PROPAGATION OF RABIES VIRUS IN TISSUE CULTURE

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It is generally agreed that rabies virus prior to 1936 had not been propagated *in vitro*. Levaditi's (1) and Imamura's (2) experiments indicated a survival but not an increase of virus; Noguchi's (3) findings have not been confirmed; Waldhecker (4) has reported only failures.

In October, 1936, however, Kanazawa (5) reported the cultivation of rabbit fixed virus in rabbit embryo brain plus Tyrode solution, and in March, 1937, published confirmatory data (6). His first communication was not convincing on the grounds that his media lacked serum, an omission which impairs cultivation of many viruses, including rabies (see below), and especially because he failed to show that his mice injected with later passage material were not succumbing to contaminants found in early passages but to rabies virus. The incubation period of his mice injected with culture material was less than that of mice given the original, supposedly virulent virus and less than that ordinarily encountered by others (Defries, 7; Webster, 8). Kanazawa's second report described a loss of virus after the tenth passage, its transfer and temporary survival in chick embryo brain, and a successful transfer back again to rabbit embryo brain and subsequent maintenance for a total of twenty-six passages. He reported cross serum protection tests demonstrating similar protective effects with culture and rabbit passage viruses, and noted similar encephalitic lesions in mice following injection.

In November, 1936, Webster and Clow (9) reported the propagation of rabies virus in mouse embryo brain plus serum-Tyrode media. They identified the flask culture with rabies virus by immunological tests and stated, furthermore, that the culture virus used as a vaccine protected mice against an intracerebral injection of street virus, and in dogs induced neutralizing antibodies promptly. The portion of this report dealing with cultivation of the virus will be described in this paper.

Technique

The method used to establish in tissue culture three strains of rabies virus was generally similar to that employed by Rivers and Ward (10) for the propagation of vaccine virus.

The origin of the three strains of virus was briefly as follows: Strain Sk_3P_6: Ammon's horn of a supposedly healthy skunk was injected into the brain of six mice (8). These animals developed typical rabies with Negri bodies on the 7th day. The brain of one prostrate mouse was removed, emulsified, and injected into two mice. In this manner the virus was passed serially through six mice and then inoculated into the culture flask. Strain R_1P_{88} was obtained from the brain of a rabid dog in New York and passed similarly through 88 mice. Strain $R_{57}P_8$ came from the brain of a rabid dog in Alabama and was passed by us through eight mice.

To Tyrode solution (NaCl, 8 gm.; KCl, 2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; NaH_2PO_4, 0.05 gm.; $NaHCO_3$, 1 gm.; glucose, 1 gm.; distilled H_2O, 1,000 cc.) was added serum from normal *Macacus rhesus* monkeys in the proportion of 1 part to 9 parts of Tyrode. This mixture was filtered through a Seitz pad, the pH checked at approximately 8.2, and it was then placed in 4 cc. quantities in 50 cc. Erlenmeyer flasks.

Embryo tissue was prepared by decapitating a 14 to 20 day pregnant Swiss mouse, removing the uterus to a glass dish, washing it twice with saline, removing embryos with fresh instruments to a second glass dish, washing them twice with saline, and finally enucleating their brains with other instruments and transferring them to a watch glass in a Petri dish. Brains were washed once and emulsified as finely as possible with small, curved scissors. They were then weighed and diluted with 2 parts of serum-Tyrode to one part of tissue. The mixture was then added in 3 drop quantities (approximately 0.2 cc.) to the 4 cc. of serum-Tyrode in each culture flask.

Inoculation of rabies virus was made into the first flask by removing a virus-containing brain from a mouse prostrate following injection, emulsifying, and diluting it 1 to 1,000 with Tyrode. 1 cc. of this brain-virus suspension was then added to the flask of media. Flasks were then stoppered with tin-foil-covered corks and incubated 3 to 4 days at 37°C. Transfer to the second flask was made by removing the 4 day culture to a centrifuge tube, allowing it to settle or centrifuging it slowly, and then transferring 1 cc. of the resulting supernatant to the second flask of media. This method of serial inoculation of culture media was repeated regularly.

Cultures were run in triplicate. Sterile technique was used throughout and tests for sterility were made by inoculating 0.2 cc. of culture into hormone blood broth.

The quantity of virus present in a flask at a given period was determined by withdrawing 0.5 cc. of supernatant, diluting it with Tyrode, and injecting 0.03 cc.

of various tenfold dilutions intracerebrally into Swiss mice. The highest dilution giving a 50 per cent mortality was regarded as the minimum lethal dose.

Proof that the flask material was rabies virus was obtained by giving mice 0.25 cc. of the undiluted material intraperitoneally and testing them 3 weeks later for resistance to an intracerebral injection of known rabies virus. A second method was to test the ability of known rabies-neutralizing sera to protect mice against the culture virus.

RESULTS

A protocol showing the establishment of the SK_3P_6 strain in culture is shown in Table I. The greatest dilution of the original inoculated virus giving 50+ per cent mortality was 10^{-5}; that of the first culture flask after 2 to 8 days' incubation, 10^{-2}. No change was observed for six serial transfers in flasks, after which the greatest effective dilution increased to 10^{-3} or 10^{-4} and has remained at that level for forty-two passages. Mice given the undiluted culture showed paralyses on the 7th or 8th days; those given the 1 to 1,000 dilution came down on the 10th to 12th days. This latter incubation period is twice that recorded by Kanazawa (5).

To identify the culture virus with rabies, a serum protection test was run with a known rabies-neutralizing serum plus control sera from normal persons and individuals immunized against St. Louis, louping ill, and Japanese encephalitis viruses. The rabies serum mixed with culture virus protected the injected mice against the usual 100 intracerebral lethal doses, while the other sera failed to protect against one lethal dose.

As an immunity test, nine mice were given an immunizing dose of thirteenth passage culture virus, 0.25 cc. intraperitoneally, and seven mice a similar dose of fifteenth passage virus. 3 weeks later these mice were tested for resistance to an intracerebral injection of known rabies mouse passage virus. Table II shows that normal mice succumbed to a 10^{-6} dilution of test virus, while the mice immunized with culture virus survived 1,000 times the fatal dose.

To check the amount of virus in a given flask at various intervals following inoculation, three flasks were each inoculated with 1 cc. of the eighteenth passage culture and titrated at various intervals. Later the test was repeated on three flasks inoculated with the twenty-fifth passage culture.

TABLE I

Propagation of Rabies Virus (Sk_3P_6) in Tissue Culture

Culture tested		Culture injected intracerebrally in 0.03 cc. amounts into two mice in dilutions						
		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mouse brain virus		—	—	6/9,* 6/9	7/9, 7/11	8/9, 8/9	†, 9/12	
Passage flask	Days at 37°C.							
1st	2	8/9, 9/11	9/12, 12/14				—	—
"	4	8/10, 8/10	8/11, 14/19	8/11,	10/12,		—	—
"	6	9/12, 10/12	8/12, 10/12	12/13, 12/14			—	—
"	8	7/10, 8/10	9/11, 10/12	12/13, 12/13			—	—
4th	4	7/11, 8/11	7/11, 8/11	8/11			—	—
6th	4	7/8, 8/10	7/10, 8/11	11/13			—	—
8th	4	7/8, 7/8	7/8, 8/9	10/11, 8/11	13/15,	13/15,	—	—
9th	4	7/8, 7/8	7/8, 8/9	8/9, 9/9	11/12	—	—	—
10th	4	†, 7/8	8/12, 9/12	10/12, 10/13	11/12, 11/13	—	—	—
16th	4	—	6/10, 6/10	7/10, 10/11	10/11, 11/13	11/13		
25th	4	—	7/8, 7/9	8/11, 8/11	9/12, 11/12	12/13		
39th	4	6/9, 6/9	7/9, 7/10	7/11, 8/11	9/11, 10/12		—	—
42nd	4	6/7, 6/7	—	6/7, 6/10	7/8, 8/11		—	—

* 6/9 = mouse paralyzed on 6th day and dead on 9th day.

— = dilution not tested.

Blank spaces indicate mice remained well.

† Mouse died of trauma within 12 hours of injection.

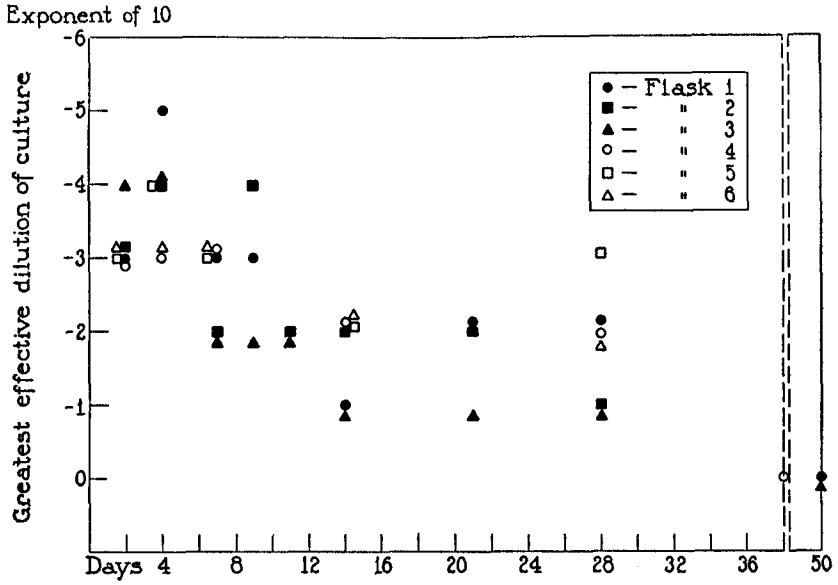
Text-fig. 1 shows that virus in the six flasks behaved relatively uniformly, being active in the 10^{-3} dilution on the 2nd day, 10^{-3} to

TABLE II

Antirabic Immunization of Mice with Culture Virus

Mice immunized with	Mice tested with standard virus 0.03 cc. intracerebrally in dilutions			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Nil-controls.....	—	6/7*, 6/7, 6/8	6/8, 6/8, 6/9	6/9, 8/9, 8/9
13th passage culture virus....	S, S, S	9/14, S, S	S, S, S	—
15th " " "	S, S, S	S, S	S, S	—

* 6/7 = mouse paralyzed on 6th day and dead on 7th day.
— = dilution not tested.
S = mouse remained well.



TEXT-FIG. 1. Titration of rabies virus in embryo mouse brain serum-Tyrode tissue culture.

10^{-5} on the 4th day, 10^{-2} to 10^{-3} on the 7th, 10^{-1} to 10^{-2} on the 14th day and remaining at this level for at least 4 weeks. At 50 days there was still active virus present in the two flasks tested.

The amount of inoculum per flask was reduced from 1 cc. to 0.5 cc. without altering the rate or amount of propagation of the established virus.

Monkey serum was replaced by horse serum without apparent effect on the culture virus. If serum was omitted from the medium, it was not adequate to propagate a newly introduced mouse brain virus, nor an already established culture virus.

Rabbit embryo brains were substituted for mouse embryo brains without alteration in titre of established culture virus.

Chick Embryo Brain Plus Monkey Serum-Tyrode.—Mouse embryo culture virus was readily established in chick embryo serum-Tyrode media (10).

The media and technique differed from that described above only in the substitution of chick embryo brains for embryo mouse brains. A 10 to 12 day chick embryo was removed aseptically from the egg, washed in sterile saline, and placed in a Petri dish. The brain was macerated in a mechanical grinder or with scissors and the resulting emulsion diluted 1 part to 2 parts of serum-Tyrode. 4 drops were added to each flask containing 4 cc. of serum-Tyrode.

After six and eight passages in the chick embryo culture media, the virus was active in the 10^{-3} dilution and readily identified as rabies.

Preservation.—The culture virus remained active in the ice box at 40° in fluid bulk or after freezing and drying. In both cases the virus was infective for 30 days when diluted 10^{-3}, and for at least 60 days when diluted 10^{-2}.

SUMMARY

Rabies virus has been propagated in serum-Tyrode solution containing either embryo mouse brain or embryo chick brain.

The culture virus reached a titre of 3×10^{-5} cc. after 4 days' incubation at 37°C., and survived at least 2 months at 5°C. in the liquid or dry state.

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