EXPERIMENTAL PNEUMONIA PRODUCED BY TYPHUS RICKETTSIAE *

M. RUIZ CASTANEDA, M.D.

(From the Typhus Laboratory, Department of Medical Research, General Hospital, Mexico City, Mexico)

After Prowazek and da Rocha-Lima demonstrated that the organism of typhus fever multiplied within the cells of the intestinal tract of infected lice, it was assumed that Rickettsiae were obligate parasites of certain animal cells. This was confirmed by Wolbach and coworkers [1] who observed intracellular Rickettsiae in the endothelium of the blood vessels of humans and animals infected with typhus. A few years later Mooser [2] discovered intracellular organisms in the mesothelial cells of the tunica vaginalis of typhus infected guinea pigs, and Zinsser and Castaneda [3] cultivated Rickettsiae in large numbers in the peritoneum of rats where the organisms multiplied readily within the serosal cells. Okamoto [4] reported that he had observed Rickettsiae in the alveolar cells of the lungs of mice infected by the intraperitoneal route. Recently Hitz,[5] in our laboratory, succeeded in cultivating Rickettsiae in minced guinea pig lung suspended in an ascitic-serum mixture. The cultures did not grow as readily as those made from the tunica vaginalis, but his findings corroborate indirectly those of Okamoto. He observed also, in cultures made from the tunica vaginalis, typical Mooser cells in close proximity to muscle fibers suggesting a relationship with the connective tissue sheaths, although the true nature of these cells has not been determined.

In a recent preliminary report [6] we showed that mice and rats could be infected by the intranasal route and that a considerable growth of Rickettsiae could be obtained in the lung. It was also stated that the lining of the bronchi was found to be parasitized with intracellular bodies. The cells were infected in such a manner that the epithelium resembled the gastro-intestinal tract of typhus infected lice.

The various types of cells in which Rickettsiae have been found

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show that the cellular requirements of the agent of typhus are not as limited as we had assumed. In this report we wish to confirm our first experiments and give further information concerning these observations.

Material and Methods

The strain of typhus used for the present experiments was our "L" orchitic strain isolated from a case of typhus in the General Hospital of Mexico City in 1936. This strain has been transferred over 150 times in male guinea pigs which have constantly shown the typical scrotal reaction.

Adult mice and young white rats were inoculated by the intranasal route by means of a 1 cc. pipette applied to the nostrils, administering very slowly a total of 0.2 cc. to the mice and 0.4 cc. or a little more to the rats. For inoculation the animals were anesthetized with ether and the anesthesia was repeated as necessary until the whole dose of inoculum was given.

The inoculum prepared from the guinea pig material was obtained by washing the tunica vaginalis with 1 cc. of an isotonic sodium citrate solution for each guinea pig used. The washings were centrifuged at low speed in order to remove gross particles. The inoculum prepared from the infected lungs of mice or rats was obtained by grinding the lungs with powdered sterile glass and emulsifying them in saline. The lungs of mice were suspended in 7 cc. and those of rats in 30 cc. each of saline. The emulsions were then centrifuged at low speed to remove the particles of glass.

Rabbits of various sizes were anesthetized with Dial (Ciba) by the intraperitoneal route in amounts of 0.55 cc. per kilo of body weight, and others with ether. When completely anesthetized 8 to 10 cc. of the inoculum was injected directly into the trachea. When desired, the body temperature of the inoculated rabbits was kept below 37° C. by repeating the injection of Dial. Three or 4 doses, each containing 75 per cent of the original dose, applied at convenient intervals of time, were sufficient to keep the animals 72 hours or more under narcosis and at low body temperature. As stated elsewhere,[7] low body temperature favors the growth of typhus Rickettsiae.

The lungs of the animals found dead or killed at various intervals of time after inoculation were placed in sterile Petri dishes

and plain or blood agar slants were smeared with small pieces of tissue. Smears and impressions on slides were made for direct examination and fragments of the lung were fixed for histological study.

The smears were stained by Giemsa's method and by our methylene blue-safranin stain. Tissues were fixed in Regaud's solution (potassium bichromate 3 per cent, sodium sulphate 1 per cent, and formalin 10 per cent). Sections were stained with a modification of one of Pappenheim's methods, recommended by Hitz,[5] as follows:

SOLUTION A

Distilled water	100 cc.
Glacial acetic acid	1 drop
May-Grünwald stain	20 cc.

SOLUTION B

Distilled water	100 cc.
Glacial acetic acid	1 drop
Giemsa's stain	5 cc.

After treating the sections with Solution A for 15 minutes, transfer without washing into Solution B and leave for 30 minutes to 1 hour. Both solutions have a better action at 37° C. Dehydrate rapidly with absolute alcohol and after clearing in xylol mount in cedar oil. This seems to be the simplest way of staining Rickettsiae in sections.

Experimental

The inoculation into mice of washings from the tunica vaginalis of typhus infected guinea pigs by the intranasal route gives rise to fatal results in a large percentage of the animals. The mice die usually about 96 hours after inoculation and show pneumonic lesions characterized by considerable hyperemia and hemorrhage of the lungs, which usually become completely involved. The affected lobes of the lungs resemble liver or spleen. The non-affected tissue shows a compensatory emphysema. If the lungs are left in a Petri dish for a few minutes there is an exudation of blood which soon coagulates.

Microscopic examination of the lungs shows that the capillaries are filled with blood and many extravasated red cells have invaded

the alveoli. There is also an infiltration by polymorphonuclear leukocytes, which are present in large numbers but do not suggest pus formation. Many leukocytes show various degrees of necrobiosis, mainly pyknosis. The cellular degeneration seems to involve the cells of the alveoli, bronchi and capillaries. The cytoplasm of many cells is swollen by considerable numbers of small organisms. Many of these cells appear to belong to the blood capillaries, but the epithelium of the bronchi is also found to be infected with the same parasite, presenting an appearance similar to that of the intestinal tract of typhus infected lice. The infected bronchial cells, as well as those scattered in the lungs, resemble the so-called Mooser cells, so characteristic of the lesions of the tunica vaginalis and the peritoneal infection of X-rayed, typhus infected rats. Extracellular organisms may also be seen but are not very numerous. In some animals ordinary bacteria are also present. Smears or impressions made from the lungs of infected animals show large numbers of small intracellular and extracellular organisms. Many polymorphonuclear leukocytes have phagocytozed these organisms. The general appearance resembles the smears made from the peritoneal exudate of X-rayed, typhus infected rats.

The inoculation of washings from the tunica vaginalis of infected guinea pigs into rats of various sizes has given very irregular results. Only a few animals have developed lesions in the lungs after inoculation. The microscopic appearance is similar to that observed in mice and Mooser cells are easily found.

Transfer of the Lung Infection by Means of a Lung Emulsion

Rats, mice and rabbits inoculated by the intranasal route with emulsions of the lung from infected animals died within 72 to 96 hours after inoculation with extensive lesions in the lung identical with those we have described.

It has been possible to transfer the infection from rat to rat for several generations with the same characteristic hemorrhagic lesions developing in the lungs. In one instance a "lung strain" was obtained from a rat which developed lesions after infection with washings from the tunica vaginalis of infected guinea pigs. This strain which killed the animals within 4 to 6 days was unfortunately lost on the 7th transfer.

Cultures on Plain or Blood Agar from Infected Lungs

Many attempts to cultivate ordinary bacteria were made, using as a medium plain or blood agar slants. For this purpose small pieces of lung were cut as far as possible from the large air ducts and were smeared on the slants. The mice infected by washings from the tunica vaginalis of infected guinea pigs showed few or no colonies on the slants after 48 hours incubation. In rats inoculated with emulsions from the lungs of mice, contaminating organisms were rarely found, but in transfers from rat to rat ordinary bacteria, usually Gram-negative, were cultivated on various occasions. These organisms were tested with typhus serum and did not give the agglutination reaction. Emulsions of the Gram-negative bacilli were inoculated into guinea pigs intraperitoneally and produced a peritoneal infection with death of the animal in from 48 hours to 8 days. The exudate showed the injected organisms, but Mooser cells were not found. The inoculation of large doses of the same Gram-negative bacteria into rats by the intranasal route failed to produce the hemorrhagic pneumonia shown by those inoculated with typhus material or with emulsion from lung transfers.

Resistance of Rats and Mice to a Second Intranasal Inoculation

The rats and mice which survived inoculation with either washings from the tunica vaginalis or emulsion of the lungs from infected mice were reinoculated 10 to 15 days later with an emulsion of the lungs from an infected mouse by the intranasal route. These animals survived the test.

Identification of the Etiological Agent Producing Hemorrhagic Pneumonia in Mice, Rats and Rabbits Inoculated with Typhus Material

Several guinea pigs were inoculated intraperitoneally with emulsions made from lungs showing typical hemorrhagic lesions and many Mooser cells. The material was obtained from rats infected with virus from the 3rd to the 7th transfer from lung to lung. As a control, typhus immune guinea pigs were injected with the same material.

In one instance both normal and immune guinea pigs died on the 3rd or 4th day after inoculation with a peritoneal infection by a Gram-negative bacillus similar to that isolated on agar cultures. No bodies resembling Rickettsiae were observed.

Two normal guinea pigs showed fever and swelling on the 4th day after inoculation and one died from peritonitis on the 6th day. The other animal was killed and material was transferred into another guinea pig. Smears from the tunica vaginalis showed typical Mooser cells, but a few bacteria were also seen. The guinea pigs inoculated with this material developed a peritoneal infection with a Gram-negative organism. The typhus immune guinea pig controls also died with peritonitis.

The inoculation of emulsion of the lung of the 5th generation from rat to rat produced in a normal guinea pig typical fever and swelling without intercurrent infection, while the typhus immune controls showed no fever or swelling. These animals were tested later with the "L" strain and showed no reaction at all.

A normal and an immune guinea pig were inoculated with an emulsion of the brain from a rat which was previously inoculated with washings from the tunica vaginalis of guinea pigs inoculated with material from the lung. The normal animal developed typical fever and swelling and was found to be immune when reinoculated with the "L" typhus strain. The immune guinea pig showed no reaction at all.

Guinea pigs were vaccinated with 2 doses of 1 cc. each, given subcutaneously, of an emulsion of organisms obtained from the lungs of infected rats. The suspensions made in formalinized saline were purified by fractional centrifugation and contained 3×10^9 organisms per cc. When the guinea pigs were tested 15 days after the first vaccination they were found to be immune to the "L" strain.

Purified emulsions of organisms found in the lungs of infected rats were submitted to microscopic agglutination tests. These were made by mixing a droplet of serum with a drop of the emulsion and adding a little methylene blue. The mixtures were placed in a hanging drop preparation and observed under the No. 40 objective. The mixtures containing normal human or guinea pig serum showed no agglutination. Those containing human convalescent typhus serum or immune guinea pig serum were aggluti-

nated within a short time. Care was taken to use the serums of guinea pigs bled before and at various intervals of time after the inoculation with orchitic typhus.

To this data we may add the further information that guinea pigs inoculated with minute amounts of emulsion of the lungs from mice inoculated with washings from the tunica vaginalis of guinea pigs, and from rats infected from such mice, invariably develop typical typhus infection.

From these various experiments we conclude that the organisms found in the lungs of rats and mice infected with typhus material and transmitted by emulsions of the lung to other animals are *Rickettsiae prowazeki* which grow in great numbers in the cells of the bronchi, the alveoli and the endothelium of the capillaries. The contaminating organisms are easily detected by cultivation on agar slants and inoculation into animals. These contaminants have a tendency to increase in proportion with the transfers from rat to rat and may finally predominate in the lungs, but are rare in mice infected with material from guinea pigs and in rats inoculated with emulsions from the lungs of mice.

Production of Large Quantities of Rickettsia Bodies from Infected Lungs

When a mouse is inoculated with washings from the tunica vaginalis of typhus infected guinea pigs sufficient amounts of Rickettsiae are produced to infect 15 medium sized rats. Whenever the infection is successful the mice die 96 hours after inoculation and the rats infected with emulsion from the lungs die with great regularity on the 3rd day after inoculation.

Rabbits were anesthetized with Dial and inoculated with an emulsion of the lungs from infected rats. Each rabbit received about one-third of a whole lung. The animals were kept at low body temperatures, which is essential to obtain abundant growth of Rickettsiae.[7] Rabbits inoculated by the intratracheal route but not submitted to a depression of temperature, developed a fatal disease with considerable involvement of the lungs and showed Rickettsiae in large numbers, but the yield was negligible compared to that obtained from animals subjected to a low body temperature.

From the lungs of medium sized rats we obtained, after grind-

ing and purifying by fractional centrifugation to remove foreign particles, about 10 gm. of packed Rickettsiae per 100 animals. This may be diluted to 5 liters or more to obtain a concentration of Rickettsia bodies suitable for vaccination. Two technicians may easily inoculate 3 lots of 100 rats a week to obtain about 15 liters of vaccine.

We have not so far calculated the production that may be obtained from rabbits kept at low body temperatures by continued narcosis with Dial, but roughly one may estimate that as much vaccine can be obtained from 1 rabbit as may be obtained from 10 rats.

The various methods of production of the Mexican vaccine first prepared by Zinsser and Castaneda enable us to obtain sufficient amounts of formalin-killed Rickettsiae which may be used advantageously as a prophylactic means against typhus.

Summary

The intranasal inoculation of mice and rats with typhus virus (orchitic variety) has given rise to hemorrhagic lesions of the lungs which kill mice in 96 hours and rats in 72 hours each. The lungs show in sections and smears considerable numbers of Rickettsia bodies which have been obtained in pure suspension by grinding and fractional centrifugation. Rabbits have also been infected by the intratracheal route with or without forcing down the body temperature. The animals develop hemorrhagic pneumonia, and Rickettsiae are present in large numbers in smears and in sections of the lungs, but the animals subjected to a low body temperature produce greater quantities of Rickettsia bodies. These rabbits die in from 48 to 96 hours after inoculation. The rabbits not submitted to a low body temperature die after a longer period of time and show lesions of the lungs which are more extensive but which contain fewer Rickettsiae.

To produce massive infection of the lungs it is necessary to inoculate considerable numbers of Rickettsiae.

This method of cultivating Rickettsiae has proved very useful for obtaining typhus vaccine for practical purposes.

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DESCRIPTION OF PLATE

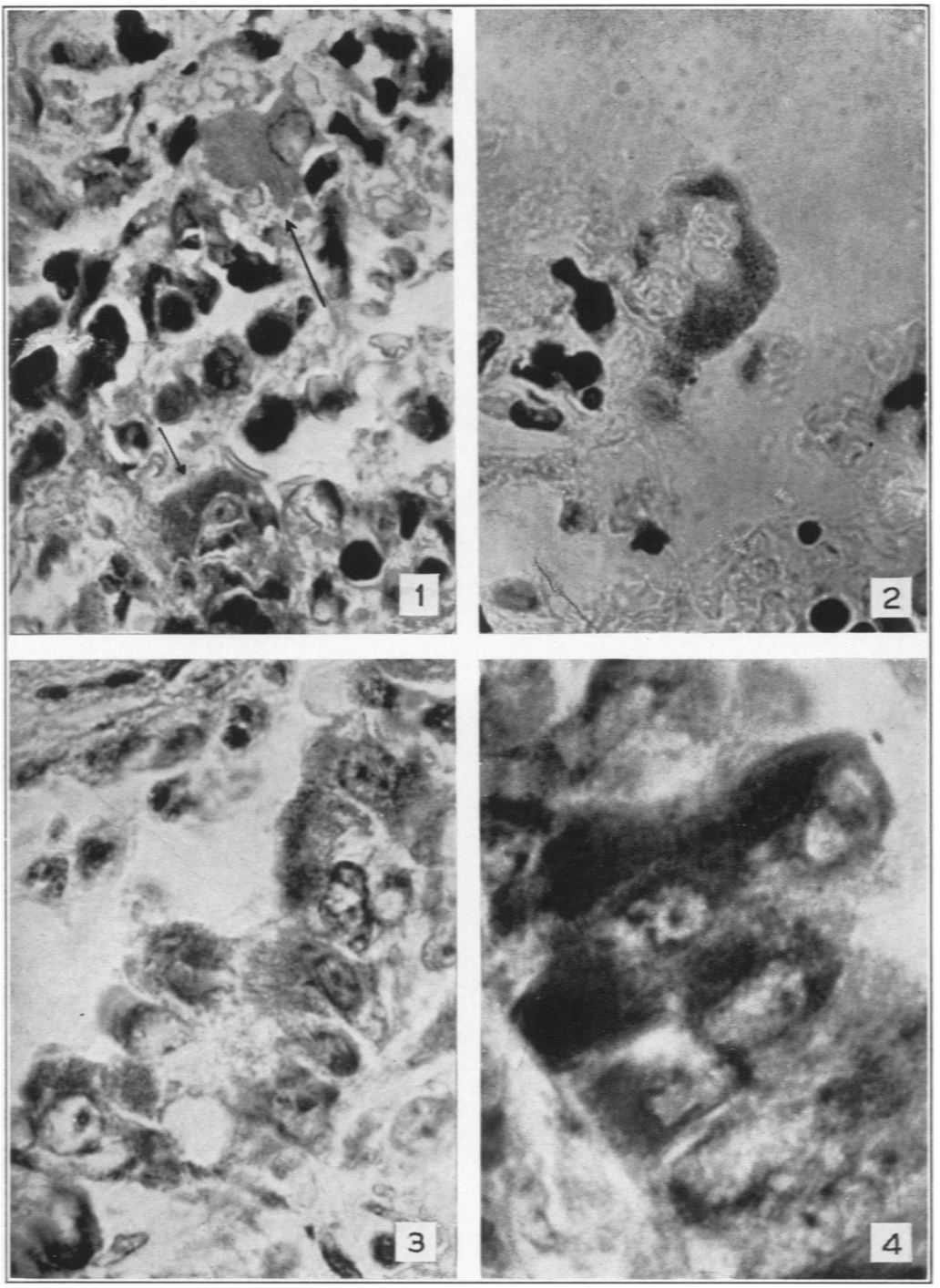
PLATE 77

FIG. 1. Lung of rat infected with typhus Rickettsiae showing two Mooser cells indicated by the arrows.

FIG. 2. Parasitized cells from the lung of a rat, apparently from a capillary.

FIG. 3. Section showing the bronchial epithelium parasitized with Rickettsiae.

FIG. 4. Higher power of Fig. 3 showing bronchial cells filled with Rickettsiae.



Castaneda

Pneumonia Produced by Typhus Rickettsiae