CULTIVATION OF VACCINE VIRUS FOR JENNERIAN PROPHYLAXIS IN MAN

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Plates 45 to 47

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In a previous communication (1) a simple medium for the cultivation of vaccine virus was described. The results of the work reported at that time clearly indicate that vaccine virus is capable of multiplication in the presence of minced chick embryo tissue suspended in Tyrode's solution. The experiments, however, were conducted with a neurovaccine virus, and, although the active agent engendered in cultures caused typical vaccinal lesions in rabbits, it was deemed best not to test it in human beings. To obtain a culture virus for Jennerian prophylaxis in man, it seemed advisable to adapt a dermal strain of vaccine virus to our method of cultivation. The present paper deals with the results of this work.

Methods and Materials

Virus.—Vaccine virus, Lot 611, prepared by the New York City Board of Health was used to initiate the cultures. In a letter to me, the original source of the virus is described by Mr. Chas. R. Tyler.

"Regarding the history of our strain of vaccine virus: the following statement appears in the Yearly Report of the New York City Board of Health for 1874–75.

'We began vaccinating with virus of the same stock as that which had been supplied by the late Dr. Loines of the Eastern Dispensary and myself for about 5 years and which had been used and sold by him for about 20 years previously. This virus was originally obtained from England by Dr. Loines and in all probability was descended from the stock furnished by Jenner. As it always developed characteristic Jennerian vesicles and as it always thoroughly protected from smallpox those upon whom it was used, Dr. Loines never thought favorably of employing any other.'

"The same strain of virus has been in use since the time of this report. The virus has been 'humanized' at various times, usually from one to three times yearly."

Tissues.—Embryonic tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (2) or that of Eagles and McClean (3). If the embryos were contaminated by yolk, they were thoroughly washed in sterile Tyrode's solution before being minced. Furthermore, the eyes were removed, because the pigment granules contained in them often led to confusion when smears were made to determine the sterility of the cultures. Finally, the embryos were placed in a sterile watchglass contained in a Petri dish, finely minced with scissors, and then distributed in proper amounts into flasks by means of a pipette.

Tyrode's Solution.—Tyrode's solution prepared according to the following formula and sterilized by filtration was used: NaCl, 8 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; NaH_2PO_4, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc. During the preparation and filtration of the solution, CO_2 is liberated, and the pH is found to be between 8.0 and 8.4. When living tissue is added to the solution, however, CO_2 is supplied by it and the pH becomes less alkaline reaching a point compatible with the survival of the embryonic tissue and the multiplication of the virus.

Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. Flasks (collar flasks) designed to meet these requirements were described in a previous paper (1) and have been found to be satisfactory for this work. Two sizes have been used, one with approximately the dimensions of a 50 cc. Erlenmeyer flask, the other with approximately the volume of a 250 cc. Erlenmeyer flask. In the small container 5 cc. of medium were placed, while in the large one 15 cc. were used. Such small amounts were placed in each flask in order to have the medium distributed as a thin layer. In case collar flasks cannot be obtained, 50 cc. and 250 cc. Erlenmeyer flasks make good substitutes. All glassware used in this type of work should be made of an alkali-free resistant glass, *e.g.*, pyrex.

Preparation of Cultures.—Approximately 1 gm. of minced chick embryo tissue to each 5 cc. of Tyrode's solution was used. Proper amounts of tissue suspended in its vehicle were distributed in flasks. The medium was then inoculated with 0.25 cc. of virus emulsion, the mouth of each flask containing its cotton plug was securely covered with several layers of tin-foil, and the cultures were incubated at 37°C. for 5 days. New cultures were made by direct transfer of 0.25 cc. of an old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations. One should be careful not to use too much tissue for a given amount of Tyrode's solution, inasmuch as it appears that an optimum ratio between tissue and fluid exists for the maximal multiplication of virus.

Tests for Sterility of the Cultures.—Early in the work, the medium was tested for sterility before it was inoculated with virus. During the interval (48 hours), the prepared flasks were stored at +5°C. Later these preliminary tests were omitted to advantage. Two separate batches of media were prepared for each

transfer in order to escape the possibility of having the work delayed by bacterial contaminations. After the cultures had been incubated for 3 days, a small amount of material was removed from each one and seeded in broth tubes and on blood agar plates. After the virus cultures had been incubated for 5 days, smears from each one were prepared, stained, and examined for bacteria. The material in the flasks that was found by cultural tests and smears to be free from bacteria was used for transfers and animal inoculations. Numerous flasks have been handled in the manner described above and only 1 in 12 became contaminated with bacteria.

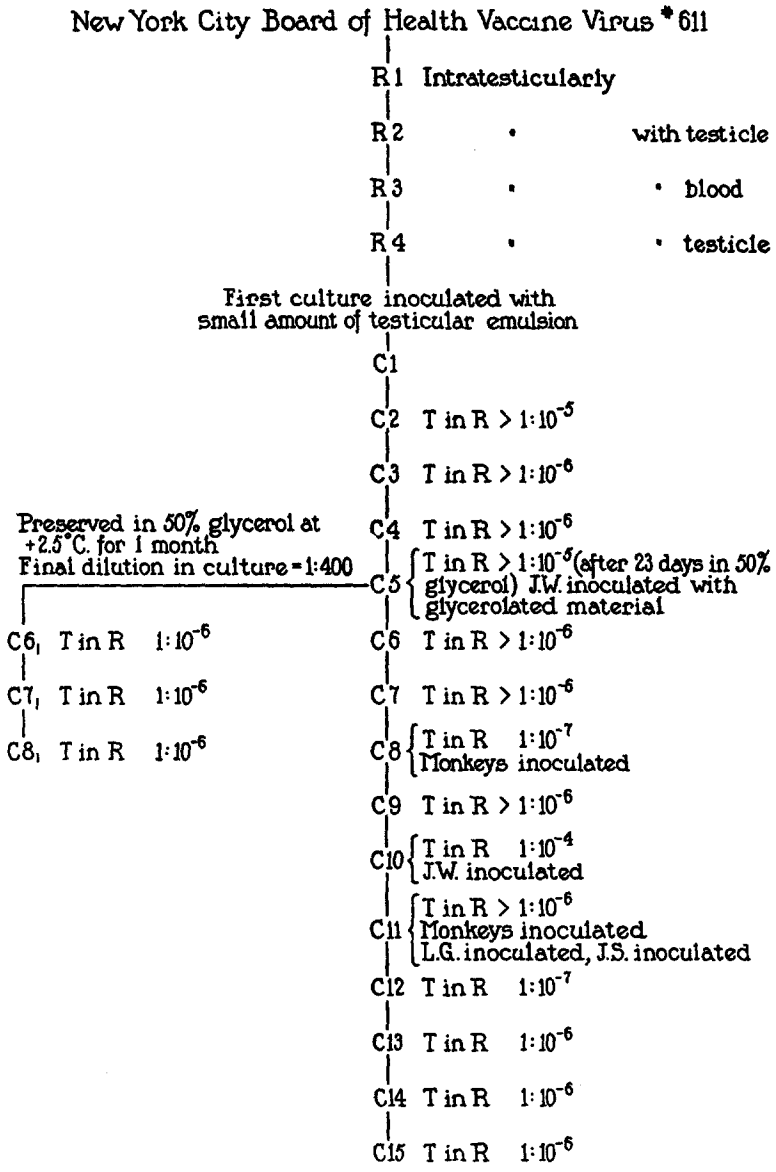
Tests for Potency of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution were injected into the shaved skin or were smeared on the scarified shaved skin of rabbits. In addition to rabbits, monkeys and human volunteers were employed for the potency tests of the virus.

Preservation of the Virus.—Many workers have stated that culture virus is difficult to preserve. This is true of viruses cultivated in certain kinds of media. It must be borne in mind, however, that no vaccine virus keeps indefinitely above 0°C. Furthermore, when glycerol is used, care must be exercised in selecting one that will not inactivate the virus. Merck's (P–W–R Analytical Chemicals) glycerol has been found suitable for this work.

EXPERIMENTAL

The vaccine virus distributed by the New York City Board of Health is prepared on calves, ripened in glycerol at about −8°C., and then treated with brilliant green. The bacterial count in such material is low. Indeed, at times, the virus seems to be entirely free from viable microorganisms. A specimen from Lot 611 was obtained from the Board of Health Laboratories, and with it cultures were initiated. At first it appeared that we had been fortunate enough to obtain a virus free from bacteria, but soon it was discovered that a very slow-growing diptheroid was present as a contaminant. Consequently we were faced with the problem of how to rid the virus cultures of the bacteria. Filtration was first considered as a means of accomplishing this, but it was not attempted because vaccine virus filters with difficulty. Then we decided to try passage through rabbits in the following manner.

Rabbit 1 received in each testicle 1 cc. of a culture containing diphtheroids and vaccine virus. 4 days later the testicles were removed and emulsified with Locke's solution. 1 cc. of the emulsion was injected into each testicle of Rabbit 2. After



TEXT-FIG. 1. Summary of experiments in which a dermal strain of vaccine virus was adapted to a culture medium consisting of minced chick embryo suspended in Tyrode's solution. T in R = titer in rabbit.

a lapse of 4 days, blood (4) was removed[1] aseptically from the heart of Rabbit 2 and immediately injected in 1 cc. amounts into the testicles of Rabbit 3. This animal developed fever on the 4th day, and small hard nodules were felt in the testicles. At this time the testicles were removed and emulsified with Locke's solution. 1 cc. of the emulsion was inoculated in each testicle of Rabbit 4. After 4 days these testicles were removed and ground in a mortar without sand. Approximately 20 cc. of Tyrode's solution were thoroughly mixed with the macerated tissue. The emulsion was tested for the presence of bacteria and was found to be sterile. Then small amounts (0.25 cc.) of the supernatant material from a centrifuged specimen of the emulsion were used to initiate a series of virus cultures (see Text-fig. 1).

Having rid the dermal vaccine virus of bacteria it remained for us to determine whether it was possible to adapt such a strain to our method of cultivation. Proceeding in the manner described above, we have been able to carry the virus through an uninterrupted series of 15 culture generations (Text-fig. 1), and there is no reason to suppose that it cannot be cultivated indefinitely. The records of the titrations of the virus shown in Text-fig. 1 leave no room for doubt concerning the multiplication of the active agent. Furthermore, fresh cultures have been initiated with glycerolated material (Text-fig. 1) stored for 30 days at +2.5°C. Such facts make it evident that vaccine virus is capable of indefinite propagation in cultures without the intervention of animal passage.

Reactions in Animals

It has been shown that a dermal strain of vaccine virus is capable of multiplication in a medium consisting of minced chick embryo suspended in Tyrode's solution. The question as to whether the virus has been altered by such treatment naturally arises. To investigate this matter, rabbits and monkeys were employed.

Material from each set of cultures, with the exception of the 1st, has been tested by intradermal inoculations in the shaved skin of rabbits. The reactions caused by the virus have not been altered through the process of cultivation, and have always been similar to those produced by a potent strain of vaccine virus unassociated with bacteria. This fact is illustrated by Fig. 1 that portrays lesions following intradermal injections of different dilutions of a culture in the 14th generation. In addition to intradermal injections, inoculations on the scarified shaved skin of

[1] All operations were performed under ether anesthesia.

rabbits have been made from time to time. Following such inoculations, typical vaccinal lesions unassociated with hemorrhage and necrosis developed. Fig. 2 represents lesions produced by different dilutions of a culture in the 6th generation. Finally, it has been shown that the culture virus smeared on the scarified skin of monkeys (*Macacus rhesus*) induces typical vaccinal lesions that heal rapidly without resultant injury to the animals.

From the above experiments it is obvious that the culture virus caused typical vaccinal lesions in animals. We then became interested in determining whether the active agent had undergone immunological changes. Consequently the following cross immunity experiments were performed.

Rabbits inoculated respectively with virus from the 6th, 8th, 10th, and 11th culture generations were allowed to recover. Then, they and 2 normal stock rabbits were inoculated with New York City Board of Health vaccine virus, Lot 617. The recovered animals were refractory while the normal ones developed typical vaccinal lesions.

The above experiment indicates that repeated cultivation of the virus in a medium consisting of minced chick embryo suspended in Tyrode's solution did not alter its antigenic properties.

Reactions in Human Volunteers

Not infrequently we receive requests for vaccination against smallpox. Since there was no valid reason for not using the culture virus in human beings, we decided, with the consent of the patients or of their parents, to substitute in certain instances the culture vaccine for calf lymph. The results obtained in 3 children are detailed below.

J. W., aged 2 years, *May 2*, was vaccinated at 2 points on the outer surface of the upper left arm. The superior inoculation was made with material from a 5th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 28 days. The inferior inoculation was made with material from a 10th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 2 days. The method of vaccination consisted of a small amount of the virus being smeared over an area of skin previously superficially scarified. *May 3*, superficial marks at points of inoculation. *May 4*, no change. *May 5*, 9:00 a.m., both lesions, the lower slightly more than the upper, show some swelling and elevation. 2:30 p.m., there have appeared, particularly over the area of the lower inoculation, crops of small vesicles (Fig. 3). No fever. *May 6*, lesions well developed. Both are small, the upper being about 0.5 cm. in diameter, the lower about 1.0 cm. (Fig.

4). Temperature rose to 101.2°F. *May 7*, lesions have increased in diameter (Fig. 5). Temperature rose to 101.6°F. *May 8*, lesions are still increasing in size and are now pustular (Fig. 6). Temperature rose to 102°F. Further development of the lesions not observed, because the child had to leave town with its parents.

L. G., aged 22 months, *May 11*, was vaccinated at one point on the outer surface of upper left arm with material from an 11th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 11 days. *May 12*, no evidence of inflammation. *May 13*, vaccinated area red but not elevated (Fig. 11). *May 14*, at point of inoculation, there is a red papule (Fig. 12). *May 15*, several small vesicles have appeared (Fig. 13). *May 16*, vesicles have coalesced and are beginning to be pustular. Narrow primary areola is present (Fig. 14). *May 17*, lesion somewhat larger, no induration, no fever. *May 18*, lesion has increased in size and is beginning to dry in center (Fig. 15); no fever. *May 19*, pustule larger (Fig. 16), no induration, no fever. *May 20*, pustule larger, drying; secondary areola 3 mm. in width; slight amount of induration (Fig. 17). Temperature rose to 100.4°F. *May 21*, pustule drying, secondary areola 4 mm. in width, induration more marked (Fig. 18). Temperature 100.0°F. *May 22*, secondary areola 1 cm. in diameter, induration more marked (Fig. 19); no fever. Patient was reinoculated on right arm with New York City Board of Health vaccine virus, Lot 617. *May 23*, primary vaccination: secondary areola has almost disappeared, induration less (Fig. 20). Secondary vaccination: skin at point of inoculation red. No fever. *May 25*, primary vaccination: lesion covered by dry scab; secondary areola and induration no longer present (Fig. 21). Secondary vaccination: slightly red. *May 26*, primary vaccination: only a dry scab remains. Secondary vaccination: negative. *May 28*, primary vaccination: the scab will drop off in a few days (Fig. 22). Secondary vaccination: negative. Patient in excellent condition and discharged from the hospital. The virus with which this patient was reinoculated was shown to be potent by a successful vaccination of a normal person. Thus, the lack of response to the secondary inoculation indicates that a state of immunity had been induced by the primary vaccination.

After it had been demonstrated that the culture virus produces without danger a typical vaccinia in man and that it induces an immunity against calf lymph, we decided, in view of the fact that the culture vaccine seemed to possess a greater potency than does ordinary calf lymph, to compare the action of the two kinds of active agent.

M. S., aged 11 months, and J. S., aged 22 months, were vaccinated; the former with New York City Board of Health virus, Lot 617, that had been stored in 50 per cent glycerol below 0°C. for more than a year, the latter with virus from an 11th-generation culture that had been preserved in 50 per cent glycerol at +2.5°C. for 21 days. Typical vaccinia developed in both children who showed no evi-

dences of illness except a local reaction and a slight elevation of temperature, 101.5°F. Certain differences, however, were noted in the course of the infection in the 2 patients, *viz.*, the child who was inoculated with the culture virus had a more prolonged febrile reaction and developed more redness and induration associated with a few secondary vesicles around the vaccinal pustule than did the infant who received the calf lymph (Figs. 7 to 10).

The experiment just described and others that will not be presented at this time indicate that the culture virus is very potent. The fact that fresh culture vaccine produces considerable reaction is not surprising, inasmuch as it has long been known that a "green" virus is more likely to give rise to redness, induration, and secondary vesicles than is one properly aged. Therefore, the culture virus should be allowed to ripen before it is used for vaccination of human beings.

DISCUSSION

The medium employed for the *in vitro* cultivation of vaccine virus is exceedingly simple, but one must remember that it contains living cells. In spite of recent reports (5) to the contrary, no one has definitely shown (6, 7) that vaccine virus is capable of pullulation in the absence of surviving susceptible cells.

Several kinds of media have been devised for the production of vaccine virus. The one described in this paper, however, is the best of the lot already proposed, because, inasmuch as neither serum nor plasma is used and since the tissue consists of minced chick embryo, it is the least likely to become contaminated by an extraneous virus injurious to man. Furthermore, the virus produced in this manner remains potent in 50 per cent glycerol at +2.5°C. for at least a month. More information, however, concerning the length of time the culture vaccine remains active under a variety of conditions should be obtained and to this end experiments are now being conducted.

SUMMARY

1. A dermal strain of vaccine virus has been adapted to a simple culture medium consisting of minced chick embryo suspended in Tyrode's solution.

2. The bacteria-free culture virus, thus obtained, produces in lower animals and in man typical vaccinia that renders them refractory to infection with ordinary vaccine virus harvested from calves.

REFERENCES

1. Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.
2. Carrel, A., and Rivers, T. M., *Compt. rend. Soc. biol.*, 1927, **96**, 848.
3. Eagles, G. H., and McClean, D., *Brit. J. Exp. Path.*, 1929, **10**, 35.
4. Ohtawara, T., *Japan Med. World*, 1922, **2**, 254.
5. Eagles, G. H., and McClean, D., *Brit. J. Exp. Path.*, 1931, **12**, 97.
6. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1929, **50**, 181.
7. Muckenfuss, R. S., *J. Exp. Med.*, 1931, **53**, 377.

EXPLANATION OF PLATES

PLATE 45

FIG. 1. Lesions produced in a rabbit by intradermal inoculations (0.25 cc.) of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions, respectively, of a 14th-generation culture of vaccine virus. × 1.

FIG. 2. Lesions induced in a rabbit by different dilutions (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of a 6th-generation culture of vaccine virus. × 1.

PLATE 46

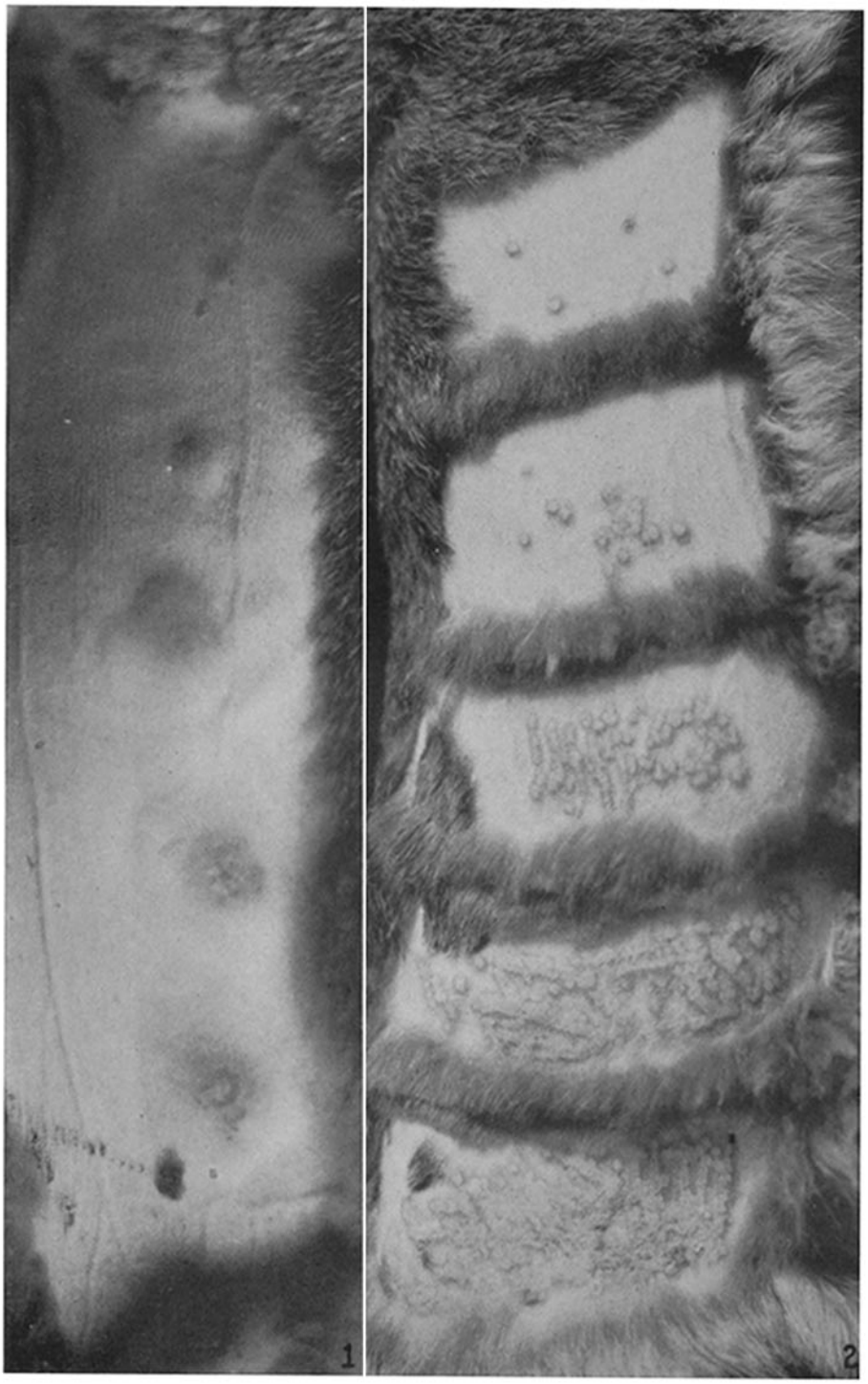
FIGS. 3 to 6. 3rd-, 4th-, 5th-, and 6th-day vaccinal lesions, respectively, produced in J. W. by culture virus: the upper resulted from an inoculation of a 5th-generation culture, the lower from an infection with a 10th-generation culture. × 1.

FIGS. 7, 9. 6th- and 9th-day vaccinal lesions, respectively, produced in M. S. by New York City Board of Health virus, Lot 617. Compare with Figs. 8 and 10. × 1.

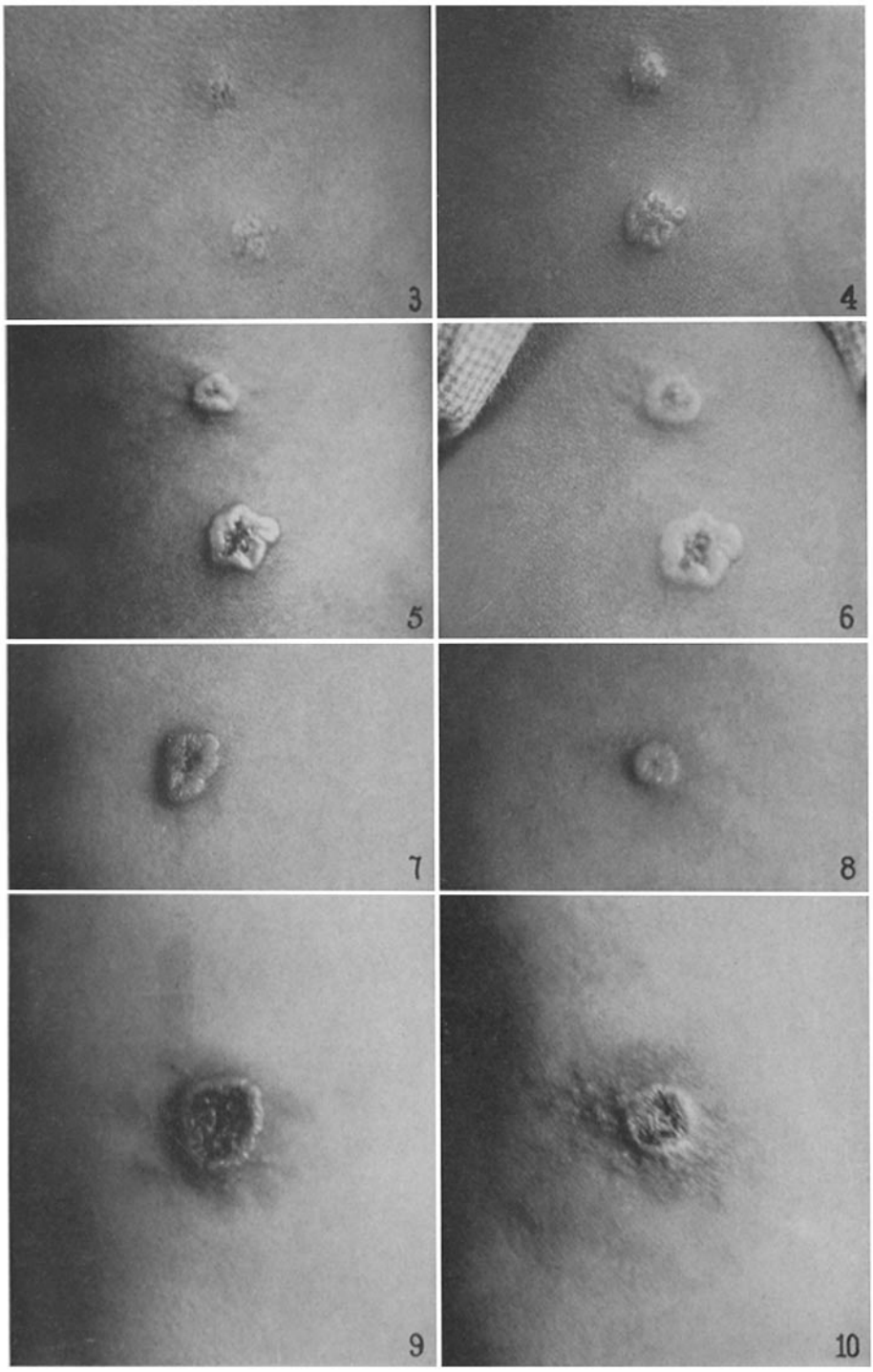
FIGS. 8, 10. 6th- and 9th-day vaccinal lesions, respectively, induced in J. S. by an 11th-generation culture. Comparison of these reactions with those in Figs. 7 and 9 shows that the culture vaccine, only 21 days old, caused more redness and secondary vesicles than did the City Board of Health virus that had been aged for more than a year. × 1.

PLATE 47

FIGS. 11 to 22. 2nd-, 3rd-, 4th-, 5th-, 7th-, 8th-, 9th-, 10th-, 11th-, 12th-, 13th-, and 18th-day vaccinal lesions, respectively, produced in L. G. by a 10th-generation culture. The photographs portray the complete evolution of a vaccinal lesion induced in man by a dermal strain of virus adapted to *in vitro* cultivation. × 1.



(Rivers: Vaccine virus for prophylaxis)



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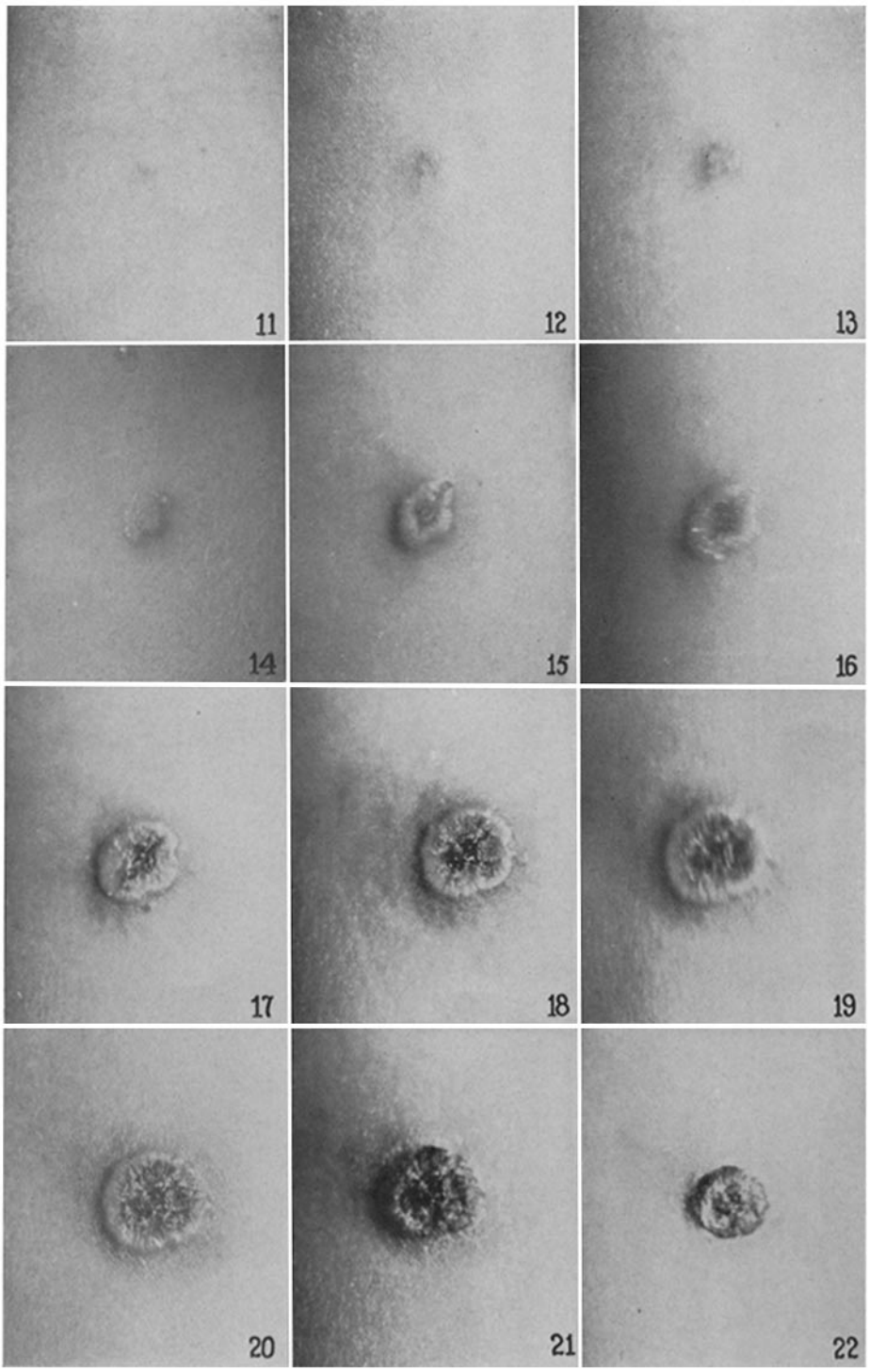

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