been isolated from the two patients whose convalescent sera were shown to possess virus-neutralizing capacity.

G) *Production of complement fixing antigen in tissue cultures.* Since it has been shown (17) that in cultures of poliomyelitis viruses antigens capable of fixing complement in the presence of specific antibodies appear in the fluid phase, tests were carried out to determine whether or not the fluids removed from cultures exhibiting cytopathic changes induced by the measles agents might behave in a similar manner. To this end the drop method of Fulton and Dumbell(18) as modified by Svedmyr, Enders and Holloway(17) was employed. As antigens crude undiluted fluids have been used. These were derived from human or monkey kidney cultures inoculated with strains isolated from either blood or throat washings of Cases 2, 3 and 4. The fluids from several cultures were collected at various intervals after inoculation of the virus, pooled, centrifuged at 1500 rpm for 5 minutes and stored at either 5°C or −16°C. Immediately before use the fluid was heated at 56°C for 30 minutes to remove any anti-complementary activity that might be present. As control antigens, fluids were taken from uninoculated cultures maintained under the same conditions as well as fluids from cultures of the agent producing changes superficially similar to those caused by the measles agents and which are mentioned below. These materials failed to fix complement with any of the sera that have been examined. Acute and convalescent phase measles sera were inactivated at 56°C and serial dilutions prepared as in complement fixation tests for poliomyelitis antibody. The results of tests that have so far been completed indicate clearly that antibodies develop during the course of measles capable of reacting with an antigen that appears in the culture fluid after the 3rd to the 7th day following inoculation of the virus. From representative data presented in Table III it is evident that the antibody may emerge at least as early as the 7th day following the appearance of the rash and continues to persist for at least 2 months in fairly high titer. By this time, however, there is some indication that the maximal concentration has been previously attained. It is noteworthy in respect to the possible etiologic relationship of these agents to measles that antibodies appeared in the blood of Cases 6

TABLE III. Results of Complement Fixation Tests on Measles Sera Employing as Antigens Fluids from Tissue Cultures Infected with Agents Isolated from Cases of Measles.

Serum source (case No.)	Serum drawn (days after rash)	CF titer (serum dilution) Antigen MKC*	Antigen HKC†
1	1	<1:8	1:8
	17	1:256	1:512
	75	1:64	1:128
2	0	<1:8	<1:8
	12	1:128	1:256
	70	1:64	1:128
3	0	<1:8	<1:8
	58	1:256	1:256
6	0	<1:8	<1:8
	7	1:32	1:32
7	1	<1:8	<1:8
	8	1:64	1:128

* MKC = Monkey kidney tissue culture fluid infected with agent from blood of case 2.
† HKC = Human kidney tissue culture fluid infected with agent from blood of case 3.

and 7 which fixed complement with the antigens from Cases 2 and 3. The latter occurred in a widely separated area and at an earlier time. A few tests have been carried out with measles antigen on sera from 3 adults giving a history of measles during childhood. Serum titers of 1:2, 1:8 and >1:16 were recorded.

Filterability and resistance to physical agents. Filtration. A portion of the pool of virus with infectivity titer of $10^{-2.5}$ (*cf.* last paragraph under E) was diluted 1:5 in beef infusion broth and passed through a sintered glass filter under a pressure of 45 mm Hg. Time of filtration was 10 minutes. The capacity of the filter to retain *Serratia marcescens* was then demonstrated. The viral filtrate was shown to be free of bacteria by addition to infusion and thioglycollate broth and blood agar media. Five cultures of monkey renal cells were inoculated with the filtrate (0.5-1 ml). Characteristic cytopathic changes were subsequently noted in all. *Thermal stability.* The cytopathogenicity of one strain was destroyed by heating at 65° for 30 minutes. The infectivity of heparinized blood or throat washings in milk as tested in tissue culture was preserved for at least 3½ hours by refrigeration at 1°C to 5°C. Agents present in tissue culture fluids remained infective after 38 days at –15°C and after at least 35 days at about –50°C to –60°C.

Other agents isolated during this study. Two agents have been isolated while the present work was in progress that appear unrelated to those we have just described. The first was recovered from the throat washings of a typical case of measles occurring in the boys' school. Its wide cytopathogenic range, the character of the cytopathic changes induced and the fact that its infectivity for tissue cultures was neutralized by herpes simplex immune rabbit serum served to define its nature. A second agent was obtained from an uninoculated culture of monkey kidney cells. The cytopathic changes it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles. But, when the cells from infected cultures were fixed and stained, their effect could be easily distinguished since the internuclear changes typical of the measles agents were not observed. Moreover, as we have already indicated, fluids from cultures infected with the agent failed to fix complement in the presence of convalescent measles serum. Obviously the possibility of encountering such agents in studies with measles should be constantly kept in mind.

Discussion. Of the numerous experiments that have been reported in the past describing the successful isolation of the etiologic agent of measles only those in which monkeys were employed as the experimental animal have been consistently confirmed by other workers. Great caution should therefore be exercised in the interpretation of any new claims that the virus has been propagated in other hosts or systems. Accordingly, the results that are summarized here must be subjected to the most critical analysis.

The following facts tend to support the hypothesis that the viruses we have described are responsible for the disease. Experimentally transmissible agents exhibiting a similar and characteristic cytopathogenic effect in cultures of human or simian epithelial cells have been isolated from either the blood or throat washings derived from 5 of 7 typical cases of measles during the early acute phase. An agent was demonstrated in the blood of 4 of the 5 cases from which specimens were ob-

tained and examined by the tissue culture method. These findings would seem to be of especial significance since it is unlikely that viruses unrelated to measles would be regularly present in the circulating blood of these individuals some of whom were geographically widely separated.

The pathologic changes induced by the agents in epithelial cells in tissue culture resemble, at least superficially, those found in certain tissues during the acute stage of measles. While there is no ground for concluding that the factors *in vivo* are the same as those which underlie the formation of giant cells and the nuclear disturbances *in vitro*, the appearance of these phenomena in cultured cells is consistent with the properties that *a priori* might be associated with the virus of measles.

The emergence of antibodies during the course of the disease capable of suppressing the cytopathogenic effect and of fixing complement in the presence of infected tissue culture fluids affords further evidence for the close association of the agents with measles. Obviously additional data to be derived from tests with sera from a large number of cases of measles as well as other infectious diseases, especially the common exanthemata, are desirable in order to eliminate any remaining doubt concerning the specificity of these serologic reactions. The accumulation of such data is now in progress.

Although we have thus already obtained considerable indirect evidence supporting the etiologic role of this group of agents in measles, 2 experiments essential in the establishment of this relationship remain to be carried out. These will consist in the production of measles in the monkey and in man with tissue culture materials after a number of passages *in vitro* sufficient to eliminate any virus introduced in the original inoculum. The recovery of the virus from the experimental disease in these hosts should then be accomplished.

Conclusion. The findings just summarized support the presumption that this group of agents is composed of representatives of the viral species responsible for measles.

Summary. Eight agents exhibiting the properties of viruses have been isolated in cultures of human or simian renal cells from the blood or throat washings of five cases of typical measles. Multiplication of the agents *in vitro* is accompanied by characteristic changes in the cells. Primarily these changes consist in the formation of syncytial giant cells wherein the chromatin assumes a marginal position and is replaced centrally by an acidophilic substance of unknown nature. The cytopathogenic effect of at least one of the agents is inhibited by convalescent phase measles sera from other patients with measles. Antigen appears during cultivation *in vitro* of the measles agents that reacts specifically in complement fixation tests with convalescent phase measles sera.

1. Enders, J. F., *Virus and Rickettsial Diseases,* Harvard University Press, Cambridge, Mass., 1940, pp237-267.

2. Van Rooyen, C. E., and Rhodes, A. J., *Virus Diseases of Man.* Thomas Nelson and Sons, New York, 1948, pp226-228.

3. Rake, G., Measles in *Viral and Rickettsial Infections of Man,* ed. T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1952, pp480-481.

4. Rake, G., and Shaffer, M. F., *J. Immunol.,* 1940, v38, 177.

5. Rake, G., Shaffer, M. F., and Jones, H. P., *J. Inf. Dis.,* 1941, v69, 65.

6. Plotz, H., *Bull. Acad. Med.,* 3rd Ser., 1938, v119, 598.

7. Shaffer, M. F., Rake, G., Stokes, J., Jr., and O'Neil, G. C., *J. Immunol.,* 1941, v41, 241.

8. Stokes, J., Jr., O'Neil, G. C., Shaffer, M. F., Rake, G., and Maris, E. P., *J. Pediat.,* 1943, v22, 1.

9. Maris, E. P., Rake, G., Stokes, J., Jr., Shaffer, M. F., and O'Neil, G. C., *ibid.,* 1943, v22, 17.

10. Shaffer, M. F., Personal communication to JFE.

11. Robbins, F. C., Weller, T. H., and Enders, J. F., *J. Immunol.,* 1952, v69, 673.

12. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.

13. Youngner, J. S., *ibid.,* 1954, v85, 202.

14. Warthin, A. S, *Arch. Path.,* 1931, v11, 864.

15. Mulligan, R. M., *ibid.,* 1944, v37, 61.

16. Bonenfant, J. L., *Arch. Fran. de Pediatrie,* 1952, v9, 497.

17. Svedmyr, A., Enders, J. F., and Holloway, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 296.

18. Fulton, F. and Dumbell, K. R., *J. Gen. Microbiol.,* 1949, v3, 97.

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Measles Virus: A Summary of Experiments Concerned with Isolation, Properties, and Behavior

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Presented here is a clarifying summarization of the many studies upon which any forthcoming advances in measles prophylaxis will be based.

Study of the etiologic agent of measles began with Home at the end of the 18th century and has been continued intermittently until the present. Among the more significant contributions to this subject in modern times was the demonstration by Goldberger and Anderson [1] in 1911 that macaque monkeys are susceptible to infection. This observation was later confirmed and extended by Blake and Trask,[2] among others. During the course of these earlier studies evidence for the filterable nature of the agent was obtained.

Using the monkey to test for the presence of virus, Plotz [3] in the 1930's reported its successful cultivation in cultures of chick embryonic tissues. In 1939–1940 Rake and Shaffer [4, 5] described the serial propagation of the agent in chick embryos and in 1941 with Jones [6] confirmed Plotz's observations. In the same year Shaffer and his associates [7] also noted that during the course

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of passages in the chick embryo the virulence of the virus for man was reduced and they attempted to determine whether this attenuated virus might be employed as a vaccine. The results were inconclusive and since that time we have seen no account of further trials with such materials, although it is our understanding that similar experiments are now in progress within the Soviet Union.[7a] Other workers in the past,[8] including one of us, were not able to induce measles in the monkey with regularity and also failed to obtain convincing evidence that the virus multiplied in living chick embryos or in chick embryonic tissues cultivated in vitro.

Such experimental discrepancies as well as the failure to make more rapid progress toward the precise definition of the nature and properties of the measles agent were largely attributable to the lack of a convenient and inexpensive technic whereby viral multiplication could be unequivocally determined. Following the demonstration of the growth and cytopathogenic effect of poliomyelitis virus in cultures of extraneural human tissues it seemed likely that application of the same method might prove effective in the case of measles virus.

Isolation of Measles Virus in Tissue Culture

Accordingly, Enders and Peebles [9] in 1954 undertook experiments in which cultures of human postnatal tissues in roller tube cultures were exposed to whole blood and throat washings obtained from a patient with measles during the first 24 hours of the exanthem. After an interval of from four to 10 days abnormal changes were observed of a character that we shall presently describe and which were shown to be induced by a virus. Comparable materials from seven other typical cases of measles subsequently tested in cultures of human renal or monkey (rhesus) renal cells have yielded agents exhibiting the same cytopathogenic properties. In three of these cases virus was demonstrated in both blood and throat washings; in two the blood was positive and washings were negative. In each of the two remaining cases in which only blood or throat washings was tested virus was also found. In addition, an agent was recovered in tissue culture from the lung of a patient dying during the acute stage of measles that was indistinguishable from the others. It is to be emphasized that these nine viruses were isolated from cases occurring in different geographic areas at two different times, i.e., in the spring of 1954 and 1955. The association with measles of viruses conforming in their characteristics with those we have isolated has been subsequently reported by Cohen and his co-workers [10] and by Ruckle.[11]

Cytopathogenic Properties of the Virus

Upon isolation and during serial passages in cultures of human renal cells our nine strains of virus have induced the formation of syncytia or multinu-

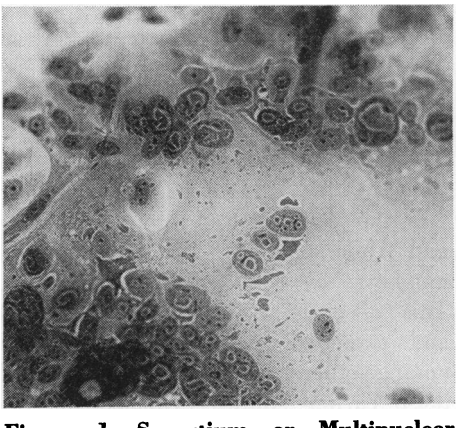

Figure 1—Syncytium or Multinuclear Giant Cell in Culture of Postnatal Human Renal Cells; 16 Days After Addition of 23rd Renal Cell Passage of Edmonston Strain of Measles Virus. H and E Stain x 430

clear giant cells within the sheet of normal epithelial elements such as those shown in Figure 1. Gradually all or nearly all the cells become involved in the process and eventually are destroyed. Several days after the syncytia are apparent, eosinophilic inclusion bodies appear in most of the nuclei and irregular masses of eosinophilic material accumulate in the cytoplasm. Examination of many preparations has convinced us that both these abnormalities are the result of viral multiplication.

When virus that has been propagated in renal cells is introduced into cultures of certain other cells essentially the same cytopathogenic effects are observed at least during a limited number of passages. In Figure 2 are shown the changes that occurred 16 days after inoculation into human amnion of the so-called Edmonston strain of virus. The agent had been previously passed 23 times in cultures of human renal cells. Following the addition of this strain to cultures of the KB line of human carcinoma cells (developed by Eagle) Dekking and McCarthy [12] in our laboratory observed the formation of multinuclear giant cells. Black and his co-workers [13] have described similar changes in cultures of the Hep-2 line of human carcinoma cells. McCarthy [14] and Rustigian [15] independently have noted the same effect in cultures of HeLa carcinoma cells. In unpublished experiments we have demonstrated the growth of virus in the Detroit 6 line of cells that Berman and Stulberg derived from human bone marrow, but we have not noted the formation of syncytia, although in stained preparations intranuclear inclusion bodies were conspicuous in individual cells. We have also seen cells of abnormal appearance in cultures consisting predominantly of human embryonic fibroblasts that were accompanied by multiplication of the virus within the system. So far we have observed no cytopathogenic effects in cultures of bovine amnion cells, chick amnion, or chorioallantoic tissues, or in chick embryonic tissues to which the virus was added.

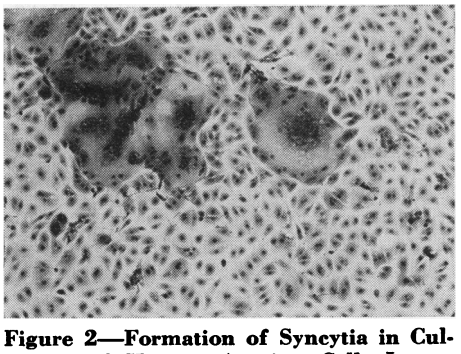

Figure 2—Formation of Syncytia in Culture of Human Amnion Cells Inoculated 16 Days Earlier with Edmonston Strain, from 24th Human Renal Cell Passage. Note Normal Appearance of Surrounding Epithelial Cells. H and E x 160

Lately we have found that after prolonged cultivation, in vitro, the cytopathogenic properties of the virus may become altered. Thus the Edmonston strain after 23 passages in human renal cells has been continuously propagated in human amnion cells. During the 14th amnion cell passage, in addition to the presence of multinuclear giant cells, increasing numbers of refractile, fusiform, or stellate cells were noted that somewhat resembled fibroblasts. Their appearance in the 19th passage is shown in Figure 3 along with typical giant cells. In successive passages these forms tended to predominate or, indeed, in certain cultures came to represent the only manifestation of viral multiplication. In stained preparations intranuclear inclusions were often found in these affected cells. This newly recognized cytopathogenic effect is of much interest from the general biological standpoint: it also led us to attempt again the cultivation of the virus in chick embryos. Later we shall summarize the results of these experiments.

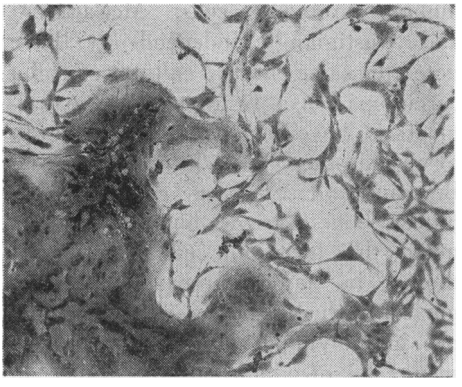

Figure 3—Cytopathogenic Effects in Human Amnion Cells of Edmonston Strain After 23 Passages in Human Renal Cells and 19 Passages in Human Amnion Cells. Note Numerous Fusiform Cells Together with Syncytia. H and E x 150

Serological Reactions

Tests for Virus-Neutralizing Antibodies—Tests of the capacity of acute and convalescent phase sera from 12 measles patients to prevent the cytopathogenic effects of the virus have been carried out using 100 ID_{50} of virus and dilutions of serum increasing by a factor of two. The results have clearly shown that antibodies develop during the course of the disease that prevent the occurrence of both types of cytopathic change in cultures of human renal and amnion cells.[16, 19] Thus in the acute phase sera of six of these 12 patients no neutralizing effect was found in the lowest dilutions of serum tested, i.e., 1:2–1:8. The mean of the convalescent phase serum titers was 1:260 with a range from 1:160 to 1:512. The antibody emerges soon after the exanthem is declared and may attain a moderate or high level within the following seven to 10 days or even earlier as appeared to be the case in the remaining six cases that were studied. Investigation of the persistence of the neutralizing antibody over prolonged periods has not been undertaken, but it can be stated that in a few cases the high concentrations developed early in convalescence are maintained for at least two to four months.

Tests for Complement-Fixing Antibodies—More extensive data on the formation of antibody during the disease have been obtained by means of the complement-fixation test, since this procedure can be carried out with greater convenience and rapidity. As antigen we have used the fluid removed from cultures of human renal or amnion cells two or three weeks after infection with the virus. By means of the drop technic of Fulton and Dumbell 25 pairs of acute and convalescent-phase serum from patients with measles have been tested.[16, 17] In all cases development of antibody or an increase in its initial concentration was demonstrated. The mean increase for these 25 cases was at least 30-fold. In seven instances the first specimen of serum was either taken several days after the onset when an elevated titer was recorded or the end point of the convalescent-phase specimen was not obtained. Excluding these, the mean acute phase serum titer of the remaining 18 cases was less than 1:4 (range: $<$1:2 to 1:8). In contrast the mean convalescent phase serum titer was 1:160 with a range from 1:32 to 1:512. These results, similar to those of the neutralization tests, not only afford strong evidence for the etiologic role of the virus in measles, but also suggest that the complement-fixation test should provide a practical diagnostic procedure.

Further evidence for the relationship of the virus to measles was obtained by comparing the results of complement-fixation tests on the sera of persons with either a positive or negative history of the disease.[17] In this study most of the subjects were children under the age of 10 years. The results are summarized in Table 1. It is clear that in a group of 54 persons giving a positive history the proportion with titers exceeding 1:8 is about six times greater than among

Table 1—Correlation of History of Measles with Results of Complement-Fixation Tests

History Positive			History Negative		
CF Titer *	No.	Per cent	CF Titer	No.	Per cent
<4	8	15	<4	12	48
4–8	6	10	4–8	10	40
>8	40	75	>8	3	12

* Reciprocal of initial dilution of serum giving fixation in presence of two units each of antigen and complement.

the group of 25 with negative history. Although the numbers in the groups are not equal, this difference is clearly significant. The relatively large proportion of sera giving positive tests in dilutions four to eight from the group with negative history suggests that in this range nonspecific reactions may often occur.

Pathogenicity of Virus for Monkeys

The results of experiments in susceptible cynomologus monkeys have indicated that two of the strains of virus tested after cultivation, in vitro, are capable of producing a mild disease comparable in most respects to measles in man.[16, 18] The criteria for determining the susceptibility of monkeys will be subsequently discussed. When animals were inoculated by both the intravenous and intranasal routes with virus of the first, second, and 23rd tissue culture passages several phenomena were noted. Viremia was established beginning on the fourth or fifth day and continuing thereafter for two to five days. An exanthem appeared soon after the beginning of the viremic phase which extended over the thorax and abdomen and was especially prominent in the axillary and inguinal regions. Slight to moderate leucopemia developed that was maximal on the ninth day. Shortly after the period of exanthem, i.e., about two weeks after inoculation of the virus, specific complement-fixing antibodies appeared in the blood and soon attained maximal concentrations. These high levels were maintained for several weeks when they usually began to diminish. Antibodies, however, were detectable in significant amounts at least eight months after inoculation of the virus. Not all these phenomena were apparent in other animals that were inoculated at the same time with portions of the same preparation of virus. In one monkey no exanthem was seen, although viremia and antibody response were comparable to those of animals exhibiting a rash; another responded only by the development of a slight leucopemia and the production of antibody. It is evident, then, that susceptible monkeys may vary considerably in their reaction to infection with this agent.

We have emphasized the fact that only susceptible animals may be expected to react in these various ways because early in our work [17] we found that a majority of normal rhesus and cynomologus monkeys that had been held for some time in three laboratories in this country possessed complement-fixing antibodies for the measles agent. In these tests the sera of 16 rhesus and eight cynomologus monkeys were examined. Antibody was found in 14 of the rhesus sera and in all the cynomologus sera. Neutralizing antibody was also detected in the few specimens that were tested. Ruckle [11] has lately reported similar findings and in addition has isolated an agent from monkey kidney tissue that so far is indistinguishable from human measles virus. The problem, however, of the origin of the agent responsible for the presence of these antibodies in apparently normal monkeys has not yet been solved. We are inclined to believe, for reasons that have been discussed elsewhere at length,[18] that it is probably identical with human measles virus and is derived either di-

rectly or indirectly from cases of the disease in man. The most cogent evidence in favor of this view lies in the fact that no complement-fixing antibodies capable of reacting with the Edmonston strain of virus were found in the sera of 31 cynomologus monkeys bled soon after their capture in the forests of Malaya and the Philippines. However this may be, it is obvious that the presence of antibodies reacting with measles virus in laboratory monkeys probably accounts for the discrepant results obtained by past workers in attempts to reproduce the disease in these animals.

Multiplication of the Virus in Chick Embryos

We referred at the beginning to the uncertainty that has continued respecting the capacity of measles virus to multiply in the developing chick embryo. Accordingly, after the means of isolating the agent and assaying its infectivity in tissue culture had been established, we proceeded to determine whether certain of our nine strains could be propagated in this host. Five of these strains maintained in renal cells since their isolation have been tested. The various routes of inoculation included the amniotic and yolk sacs and the chorioallantoic membrane. An incubation period of from four to six days was adopted following the procedure of Rake and Shaffer.[5] In our experiments two or three egg passages were made in the case of each strain and tests for the presence of virus in embryonic materials were carried out in cultures of human renal cells. In no instance was evidence of viral multiplication obtained.

Subsequently, when the variation in cytopathogenic properties of the Edmonston strain occurred, the possibility arose that this change might be accompanied by an alteration in the capacity of the virus to multiply in the chick embryo. A series of egg passages was therefore initiated. The inoculum consisted of virus from the 28th passage in human amnion cell and was introduced into the amniotic sac of the chick embryo. This route was selected with the thought that virus adapted to growth in human amnion cells might more readily proliferate in the analogous cells of the developing chick. After inoculation the embryos were incubated for nine days at 35° C—a longer period than previously allowed. The inoculum used as routine in subsequent egg passages consisted of amniotic fluid. In collateral passage lines suspensions of amniotic and chorioallantoic membranes mixed with the corresponding fluid were also employed. In each passage after the first the concentration of virus in various embryonic constituents was determined by titration in cultures of human amnion cells. The agent has now been maintained throughout seven serial passages. It continues to induce the formation of fusiform and stellate cells when transferred to cultures of human amnion cells (see Figures 4 and 5). In each of the last four passages multiplication was demonstrated by the titrational data. For example, the mean number of 50 per cent tissue culture infecting doses intro-

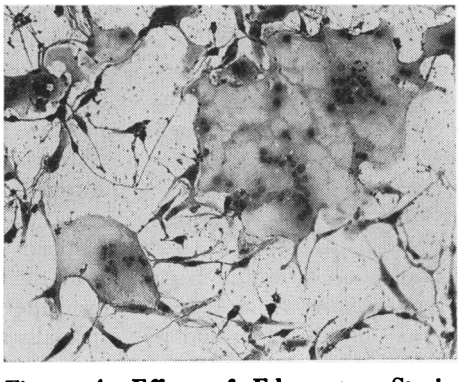

Figure 4—Effect of Edmonston Strain from Third Chick Embryo Passage in Human Amnion Cells, 14 Days After Inoculation of Virus. H and E x 180. Note Fusiform Cells and Syncytium

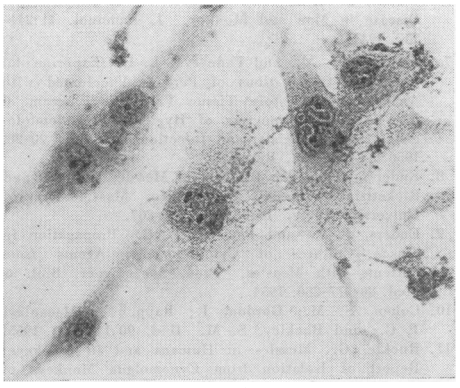

Figure 5—Cells from Same Culture Shown in Figure 4 x 750. Note Inclusion Bodies in Fusiform Cells

duced in the amniotic fluid employed as inoculum was 16, whereas on the average about 2,000 TC ID_{50} per 0.1 ml were found in the amniotic membrane at the time of harvest. The identity of the agent recovered from the fourth egg passage was confirmed in neutralization tests using human and monkey acute and convalescent phase sera as well as in complement-fixation tests in which the antigen consisted of fluid from infected human amnion cell cultures inoculated with the egg-passaged virus. A complete account of these experiments is in preparation.[19]

When it was found that multiplication of this strain occurred in the developing chick it obviously became of interest to determine whether it could be cultivated in cultures of chick embryonic tissues. Experiments of this sort are now in progress and the results so far suggest that multiplication does occur under these conditions.

Discussion

In conclusion, we shall offer brief comment on the results of the investigations that have here been summarized. We believe that they have provided tools whereby the virus can be readily isolated, its infectivity accurately determined, and its pathogenic, immunogenic, and other biologic properties analyzed. Using these tools progress already has been made toward the elucidation of certain long-standing problems, such as the cause of variation in the natural resistance of monkeys and the question of the susceptibility of the chick embryo. Among others that remain to be explored is the nature of the relationship of the virus to postmeasles encephalitis, determination of the true incidence of inapparent infection and of second attacks of measles, the duration of immunity as indicated by the persistence of antibody in the blood, and the determination of whether dermal hypersensitivity to the virus develops after infection.

From the more immediate, practical standpoint it is evident that methods of directly assaying the antibody content of different preparations of gamma globulin or other materials used in the production of passive immunity are already at hand.[16, 17] It also seems that groundwork has been established for future studies directed toward the development of vaccines whether composed of attenuated or inactive virus. We are encouraged to believe that progress toward this objective was made when we succeeded in propagating the agent in chick embryos. In our opinion virus grown under these conditions or in cultures of chick tissue would represent the most suitable material for the preparation of vaccine. There is a potential risk in employing cultures of primate cells for the production of vaccines composed of attenuated virus, since the presence of other agents possibly latent in primate tissues cannot be definitely excluded by any known method. For the preparation of inactive virus vaccine chick embryonic tissue would also appear to be advantageous. Not only would it afford relative assurance of the absence of extraneous viruses in the starting material, but also greatly reduce the cost of manufacture.

Much investigation, however, will be necessary before such considerations become of major importance, since first it must be determined whether multiplication of the virus in chick tissue will continue indefinitely, whether sufficient viral antigen is produced under these circumstances to be immunogenic when inactivated, or whether attenuation of pathogenicity for monkey and man may occur as a result of passage in chick tissue. All these questions future investigation must answer.

REFERENCES

1. Goldberger, J., and Anderson, J. F. An Experimental Demonstration of the Presence of the Virus of Measles in the Mixed Buccal and Nasal Secretions. J.A.M.A. 57:476–478, 1911.
2. Blake, F. G., and Trask, J. D. Studies on Measles. II Symptomatology and Pathology in Monkeys Experimentally Infected. J. Exper. Med. 33:413–422, 1921.
3. Plotz, H. Culture "in vitro" du virus de la rougëole. Bull. Acad. de méd., Paris 119:598–601, 1938.
4. Rake, G., and Shaffer, M. F. Propagation of the Agent of Measles in Fertile Hen's Egg. Nature 144:672–673, 1939.
5. Rake, G., and Shaffer, M. F. Studies on Measles I. The Use of the Chorio-Allantois of the Developing Chick Embryo. J. Immunol. 38:177–200, 1940.
6. Rake, G.; Shaffer, M. F.; and Jones, H. P. Studies on Measles. III The Use of Tissue Culture in Propagation of Measles Virus. J. Infect. Dis. 69: 65–69, 1941.
7. Shaffer, M. F.; Rake, G.; Stokes, J., Jr.; and O'Neil, G. C. Studies on Measles. II Experimental Disease in Man and Monkey. J. Immunol. 41:241–257, 1941.

7a. Zhdanov, V. M., and Fadeyeva, L. L. Experimental Data and Observations of Persons Vaccinated with Antimeasles Allantois—Tissue Vaccine. Program of XIII All-Union Congress of Hygienists, Epidemiologists, Microbiologists, and Infectionists. June 20–28, 1956. Leningrad, Russia.

8. Enders, J. F. The Etiology of Measles in Virus and Rickettsial Diseases. Cambridge, Mass.: Harvard University Press. 1940, pp. 237–267.
9. Enders, J. F., and Peebles, T. C. Propagation in Tissue Cultures of Cytopathogenic Agents from Patients with Measles. Proc. Soc. Exper. Biol. & Med. 86:277–286, 1954.
10. Cohen, S. M.; Gordon, I.; Rapp, F.; Macaulay, J. C.; and Buckley, S. M. Ibid. 90:118–122, 1955.
11. Ruckle, G. Measles in Humans and in Monkeys: Report of Isolation from Cynomolgus Monkeys of an Agent Immunologically Related to Human Measles Virus. Federation Proc. 15:610, 1956.
12. Dekking, F., and McCarthy, K. Propagation of Measles Virus in Human Carcinoma Cells. Proc. Soc. Exper. Biol. & Med. 93:1–2, 1956.
13. Black, F. L.; Reissig, M.; and Melnick, J. L. Propagation of Measles Virus in a Strain of Human Epidermoid Cancer Cells (Hep-2). Ibid. 93:107–108, 1956.
14. McCarthy, K. Unpublished data.
15. Rustigian, R. Personal communication to the authors.
16. Enders, J. F. Observations on Certain Viruses Causing Exanthematous Disease in Man. Am. J. M. Sc. 231:622–637, 1956.
17. ————. Recent Observations on the Behavior in Tissue Culture of Certain Viruses Pathogenic for Man. Fourth R. E. Dyer Lecture (1954). Public Health Service Publication No. 467. Washington, D. C.: Gov. Ptg. Office.
18. Peebles, T. C.; McCarthy, K.; Enders, J. F.; and Holloway, A. J. Immunol. (In press.)
19. Milovanović, M.; Enders, J. F.; and Mitus, A. To be published.)

Mental Health Week Begins April 28

Mental Health Week has been scheduled for the week of April 28. As in the past the National Association for Mental Health is directing this nation-wide observance in cosponsorship with the National Institute of Mental Health, Public Health Service. The week of concentrated attention to mental health problems is designed to get year-round and concerted public support for the care, treatment, and understanding that the mentally ill require in order to come back.

The 1957 slogan is "The Mentally Ill Can Come Back—Help Them—Give" (of understanding, service, and funds). The Association has prepared a kit of materials that is available for publications such as state health bulletins as well as for newspapers and magazines. 10 Columbus Circle, New York 19.