FURTHER OBSERVATIONS ON THE CULTIVATION OF VACCINE VIRUS IN LIFELESS MEDIA

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In a previous paper (1) we reported the results of attempts to cultivate vaccine virus in a "cell-free" medium described by Eagles and McClean (2, 3). We were unable to confirm the findings of these workers. Following the presentation of our paper for publication, a report by Eagles and Kordi (4) appeared in which they stated that they also were able to cultivate the virus in the absence of cells. Inasmuch as these investigators used a medium somewhat different from that employed by Eagles and McClean, it seemed advisable for us to determine whether we were able to confirm their findings. This paper records the results of our work dealing with the matter.

EXPERIMENTAL

Eagles and Kordi (4) stated that they were able to obtain growth of vaccine virus in a medium consisting of a mixture of Tyrode's solution, rabbit serum, and an extract prepared from rabbit kidney tissue by means of a hypertonic salt solution and freezing. We have repeated their experiments according to the directions given in their paper. In view of the fact that vaccine virus multiplies more consistently in media containing bits of rabbit testicles or chick embryos than it does in media containing minced rabbit kidney (1), it seemed expedient to supplement Eagles and Kordi's experiments by the use of extracts of these tissues. Furthermore, it seemed advisable to use at least two strains of vaccine virus.

We say that we repeated Eagles and Kordi's (4) experiments according to the directions given in their paper. We realize, however, that we may not have accomplished this, because these workers, as did

Eagles and McClean (2, 3), failed to provide adequate descriptions of their methods. For instance, they give minute details about the method of obtaining the "cell-free" kidney extract but say nothing of the manner in which the rabbit serum and the fresh testicular virus were freed from cells. Apparently all cells were not removed from the serum, because they state that in hanging drop preparations of the medium "occasional red blood cells from the rabbit serum" were seen. Furthermore, the amounts of the different ingredients in their medium are described in one ambiguous sentence: "Equal parts of fresh rabbit serum and Tyrode's solution were added to the kidney extract to make a dilution of 1 in 50 of the virus seeding." This sentence can be interpreted in several ways, but, in view of the work on the cultivation of vaccine virus that has been described by different investigators, we have chosen to interpret it in the following manner: 1 part of Tyrode's solution, 1 part of rabbit serum, and 1 part of kidney extract were mixed and to this mixture sufficient vaccine virus was added so that the dilution of the virus was 1 in 50.

Virus.—Two strains of vaccine virus were used in the experiments. One was a dermal strain (5) obtained from the New York City Board of Health, the other was the Levaditi neuro-vaccine virus. Both strains can be propagated without difficulty by passages through rabbit testicles or by cultivation in modified tissue cultures (5, 6) and are free from ordinary aerobic and anaerobic bacteria. When a virus was used to initiate a series of cultures, care was taken to remove or to kill all cells in the virus emulsion by thorough centrifugation followed by repeated (3 times) freezing and thawing of the supernatant virus-containing material. CO_2 snow (−78°C.) was used for freezing.

Tyrode's Solution.—Tyrode's solution prepared according to the following formula was sterilized by filtration: NaCl, 8.0 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.*, 1,000 cc.

Serum.—Serum obtained from defibrinated rabbit blood was used in accordance with Eagles and Kordi's instructions. We were careful, however, to remove as many cells as possible by centrifuging it twice, 1 hour on each occasion, at approximately 3,600 R.P.M.

Tissues.—In our experiments, media containing viable cells were used as controls. Testicular and renal tissues were obtained from young rabbits (half-grown). Chick embryo tissue was obtained from eggs incubated 11 or 12 days. Inasmuch as only small quantities of tissue were required for the controls in each experiment, the remainder of the kidneys, testicles, or chick embryos was used for the preparation of tissue extracts.

Extracts of Renal Tissue.—Extracts of renal tissue were made in the following manner: Both kidneys from a half-grown rabbit were removed aseptically. After the fat and pelvic tissue had been removed, the remaining kidney tissue, which weighed approximately 10 or 11 gm., was divided into equal parts, placed in mortars, and thoroughly ground without the addition of an abrasive. Then each portion was mixed with 1 cc. of a 9 per cent NaCl solution. One portion was allowed to stand 1 hour at room temperature after which time 9 cc. of sterile distilled water were added. The other portion was placed in the freezing compartment of a frigidaire (approximately −10°C.) where it was allowed to remain for 1 hour, during which time it became frozen. After it had been removed from the ice box and rapidly thawed, 9 cc. of sterile distilled water were added. Each portion was then placed in a tube and centrifuged for 1 hour at a speed of approximately 3,600 R.P.M. The supernatant fluids were removed and again centrifuged for an hour at high speed. The supernatant fluids after the second centrifugation constituted the kidney extracts used in the preparation of the media. In each instance the supernatant fluids were carefully removed by means of 10 cc. pipettes, the tips of which were made of medium size capillary tubing. Such pipettes were used in order to avoid the fat floating on the surface of the fluids as well as the sedimented cells.

Extracts of Testicular Tissue and Chick Embryo Tissue.—Testicular extracts and extracts of chick embryo tissue were prepared in a manner similar to that used for the preparation of renal extracts with the exception that 4 rabbit testicles (approximate weight 7–9 gm.) or 4 chick embryos (11 days old and weighing approximately 8–9 gm.) were used instead of 2 kidneys.

Preparation of Cultures.—Inasmuch as two kinds of extracts were made from the tissues, *e.g.* one made at room temperature, the other obtained by freezing and thawing, two sets of cultures were prepared in each experiment. The cultures were prepared in the following manner: 1 part of extract centrifuged twice, 1 part of serum centrifuged twice, and 1 part of Tyrode's solution were mixed and seeded with vaccine virus (the amount used was sufficient to insure the presence of considerable virus) from which viable tissue cells had been removed or killed by centrifugation and repeated freezing and thawing. The material was then distributed in 2 cc. amounts to Carrel D flasks. Usually there were 3 flasks for each kind of medium.

Incubation of Cultures.—The cultures were incubated at 37°C. for 3 or 4 days.

Titration of Virus in Cultures.—The titer of the virus in the cultures before incubation was determined by means of intradermal inoculations (0.2 cc.) in rabbits of dilutions of the cultures. After incubation the titer was again determined in the same manner. At first the material in each flask was titered. It was found, however, that such a procedure was not essential for this work and then all the flasks (usually 3) containing similar cultures were pooled before titration.

Controls.—Each time extract cultures were prepared, a number of control cultures were made and usually consisted of (1) bits of minced viable tissue, (2)

bits of ground tissue, (3) bits of ground tissue treated 1 hour at room temperature with hypertonic salt solution, (4) bits of ground tissue treated with hypertonic salt solution and then frozen and thawed once, suspended in mixtures of serum (1 part), Tyrode's solution (2 parts), and vaccine virus; (5) supernatant fluids (1 part), obtained after the first centrifugation of the extracts described above, mixed with rabbit serum (1 part), Tyrode's solution (1 part), and vaccine virus. These controls were handled, titered, incubated, and again titered in a manner similar to that used for cultures set up with the supernatant fluids obtained after the second centrifugation of the tissue extracts.

In certain instances, in addition to the controls already enumerated, plasma-tissue preparations of the minced tissues, the ground tissues, the tissues treated with hypertonic salt solution at room temperature, and the tissues treated with hypertonic salt solution and then frozen and thawed once were also made to determine if the treatment with the salt solution followed by a single freezing and thawing really killed the cells. These controls were made when the extracts were prepared from rabbit testicles and chick embryos because such tissues are highly suitable for cultivation in plasma. The usual cover-slip method was employed in which bits of tissue were embedded in thoroughly centrifuged rabbit plasma undiluted or diluted with an equal amount of Tyrode's solution and clotted with chick embryo extract that had been freed from viable cells by means of centrifugation and repeated freezing (3 times with CO_2 snow) and thawing. The cultures were incubated at 37°C. for a week and were examined daily for evidences of growth of cells.

Sterility of Cultures.—Each culture was tested for the presence of ordinary aerobic and anaerobic bacteria, and all cultures contaminated with such organisms were discarded.

Rabbit Kidney Extracts

Three attempts to cultivate a dermal strain of vaccine virus in kidney extracts prepared in the manner described above were unsuccessful. The results of one experiment are summarized in Table I and show that the virus did not grow in the extracts nor in the presence of viable kidney tissue suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts), but did increase in amount in the medium composed of bits of testicular tissue suspended in a mixture of kidney extracts (1 part), serum (1 part), and Tyrode's solution (1 part). One attempt to cultivate the Levaditi neuro-vaccine virus in kidney extracts was also unsuccessful. The results of this experiment are summarized in Table II and reveal that the virus multiplied abundantly in the extract media if bits of viable testicular tissue were added, but failed to increase in amount in any of the other media.

TABLE I

Cultivation of Vaccine Virus in Kidney Extracts

Time of titration	Mixed kidney extracts plus testicular cells, serum, and Tyrode's solution	Serum, Tyrode's solution, and kidney cells	Kidney extract prepared at room temperature plus serum and Tyrode's solution	Kidney extract prepared by freezing plus serum and Tyrode's solution
Before incubation	20,000	20,000	20,000	20,000
After incubation for 4 days	2 flasks pooled 100,000	3 flasks pooled 1,000	Flask 1 100 " 2 100 " 3 100	Flask 1 100 " 2 100 " 3 100

Summary of results obtained in an attempt to cultivate a dermal strain of vaccine virus in extracts of rabbit kidney. The strain of virus employed has been cultivated for several years in modified tissue cultures. The virus multiplied in the medium containing testicular cells but decreased in amount in the extract media and in the medium containing kidney cells. The titer of the virus varied very little, if any, in the different flasks containing extract media.

TABLE II

Cultivation of Vaccine Virus in Kidney Extracts

Time of titration	Mixed kidney extracts plus minced testicular tissue	Minced kidney tissue	Ground kidney tissue	Kidney tissue salted at room temperature	Kidney tissue salted and frozen	1st supernatant from kidney tissue salted at room temperature	1st supernatant from kidney tissue salted and frozen	2nd supernatant from kidney tissue salted at room temperature	2nd supernatant from kidney tissue salted and frozen
Before incubation	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000
After 4 days incubation. 3 flasks pooled	1,000,000	10,000	1,000	10	10	100	1,000	100	100

Summary of results obtained in an attempt to cultivate the Levaditi neuro-vaccine virus in extracts from rabbit kidney tissue. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. Multiplication of the virus occurred in the extract medium containing viable testicular cells; the virus maintained its original titer in the medium containing minced kidney tissue; the titer decreased in all other media.

Rabbit Testicle Extracts

One attempt to cultivate a dermal strain of vaccine virus in rabbit testicular extracts was unsuccessful. The results of the experiment, summarized in Table III, show that the virus multiplied in media containing minced and ground tissues, but failed to grow in other kinds of media. Plasma cultures of the minced tissue, the ground tissue,

TABLE III

Cultivation of Vaccine Virus in Testicular Extracts

Time of titration	Minced testicle tissue	Ground testicle cells	Testicle cells treated with salt at room temperature	Testicle cells treated with salt and frozen	1st supernatant from medium prepared at room temperature	1st supernatant from medium prepared by freezing	2nd supernatant from medium prepared at room temperature
Before incubation	1,000	1,000	1,000	1,000	1,000	1,000	1,000
After 4 days incubation. 3 flasks pooled	1,000,000	10,000	Undiluted	1,000	100	10	10
Percentage of plasma preparations showing growth of cells	100	37.5	12.5	5.5			

Summary of results obtained in an attempt to cultivate a dermal strain of vaccine virus in extracts of testicular tissue. The strain of virus has been propagated by serial testicular passages in rabbits and a testicular emulsion containing the virus was used to inoculate the cultures. The cultures contained the material listed at the head of each column plus serum and Tyrode's solution. The virus multiplied in the media containing minced testicle and ground testicle. The multiplication, however, was distinctly less marked in the latter. No multiplication occurred in any of the other media. It will be noted that grinding injured the cells considerably but that treatment with salt and freezing (once) did not kill all of the cells.

and the tissues treated with hypertonic salt solution and freezing and thawing once were made. The percentage of slides that showed growth of cells is also recorded in Table III and indicates that all of the cells in testicular tissue were not killed by the method employed by Eagles and Kordi (4) in the preparation of their extracts. An attempt to cultivate the Levaditi neuro-vaccine virus in testicular extracts was

also unsuccessful. In this experiment one set of subcultures was made and the results of the work recorded in Table IV show that the virus multiplied only in the media containing minced testicular tissue.

Chick Embryo Extracts

One attempt to cultivate a dermal strain of vaccine virus in extracts of chick embryos was unsuccessful. In this experiment a set of sub-

TABLE IV

Cultivation of Vaccine Virus in Testicular Extracts

Time of titration	Minced testicular tissue	Ground testicular tissue	Testicular tissue salted at room temperature	Testicular tissue salted and frozen	1st supernatant from testicular tissue salted at room temperature	1st supernatant from salted and frozen testicular tissue	2nd supernatant from testicular tissue salted at room temperature	2nd supernatant from salted and frozen testicular tissue
Before incubation	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000
1st culture. 3 flasks pooled	10,000,000	100	100,000	100	10,000	10,000	10,000	10,000
2nd culture. 3 flasks pooled	10,000,000	Undiluted	10	—	—	—	—	—

Summary of results obtained in an attempt to cultivate the Levaditi neurovaccine virus in extracts from rabbit testicular tissue. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. Each set of cultures was incubated 4 days. Multiplication of the virus occurred only in the medium containing minced testicular tissue.

At the time transfers were made, the virus underwent a twenty-fivefold dilution.

— indicates that no virus was demonstrable.

cultures was also made. Plasma cultures of the minced tissue, the ground tissue, and the tissues treated with hypertonic salt solution and a single freezing and thawing were set up. The results obtained are recorded in Table V and reveal (1) that the virus multiplied only in the media containing bits of minced tissue, ground tissue, or tissue treated with hypertonic salt solution for 1 hour at room temperature; (2) that all chick embryo cells were not killed by grinding and treatment with hypertonic salt solution and freezing and thawing once.

Figs. 1 and 2 are photographs of the growth of cells that had been treated in such a manner.

Two attempts to cultivate the Levaditi neuro-vaccine virus in extracts of chick embryos were unsuccessful. In each instance, a fresh testicular virus emulsion was used to initiate the cultures and one set of subcultures was made. In addition to the control cultures usually prepared, one was made with cells frozen (CO_2 snow) and thawed 5

TABLE V

Cultivation of Vaccine Virus in Chick Embryo Extracts

Time of titration	Minced embryo tissue	Ground embryo tissue	Cells salted at room temperature	Cells salted and frozen	1st supernatant from cells salted at room temperature	1st supernatant from cells salted and frozen	2nd supernatant from cells salted at room temperature	2nd supernatant from cells salted and frozen
Before incubation	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000
1st culture. 3 flasks pooled	100,000	100,000	>100,000	>100,000	1,000	100	1,000	100
2nd culture. 3 flasks pooled	1,000,000	1,000,000	1,000,000	100	—	—	—	Undiluted
Percentage of plasma preparations showing growth of cells	66	100	72.5	81.2				

Summary of results obtained in an attempt to cultivate a dermal strain of vaccine virus in extracts from chick embryos. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. Multiplication of the virus occurred in media containing viable cells; none occurred in any of the other media. The plasma preparations showed that treatment of chick embryo cells with salt and a single freezing did not kill all of the cells.

At the time transfers were made, the virus underwent a twenty-fivefold dilution.

The first set of cultures was incubated 3 days; second set, 4 days.

— indicates that no virus was demonstrable.

times. Plasma cultures of the minced tissue, the ground tissue, the tissue treated with hypertonic salt solution and freezing (ice box) and thawing once, and the tissue frozen (CO_2 snow) and thawed 5 times were also set up. The results obtained in one of the experiments are summarized in Table VI and show (1) that the virus multiplied in 2 successive cultures only in the medium containing bits of minced tissue; (2) that growth of cells was found in plasma preparations of

minced tissue, ground tissue, and tissue treated with hypertonic salt solution and freezing (ice box) and thawing once, while no growth was observed in preparations of cells frozen (CO_2 snow) and thawed 5 times.

TABLE VI

Cultivation of Vaccine Virus in Chick Embryo Extracts

Time of titration	Minced embryo tissue	Ground embryo tissue	Cells salted at room temperature	Cells salted and frozen in ice box	Cells salted and frozen with CO_2 snow	1st supernatant from cells salted at room temperature	1st supernatant from cells salted and frozen in ice box	2nd supernatant from cells salted at room temperature	2nd supernatant from cells salted and frozen in ice box
Before incubation	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000
1st culture. 3 flasks pooled	>1,000,000	>1,000,000	>1,000,000	100	100	1,000	1,000	100	1,000
2nd culture. 3 flasks pooled	1,000,000	100	100	—	—	—	—	10	—
Percentage of plasma preparations showing growth of cells	100	83	78	67	0				

Summary of results obtained in an attempt to cultivate the Levaditi neuro-vaccine virus in extracts from chick embryos. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. One set of subcultures was made. Multiplication of the virus in 2 successive sets of cultures took place only in the medium containing minced chick embryo tissue. The plasma preparations showed that treatment of chick embryo cells with salt and freezing (once at −10°C.) did not kill all the cells, while no growth of cells was obtained from tissue that had been frozen (CO_2 snow) and thawed 5 times.

At the time transfers were made, the virus underwent a twenty-fivefold dilution.

The first set of cultures was incubated 4 days; the second set, 3 days.

— indicates that no virus was demonstrable.

SUMMARY AND CONCLUSIONS

We have made ten attempts to cultivate vaccine virus in tissue extracts prepared according to the method described by Eagles and Kordi (4). Renal, testicular, and chick embryo extracts were employed with a dermal strain of vaccine virus and with the Levaditi strain of neuro-vaccine virus. In no instance were we able to show that the virus multiplied in the extract media. Both of these strains of virus, however, multiplied in media containing bits of minced viable tissue.

Furthermore, treatment of rabbit testicular tissue and chick embryo tissue in the manner described by Eagles and Kordi for the preparation of the extracts leaves some cells not only alive but capable of proliferation. Although the results of our work are not in accord with those obtained by Eagles and Kordi, we offer no explanation for the discrepancy. Nevertheless, one cannot examine the results of our work recorded in the six tables without recognizing the fact that in the types of media used the presence of viable cells appears to be essential for the multiplication of vaccine virus. Rabbit testicular tissue and bits of chick embryos support the regeneration of the active agent more efficiently than does rabbit renal tissue.

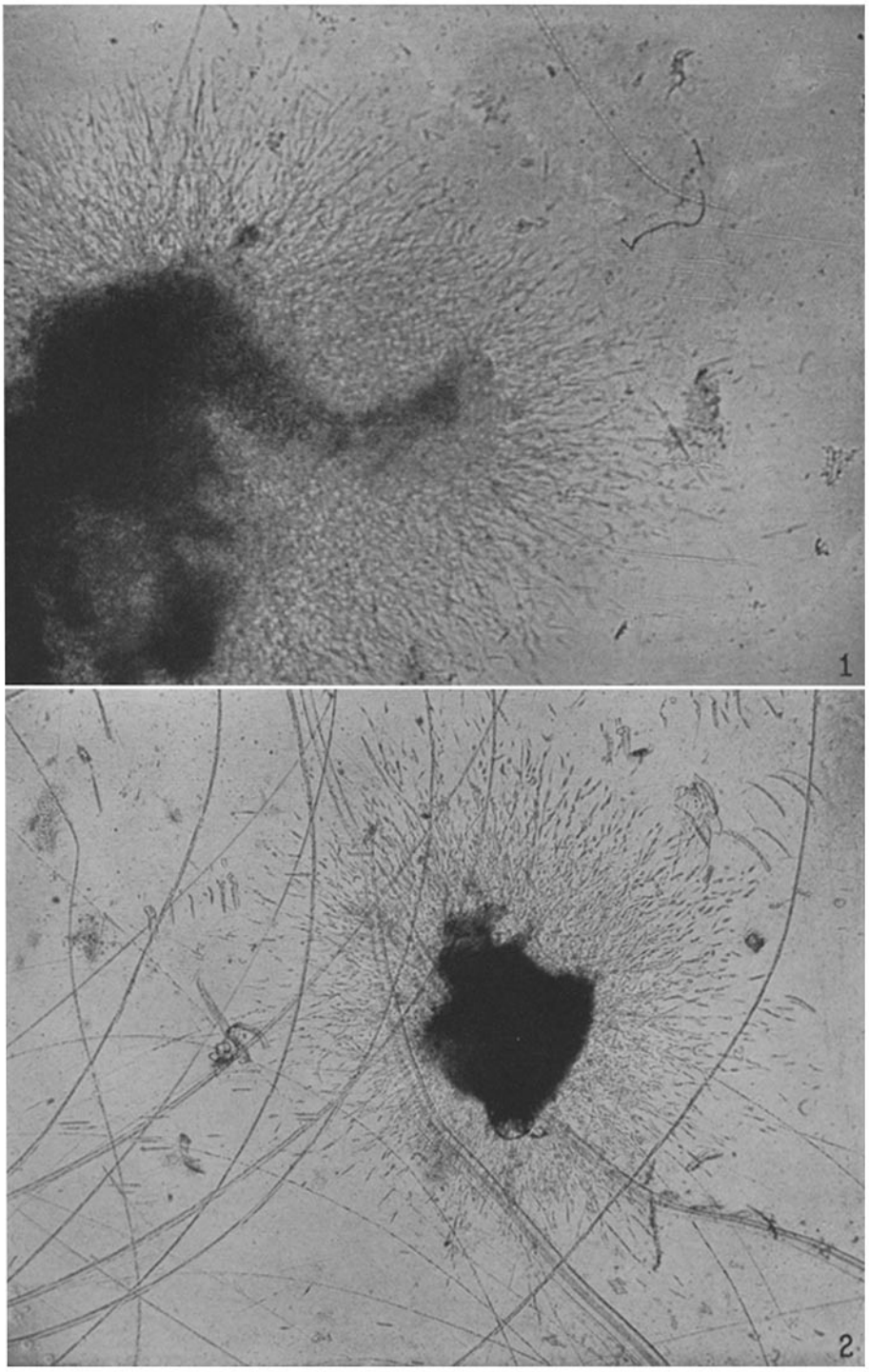
REFERENCES

1. Rivers, T. M., and Ward, S. M., *J. Exp. Med.*, 1933, **57**, 51.
2. Eagles, G. H., and McClean, D., *Brit. J. Exp. Path.*, 1930, **11**, 337.
3. Eagles, G. H., and McClean, D., *Brit. J. Exp. Path.*, 1931, **12**, 97.
4. Eagles, G. H., and Kordi, A. H. H., *Proc. Roy. Soc. London, Series B*, 1932, **111**, 329.
5. Rivers, T. M., *J. Exp. Med.*, 1931, **54**, 453.
6. Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.

EXPLANATION OF PLATE 45

FIG. 1. Photograph of a tissue culture prepared from chick embryo tissue that had been treated with hypertonic salt solution for 1 hour at room temperature. Note the evidence of active growth of cells. Unstained. × 30.

FIG. 2. Photograph of a tissue culture prepared from chick embryo tissue that had been treated with hypertonic salt solution followed by freezing (ice box) and thawing once. Note the evidence of active growth of cells. Unstained. × 30.



Photographed by Louis Schmidt

(Rivers and Ward: Vaccine virus in lifeless media)