FURTHER STUDIES ON TYPHUS FEVER

On Homologous Active Immunization against the European Strain of Typhus Fever

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(Received for publication, June 23, 1936)

It appears justified to conclude from the studies of Weigl (1), and from our own work with Castaneda, that active immunization against typhus fever with killed *Rickettsiae* can be achieved, provided a sufficient accumulation of the organisms can be obtained for vaccine preparation. Such mass production of *Rickettsiae* is also an indispensable preliminary for the immunization of horses, donkeys, or goats in the preparation of sera for temporary prophylaxis and for therapeutic application in man.[1] With the murine strains of typhus, as we have reported in preceding papers (2), such *Rickettsia* accumulation has been achieved by a number of methods, all of which depend upon the intraperitoneal infection of rats in which resistance had been reduced by a variety of methods. All such attempts, even the use of preliminary X-ray radiation of the animals (the most satisfactory of the methods employed), have completely failed when the European virus is used. Indeed, our persistent failures in this regard are one of the most convincing reasons for our belief, elsewhere set forth, that the agents of the murine and European infections—so closely related in many of their attributes—are nevertheless distinct and biologically fixed varieties, not permanently convertible one into the

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[1] In this respect, the situation appears to be not unlike that revealed by recent investigations for virus immunization—namely, that for the production of any degree of immunity with killed virus, large quantities of the material are required. It is not impossible that this point of similarity between infectious agents otherwise so far apart in biological attributes may be related to the tendency for intracellular localization which characterizes the pathogenic behavior of both.

other by temporary environmental or experimental manipulations. These matters have been discussed by one of us in another place (3). The important practical consideration which follows from our observations is the fact that for homologous vaccine production with the European *Rickettsiae*, the rat technique is of no value.

In regard to the methods at present available for artificial immunization against the European disease, it appears quite likely that Weigl's phenol-killed louse vaccine is effective in producing a demonstrable degree of immunity. But Weigl's vaccine production necessitates the passage of the virus through large numbers of lice and appears obviously unsuited to the mass immunization desirable for practical purposes in times of epidemic spread. Our own vaccines prepared with the murine strain from rats can be produced in quantity and do protect guinea pigs to a measurable degree from European infection. But the protection is not as complete as similar vaccination against the homologous strain. There is also some protective action (possibly also some therapeutic effect) of the antimurine serum against the European infection. Guinea pig experiment has demonstrated this (4). But the few human cases of the European disease treated with this serum do not justify even a preliminary opinion. That there should be some overlapping in these respects is to be expected from the cross immunity observed in animals which have recovered from infection with the respective viruses and from the serological overlapping observed by one of us with Castaneda (5). But in view of the seriousness of the endemic and epidemic problems of the European disease, it would nevertheless be of the greatest practical importance were it possible to develop a method applicable on a large scale and not too complicated, for homologous immunization with the *Rickettsiae* of the European disease.

In endeavoring to find other methods of approach to the problem of active and eventually passive immunization against this disease we have followed two directions of study: 1. The development of tissue culture vaccine. 2. Serovaccination.

1. Tissue Culture Vaccines

In preparing tissue cultures of the European *Rickettsiae*, we have followed with little modification the Maitland method successfully

adapted to this purpose by Nigg and Landsteiner (6). In attempting to improve this method a considerable amount of work has been done which may be briefly summarized, since it may serve to save time for others working along similar lines.

Rickettsiae are extremely selective in regard to the tissue. No growth has been obtained when guinea pig kidney, liver, brain, or heart muscle was substituted for tunica scrapings. Slight growth was obtained with scrapings of the lining cells of the peritoneum. Rabbit tissues have given negative results. Chick embryo likewise.

It was at first thought that guinea pig serum was essential. We have recently found, however, that one part in four of either horse serum or human serum mixed with Tyrode solution before filtration gives even better growth than the guinea pig-serum Tyrode mixtures. It is still to be determined whether there is active metabolism as determinable by oxygen consumption on the part of the guinea pig tissue in horse serum and human serum mixtures. No growth has been obtained in Tyrode solution without serum. Filtered ascitic fluid, substituted for Tyrode solution, if free from bile, gives much heavier growth than the Tyrode-serum mixtures, though individual ascitic fluids vary.

Like Nigg and Landsteiner, we have found that the cultures remain virulent and *Rickettsiae* survive when the flasks are stored without removal of the stoppers. In this manner, a tissue culture of the Mexican strain has been found virulent after 7½ months and a European culture for 4½ months. One of our students, Dr. C. J. Wu, took a sealed murine culture to China, without precautions of refrigeration, and writes us that he obtained successful inoculation on arrival.

The *Rickettsiae* will not grow on tunica tissue heated to 50°C. for 15 minutes. Nigg and Landsteiner (7) reported occasional slight temperature reactions and immunization in guinea pigs after the inoculation of a third generation culture on tissue thus heated, although no *Rickettsiae* could be seen in the material inoculated. They reported the facts without drawing conclusions, but Laigret and others have taken this to indicate the presence of an invisible form of typhus virus, perhaps in cyclic relation with the *Rickettsiae*. We have repeated these experiments a number of times and have in no case obtained either temperature reactions or subsequent immunity in guinea pigs inoculated with materials from the second or third heated tissue culture generations. We venture to suggest that Nigg and Landsteiner's results may have been due to the survival of a few *Rickettsiae* carried over from flask to flask.

Although tissue in such cultures ceases to respire after about 40 hours, as measured in the Warburg apparatus, and the viability of the cells is unlikely to last longer than a week, the most active proliferation of *Rickettsiae* appears to take place after the 4th day. An equilibrium of some kind seems to be established which creates conditions favorable for growth. These conditions, as far as respiration, reaction, and oxidation-reduction potentials are concerned, are being investi-

gated. Assuming the establishment of such an equilibrium, uninoculated tissue cultures were preserved in the incubator for a varying number of days and then inoculated, but no successful cultures were thus obtained.

Efforts are being continued to adapt the Nigg and Landsteiner method to larger quantitative yields by the use of specially designed flasks. It appears that for successful cultivation a definite relationship between the square surface exposed by the fluid and the cubic air space of the closed flasks must be maintained.

For the present studies our methods of tissue culture vaccine production have not varied—except for the frequent substitution of ascitic fluid for Tyrode solution, from the Nigg-Landsteiner procedure.

The source of the culture virus—in three separate isolations, was the material obtained by mincing or by scraping the tunica vaginalis of guinea pigs injected intraperitoneally with large doses of blood and brain mixtures from previously infected animals. Such guinea pigs show, in about 20 per cent of the inoculated, slight scrotal swelling with a few *Rickettsiae* in cell smears. The cultivated strains have been carried on in our laboratory for about 2 years.

The optimum time for the harvesting of such cultures lies between the 8th and the 10th days.

The production of the vaccines has been more or less the same as that employed for the same purpose by Kligler and Aschner (8). Culture flasks are pooled and centrifugalized. The supernatant fluid—in which the numbers of organisms are negligible—is discarded. The bits of tissue are shaken in salt solution containing 0.2 per cent formalin. This removes most of the obtainable *Rickettsiae*. The tissue is again thrown down in the centrifuge and can be further disintegrated by freezing and thawing in a beaker of solid carbon dioxide in ethylene-glycol-monomethyl ether—the solution used with the Florsdorf-Mudd apparatus. This is done in pointed centrifuge tubes accompanied by grinding. The ground tissue is added to the washings previously obtained. Standardization of such vaccine has so far been impossible on any basis sufficiently accurate to justify description. The amounts of vaccine employed are based on the assumption that the cultures contain approximately equivalent numbers of *Rickettsiae*. 1 cc. represents the yield of about one-fifth of a small (25 cc.) flask, containing a total volume of 2.5 to 3 cc. of culture fluid.

With such vaccines, we have carried out a number of immunization experiments. All vaccine injections were subcutaneous. The results of our first fifteen vaccinations were as follows:

One Injection of Vaccine.—A single vaccine injection of 1 cc. Immunity test 1 month later, with brain virus. Temperature touching 104°F. on 8th, 9th, and 10th days. Mild typhus.

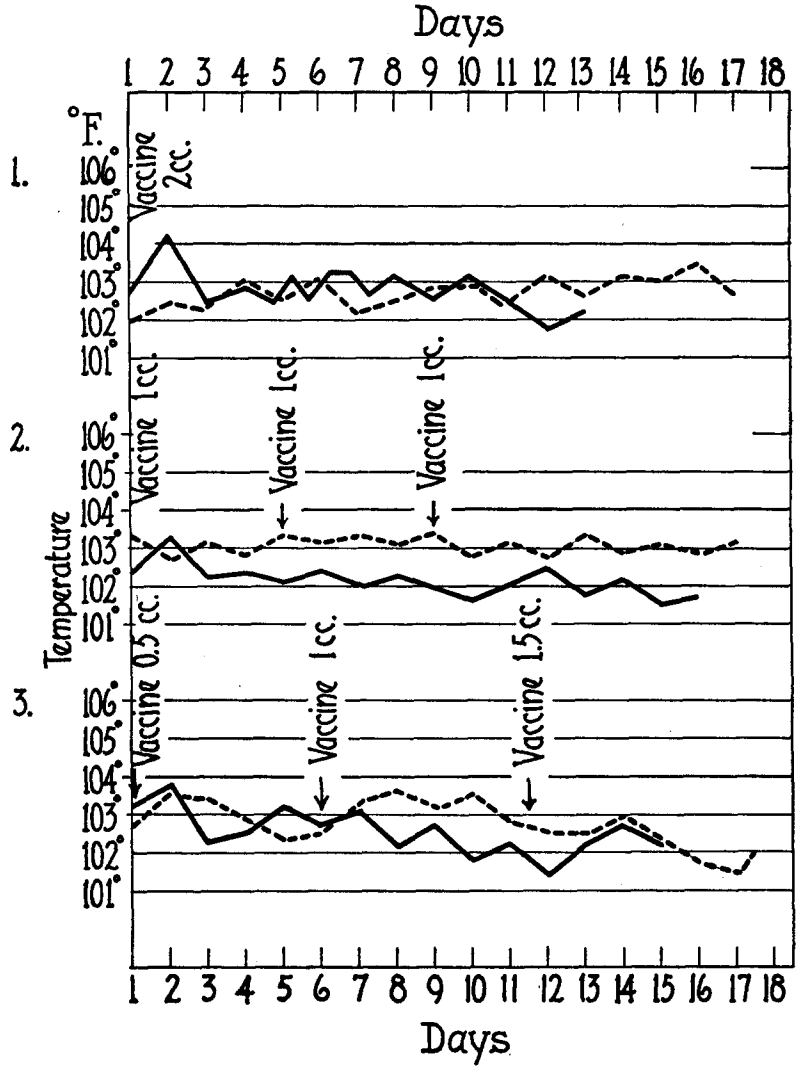

CHART 1. The results of the subcutaneous injection of formalinized tissue culture vaccine in three different types of experiment. The arrows indicate the time of vaccine injections and the amounts. The broken lines represent the results of immunity tests carried out by intraperitoneal inoculation of doses of European brain virus, which gave severe typhus in controls. In curve 1, the immunity test was carried out 33 days after vaccination; in curve 2, 28 days, and in curve 3, 37 days after vaccination.

Two guinea pigs. *A single vaccine injection of 2 cc.* Immunity test 1 month later. No typhus.

Two Injections of Vaccine.—Five animals were given two vaccine injections, 5 days apart, 1 cc. at each injection. Three of these animals were reinjected after 2 months with heavy doses of brain virus (3 to 5 cc.). All showed mild typhus temperature curves, rising above the critical point of 104°F. for 2, 3, and 4 days respectively. There was apparently a moderate increase of resistance but no immunity.

The other two were similarly reinfected 6 weeks after vaccination and were found immune.

Three Injections of Vaccine.—Eight animals received three injections of the culture vaccine, the intervals varying in the individual experiments from 3 to 4 days. In three of these guinea pigs the dosages were 0.5 cc. at each injection. In others the doses were 1 cc. each and in four animals the doses were 0.5 cc., 1.0 cc., and 1.5 cc. respectively. Intervals between vaccination and immunity tests were from 30 to 38 days. All of these animals were found immune when reinoculated with virus controlled and found potent in normal animals.

Chart 1 shows the results of tissue culture vaccination in a number of animals.

From these experiments it is clear that, as Kligler and Aschner have reported, it is quite possible to obtain effective active immunization in guinea pigs against the classical European virus with formalinized homologous tissue culture vaccines. The experiments further indicate that such immunity may last at least a month and that successful vaccination requires a definite minimum of killed *Rickettsia* material. That a single sufficiently large dose of virus may suffice is indicated in Chart 1. The best results were obtained when three separate injections of vaccine were administered.

2. Serovaccination

While it is always preferable to make use of killed rather than of living infectious agents for immunizations which are intended for eventual application to diseases of man, it has, nevertheless, appeared important to us to explore all the possibilities which offer some hope of practical solution of such large scale problems as those involved in epidemics of European typhus fever.

Our attention was turned to serovaccination by an incidental observation made by one of us, with Batchelder, in 1930 (9). At that time, a guinea pig which had been used for neutralization tests of conva-

lescent guinea pig serum against homologous (murine) virus and found fully protected, was reinoculated several weeks later, and failed to exhibit any symptoms of typhus.

Serovaccination has given encouraging results in the past in the immunization of animals against a variety of diseases caused by filtrable virus agents and, within recent years, has been successfully employed in the case of yellow fever, by Sawyer and his collaborators (10). To be sure our own experience with herpes, where fully neutralized virus invariably failed to induce even a partial immunity, was not encouraging. But here, as in poliomyelitis, the immunological problem is complicated by the unsolved difficulties involved in neural transport and in the blood-brain barrier. Between those virus diseases, in which these factors do not enter into consideration, and the *Rickettsia* diseases, there is a considerable immunological similarity, possibly because of the analogous intracellular localization of the responsible infectious agents.

Basing our efforts on these considerations and on the observation cited above, we accordingly proceeded to treat series of guinea pigs with a variety of adjusted mixtures of European typhus virus and protective sera, employing combinations of the two in the following forms.

1. Virus in the form of infectious defibrinated guinea pig blood, or of virulent guinea pig serum, plus convalescent guinea pig serum taken 4 or 5 days after defervescence.

2. Similar defibrinated blood, plus antimurine (heterologous) immune horse serum.

3 and 4. Virulent brain suspension with each kind of serum respectively.

5 and 6. Tissue culture virus with each kind of serum respectively.

The technique, except in those cases in which brain virus was used, consisted in the preparation of measured mixtures of virus and serum, allowing these to stand for 10 minutes at room temperature, and then injecting intraperitoneally.

A major difficulty encountered in some of these studies was the fact that our original typhus horse, which had been yielding a potent antityphus serum, died at this time and all our stocks of active serum contained preservatives. The new horse was delivering a serum of

which the Weil-Felix reaction had not yet exceeded a titer of 1 to 80 (++++), 1 to 160 (+). With a stronger serum it might have been possible to make more accurate adjustments and this, of course, will be done later.

Experiments in which infectious brain was used as virus were on the whole unsatisfactory because, as we have reported in a preceding publication, in order to protect against brain virus in which the injected *Rickettsiae* are presumably intracellular, it is necessary to administer the protective serum in several injections from 24 to 48 hours after inoculation of the virulent material. The probable reasons for this have been discussed elsewhere (4). By following this technique it has indeed been possible, in two experiments of the present series, to immunize guinea pigs by inoculations of brain virus completely neutralized by serum given 24 and 48 hours later as indicated. This method is, however, less certain and more complicated than other procedures to be reported.

Two early experiments gave no information because we used doses of virus so small that even the control animals failed to show the usual symptoms of experimental typhus infection.

As the amounts of virus were increased to points at which the unprotected controls showed late and short lived typhus reactions, the serovaccinated animals, fully protected as regards the vaccination mixtures, did not prove to be immune. (Two experiments.) It was obviously necessary, for successful serovaccination, to make use of mixtures which contained enough virus to produce vigorous experimental typhus in the controls.

In subsequent experiments, therefore, the amounts of virus in the mixtures were so adjusted that the control animals in which normal serum and salt solution were used, reacted with a severe and prolonged experimental typhus. When this technique was employed the results were of two types which we may designate as complete and partial, these terms referring to the degrees of neutralization of the original mixtures.

In the complete experiments, of which there are four to date, the animals receiving the serum-virus mixtures remained entirely normal as far as the usual criteria of experimental typhus are concerned, while the normal serum and salt solution virus controls reacted severely.

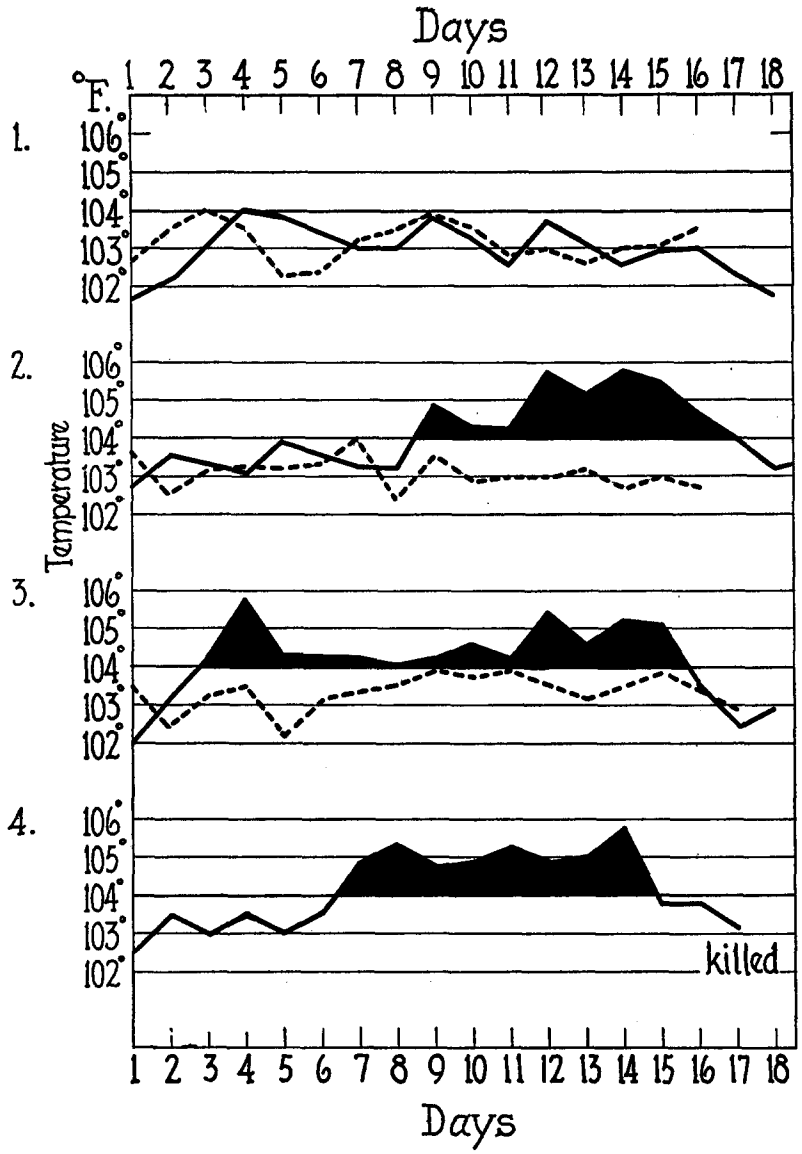

CHART 2. In this experiment serovaccination was carried out on four guinea pigs with mixtures containing, respectively, 0.5 cc. of tissue culture virus (representing about 0.1 cc. of a tissue culture) and 1 cc. of convalescent guinea pig serum, taken from an animal that had passed through a typical European typhus, on the 4th day after defervescence. The mixtures were allowed to stand for 10 minutes at room temperature and injected intraperitoneally.

In this chart, the first curve represents one of three serovaccinated animals. One only is charted, since they were all alike and it was considered particularly important to chart all the controls. In all the curves, the solid blocks represent temperatures above 104°F. The broken lines represent the temperatures after reinoculation with virulent brain emulsion for immunity test. Curve 2 represents the normal serum control. Curve 3 represents the salt solution control. Curve 4 is the control of the brain virus used for immunity test 1 month after the serovaccination.

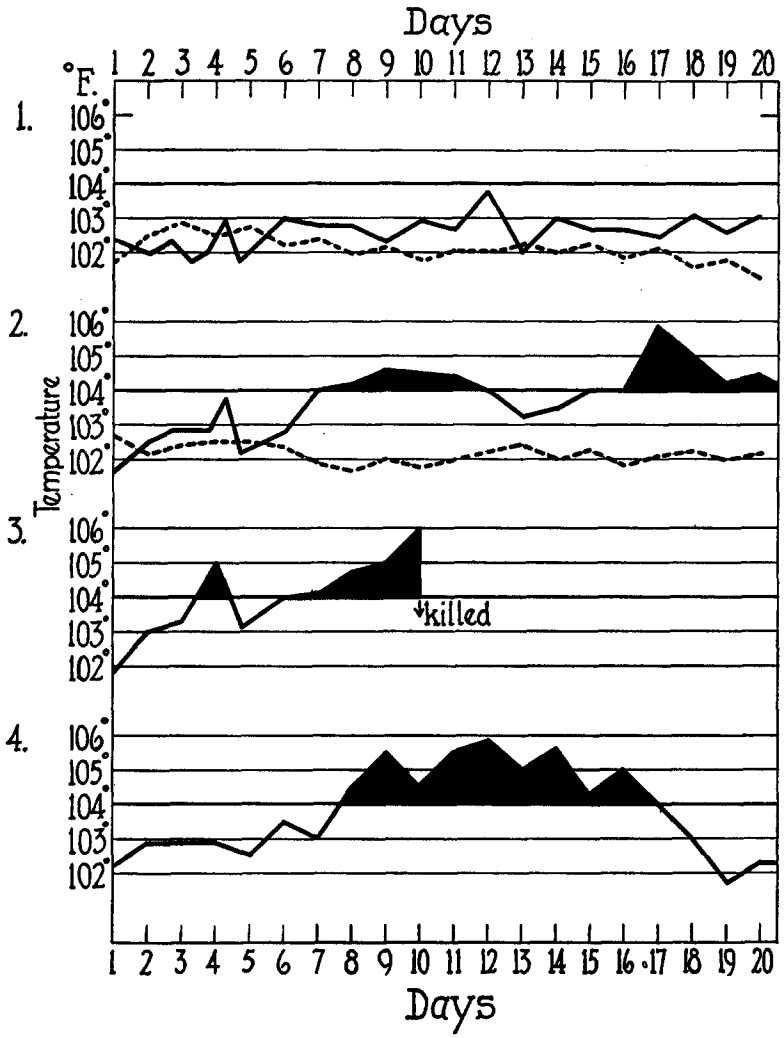

CHART 3. In this experiment, two guinea pigs received 1.5 cc. of virulent defibrinated guinea pig blood mixed with 0.5 cc. of the serum of our second horse, which was being immunized with murine (heterologous) *Rickettsiae* obtained by the X-ray rat method. For reasons stated above, the Weil-Felix titer of this serum did not exceed 1–80 at the time of this experiment. The mixtures were allowed to stand for 10 minutes at room temperature and injected intraperitoneally. One only of the serovaccinated animals is charted, since both were essentially alike in result and it appeared important to chart the controls completely. Temperatures above 104°F. are blocked in black. Broken lines represent the results of immunity tests carried out with virulent brain emulsion of European typhus, intraperitoneally injected 2 months after serovaccination. Curve 2 is the vaccination control in which the virulent blood was mixed with 0.5 cc. of normal horse serum. Curve 3 is the saline control of the virus used for the original vaccination. Curve 4 proves the virulence of the virus employed for reinoculation.

The test animals proved immune when reinoculated with large doses of virus a month or so later. Charts 2 and 3 illustrate such experiments.

In the partial experiments, of which four have been completed at the present time, the relative amounts of virus and of serum in the vaccinating mixtures were such that there was a slight excess of virus over neutralizing power. From the practical point of view these experiments seem to us of considerable importance in that they show that even when complete neutralization is not achieved in the experimentally difficult adjustment between the two reacting factors, the severity of the infection is almost always mitigated, sufficiently to prevent serious infection. A simple table will serve to illustrate these conditions as follows:

Type of serovaccination	Serovaccinated		Normal serum and virus controls	
	Incubation	Duration of fever	Incubation	Duration of fever
	days	*days*	*days*	*days*
1. Virus defibrinated guinea pig blood 2 cc.	16	1	7	10
Immune horse serum 0.5 cc.	16	2	8	8
2. Tissue culture virus 0.1 cc.	11	4	5	10
Convalescent guinea pig serum 0.5 cc.	11	2	5	8
	11	2	—	—
3. Tissue culture virus 0.1 cc.	No fever whatever		3	10
Immune horse serum 0.5 cc.	11	1	5	8
	11	1		
4. Tissue culture virus 0.05 cc.	Two "spikes" of temperature on 4th and 14th days		6	7
Convalescent guinea pig serum 1 cc.				
	13	1	3	10
	11	4	3	11
	11	4		

A chart of one of these partial experiments further illustrates these conditions (Chart 4).

It is clear from the experiments recorded that guinea pigs can be immunized against European typhus virus by preliminary injections of mixtures of living virus and protective serum so adjusted that

no experimental typhus results from the injections. It has further been shown that when the perfect balance between the two active factors is not achieved and a moderate excess of virus is present, the serum almost invariably reduces the severity of the infection.

An important question arises in connection with the serovaccination experiments in regard to the fate of the living virus injected in such cases. It is quite conceivable that although such virus is neutralized by the serum with which it is mixed and thereby prevented from causing experimentally determinable infection, it might still remain

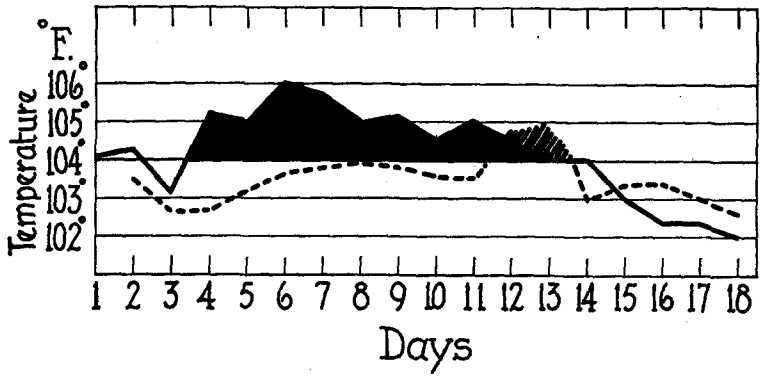

CHART 4. One of the partial serovaccination experiments. The temperature curve represented in broken lines is that of a guinea pig which received, intraperitoneally, 0.1 cc. of tissue culture virus mixed with 1 cc. of homologous convalescent guinea pig serum. That charted in solid lines represents the reaction in a guinea pig injected with the same amount of virus mixed with normal guinea pig serum.

alive and convert the vaccinated animals into carriers. The importance of such a possibility in connection with any attempts to apply serovaccination to man is obvious. In attempting to approach this problem experimentally we have injected large quantities of brain and blood (½ brain plus 4 cc. of defibrinated blood) from a successfully serovaccinated animal, killed on the 24th day after the vaccination, into other guinea pigs. The animals so injected showed no reactions whatever. Such tests will require elaboration and, whatever the outcome, it must be borne in mind that in this regard particularly no generalization from guinea pig experiment to man is permissible.

DISCUSSION

Although the manner of transmission and the rôles played by insects and by rodents in the dissemination of the typhus fevers is fairly well understood, experience has shown that sanitary measures alone do not, as yet, suffice to control these diseases when circumstances favor epidemic spread. The situation would be materially improved were methods available for specific prophylaxis applicable to infected populations on an adequate scale. In the case of the Mexican (murine) type, vaccine and serum production by methods which depend upon the accumulation of *Rickettsiae* in rats in which resistance has been experimentally reduced have been elsewhere described and are giving encouraging results. But while these murine products exert a certain degree of overlapping protective action on infection with the European virus, their effects in this respect are partial and imperfect.

While, in our opinions, the Weigl louse vaccine is about as effective as any that can be at present obtained, it is quite unlikely that a vaccine of this kind can ever be produced in quantities large enough to be of practical importance in epidemics.

The experiments above recorded have confirmed the effectiveness of formalinized European tissue culture vaccines in the guinea pig experiment. Such vaccines can be produced on a reasonable scale by a trained staff in a well equipped laboratory. Judging from a now extensive experience with injections of formalinized murine vaccines into man, human application of the tissue culture vaccines, with proper sterility control, would seem to be without danger. Our own efforts at present are being exerted toward the development of methods for increased quantity production, in observations on the duration of the active immunity obtained in guinea pigs and on the storage periods for which the vaccines will remain potent. In the meantime, by the now available methods, sufficient amounts of the tissue culture vaccines can be freshly produced for thorough testing in man.

It is not likely, however that, until one succeeds in increasing quantity production and in simplifying *Rickettsia* tissue culture methods, these vaccines can be freshly produced in amounts adequate to meet the serious emergencies of typhus epidemics. It is this consideration which has encouraged our interest in serovaccination. That such a method can be applied effectively is shown by our experiments. It is

equally obvious, however, that as yet it is quite impossible to set up any standard which will insure against moderate excess of virus over serum potency. Nevertheless, the materials for certain types of serovaccination (guinea pig blood and guinea pig convalescent serum) can be made available in considerable amounts with almost no equipment. The method is therefore at least worthy of further study for possible application in the face of destructive epidemics. Our efforts to overcome some of the difficulties involved in the adjustment of virus and serum for serovaccination, by the desiccation of tested virus and virus-serum mixtures *in vacuo* by the Florsdorf-Mudd method, have thus far failed. Such failure has been due to the deterioration of the European virus under these conditions, within 50 days, a period during which the murine virus, similarly desiccated, appears to retain full potency. These experiments are being continued.

SUMMARY

1. Guinea pigs can be actively immunized against European typhus fever with homologous formalinized *Rickettsia* tissue cultures, provided sufficient amounts are injected. The method is suggested for practical application in man.

2. Serovaccination against European typhus fever can be successfully applied to guinea pigs by a variety of methods, the simplest of which consists of the injection of mixtures of virulent defibrinated guinea pig blood and convalescent guinea pig serum taken from 3 to 5 days after defervescence. Similar results can be obtained with mixtures in which tissue culture virus, either with convalescent guinea pig serum or with antimurine horse serum, is used. There is no indication so far that such animals become carriers.

Possible application of these methods to typhus epidemics is discussed.

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