STUDIES ON THE CULTIVATION OF THE VIRUS OF LYMPHOGRANULOMA VENEREUM*

By MURRAY SANDERS,** M.D.

(From the Departments of Dermatology and Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

PLATE 8

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Previous attempts to cultivate the virus of lymphogranuloma venereum *in vitro* have yielded inconsistent results. It has usually not been possible to maintain the virus through repeated subcultures and there has been no clear demonstration of an increase in virus potency after tissue culture passage.

In 1932, Meyer and Anders (9) reported passage of the virus through 6 generations in cultures made of guinea pig kidney, testis and 25 per cent guinea pig serum in Tyrode's solution. The 6th passage produced an inguinal adenitis in only 10 of 20 guinea pigs and failed to give positive reactions when used as Frei antigen in human cases of lymphogranuloma. Miyagawa and his collaborators (10) repeated this work with no success.

Tamura (17–19) inoculated diluted pus from the lesions of lymphogranuloma into test tubes containing a piece of guinea pig liver or kidney in Tyrode's solution. He stated that turbidity developed in the supernatant fluid while uninoculated control tubes remained clear. The turbidity persisted after filtration through a Berkefeld N filter and could be transferred through as many as 24 subcultures. Fluid from the 23rd culture generation gave a skin reaction in man similar to a positive Frei test. When 1 cc. of the supernatant culture fluid was inoculated into the groins of guinea pigs, a transient adenitis resulted in 2 or 3 days, and skin sensitivity developed in 3 or 4 weeks. D'Aunoy, von Haam and Lichtenstein (2), using Tamura's technique, reported obtaining 3 successful culture passages, but Voet (20) was unable to demonstrate an increase

* Since this paper was submitted for publication, Gey and Bang (4 *b*) have reported maintenance and growth of the lymphogranuloma virus by the roller tube culture method. The media consist of human placental serum 40 per cent, bovine extract 10 per cent, balanced salt solution (Gey) 10 per cent, chicken plasma 40 per cent. Supernatant fluid media (minus plasma) were replenished every 3 or 4 days, and in this manner the cultures and virus were maintained for 7 months. Quantitative increases in potency were not determined. The original inoculum produced a 60 per cent mortality in mice after 7 days. After 53 days the culture fluid killed 5 of 5 mice in 13 days. Later culture material lost its virulence for mice, although positive Frei tests were obtained with the same material.

** With the technical assistance of Miss Frieda Gersh.

in virus potency by this method, and Miyagawa *et al.* (11) failed entirely to confirm Tamura's findings.

Miyagawa, Mitamura, Yaoi, Ishii and Okanishi (10) inoculated material from infected mouse or squirrel brain into large hanging drop preparations containing adult mouse tissue (testicle, brain or spleen) in guinea pig plasma and mouse spleen extract. Second culture generations were infective for mice and produced Frei-like skin reactions of varying degrees. Although stainable intracellular "granulocorpuscles" were described in cultured tissue, no further culture passages were done. Malamos (8) also reported inclusion bodies in hanging drop cultures.

Materials and Methods

During the past year we have attempted to find a tissue culture preparation which would allow consistent *in vitro* propagation of the lymphogranuloma virus. In all cases the fluid type of culture in 50 cc. Erlenmeyer flasks was utilized. The tissues consisted of minced embryonic guinea pig brain or embryonic chick cells; the mediums, of a buffered salt solution (similar to Tyrode's), an ultrafiltrate of ox serum, or human fetal serum. The three mediums were used together and separately with each type of tissue. Calf brain was tried in place of the other tissues, but was found to have no advantage over embryonic guinea pig brain. We have also varied incubation temperature from 37°C. to room temperature (23°C. with a range of approximately ± 5°), and oxygen tensions from anaerobiosis to aerobiosis.

Fluid mediums, used in 8.0 cc. amounts, consisted of the following combinations: (*a*) buffered salt solution, 8.0 cc.; (*b*) buffered salt solution, 7.5 cc. + fetal serum, 0.5 cc.; (*c*) serum ultrafiltrate, 2.66 cc. + buffered salt solution, 5.33 cc.; and (*d*) serum ultrafiltrate, 2.5 cc. + buffered salt solution, 5.0 cc. + fetal serum, 0.5 cc. The tissue culture system was completed by the addition of minced living tissue to one of the above mediums under rigidly aseptic conditions. All flasks before inoculaton were incubated at 37°C. for at least 24 hours to insure their sterility. Flasks not used immediately after incubation were stored in the refrigerator.[1]

Embryonic Guinea Pig Brain.—This was obtained in the following manner. A pregnant animal was killed by a quick blow on the neck. The abdomen was shaved from sternum to pubis and swabbed with generous amounts of iodine (10 per cent) and then with alcohol (75 per cent). The embryo was delivered by hysterotomy and its head severed and placed in a sterile Petri dish. The brain was removed, washed twice in 10 cc. of buffered salt solution and minced so that the largest pieces of tissue did not exceed 2 mm. This tissue was added to the flasks in rather large amounts (about 0.5 to 0.75 cc.).

Chick Tissues.—When chick tissues were used, the whole embryo was minced. Smaller amounts of this tissue (0.3 to 0.5 cc. per flask) were required, perhaps because the metabolism of the chick cells *in vitro* was much more rapid than that of the embryonic guinea pig brain cells.

Buffered Salt Solution.—The buffered salt solution of Simms is made up in two concentrated solutions which are combined and diluted as needed.

[1] Since this experiment was done it has been found that if the cultures are kept at room temperature the pH remains constant. For this reason they are now stored at room temperature rather than in the refrigerator.

Their composition is listed in Table I, with Tyrode's solution given for comparison.

Solution A is autoclaved. Solution B is filtered through a neutral, sintered 5/3 Jena glass filter[2] and kept stoppered in the refrigerator. A dilution made by adding 50 cc. of solution A to 900 cc. of redistilled water is autoclaved, and 50 cc. of solution B added aseptically. The Simms salt solution has been found to maintain tissues well even without the addition of serum ultrafiltrate or whole serum. It appears to be more stably buffered than Tyrode's solution as indicated by the phenol red which allows an observation of pH at all times.

TABLE I

Simms' Buffered Salt Solution

Solution A	Concentrate	Final concentration	Equivalent values in Tyrode's (final concentration)
	gm. per liter	*gm. per liter*	*gm. per liter*
Sodium chloride (NaCl)	160	8.0	8.0
Potassium chloride (KCl)	4.0	0.20	0.20
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	2.94	0.147	0.265
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	4.06	0.203	0.214
Solution B			
Sodium bicarbonate $NaHCO_3$	20.2	1.01	1.0
Disodium phosphate Na_2HPO_4	4.26	0.213	—
Sodium acid phosphate $NaH_2PO_4 \cdot H_2O$	—	—	0.057
Glucose	20.0	1.0	1.0
Phenol red	1.0	0.05	0.05

Serum Ultrafiltrate.—This was made from ox blood serum which was filtered under pressure through collodion membranes in an apparatus modified from the one described by Simms and Stillman (15).[3]

The rationale for applying the serum ultrafiltrate to the cultivation of a virus is as follows: Simms and Stillman (15) have shown that serum ultrafiltrate contains a growth-promoting factor, and that cultures of normal adult mammalian cells can be grown in Carrel flasks with no other nutrient material than serum ultrafiltrate diluted with two parts of the salt solution described above. Moreover, it is known that serum may be useful in propagating viruses and that it is essential for growth in at least two instances, *i.e.*, yellow fever (Haagen, 5) and rabies (Webster and Clow, 21). Since serum ultrafiltrate can be used in much higher concentrations (33 per cent or more) than can serum

[2] Schott and Gen., Jena (sold in New York by Fish-Schurman Corporation).

[3] We wish to thank Dr. Simms for his cooperation in providing serum ultrafiltrate for these experiments.

itself, it seemed possible that accessory factors for virus growth might thus be supplied more effectively. It may also be noted that the active agent in serum ultrafiltrate (called the A factor (15)) is stable in neutral solution, and can be stored for several months in the refrigerator without appreciable loss of activity. Finally, the absence of protein in the serum ultrafiltrate is obviously advantageous to the cultivation of those viruses from which vaccines might be made.

Fetal Serum.—Evidence has been presented by Gey and Gey (4*a*) that human fetal serum contains growth-sustaining substances for cells in culture. On the other hand, Carrel and Ebeling (1) have shown that serum from old individuals exerts an inhibitory effect on tissue cultures. Fetal serum was used successfully in the early part of this work but later was replaced by serum ultrafiltrate which was more convenient and more effective for extended cultivation of tissues.

Instead of collecting placental blood by venous puncture after the placenta has been expressed (Gey and Gey, 4*a*), the following method was devised to reduce the risk of contamination. A 100 cc. centrifuge tube is fitted with a cork carrying a small funnel; the funnel is covered tightly with a piece of linen which is perforated and secured to the sides of the cork with thumb tacks (Fig. 1). The device is wrapped in a towel and autoclaved. After completion of the second stage of labor, and before separation of the placenta, the distal end of the umbilical cord is inserted through the perforation in the linen cover and the blood from the placenta allowed to flow into the tube. The cork is then replaced by a sterile cotton plug. From this point, the directions given by Gey and Gey are followed. The blood is placed in the ice box for about 1 hour; the clot is then ringed and the tube returned to the ice box. An hour later, the blood is centrifuged at 3000 R.P.M. for at least 15 minutes and the serum is removed and tested for bacterial contamination.

Experiment 1

A white patient, G. K., had a left inguinal bubo, egg-sized, which had been present for 2 weeks; he reacted strongly to human Frei antigen and gave a history of having had sexual intercourse with a Negress a short time before the appearance of symptoms. The Wassermann test and skin tests for chancroid infection were negative. Glandular material and approximately 1.5 cc. of pus were removed from the bubo and macerated with an equal volume of physiological salt solution. The emulsion was passed through sterile gauze and tested for bacterial contamination. A portion of the emulsion, heated at 58°C. for 1 and 2 hours on successive days, was used as Frei antigen, controlled with known antigens. The emulsion gave consistently typical reactions in patients with known lymphogranuloma infections and negative reactions in normal patients.

Fresh emulsion was inoculated intracerebrally into 2 *Macacus rhesus* monkeys and 8 mice in dosages of 1 cc. and 0.03 cc. respectively. None of these animals showed any symptoms during an observation period of 2 months. At the same time, 3 drops of the mixture were inoculated into tissue culture preparations of embryonic calf brain, fetal serum and buffered salt solution. The cultures were incubated for 10 days at 37°C. On the 5th, 7th and 10th days 0.03 to 0.04 cc. of an emulsion of the cultures was inoculated intracerebrally into mice, control mice receiving emulsions of uninoculated tissue cultures.

Mice that had received the 5 and 7 day and control cultures remained free

from symptoms, but of 3 mice inoculated with 10 day culture material 2 showed symptoms in 4 days, and one of these died on the 5th day. The symptoms in both animals suggested infection due to lymphogranuloma virus: ruffled coat, humped back, emaciation, marked ataxia, paresis and paralysis of the hind legs. Brain emulsions of these animals were bacterially sterile in aerobic and anaerobic cultures and gave skin tests similar to positive Frei reactions in a patient known to be infected with

TABLE II

(Experiment 1)

Potency of Culture Virus in Successive Passages

Culture generation	Incubation period	Symptoms in mice	Frei reaction
	days		
1st	5	−	−
	7	−	−
	10	+	+ (Emulsified whole tissue culture and mouse brain emulsions)
6th	30	+	
7th	7	−	
8th	5	−	
	22	+	+ (Emulsified whole tissue culture and mouse brain emulsions)
9th	3	−	
	26	+	
10th	5	+	
12th	9	− (Supernatant fluid from culture) + (Emulsified whole tissue culture)	− (Supernatant fluid from culture) + (Emulsified whole tissue culture)

lymphogranuloma venereum. Sections of the mouse brains revealed a meningo-encephalitis characterized by round cell infiltrations in the meninges and around the distended cerebral vessels. 0.5 cc. of brain emulsion was inoculated into tissue cultures which were passed with 0.5 cc. inocula through 12 subcultures at intervals of 3 to 30 days without intercurrent animal passage. The final dilution of the original inoculum was about $1:18^{12}$. At intervals the potency of the culture virus was determined by Frei tests on human cases known to be infected with lymphogranuloma and by mouse inoculation, the mice being examined both for gross symptoms and microscopic histopathology. As shown in Table II, the potency of the

final culture generation was demonstrated in emulsified whole culture but not in the supernatant fluid, while in intervening culture passages virus was demonstrated only in cultures incubated at 37°C. for more than 7 days.

Experiment 2

Gland material and approximately 2 cc. of pus were removed from a left inguinal bubo of 2½ weeks duration. This patient (W. S.) reacted positively to human Frei antigen and gave negative Kline, Wassermann and chancroid skin tests. The infectious material was treated in the same manner as in the first experiment and both animals and tissue cultures were inoculated. The culture preparations consisted of embryonic guinea pig brain tissue in serum ultrafiltrate diluted with two parts of buffered salt solution. A portion of the original material used as Frei antigen produced positive reactions in known lymphogranuloma patients and evoked no response in normal patients.

The tissue culture inoculated with 0.75 cc. of the gland mixture was incubated for 6 days at 37°C., emulsified and injected into mice intracerebrally (0.03 cc.) without producing symptoms. However, a subculture of this flask when incubated for 34 days at 37°C. produced severe characteristic symptoms.

Of the 12 mice inoculated with the original mixture of gland material, pus and salt solution, only one showed symptoms. Death occurred on the 6th day and an emulsion of the brain was used for further inoculation of a tissue culture (1 cc.) and injection into mice. The mice which received this inoculum appeared to be as unaffected as the controls which received an equal dose (0.03 cc.) of a normal mouse brain emulsion.

It has been possible to maintain the tissue culture for 21 serial transfers without intercurrent animal passage with a final dilution of the original inoculum of $1:9.5^{21}$. As is apparent from Table III, evidence of the presence of the virus has been demonstrated in various culture transfers. Not only did an emulsion of the last subculture produce symptoms in mice, but its action on human skin was similar to that of Frei antigen (9 patients).

An increase in potency was apparent after the 14th culture generation when the transfer inoculum was changed from whole culture to emulsified culture. This is not surprising since the possibility of virus propagation occurring intracellularly must always be borne in mind (yellow fever (Haagen, 5), foot and mouth disease (Hecke, 6), influenza (Smith, 16)) and may very well account for the inability of Meyer and Anders (9) to maintain their strain of lymphogranuloma virus *in vitro* or to obtain a high level of potency. (The transfer inoculum used by these authors consisted of the clear supernatant fluid present after centrifuging whole cultures for 2 minutes.)

In order to determine the importance of oxygen in the *in vitro* propagation of the virus of lymphogranuloma venereum, material from the bubo of the second patient (W. S.) was inoculated into Dochez-Mills-Kneeland tube cultures (3). This medium consisted of minced chick embryo, spe-

cial peptone broth, cysteine hydrochloride, and a layer of vaseline to insure anaerobiosis. Two parallel series were run. In one, the method used by Dochez, Mills and Kneeland (3) for propagation of the virus found in the upper respiratory tract was duplicated. In the other, partial aerobiosis was obtained by removal of the vaseline.

In the anaerobic series no evidence of the survival of the virus was apparent when the 3rd culture generation was inoculated into mice. However, the series was continued and tested in the 8th generation, again with negative results. In the aerobic cultures the virus was noted in the 3rd

TABLE III

(Experiment 2)

Potency of Culture Virus in Successive Passages

Culture generation	Incubation period	Symptoms in mice	Frei reaction
	days		
2	3	Mild symptoms	
3	2	+	+ Mouse brain emulsion
4	4	+	
5	2	+	
13	4	Mild +	−
14	21	− Supernatant fluid from culture	− Supernatant fluid from culture
		+ (mild) Emulsified whole tissue culture	−Emulsified whole tissue culture
*15	10	−Supernatant fluid from culture	− Supernatant fluid from culture
		+ Emulsified whole tissue culture	+ Emulsified whole tissue culture
18	12	+	
21	11	+	†+ Emulsified whole culture and mouse brain emulsion

No reactions were visible in 48 hours at the site of inoculation in 3 control patients. These findings were confirmed by using a human antigen as control.

* Transfer inoculum of cultures changed at this point to emulsified whole culture.

† Large infiltrated papules occurred in 48 hours at the site of inoculation in the skin of 6 patients known to be suffering from lymphogranuloma venereum.

and 8th generations, but a decreasing potency was apparent and by the 12th generation the virus had disappeared.

Experiment 3

A strain of virus which could be maintained consistently in animals was developed when the patient (W. S.) who had supplied material for Experiment 2 returned to the Vanderbilt clinic with a recurrence 47 days after excision of the first bubo. On aspiration of the egg-sized swelling, 1 cc. of pus was obtained. It was diluted with an equal part of physiological saline

and injected intracerebrally into mice without further manipulation.[4] Of 7 mice thus inoculated, 6 showed marked characteristic symptoms. The strain was maintained for 27 mouse passages and showed a fairly consistent pathogenicity. There was a tendency for it to decrease in virulence in the later passages and the pathologic changes were less prominent than in the earlier series.

As a routine test for specificity, Frei tests under controlled conditions were done in lymphogranulomatous and normal patients by use of brain emulsions from animals inoculated with this strain. Although there was an occasional doubtful reaction (false positive), on the whole the results were strongly in favor of the presence of the lymphogranuloma virus. However, since this strain was to be used as a source for quantitative tests of potency under varied conditions, we considered periodic verification of viral specificity essential. This was done by attempting to neutralize the virus with convalescent serum and by producing skin sensitivity in guinea pigs.

Neutralization with Convalescent Serum.—0.5 cc. of a 1:5 dilution of mouse brain emulsion from the 10th passage was mixed with 3.5 cc. of serum taken from a patient (J. D.), who had contracted the disease about 7 years ago and now reacted strongly to human antigen. As a control, normal adult serum was used in the same manner. After thorough shaking, part of each mixture was inoculated into separate tissue cultures (serum ultrafiltrate and embryonic guinea pig brain). The remainder was left in the ice box overnight and then inoculated intracerebrally into two groups of 4 mice. All the animals receiving the mixture of normal serum and virus showed severe symptoms by the 4th day, one death occurring on the 7th day. The others continued to show marked symptoms for 2 weeks after inoculation and then slowly recovered. In the group receiving the convalescent serum mixture, 2 animals were slightly sick by the 4th day, one showed severe symptoms on the 7th day. However, no deaths occurred and recovery was so rapid that all the animals were alive and well by the 11th day.

The two tissue cultures which had received the mixtures of convalescent or normal serums and virus were incubated at 37°C. for 4 days. Upon inoculating the culture with the normal serum into mice, 2 deaths with characteristic symptoms were seen in 2 and 18 days. The 2 remaining animals showed severe symptoms in 2 days with recovery after 20 days. The culture with convalescent serum produced 1 death in 3 days (with severe symptoms). In 5 days the 3 remaining mice showed less severe symptoms (than the group receiving normal serum and virus mixture) and recovered in 13 days.

It is apparent that a much better degree of neutralization occurred in the test tube mixture of convalescent serum and virus than in the culture, al-

[4] On ten previous occasions we had injected fresh pus from buboes into mice without production of symptoms. Before inoculation the pus had been transported for more than an hour and then diluted and centrifuged. For this material we are indebted to Dr. Boris Kornblith of the New York Board of Health (Dr. Louis Chargin's service).

though at best neutralization could not be considered complete. The fact that it occurred to a lesser extent in tissue culture suggests again the possibility of the intracellular site for virus activity.

The results obtained in the culture experiments are somewhat in agreement with the observations made by Rivers and Ward (12) who studied immune reaction of the vaccinia virus in tissue cultures. These authors found that neutralization occurred only if immune serum was kept in contact with the virus before the virus had a chance to invade the explanted cells.

Guinea Pig Sensitization.—A brain emulsion of the 13th mouse passage was inoculated subcutaneously into the inguinal region of guinea pigs. A transient adenitis occurred with a subsequent marked sensitivity of the skin similar to that seen in patients. This fact has been noted and amply documented by other investigators (7).

Quantitative Estimates of Potency.—An effort was now made to estimate quantitatively the increase in potency that occurred when an inoculum of known virulence was put into tissue culture. Two parallel series were tested, one incubated at 37°C. and one kept at room temperature.

Augmented potencies were determined by inoculating mice intracerebrally with increasing dilutions (groups of 3 mice for each dilution) of infected mouse brain emulsions. At the same time 1 cc. of a known dilution was put into the tissue cultures which were to be tested. By incubating these cultures for various periods and injecting mice with dilutions of whole culture emulsions, potency of the virus before and after inoculation into tissue cultures could be determined on a more or less quantitative basis.

The standards for increased potency which we followed were threefold. The first was the appearance of a definite and characteristic syndrome. The second was a shortened incubation period, and the third the number of deaths occurring in each group. All deaths 24 to 36 hours after inoculation were regarded as being due to post-inoculation trauma. Deaths in cases where the symptoms were not clear cut or characteristic were also disregarded. For example, if a mouse appeared healthy one day (or showed only a rough coat) and was found dead on the next, its death was not considered a specific one.

Because lymphogranuloma infection in mice may not be fatal and may be manifested only by a transitory symptomatology (depending on the strain), death was considered an important indication of increased virulence. Wherever possible, histological sections and tests for bacterial contaminations were made from the brains of recovered mice and of animals that died under observation. The large number of animals involved and the small number of lymphogranuloma patients available precluded more than an occasional test of antigen from these mice.

Routine procedures during the various phases of these experiments included rigid tests for bacterial contamination of all preparations investigated. As controls, we used two types of inoculums consisting of normal mouse brain emulsion and inactivated emulsion from the original inoculum (heated at 58°C. for 3 hours). The control inoculums

were subjected to the identical experimental conditions as the original infective inoculums and were studied simultaneously with them.

In Table IV a comparison has been made between infected tissue cultures incubated at room temperature and at 37°C. In Table V comparisons of

TABLE IV

(Experiment 3)

Quantitative Estimate of Increased Potency by Inoculating Tissue Cultures with Mouse Brain Emulsion of Known Virulence. A Comparison between Maintenance of Cultures at 37°C. and at Room Temperature

Preparation	No. of mice inoculated	No. of deaths with symptoms	Time of death	No. of mice with symptoms	Dilution activity	Increase in potency
			days			
(*a*) Original mouse brain emulsion (5th mouse passage)	12 (2 dead post-inoculation)	1	3	3	1:50	—
(*b*) Tissue culture inoculated with (*a*) and incubated at 37°C. for 8 days	12 (2 dead post-inoculation)	2	7	8	1:5000	100x
(*c*) Tissue culture inoculated with (*a*) and maintained at room temperature for 8 days	12	7	3–7	10	1:50,000	1000x

Note on Tables IV to VI.—It will be noted that activity of the brain emulsion in the first series showed a comparatively low original dilution activity (1:50) as compared to the activity of the original inoculum in the second series. This can be explained on three bases. In the first group an earlier mouse passage brain was used (5th passage), the emulsion was made by grinding in a mortar and pestle, diluting with physiological salt solution and allowing the sediment to settle. The pooled inoculum, on the other hand, was made up largely of later mouse passages when the strain was more potent (in this respect it is interesting to note that potency has decreased since the 22nd mouse passage). Also, a much better emulsion was obtained by grinding the tissues with steel balls in a thick walled glass rotating chamber. Finally, buffered salt solution was used as diluent.

other factors have been made by varying the tissue and mediums in the cultures. A typical control chart used in our experiments is also given.

In considering the data noted on the charts it will be seen that the virus of lymphogranuloma venereum in tissue cultures consistently underwent a greater increase in potency at room temperature than at 37°C. Of all the various types of culture preparations, the most effective was found to

be serum ultrafiltrate with embryonic guinea pig brain tissue. We have repeated this phase of our work by incubating various culture preparations

TABLE V

(Experiment 3)

A Comparison of Various Tissue Culture Preparations Inoculated with Mouse Brain Emulsion of Known Potency

Also a Comparison between Maintenance at Room Temperature and at 37°C.

Preparation (tissue cultures were incubated for 17 days)	No. of mice inoculated	No. of deaths with symptoms	Time of death	No. of mice showing symptoms	Dilution activity (highest dilution 1:1,000,000)	Increase in potency
			days			
*Pooled mouse brain emulsion (used as inoculum in cultures)	15	3	3–13	8	1:10,000	—
Serum ultrafiltrate with embryonic guinea pig brain. Kept at room temperature	15	9	3–11	15	1:1,000,000	100x
Serum ultrafiltrate with embryonic chick tissue. Kept at room temperature	15	7	4–6	14	1:1,000,000	100x
Buffered salt solution with embryonic guinea pig brain. Kept at room temperature	15	5	3–6	13	1:1,000,000	100x
Serum ultrafiltrate with embryonic guinea pig brain. Incubated at 37°C.	15	4	3–4	13	1:1,000,000	100x
Buffered salt solution with embryonic chick tissue. Kept at room temperature	15	4	3–6	13	1:1,000,000	100x
Serum ultrafiltrate with embryonic chick tissue. Incubated at 37°C.	15	3	4–5	11	1:1,000,000	100x
Buffered salt solution with embryonic chick tissue. Incubated at 37°C.	15 (2 dead post-inoculation)	3	4–6	10	1:100,000	10x
Buffered salt solution with embryonic guinea pig brain. Incubated at 37°C.	15	2	5–6	11	1:100,000	10x

* 8th mouse passage strain III, 1 brain.
16th " " " " 2 brains.
15th " " " " 6 "
15th tissue culture passage strain II, 1 brain.

for 8 and for 30 days. At the end of both incubation periods there was a definite increase of virus in tissue cultures but in neither case did the level

of potency attain that seen in the 17 day incubation period. However, on both occasions (8 and 30 days incubation) the order of effectiveness of culture preparations was the same as in the 17 day experiment (Table V).

Long Term Cultures.—A prominent feature of this study was the ability of the serum ultrafiltrate to maintain tissues for long periods of time with little change in the external appearance of the cultures. To determine what effect extended cultivation might have on the virus, preliminary experiments were done with cultures kept at room temperature and at

TABLE VI

(Controls)

Three Mice Inoculated in Each Group

Days	Normal mouse brain Inoculation Apr. 10, 1939	Tissue culture (embryonic guinea pig brain and serum ultrafiltrate) inoculated with emulsion (*a*) (Table IV) which had been inactivated by heating. Culture was kept at room temperature for 8 days and injected into mice	Tissue culture inoculated with normal mouse brain emulsion and incubated for 8 days at 37°C.
		1 dead postinoculation	
2	1 rough coat 2 alive and well	2 alive and well	1 rough coat 2 alive and well
4	1 rough coat (active) 2 alive and well	" " " "	3 alive and well
6	3 alive and well	" " " "	" " " "
8	" " " "	" " " "	" " " "
10	" " " "	" " " "	" " " "

37°C. Two flask cultures (embryonic guinea pig brain in serum ultrafiltrate and buffered salt solution), kept under these conditions for 30 days, received 1 cc. of a brain emulsion from the 5th animal passage. They were maintained at the same temperatures as before for an additional period of 12 days and were then injected into mice. The process was repeated with two more cultures from the same groups, but the period of maintenance after infection was extended to 15 days, making the total age of the culture 45 days instead of 42 days. In each case, marked activity of the virus was noted, but a quantitative estimate of potency was not done.

We should like to emphasize that in these experiments all the preparations during the long periods of cultivation maintained an unusually con-

sistent pH close to the neutral point, and presented an appearance so similar to freshly made cultures that the two groups could not be distinguished from each other in the gross. When tissues were removed from flasks after 42 and 45 days and were stained with hematoxylin and eosin, the cells were found to be in excellent condition. The nuclei were clearly outlined and stained well. Pyknosis and karyorrhexis were infrequent. Necrosis occurred only to a negligible extent and seemed to depend upon the size of the tissue mass rather than the length of time of incubation (Figs. 2 *a*, *b*, 3). In this respect it is interesting to note that Simms (14) was able to show a Carrel flask preparation of adult chicken aorta tissue to be alive after 47 days. To the tissue which had been incubated in serum ultrafiltrate at room temperature he added a serum high in fat-producing factor (B factor) and noted the formation of fat granules by the cultivated cells.

DISCUSSION

In attempting to demonstrate *in vitro* growth of the agent of lymphogranuloma venereum, we have followed the five standards previously formulated (13) for the propagation of a virus. Briefly stated these standards are:

1. Extension of serial passage so as to preclude the possibility of infection caused by the original inoculum in the tissue cultures.
2. Demonstration of quantitative increases in virus potency after incubation in tissue cultures.
3. Demonstration of inclusion bodies in culture preparations (this is not applicable to all viruses but when applicable is strong evidence of propagation).
4. Elimination of contamination by bacteria, or by other viruses (this includes "spontaneous" viruses) through periodic identification of the virus under investigation.
5. Confirmation of the method and its results by one or more investigators.

We feel that the first two standards have been met. In addition, there has been evidence for the effectiveness of tissue cultures as a means for increasing the potency of lymphogranuloma strains. Not only was fresh material frequently incapable of producing infection in animals, but it was found that strains I and II would not remain virulent for mice unless the virus was put back into culture. Thus, on 9 occasions symptoms in mice which had been inoculated with tissue culture material could be transmitted for only 2 successive animal passages, in 4 other instances

symptoms were produced for only 3 mouse generations. In each of the 13 cases reinoculation into tissue culture of the material from the brains of the last mouse passage producing symptoms, caused an increase in potency (as evidenced by the ability of this tissue culture to infect mice).

So far as inclusion bodies are concerned, we have been unable to demonstrate their presence in cultures or in mouse brains. On a few occasions, when inclusion bodies seemed to be present, similar formations were observed in the control preparations. Tissue from culture flasks, mouse brain sections and touch preparations were examined.

The identification of the virus under investigation has been periodically carried out by inoculating mice, testing antigens on human cases and sensitized guinea pigs, and on two occasions by neutralization experiments with the use of convalescent serum.

It is obvious that the fifth standard, namely confirmation, must necessarily be dependent on time. We have repeated almost every phase of this work and in no case have there been conflicting results.

The fact that the fluid mediums used in our experiments have shown a surprising capacity for maintaining tissues over long periods of time[5] has enabled us to employ a new principle in cultivating viruses, namely, the use of old tissue cultures as vehicles for virus propagation. Although tissues in cultures for as long as 70 days remained in excellent morphologic condition, as routine we used for transfers or inoculation with virus, preparations varying in age from 7 to 21 days. It is to be hoped that with further investigation of the factors involved, for example, determination of optimum ratios of tissue to fluid, the period during which cultures can be stored will be greatly increased. This is desirable for virus work in general because of the simplification of technique and the possibility of producing large amounts of virus in protein-free mediums. Finally, the fact that this method appears to be efficacious at room temperature gives rise to the hope that it may be useful for field work with certain viruses.

CONCLUSIONS

Two strains of the lymphogranuloma venereum virus were maintained in tissue cultures for 12 and 21 generations respectively.

In a third strain a quantitative increase in potency as high as 1000 times was obtained by inoculating tissue cultures with infected emulsions of known virulence.

Greater increases in potency were consistently obtained by maintaining tissue cultures and virus at room temperature (23°C. with a range of ap-

[5] Without washing or transfer of tissue.

proximately ± 5°C.) than by incubating them at 37°C. The virus did not survive in the absence of oxygen.

Embryonic guinea pig brain and serum ultrafiltrate were found to be the most effective vehicles for propagation of the lymphogranuloma virus.

There is evidence that the site of activity for this virus is intracellular.

Embryonic guinea pig brain cells were maintained in the serum ultrafiltrate diluted with buffered salt solution in good (morphologic) condition for as long as 70 days. Not only could old cultures be successfully inoculated with the virus of lymphogranuloma, but high titres could be maintained over extended periods.

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EXPLANATION OF PLATE 8

FIG. 1. A simple apparatus for collecting placental blood. Fetal serum may subsequently be obtained with a minimum of contamination.

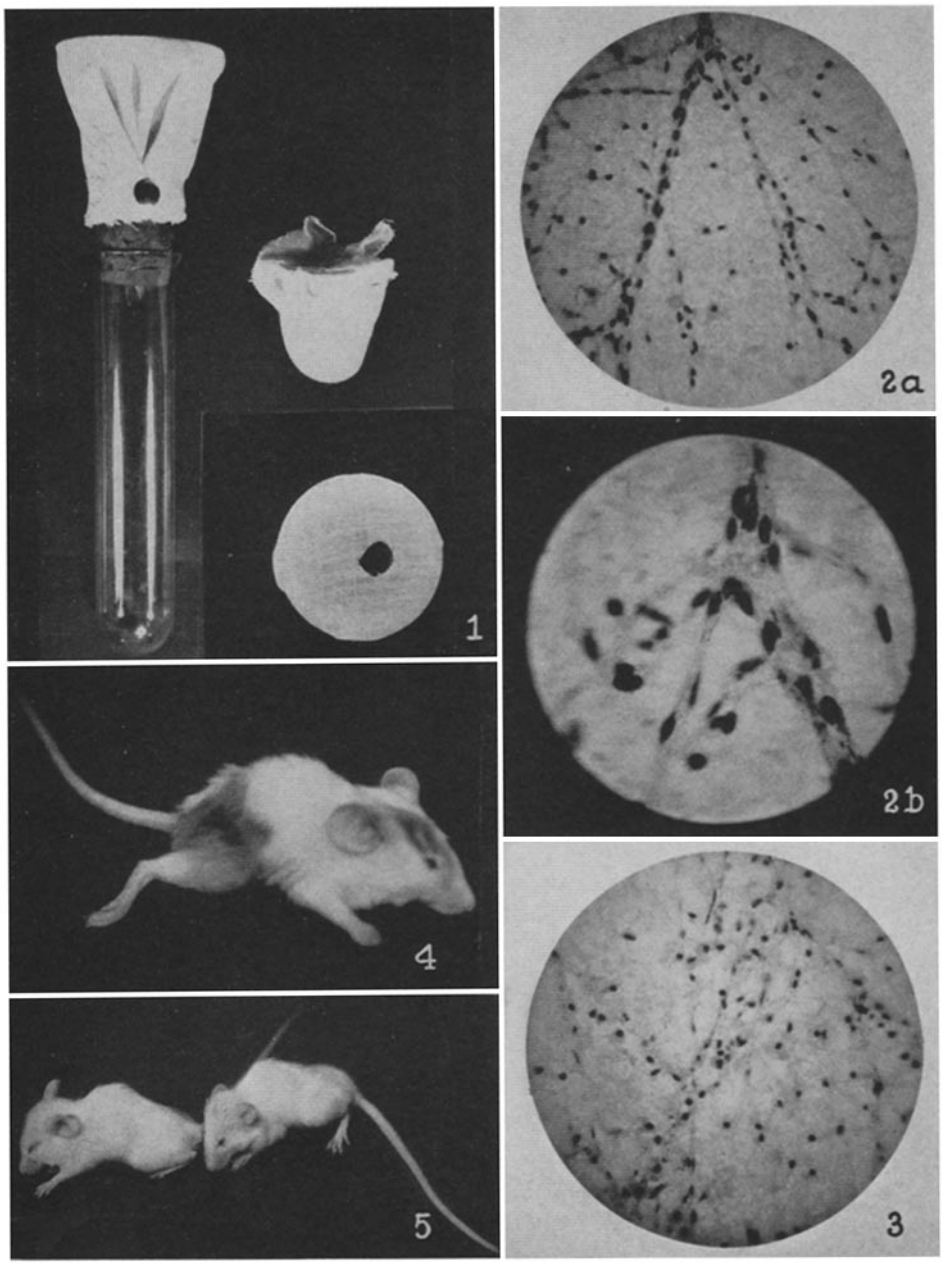
FIG. 2*a*. Embryonic guinea pig brain in serum ultrafiltrate incubated for 45 days at room temperature. Stained with hematoxylin and eosin.

FIG. 2*b*. Higher magnification of Fig. 2*a*.

FIG. 3. Embryonic guinea pig brain in serum ultrafiltrate incubated for 45 days at 37°C. Stained with hematoxylin and eosin.

FIG. 4. Mouse inoculated 5 days previously with the 21st subculture (Experiment 2). Dilution of original inoculum of virus was $1:9.5^{21}$. Positive Frei reactions were obtained with emulsions of the culture inoculum and of the mouse brain (see Table III).

FIG. 5. Mice inoculated 6 days previously with 1:100,000 dilution of emulsified tissue culture (serum ultrafiltrate and embryonic guinea pig brain) maintained at room temperature.



(Sanders: Cultivation of virus of lymphogranuloma venereum)