The Replication of Poliovirus in Primary Kidney Cell Cultures of Thirteen-Lined Ground Squirrels (35954)

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The catalog of cell strains and lines capable of supporting the replication of several viruses of animal origin has grown fantastically since the discovery by Enders *et al.* (1) that poliovirus would replicate in cell cultures of various nonneural human embryonic tissues. Between the years 1955 and 1965, several reports have appeared claiming poliovirus replication in cell cultures of nonprimates, such as murine tumors (2), rabbit kidney (3–5), chick embryos (6, 7) guinea pig spleen and liver (8), bovine kidney (9), fathead minnow (10). Some of these cell lines were subsequently shown not be to be of the supposed species of origin. Thus the cells of presumed rabbit origin (3–5) were proven to be of human origin by a variety of serologic technics and karyotypic analysis (11–14). No further studies have been reported concerning the species specificity of the other cells mentioned above (6–10) except that Solis and Mora (15) found that the fathead minnow cell line was refractory to infection by poliovirus.

During the course of a larger study in depressed metabolism in this laboratory, it was found by Cashon *et al.* (16) that primary kidney cell cultures of the thirteen-lined ground squirrel (*Citellus tridecemlineatus*) would support the replication of poliovirus, type 1. The finding was quite unexpected in view of overwhelming evidence that only cells of primate origin could support the replication of poliovirus (17). This report presents evidence that all three serotypes of poliovirus will replicate in *primary* kidney cell cultures of a nonprimate.

Materials and Methods. Animals and kidney cell cultures. Ground squirrels (*Citellus tridecemlineatus*) were taken from stocks maintained as described previously (18). Primary kidney cell cultures (GSK) were prepared from randomly chosen animals as described earlier (16).

Poliovirus stocks. Poliovirus 1 (Brunhilde) was provided through the courtesy of Dr. H. A. Wenner, University of Kansas. Poliovirus 2 (Lansing), poliovirus 3 (Leon), and poliovirus 2 and 3 (oral vaccine strains) were provided through the courtesy of Dr. R. Sullivan, United States Public Health Service, Cincinnati. Poliovirus 1 (Lsc 2ab) was kindly provided by Dr. A. B. Sabin, then at the University of Cincinnati.

Stock viruses were propagated in monolayers of either Hela or Hep-2 cells, and stored at −70°.

Poliovirus antisera. Antisera were produced by the intravenous inoculation of rabbits with each of the 3 attenuated poliovirus strains. Each animal received 1.0 ml of infective monotypic tissue culture fluid intravenously every other day for a total of 5 injections. Animals were exsanguinated by cardiac puncture 1 week after the last injection. Separated sera were stored in small portions at −20°. These were designated SSRC antisera.

Reference poliovirus antisera were also obtained from the Research Reference Reagents Branch, NIAID, NIH. Poliovirus antiserum type 1 (NIH) (strain Lsc 2ab) was of bovine origin. The antiserum was reported to titer at 1:4000–1:16,000 against 40–200 $TCID_{50}$ of type 1 poliovirus in neutralization tests. No cross neutralization was detected.

Poliovirus antiserum type 2 (NIH) (strain P-712) was of equine origin. In serum neutralizations performed in rhesus monkey kidney tissue cultures, the antiserum was reported to titer at 1:10,240 against 100 $TCID_{50}$

of type 2 poliovirus. Cross neutralization was reported with poliovirus type 1 at 1:58 and poliovirus type 3 at 1:20.

Poliovirus antiserum type 3 (NIH) (strain Leon) was also of equine origin. In serum neutralizations performed in rhesus money kidney tissue cultures, the antiserum was titered at 1:4960 against 30 $TCID_{50}$ of type 3 poliovirus. No cross neutralization was reported.

Viral assays. Stock viruses and viral harvests from infected GSK cells were titrated in Hela cells using the standard $TCID_{50}$ titration (19).

Poliovirus neutralization tests. Neutralization tests were performed according to the method of Schmidt (19) with slight modifications. All poliovirus antisera were inactivated at 56° for 30 min. The SSRC antisera were diluted 1:5 and 1:25 in BME containing 2% inactivated calf serum with antibiotics; the NIH antisera were diluted 1:50 and 1:250 in the same diluent. Each poliovirus to be tested was diluted in BME (2% inactivated calf serum and antibiotics) to give 200 $TCID_{50}$/1.0 ml volume.

Three milliliters of each dilution of antiserum were mixed with 3.0 ml of each diluted test virus. This resulted in final concentation of: (a) 1:10 and 1:50 for SSRC antisera; (b) 1:100 and 1:500 for NIH antisera; and (c) 100 $TCID_{50}$ for each test virus. Three milliliters of the lowest dilution of each antiserum and 3.0 ml of each diluted test virus were mixed with 3.0 ml of BME containing 2% inactivated calf serum and antibiotics to serve as antiserum and virus controls.

All of the antiserum–virus mixtures, antiserum and virus controls, and a 6.0 ml volume of BME (2% inactivated calf serum and antibiotics) were incubated at 37° for 30 min. Then each antiserum–virus mixture, virus and antiserum controls were inoculated in 1.0 ml volumes into each of 4 roller tubes of Hela cells. Four cell controls were each inoculated with 1.0 ml of the BME, which had also been incubated. The tubes were then incubated at 37° and observed microscopically for the appearance or inhibition of cytopathic effect.

Mixed agglutination tests. Rabbits were immunized with 5 ml of a 10% suspension of washed ground squirrel or human red cells by ip injection every other day for a total of five injections. Animals were bled 13 days after the last injection; and the inactivated sera were used in mixed agglutination tests performed on uninfected GSK cultures each time a set of GSK cultures was infected with poliovirus.

Experimental Procedures. GSK cultures in Leighton tubes were inoculated with poliovirus as described previously (16). To provide sufficient cell density for mixed agglutination tests, GSK cells were also seeded at 1.5×10^6/ml in 1 oz prescription bottles. The cells in these bottles were treated in every way the same as the tube cultures except for infection with virus. Cells for the mixed agglutination tests were removed from the glass with rubber policemen, and the tests were performed as outlined by Coombs *et al.* (20).

Results. After 3–5 weeks, nearly confluent monolayers consisting of a mixture of epithelial and fibroblastic cells had developed on about 60% of the coverslips (Fig. 1). The cultures could be maintained without degeneration for at least 4 days in No. 199 with antibiotics.

The effects of poliovirus 1, Brunhilde strain, on the GSK cell cultures began to appear at 48 hr postinoculation. Slight rounding of cells was noted at this time, and at 72 hr rounded cells and granulation could be noted in many areas. Dense granular foci and cell retraction predominated throughout most of the monolayers by the end of 96 hr (Fig. 2).

The CPE caused by the Lansing strain, poliovirus 2, appeared 24 hr postinoculation. At 48 hr, definite rounding had occurred and by 72 hours, marked cell retraction and granulated foci were noted throughout entire monolayers. Extensive sloughing had occurred at 96 hr and the cells remaining attached to the coverslips appeared as densely stained granular foci (Fig. 3).

Some rounding of cells in GSK cultures inoculated with the Leon strain, poliovirus 3, was also noted at 24 hr. By 48 hr, rounding was more pronounced, and at 72 hr postinoc-

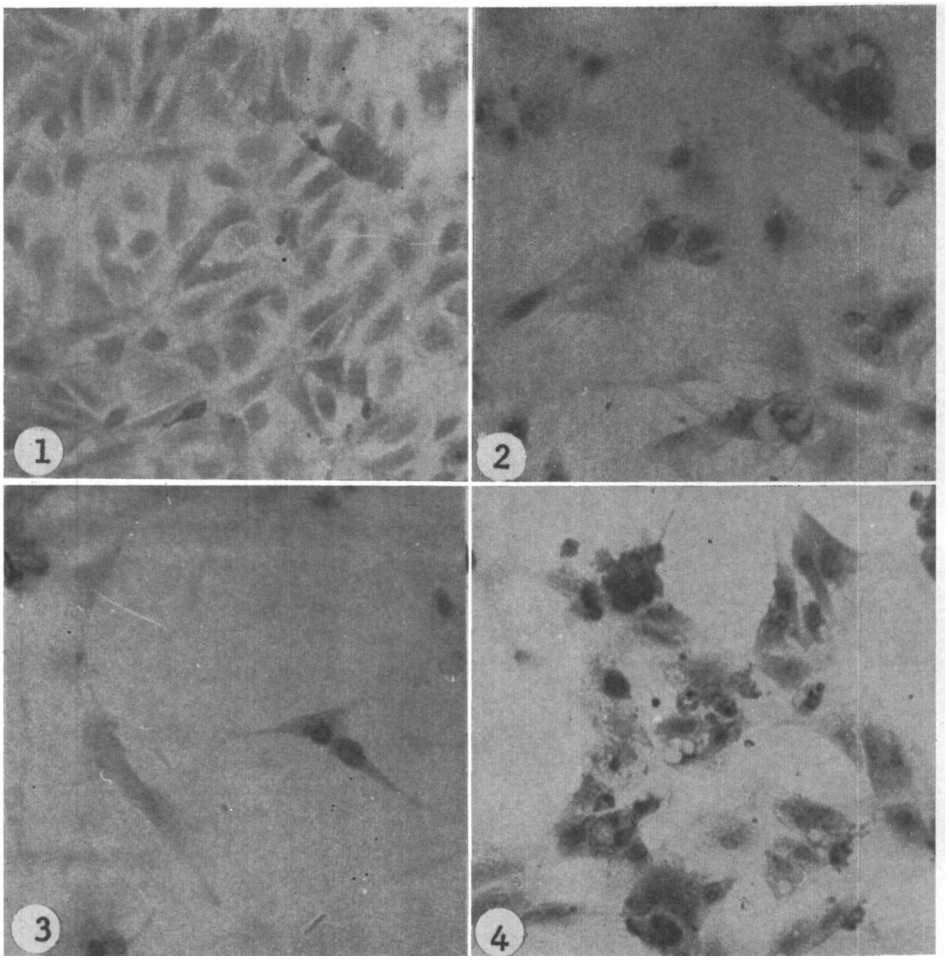

FIG. 1. Noninfected GSK cell culture (H & E stain); ×112.

FIG. 2. GSK cell culture infected with $10^{4.0}$ $TCID_{50}$ of poliovirus 1 (Brunhilde strain) for 4 days (H & E stain); ×112.

FIG. 3. GSK cell culture infected with $10^{5.3}$ $TCID_{50}$ of poliovirus 2 (Lansing strain) for 4 days (H & E stain); ×112.

FIG. 4. GSK cell culture infected with $10^{5.5}$ $TCID_{50}$ of poliovirus 3 (Leon strain) for 4 days (H & E stain); ×112.

ulation, cell retraction and granular foci began appearing in most areas. Some sloughing had occurred by 96 hr; however, most cells still remained attached to the coverslips. Entire fields appeared as retracted cells with densely staining foci (Fig. 4).

The CPE observed in GSK cell cultures inoculated with these 3 strains of poliovirus was typical of that produced by a poliovirus, *i.e.*, cell retraction, rounding, occasional ballooning, and lysis leaving granular debris.

Infectivity titrations of stock viruses and supernatant fluids of GSK cell cultures infected with each of the 3 serotypes are given in Table I. Two of 3 log increases in infective virus were obtained with all 3 serotypes following infection of GSK cell cultures. The observance of characteristic CPE coupled with the increase in infective virus confirmed the earlier report of Cashon *et al.* (16) that poliovirus would replicate in primary GSK cell cultures.

The results of poliovirus stock neutralizations and the supernatant fluids from infect-

TABLE I. Results of Infection of GSK Cultures with Polioviruses.

Virus	Stock virus[a]	Infecting dose	Titer achieved in GSK cultures
Poliovirus 1 (Brunhilde)	$10^{7.0}$	$10^{4.0}$	$10^{6.5}$
Poliovirus 2 (Lansing)	$10^{7.3}$	$10^{5.3}$	$10^{8.3}$
Poliovirus 3	$10^{7.5}$	$10^{6.5}$	$10^{7.6}$

[a] All titers expressed as $TCID_{50}$/0.2 ml in Hela cells.

ed GSK cultures are given in Table II. The virus controls of 100 $TCID_{50}$ of all stock and harvested virus produced typical poliovirus CPE. Antisera controls of the lowest dilutions of NIH and SSRC antisera exhibited no CPE. Ground squirrel kidney cell controls were also normal.

Some cross neutralization of poliovirus 1 (both harvest and stock) was observed with type 2 (NIH) antiserum. Cross neutralization was not observed with the Lansing stock and harvest or the Leon stock and harvest. The cross neutralization might be explained by a common antigen shared by poliovirus 1 and 2. But in every instance, each virus stock and harvest was neutralized more strongly by the homotypic antiserum.

The mixed agglutination test utilized was a modification of the procedure of Coombs *et al.* (20). Human red cell antiserum and ground squirrel red cell antiserum were inactivated at 56° for 30 min. Each antiserum was then diluted 1:10 in 0.85% saline. Ground squirrel kidney cells were obtained by scraping 3 cell monolayers (1 oz prescription bottles) into the growth medium and centrifuging the suspensions at 200*g* for 3 min. The pellets were resuspended in 1.0 ml of saline. Two-tenths milliliter of ground squirrel kidney cells was then added to each of 4 tubes. To 2 of these tubes, 0.2 ml of the 1:10 dilution of ground squirrel red blood cell antiserum was added; to the remaining 2 tubes, 0.2 ml of the 1:10 dilution of human red cell antiserum was added. The 4 tubes were incubated at 37° for 30 min, centrifuged at 200*g* for 3 min, and the cell–antiserum mixtures were washed twice with 0.85% saline. The supernatant fluids were decanted and 0.2 ml of a 0.5% suspension of human red blood cells was added to 1 tube of each two-tube set and 0.2 ml of a 0.5% suspension of ground squirrel red blood cells was added to the other tube of each set. Several drops from each tube were then placed on a slide, a coverslip was added, and the area was observed for mixed agglutination by phase-contrast microscopy.

The mixed agglutination test affirmed the identity of the GSK cells (Table III). The only system showing mixed agglutination was that in which GSK cells were reacted with ground squirrel red cell antiserum and subsequently with ground squirrel red cells (Fig. 5–8).

Discussion. The unusual finding of Cashon *et al.* (16), that poliovirus 1 (Brunhilde strain) appeared to propagate in GSK cells was significant enough to warrant further investigation. The two points in question were the actual identity of the viruses utilized and the species of origin from which the kidney cell cultures were derived.

Neutralization of the viruses, both stock

TABLE II. Neutralization Tests in Hela Cells.[a]

Poliovirus antiserum	Virus stock serotype 1	2	3	GSK harvest serotype 1	2	3
(1)[b] 1:100	+	—	—	+	—	—
NIH 1:500	±	—	—	—	—	—
(1) 1:10	Not done			+	—	—
SSRC 1:50				±	—	—
(2) 1:100	±	+	—	±	+	—
NIH 1:500	—	+	—	—	+	—
(2) 1:10	Not done			—	±	—
SSRC 1:50				—	—	—
(3) 1:100	—	—	+	—	—	+
NIH 1:500	—	—	±	—	—	±
(3) 1:10	Not done			—	—	+
SSRC 1:50				—	—	+

[a] + Complete neutralization (4 tubes without CPE); ±, partial neutralization (1 or 2 tubes with CPE); —, no neutralization (4 tubes with CPE).

[b] Serotype in parentheses.

TABLE III. Specied Specificity of GSK Cells by Mixed Agglutination.

Cultured cells (agglutinogen)	RBC antiserum/indicator RBC			
GSK	GSK/GSK	GSK/Human	Human/GSK	Human/human
	+	—	—	—

and GSK-passed, with poliovirus antisera (NIH and SSRC) in HeLa cells as outlined by Schmidt (19) was utilized to confirm virus identity. The Brunhilde stain was neutralized most strongly by the type 1 poliovirus antisera, the Lansing strain was neutralized by the type 2 antisera, and the Leon strain was neutralized by the type 3 antisera. Thus the serotype of each poliovirus used in these experiments was affirmed by neutrali-

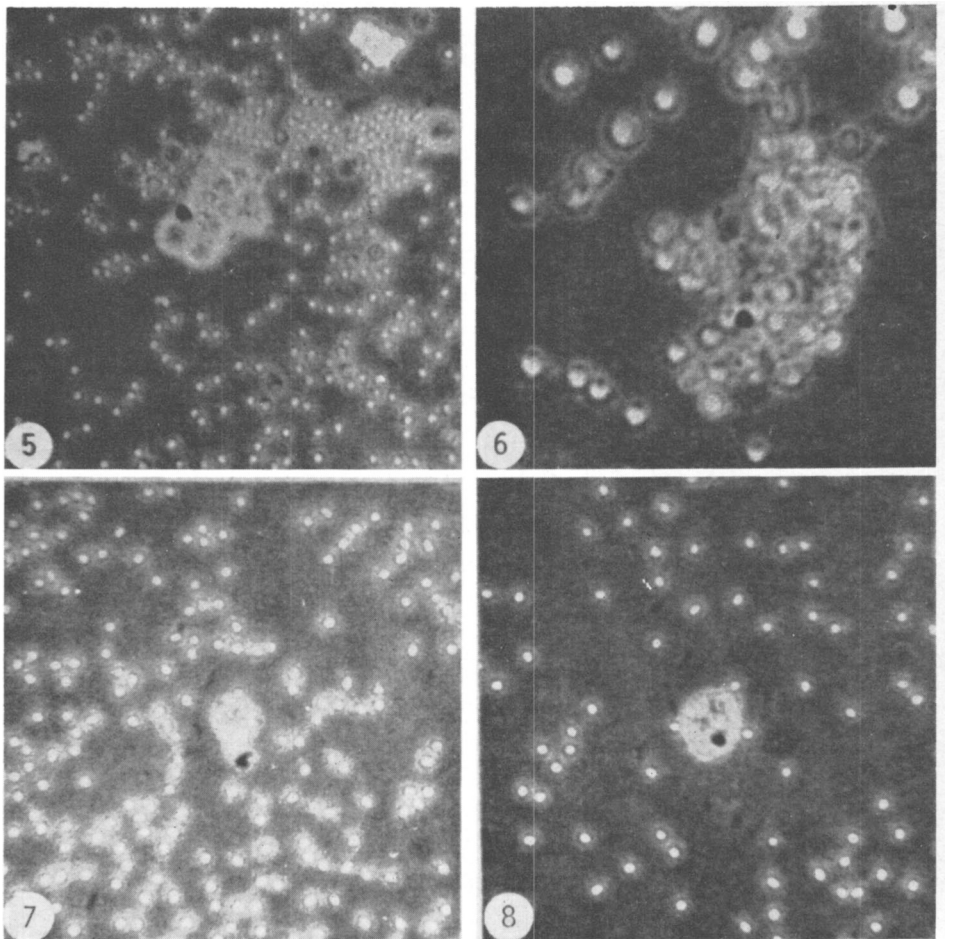

FIG. 5. GSK cells with ground squirrel red blood cells and antiserum to ground squirrel red blood cells (phase contrast); ×350.

FIG. 6. GSK cells with ground squirrel red blood cells and antiserum to ground squirrel red blood cells (phase contrast); ×625.

FIG. 7. GSK cells with ground squirrel red blood cells and antiserum to human red blood cells; ×350.

FIG. 8. GSK cells with human red blood cells and antiserum to human red blood cells; ×350.

zation with its homotypic antiserum. Cross neutralization was of the same order as reported for the NIH antisera.

Since the cell lines of presumed rabbit origin (3–5) apparently supporting the replication of poliovirus were shown in fact to be of human origin (11–14), we felt that we might be dealing with the same phenomenon. Extensive precautions were taken to eliminate any chance of cell contamination. First, all work with the GSK cell cultures was performed in a sterile room in which no other cells were present at the same time. The growth medium used for the GSK cells was BME with 10% horse serum, since Steffens and McKenna (unpublished observations) had shown that only horse serum would support their multiplication. Other cells used in this laboratory are routinely grown in calf serum. The cell cultures utilized by other workers in the earlier reports were, however, not primary, but were early passages of cell lines. These cell lines of various origins had all been noted to have undergone a morphological transformation after which they were susceptible to poliovirus infection, whereas the primary cell cultures had been found to be refractory to poliovirus replication. Further, no passages of the GSK cells in our work, once planted, were made. Only the number of cells forming monolayers in the original culture vessels was used. In addition, the results of the mixed agglutination tests definitely determined that the cells we used were indeed of ground squirrel origin. This technic has been used by Coombs *et al.* (20) and McKenna *et al.* (21) to establish the species of origin of many cell strains and lines and is most reliable.

The basis underlying poliovirus susceptibility of cells was investigated by McLaren *et al.* (22) and Holland and McLaren (23) in their studies on host cell–virus relationship. The ability of a cell to adsorb and eclipse poliovirus with subsequent replication of the virus was found to reside in specific surface receptor sites occurring only on susceptible cells. In contrast, the small amounts of virus absorbed by nonsusceptible cells did not enter an eclipse phase and did not replicate. The pattern which emerged was that only primate cells possessed the specific receptors to allow poliovirus replication. Nonprimate cells did not. A third study by Holland *et al.* (24, 25), bypassed the necessity of a receptor site and eclipsing by exposing nonsusceptible, nonprimate cells to infectious poliovirus RNA. The results indicated that these nonsusceptible cells had the capacity to support poliovirus replication once infectious RNA entered the cell.

One might suspect the possibility in our work of cell fusion to form viable multinucleate cells with subsequent replication of poliovirus in those cells as shown by Enders *et al.* (26), Neff and Enders (27), and most recently by Green *et al.* (28). This phenomenon was not seen to occur spontaneously in any of our GSK cultures, and no GSK cells were knowingly infected with any virus other than poliovirus.

In view of the evidence presented here, it would appear that *primary* kidney cell cultures from *Citellus tridecemlineatus* have the specific receptor sites for poliovirus on the cell membranes, which sites were formerly thought to be confined to primate cells. The possession of these receptor sites allows for the adsorption, penetration, apparent eclipse, and subsequent replication of the three serotypes of poliovirus producing characteristic CPE in these cells.

No extrahuman reservoirs have been found for polioviruses, although many attempts have been made. It may be of interest to observe that the GSK cells, which we have found to support poliovirus replication, were derived from a mammal capable of hibernation. The pattern of seasonal variations in the outbreaks of poliomyelitis in the temperate zones corresponds to the seasonal physiologic habits of these mammals, and it may not be unreasonable to indict these, and perhaps other hibernating homeotherms as possible animal reservoirs for a virus generally believed to be exclusively human in origin.

Summary. Evidence presented confirmed the propagation of polioviruses 1 (Brunhilde strain), 2 (Lansing strain), and 3 (Leon strain) in primary kidney cell cultures derived from normothermic ground squirrels (*Citellus tridecemlineatus*). Virus identity

was validated by neutralization with homotypic antiserum. A mixed agglutination procedure revealed that the kidney cell cultures were of ground squirrel origin. The basis underlying this phenomenon and its implications were also discussed.

1. Enders, J. F., Weller, T. H., and Robbins, F. C., Science **109**, 85 (1949).

2. Kret, A., Arch. Gesamte Virusforsch. **6**, 326 (1955).

3. Drew, R. M., Science **126**, 747 (1957).

4. Sheffield, F. W., and Churcher, G. M., Brit. J. Exp. Pathol. **38**, 155 (1957).

5. McCarthy, F. J., and Tytell, A. A., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **17**, 525 (1958).

6. Dunham, W. B., and Ewing, F. M., Proc. Soc. Exp. Biol. Med. **95**, 637 (1957).

7. Prier, J. E., and Sullivan, R., Science **129**, 1025 (1959).

8. Mascoli, C. C., Stanfield, L. V., and Phelps, L. N., Science **129**, 894 (1959).

9. Vieuchange, J., De Brion, G., Maurin, J., and Gruest, J., C. R. Acad. Sci. **249**, 863 (1959).

10. Gravell, M., and Malsberger, R. G., Ann. N.Y. Acad. Sci. **126**, 555 (1965).

11. Melnick, J. L., and Habel, K., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **17**, 526 (1958).

12. Brand, K. G., and Syverton, J. T., J. Nat. Cancer Inst. **28**, 147 (1962).

13. Coombs, R. R. A., Daniel, M. R., Gurner, B. W., and Kelus, A., Nature (London) **189**, 503 (1961).

14. Clausen, J. J., and Syverton, J. T., J. Nat. Cancer Inst. **28**, 117 (1962).

15. Solis, J., and Mora, E. C., Appl. Microbiol. **19**, 1 (1970).

16. Cashon, G. W., McKenna, J. M., and Cashon, C. S., Appl. Microbiol. **20**, 593 (1970).

17. McLaren, L. C., Scaletti, J. V., and James, C. G., Biol. Prop. Mammalian Surface Membrane, Symp., **1967**, 123 (1968).

18. McKenna, J. M., and Musacchia, X. J., Proc. Soc. Exp. Biol. Med. **129**, 720 (1968).

19. Schmidt, N. J., *in* "Diagnostic Procedures for Viral and Rickettsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.), 3rd ed., p. 78. Amer. Pub. Health Ass., New York (1964).

20. Coombs, R. R. A., Daniel, M. R., Gurner, B. W., and Kelus, A., Immunology **4**, 55 (1961).

21. McKenna, J. M., Sanderson, R. P., Davis, F. E., and Blakemore, W. S., Cancer Res. **26**, 984 (1966).

22. McLaren, L. C., Holland, J. J., and Syverton, J. T., J. Exp. Med. **109**, 475 (1959).

23. Holland, J. J., and McLaren, L. C., J. Exp. Med. **109**, 487 (1959).

24. Holland, J. J., McLaren, L. C., and Syverton, J. T., Proc. Soc. Exp. Biol. Med. **100**, 843 (1959).

25. Holland, J. J., McLaren, L. C., and Syverton, J. T., J. Exp. Med. **110**, 65 (1959).

26. Enders, J. F., Holloway, A., and Grogan, E. A., Proc. Nat. Acad. Sci. U.S.A. **57**, 637 (1967).

27. Neff, J. M., and Enders, J. F., Proc. Soc. Exp. Biol. Med. **127**, 260 (1968).

28. Green, H., Wang, R., Basilico, C., Pollack, R., Husano, T., and Salas, J., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **30**, 930 (1971).

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