THE STANDARDIZATION OF LONGEVITY AGAINST DOSE IN EXPERIMENTAL TUBERCULOSIS BY INTRACEREBRAL INOCULATION

BY KENNETH C. SMITHBURN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 42 TO 44

(Received for publication, June 20, 1936)

Progress in experimental investigation of tuberculosis has been retarded by the chronicity of the disease and by the lack of regularity in the course pursued in experimental animals. Whereas in some other infectious diseases it has often been possible to control factors of dosage of culture and host resistance so that a standardized result was obtainable, such has not been the case in tuberculosis. Final results in animal experiments on tuberculosis have not been obtainable in less than a few months, and not infrequently a single experiment has covered a year or more. Thus, in our experience, in ten experiments including 120 rabbits, each inoculated intravenously with a standard dose of 0.1 mg. of B-1 bovine strain tubercle bacilli (then only moderately virulent) and subjected to no therapeutic procedure, the first animals succumbed in the 3rd week after inoculation (15 to 20 days) and several survived more than a year, while one animal lived 738 days. In nine of the ten experiments, maximum survival ranged from 290 per cent of minimum survival up to 4500 per cent. Our results with other, more virulent bovine strains and with the H-37 human strain have been similarly variable, although somewhat less so. Obviously such variations have made interpretation of results difficult and permitted unavoidable intercurrent factors to play a part in the end-result.

In order to overcome these difficulties, Bogen (1) has adopted the procedure of killing all inoculated animals after about 90 days and making quantitative estimates of the amount of existing disease. Animals dying before this time were excluded as having died from or...
with extraneous causes. He has shown that his method is adaptable to statistical analysis, especially when very large numbers of animals are used. Even with such procedure, however, the time necessary for a single experiment is great, and the cost of animal maintenance also large.

It is readily apparent from the facts just mentioned that a standardizable infection would be a valuable new tool in tuberculosis research. It was toward this end that the experiments to be reported were directed. It was considered that the optimum result in untreated cases would be invariably death, preferably in a short time after inoculation, that a significant result should be obtainable with minimal doses of bacteria, and that all individuals of comparable stock similarly treated should succumb at approximately the same time. Having adopted these standards, three avenues of procedure were apparent: (a) search for a highly susceptible animal host; (b) use of an adjuvant (mucin) to enhance the virulence of tubercle bacilli; and (c) study of various routes of inoculation to determine which gave the most uniform result. Suffice it to say that all our experiments in the first two categories were unsatisfactory; but it was found that the intracerebral route of inoculation afforded a method for obtaining a standardizable result in guinea pigs.

During recent years several investigators have employed the intracerebral route of inoculation in their studies on tuberculosis. Krause (2) made intrathecal injections by the postorbital route in his studies on hypersensitiveness. His method was used by others in subsequent experiments (12).

Manwaring (3) trephined the skulls of dogs, inserted a paraffin plug, allowed the wound to heal, and made inoculations through the paraffin plug. He did no titration experiments but found that injections of leucocytes prolonged both the incubation period and survival time. Austrian (4) inoculated rabbits by a method similar to that of Manwaring (3) and also by the lumbar intrathecal route. Austrian's description of the clinical course and macroscopic necropsy findings is among the best on record. He suggested use of the method for diagnostic purposes. A few years later, Foot (5) introduced tubercle bacilli intracerebrally in rabbits to study the formation of lesions in the meninges. Kasahara (6) introduced tubercle bacilli into the subarachnoid space through the atlanto-occipital membrane and studied the chemistry and cytology of the cerebrospinal fluid. He called attention to the similarity of the disease so produced to the clinical infection in man. Soper and Dworski (7, 8) inoculated normal and previously vaccinated rabbits with varying doses of viable tubercle bacilli. They inoculated also
through the atlanto-occipital membrane. They found that superinfection of vaccinated animals with large doses resulted in early death, whereas when the superinfecting dose was small (8), the animals lived longer, demonstrating a protective effect of the primary inoculation. Bickford (9) also used the cisternal route of inoculation, making the observation that with small doses of organisms (800) the incubation period was longer than after a larger dose (500,000). A very interesting observation was made in 1929 by Shope and Lewis (10) and Lewis and Shope (11). They found a high incidence of paralysis in a group of guinea pigs inoculated subcutaneously with a strain of human tubercle bacilli isolated from sputum. They transmitted the disease in guinea pigs by serial intracerebral inoculations of brain suspensions, showed that the paralysis was caused by the tubercle bacilli, gave a good account of the clinical and pathological course of the disease, and described certain changes in properties of the organisms incident upon their residence in the central nervous system. The apparent special affinity of their strain of organisms for nervous tissue was unexplained.

An outstanding fact regarding the reports summarized above is that none of the investigators mentioned has done titration experiments and that for the most part very large doses of organisms were used. Several workers made note of the fact that animals of a group receiving the same dose died at approximately the same interval after inoculation (4, 5, 7). Moreover, all these workers indicated, as does Calmette (12) that the infection induced by intracerebral inoculation is acute and uniformly fatal. The experiments reported in the following paragraphs indicate also that it is a readily standardizable infection by means of which greater precision may be introduced into tuberculosis research.

**Materials and Methods**

*Bacteria.*—Six strains of tubercle bacilli were used in the experiments. They were: avian TS strain, isolated in 1933 by the late Dr. Theobald Smith; bovine strain 36, isolated from a cow in April, 1929, also by Theobald Smith; bovine strain B-1, isolated by Dr. E. R. Baldwin at Saranac in 1904; human strain H-37, also isolated by Dr. E. R. Baldwin in 1905; and human strains O'Donnell and Fox, isolated from human patients in 1935 by the author. Each of these strains, except the bovine B-1, was known to be pathogenic for susceptible animals. The B-1 strain, formerly virulent, is now almost wholly avirulent. Relatively young stock cultures of each strain grown on Corper’s medium (13), adjusted to pH 6.8, were employed. Suspensions were prepared by first weighing the organisms immediately after removal from the tube, then grinding in a mortar with sterile physiological saline sufficient to make 1 mg. (moist weight of bacteria) per cc.
Dilutions were then prepared so that 0.1 cc. contained the desired number of organisms. When mucin was employed, only the final decimal dilution was made with mucin; the suspension therefore contained 90 per cent of the mucin preparation.

The heat-killed suspension of H-37 was prepared in the usual manner, placed in a tube of 50 cc. capacity, the top of which was well heated in a Bunsen flame, the tube sealed with a rubber cap, immersed in a water bath well beyond the level of the fluid suspension, and heated for 30 minutes while the bath was vigorously boiling.

Animals.—Guinea pigs purchased in the open market were used throughout. They were closely inspected and only vigorous, normal animals were selected. In most instances they were isolated for a few days prior to the experiment and observed for evidence of any disease. Male animals have been used for the most part, although females were also used in one experiment for comparison. Weights of the animals varied from 350 to 740 gm., the average being 479 gm. in the two larger experiments. In the quantitative experiments, groups of animals with closely matched individual and total weights were used, the average in one experiment being 420 gm. and in another experiment, 505 gm. per animal. Young animals weighing about 400 gm. were found to be most suitable for the trephining operation as the skull bones were soft enough to permit this operation to be done very easily.

Mucin.—In our first experiment with intracerebral inoculation of guinea pigs, half the animals were inoculated with organisms suspended in saline, the remainder with organisms suspended in mucin. The latter was prepared by Dr. G. Rake of The Rockefeller Institute, according to the method described by him (14). The bacteria were suspended in mucin by placing one volume of bacterial suspension and nine volumes of mucin in a Petri dish and stirring with a sterile rod or pipette.

Technique of Inoculation.—The hair is clipped from the scalp of the animal. Ether is administered to the point of full surgical anesthesia. The scalp is then painted with full strength tincture of iodine. Using aseptic procedure, a longitudinal incision, about 8 to 10 mm. long, is made in the skin about 3 mm. to the left of the midline. At a point in this plane not exceeding 4 mm. posterior to a line connecting the posterior commissures of the eyes, the skull is trephined. The instrument we have used is a No. 60 carpenter’s drill fixed in a carpenter’s hollow-handled pin vise, the drill being so fixed in the chuck of the vise that the latter prevents penetration beyond the desired point. The injection is made from a tuberculin syringe fitted with a 3⁄4 inch, 27 gauge needle, the amount injected being 0.1 cc. Closure of the skin incision is made with a single metal clip. The animals regain consciousness within a few minutes and, beyond being a little listless, show no untoward effects. With adequate assistance and a little experience, the entire operation requires less than a minute after the animal is anesthetized. The skin

1 L. S. Starrett and Co., Athol, Mass., No. 162 B. The No. 60 drill has a diameter of 0.04 inch or approximately 1 mm.
clip is shaken out by the animal when the wound is healed. Infection of the wound is not encountered if the closure is properly done. An occasional animal shows torticollis and circular movements (two in 226 instances to date) but recovery is prompt and complete. We have had two deaths from operative trauma among 226 animals, so that the hazard of the method itself is small. Death from intercurrent disease or extraneous causes has occurred seven times in the same groups of animals: twice from peritonitis due to perforation of the gut while taking the temperature, twice from purulent meningitis (both in cases in which the skin wound was improperly closed), and three times from streptococcal pneumonia.

Pathological Examinations.—All animals were either allowed to die or were killed with chloroform when it was evident that exitus was but a matter of a few hours. Surveys of the pathology in the visceral organs and brains were made in the gross, the latter then being fixed in 10 per cent formalin and the other tissues in Helly's fluid. Paraffin sections were then prepared in the usual manner and stained with hematoxylin and eosin. Sections were stained for bacteria with hematoxylin and anilin-fuchsin and counterstained with light green. Sections of each hemisphere of the brain were cut in the sagittal plane. Estimates on the basis of 0 to ++ + + + were made of the extent of macroscopic and microscopic pathology and of the number of bacteria in the tissues. Sections of brain and spleen were examined as routine for the presence of bacteria, the other tissues only when it was especially desirable to do so.

EXPERIMENTAL

First Experiment.—Purpose: (a) to determine the acuity of infection induced by intracerebral inoculation of a standard dose of virulent avian, bovine, or human tubercle bacilli; (b) to ascertain whether mucin enhances the pathogenic properties of either of these organisms when introduced into the brain of guinea pigs.

Twelve guinea pigs were used in the experiment. Four individuals received avian, four bovine, and four human type organisms. Of the four inoculated with each strain, two received organisms suspended in saline, and two received the organisms suspended in mucin. In this experiment the dose of each organism was 0.15 mg. in 0.15 cc. of fluid introduced intracerebrally as described above.

Results.—Each of the twelve animals succumbed to the disease, as shown in Table I. The time of death was very irregular in the case of the four animals inoculated with avian organisms, namely, from 11 to 49 days. This was not surprising, however, as the susceptibility of guinea pigs to infection with the avian type bacilli is very low. The animals which received mammalian organisms, on the contrary,
lived but a short time. Those receiving bovine type bacilli survived from 10 to 13 days and those receiving human tubercle bacilli lived from 17 to 19 days. From Table I it may be seen that mucin did not effect an appreciable alteration of the survival time in any instance.

Later a discussion will be given of the clinical course of the disease, together with weight and temperature curves and pathological findings.

### TABLE I
Survival Time and Pathological Findings in First Group of Guinea Pigs Inoculated Intracerebrally

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Strain of tubercle bacilli</th>
<th>Suspended in</th>
<th>Survival (days)</th>
<th>Extent of tuberculosis in Lung</th>
<th>No. of tubercle bacilli in Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>4451*</td>
<td>Avian TS</td>
<td>Saline</td>
<td>11</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4450</td>
<td>“”</td>
<td>Mucin</td>
<td>37</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4452</td>
<td>“”</td>
<td>Saline</td>
<td>37</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4459</td>
<td>“”</td>
<td>Mucin</td>
<td>49</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4453</td>
<td>Bovine 36</td>
<td>“”</td>
<td>10</td>
<td>+ + ± +</td>
<td>+</td>
</tr>
<tr>
<td>4454</td>
<td>“”</td>
<td>Saline</td>
<td>10</td>
<td>+ + ± +</td>
<td>0</td>
</tr>
<tr>
<td>4455</td>
<td>“”</td>
<td>Saline</td>
<td>11</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4456</td>
<td>“”</td>
<td>Saline</td>
<td>13</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4457</td>
<td>Human (Fox)</td>
<td>Mucin</td>
<td>17</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4459</td>
<td>“”</td>
<td>Saline</td>
<td>17</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4458</td>
<td>“”</td>
<td>Mucin</td>
<td>19</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
<tr>
<td>4460</td>
<td>“”</td>
<td>Saline</td>
<td>19</td>
<td>+++ ± +</td>
<td>+</td>
</tr>
</tbody>
</table>

* These are serial numbers of animals used in this laboratory over a period of years.

† ± is used to designate half the value of +. When used alone it indicates a minimal quantity.

This experiment confirms the observation of previous workers that intracerebral inoculation of tubercle bacilli induces an acute and rapidly fatal disease. In addition, it is shown that avian tubercle bacilli introduced by this route cause death in guinea pigs, but the length of survival varies greatly. With mammalian organisms, animals inoculated with the same dose of the same strain survive approximately the same number of days.
The question at once arose as to whether this regularity of the time of death would maintain with any dose of mammalian organisms; and what the effect of smaller dosage would be on the incubation period as well as survival time. The next experiment elucidates this point.

Second Experiment.—Purpose: To determine the effect of diminishing the intracerebral dose of tubercle bacilli on incubation period and survival time.

This experiment was done in two parts. In the first part, forty-eight guinea pigs were divided into twelve groups of four animals each. Each group included one animal heavier, one lighter, and two very near the average weight which was 505 gm. Three strains of tubercle bacilli, including two human type (Fox and O'Donnell) and one bovine (strain 36) were used, four groups of animals receiving any one strain. The volume of the inoculum was constant: 0.1 cc., given intracerebrally as before; but four different doses of each organism were given. The doses were $10^{-1}$, $10^{-3}$, $10^{-5}$, and $10^{-6}$ mg. and represent respectively, according to Baldwin, Petroff, and Gardner (15) and Calmette (12), 5,000,000, 50,000, 500, and 50 bacteria. By this simple titration it was hoped that an end-point of dosage would be reached at which some animals would survive. Following inoculation, those receiving bovine organisms were kept in a separate room from those receiving human type strains. Otherwise maintenance was identical. The temperature of each animal was taken by rectum daily in the forenoon beginning on the 13th day after inoculation. Each animal was weighed once weekly and again at death.

In the second part of the experiment, twenty-four animals were used and again divided into groups of four each. The average weight was 420 gm. and all animals weighed within 60 gm. of this figure. The total weight of each group was as nearly as possible the same. In this instance the H-37 human strain was used and the organisms were suspended first in saline solution, then the final dilution was made with serum. The inoculating dose was therefore contained in 10 per cent physiological saline and 90 per cent sterile serum, either normal horse serum or normal rabbit serum. The doses of tubercle bacilli were $10^{-1}$ mg. (5,000,000), $10^{-3}$ mg. (50,000), and $10^{-5}$ mg. (500). Each dose in normal horse serum was administered to one group of four animals; and each dose contained in normal rabbit serum was given to one group of four. The temperature of each animal receiving $10^{-5}$ mg. was taken daily. Weights were determined on all animals twice weekly.

Results. Temperature.—It was found that the temperature was the best index of the condition of the animal at any period of the disease. Contrary to the observation of Shope and Lewis (10) who observed...
no temperature reaction in their animals, we observed a quite characteristic temperature curve as follows. On the 1st day or 2 after inoculation, the temperature was usually elevated to 103.0° or 104.0°F. Thereafter the temperature was normal for a few days, or nearly so. Following this there was a rise in temperature, a little greater each day, for about 4 days to a peak of 104.5-106.0°F. No symptoms were usually noted until about the time of the highest fever. The peak of fever was maintained for a day or so, then there was a gradual decline in temperature with concomitant increasing severity of symptoms. The decline in temperature was sometimes abrupt; at other times it lasted 4 or 5 days. But in most instances the temperature during the final 24 hours of life was subnormal, and sometimes markedly so, temperatures as low as 87.0°F.² having been observed. With diminishing doses of organisms, all phases of the temperature curve were elongated except the original post-inoculation rise. It was stated above that the temperature curve was the most reliable index because

² In order to obtain such low temperature readings, it was necessary to shake the mercury down as far as possible, insert the thermometer, then determine the reading (below the graduated scale) with calipers.
of the fact that there was invariably a rise in temperature before any other evidence of disease developed. Chart 1 shows the temperature curves of four animals, each receiving different doses of the O'Donnell strain of human tubercle bacilli. As will be seen (Chart 1), these records began on the 13th day of the disease. The first three animals were individuals whose survival time was nearest the average of the group. The fourth animal, guinea pig 81 J, survived longer than the average of the group but illustrates well the temperature curve observed in animals receiving a minimal dose of organisms. Chart 1 also shows that three of the four animals exhibited a terminal

![Chart 1](image1)

subnormal temperature, while the final reading on the fourth animal was about normal. On Chart 2 may be seen the temperature and weight records of guinea pig 235 J, which received $10^{-3}$ mg. of the H-37 strain. The initial post-inoculation rise in temperature is shown. Also note that the temperature was elevated for several days before the animal began to lose weight.

**Loss of Weight.**—There was frequently a moderate loss of weight during the first 2 or 3 days after inoculation. This was almost invariably regained, however, and most of the animals gained a little weight, or at least maintained their pre-inoculation weight until
about the time the peak of fever was reached. Thereafter there was a sharp loss of weight which was progressive until death occurred. The average loss of weight computed from the pre-inoculation weight and that at death in 65 animals with all doses and all strains of organisms was 32.36 per cent of the body weight. Of these 65 animals, only two failed to show a loss in weight, and these were animals which received an inoculating dose of 0.000001 mg. and survived about 1 month each. In some animals emaciation was extreme, due in part to the fact that they were paralyzed and unable to eat during the last hours of life. The fact that loss of weight did not begin until about the time of maximum fever is illustrated on Chart 2 which shows the weight and temperature curves of the same animal. It will be noted that this animal had temperatures exceeding 104°F. on 5 successive days before weight loss was initiated.

**Clinical Course of the Disease.**—The first noticeable symptom was listlessness. The animals became less active in voluntary movements, and were infrequently heard to make a sound. Appetite at this time was usually well maintained. Next the gait became abnormal; it was of a waddling type, unsteady and hesitating, and marked weakness of the hind quarters was evident. The coat became markedly ruffled in most instances. Paralysis, usually spastic, often but not invariably ensued. This was manifest in one or both hind legs but rarely in the fore legs, so that the animals were capable of locomotion by means of the fore legs. An occasional animal exhibited circular movements to one or the other side. Convulsions were noted in perhaps half the animals but occurred only at a late stage in the disease. Preceding death by 12 to 24 hours there was usually stupor, the animal lying on its side but able to make incoordinated movements when aroused. The respirations were usually somewhat accelerated and deep at this time, the temperature subnormal, and convulsions or rigors were noted.

**Pathological Findings.**—If the animal had lived as long as 14 days after inoculation, the scalp wound was healed and the skin clip usually missing. Even the trephine opening in the skull was frequently healed. Some, but not all animals showed a tiny yellow nodule in the meninges opposite the point of inoculation. There was marked hyperemia of the meninges and brain, most marked at the base of the brain and over the cerebellum. Macroscopic tubercles were not usually seen, and when they occurred were extremely small and opalescent. The meninges were usually moderately thickened.
The cervical lymph nodes were invariably involved, although tubercles were not visible to the unaided eye in about one-third of the cases. Other lymph nodes were not ordinarily involved.

The spleens were not enlarged but usually showed a few pin-point tubercles. Microscopically the spleen was tuberculous in almost every instance. In Experiments 1 and 2, only four spleens failed to show microscopic tubercles and three of these were from animals (in Experiment 1) which died in 10, 10, and 11 days respectively. Those animals which survived longest usually exhibited the more extensive tuberculosis of the spleen. But it must be emphasized that involvement of the spleen did not cause splenomegaly, as is the case in animals inoculated by other routes than the intracerebral.

Metastatic lesions were next most frequently seen in the liver, but were for the most part microscopic, small, interlobular aggregations of epithelioid cells without giant cells. Of the animals in Experiments 1 and 2, ten only showed absence of tubercles in the livers. Lesions in this organ were more constant and more extensive after human than after bovine tubercle bacilli.

Other organs than the above were not regularly involved.

Of the adrenals from 54 animals, only four showed lesions. These were always microscopic. Only four of the same 54 animals showed pulmonary tuberculosis and two of these were the same animals which showed lesions in the adrenals. Pulmonary lesions were minimal in extent and always microscopic. In thirty-eight animals we obtained two to four sections of lungs, a total of 92 sections, and studied likewise the tracheal lymph nodes from the same animals. Whereas only four of the thirty-eight lungs (92 sections) showed tuberculosis, the tracheal lymph nodes of twenty-four of the same animals were tuberculous. The lung sections from one animal showed lesions when the lymph node was negative (probably from the opposite side). In the other three animals showing pulmonary tubercles the lymph node was also involved; so that there were twenty-one instances in which the tracheal node was tuberculous and the lung not. From these twenty-one animals we examined forty-eight sections of lungs, —not less than two from any animal. This result might seem to indicate some lymphatic connection between the cervical nodes (always
LONGEVITY IN EXPERIMENTAL TUBERCULOSIS

tuberculous in these experiments) and the tracheal nodes. The only other explanation for such a result would appear to be that these lungs contained small tuberculous foci not included in the sections.

Sections of the kidney from each animal failed to show tubercles. The reproductive organs and intestines were likewise invariably negative.

Microscopically the sections of the brains showed extensive generalized meningitis involving principally the pia at the base of the brain, over the medulla and cerebellum, and extending into the brain substance along the blood vessels. Lesions over the vertex were less extensive than elsewhere; but the right hemisphere was usually as extensively involved as the left. Formed tubercles were the exception, not the rule. Rather the lesions consisted of a diffuse reaction of granulocytes and large mononuclear cells (monocytes and epithelioid cells, Fig. 11), with smaller numbers of lymphocytes, particularly in the older lesions. Necrosis occurred in small foci which were relatively remote from the small arteries (Fig. 8); that is to say, the tuberculous tissue around these vessels rarely showed necrosis. But everywhere were polymorphonuclear cells showing degenerative change. Fig. 1 shows a characteristic field in the meninges over the occipital lobe. It may be seen that the dura is intact, while the underlying structures are massively involved in the tuberculous process. Fig. 2 shows the perivascular infiltration with mononuclears and granulocytes in the brain substance adjacent to the lateral ventricle. Fig. 3 shows similar perivascular infiltration at the base of the frontal lobe.

The lesions involving the cerebellum were formed by direct extension from tuberculosis in the meninges, or from lesions along the blood vessels. Cerebellar lesions were very marked in some animals. Characteristic foci of pathology in the cerebellum are shown in Figs. 4 and 5. Giant cells were very infrequently seen in the lesions.

The possible means by which the infection spreads from the brain to remote organs were: by the lymphatic extension and by way of the blood stream. That spread occurred through the lymphatics is certain from the fact that the cervical lymph nodes were invariably tuberculous. That metastatic lesions may also have arisen by direct invasion of the blood stream is indicated by the fact that occasionally
tuberculous lesions were seen which perforated to the lumen of meningeal veins. Fig. 6 shows a vein at the base of the brain cut longitudinally, with its lumen occluded at one point by tuberculous tissue. Some of the cells in this tissue contained acid-fast bacilli; in fact some cells containing acid-fast bacilli were seen adjacent to the blood in the open portion of the vessel. Fig. 7 shows a vein in the ventricle similarly involved.

Sections of the brains stained with hematoxylin and anilin-fuchsin showed many acid-fast bacilli in the lesions. In animals receiving the largest doses, the bacilli were very numerous, as may be seen in Fig. 8, from an animal injected with 0.15 mg. bovine strain 36. In general the animals receiving bovine organisms tended to show somewhat greater numbers of organisms in the lesions than those receiving human type strains. As an example, Fig. 8, showing many bacilli, may be compared with Fig. 9, showing fewer, the latter from an animal receiving 0.15 mg. of the Fox human strain. This may have been due to the fact that there were greater numbers of organisms per unit weight in the suspensions of bovine organisms inoculated, or to more rapid multiplication of the bovine organisms in vivo. That multiplication of organisms in vivo did take place may be seen from the number of organisms in Fig. 10, from an animal inoculated with $10^{-6}$ mg. (approximately 50 bacteria). Many fields in this section showed as many tubercle bacilli as were inoculated. Many of the bacilli were intracellular, while others were obviously extracellular. In which situation multiplication occurred can only be surmised. However, short chains of two or three bacilli lying end-to-end, which we interpret as evidence of multiplication, occurred both intra- and extracellularly. Fig. 11 shows intracellular bacilli in short chains. This photograph represents an area in the meninges over the cerebellum.

Not only were tubercle bacilli found without difficulty in the lesions of the brain, but they were also numerous in metastatic lesions. In the spleens of animals inoculated intracerebrally, bacilli were often more numerous than it has been our experience to find them in animals inoculated subcutaneously.

Survival Time.—Perhaps the most important features of the disease produced by intracerebral inoculation of tubercle bacilli were the acuity of the disease and the uniformity of survival time in comparable
animals. First, as to the acuity of the infection, it may be stated that, of all animals included in Experiments 1 and 2, only three fatalities occurred after the 5th week and two of these were on the 36th day. The experiments were therefore completed in a relatively short time. But more important still is the fact that all animals receiving a given dose of a given strain of bacilli survived approximately the same number of days. For example, of the animals receiving the bovine

TABLE II
Survival Time in Days of Guinea Pigs Inoculated with Various Doses of Mammalian Tubercle Bacilli Suspended in Saline Solution

<table>
<thead>
<tr>
<th>Dose</th>
<th>Human O'Donnell</th>
<th>Human Fox</th>
<th>Bovine 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>Survival</td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>15, 16, 16, 21*</td>
<td>19, 19, 20, 28</td>
<td>11, 13, 13, 15</td>
</tr>
<tr>
<td>Average</td>
<td>17</td>
<td>21.5</td>
<td>13</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>21, 21, 23, 25</td>
<td>22, 23, 25, 32</td>
<td>18, 19, 19, 22</td>
</tr>
<tr>
<td>Average</td>
<td>22.5</td>
<td>25.5</td>
<td>19.5</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>25, 27, 30, 36</td>
<td>26, 32, 33, S</td>
<td>22, 24, 29, 32</td>
</tr>
<tr>
<td>Average</td>
<td>29.5</td>
<td>30.3+</td>
<td>26.75</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>23, 53, S</td>
<td>27, 31, S, S</td>
<td>30, 31, 36, S</td>
</tr>
<tr>
<td>Average</td>
<td>38+</td>
<td>29+</td>
<td>32.3+</td>
</tr>
</tbody>
</table>

One animal inoculated with $10^{-8}$ mg. O'Donnell strain died of intercurrent disease and is not included in these data.

* Each figure represents one animal. S indicates that the animal survived.

strain 36 (Table II), those inoculated with 0.1 mg. died in 11 to 15 days, those with 0.001 mg. died in 18 to 22 days, those with 0.00001 mg. died in 22 to 32 days, and of those with 0.000001 mg., three died between the 30th and 36th days, while one survived. The average survival times were: 13 days after inoculation of 0.1 mg., 19.5 days after 0.001 mg., 26.75 days after 0.00001 mg., and 32.3+ days after 0.000001 mg. Comparable results were obtained with the Fox and O'Donnell (human) strains, as seen in Table II, and with
the H-37 human strain, as seen in Table III. The fact that 0.000001 mg. is near the end-point of virulence for at least three strains of tubercle bacilli may also be seen in Table II, since with this dose one or two animals in each group survived. The prolongation of the incubation period, and of the disease itself, brought about by diminishing doses, is such that a one hundredfold dilution of dosage allowed an increase of 4 to 7 days in the survival time.

*Third Experiment.—Purpose: (a) to compare the effects of virulent and attenuated tubercle bacilli when introduced intracerebrally; and

\[ \text{TABLE III} \]

<table>
<thead>
<tr>
<th>H-37</th>
<th>Serum</th>
<th>Normal horse</th>
<th>Normal rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td></td>
<td>Survival</td>
<td>Survival</td>
</tr>
<tr>
<td>(10^{-3})</td>
<td></td>
<td>11, 17, 19, 24</td>
<td>10, 15, 15, 17</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>17.75</td>
<td>14.75</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td></td>
<td>18, 19, 19, 22</td>
<td>18, 18, 23*</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>19.5</td>
<td>19.6</td>
</tr>
<tr>
<td>(10^{-5})</td>
<td></td>
<td>24, 26, 27, 31</td>
<td>21, 26, 27, 31</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>27.0</td>
<td>26.25</td>
</tr>
</tbody>
</table>

* One animal died with pneumonia and is excluded.

(b) to compare the effects of viable and heat-killed tubercle bacilli of the same virulent strain.

Three male guinea pigs, average weight 543 gm., were each inoculated intracerebrally with 0.1 mg. of H-37 human tubercle bacilli (virulent). Three male guinea pigs, average weight 530 gm., were each inoculated on the same day, intracerebrally with 0.1 mg. bovine tubercle bacilli strain B-1 (attenuated).

At a later date two male guinea pigs, weighing 360 and 400 gm., each were inoculated intracerebrally with 0.1 mg. of living tubercle bacilli, strain H-37. At the same time, two other male guinea pigs, weighing 380 and 390 gm., were inoculated intracerebrally with 0.1 mg. of the same suspension of H-37 but which had been heated 30 minutes in a boiling water bath.
Results.—One of the three animals inoculated with bovine B-1 strain died from general peritonitis following accidental perforation of the intestine with a thermometer. This animal will be excluded from further consideration. A second animal became paralyzed 59 days after inoculation and died on the 64th day. Sections of the brain showed a single tubercle at the base of the brain with marked evidence of healing. No tubercle bacilli could be found in the lesion. There was a small group of giant cells in the cerebellum at one point—also without bacilli. The cervical lymph nodes were extensively tuberculous but all other organs were normal. The other animal receiving 0.1 mg. of bovine B-1 showed no marked temperature reaction, gained weight, and is alive and healthy 117 days after inoculation. The three animals receiving H-37 at the same time died on the 15th, 17th, and 24th days with typical tuberculous meningitis.

The two animals receiving heat-killed H-37 remained well and gained weight, whereas those receiving the same dose of viable bacilli died on the 26th and 31st days respectively.

This experiment demonstrates that the clinical course and fatal issue are brought about by the properties of virulence associated with the inoculated bacilli, since the result is quite different when viable attenuated bacilli or heat-killed virulent organisms are used.

DISCUSSION

In any problem selected for experimental investigation it is highly desirable that the methods used and results obtained be capable of standardization. In research in the field of infectious disease, the result of any inoculation of animals which is least subject to experimental error is survival or death from effects of the inoculation. The value of the result is likewise greatly enhanced if the fatal result occurs at precisely or approximately the same time in individuals receiving the same inoculum and the same post-inoculation therapeutics. Such an optimum result has by no means been obtainable heretofore in tuberculosis research; the method described in this paper, however, goes far toward standardization of experimental tuberculosis.

The culture with which these animals were inoculated was of a line which is slightly attenuated, thus the longer survival than in previous animals receiving the H-37 strain.
The technique of operation in order to make the inoculation intracerebrally is simple and the cost of instruments negligible. The hazard of the operation to the experimental animal is small. The time consumed by the operation and inoculation is little or no greater than that necessary for an intravenous inoculation. The results obtained by this method have many advantages. First, the acuity of the disease is such that experiments are terminated rather quickly with consequent considerable saving in time, and in cost of animal maintenance. A fatal result is obtainable with sufficiently small doses of organisms that the method gives promise of service as a test of prophylactic or therapeutic measures. But finally and most important of all perhaps, is the fact that the infection induced by intracerebral inoculation of tubercle bacilli in guinea pigs is standardizable, and therefore permits the performance of quantitative titration experiments. The results of such experiments, expressed in terms of survival time, should be readily applicable to statistical methods of analysis.

Certain possible applications of the method herein outlined are at once apparent. It seems not unreasonable to believe that it might be of value in testing the virulence of tubercle bacilli, for tests of the efficacy of prophylactic or therapeutic measures, and in testing antisera to determine whether antibody against tubercle bacilli or antibody against any antigens derived from them have protective properties. Certain of the above possibilities are under investigation in this laboratory at present, and it may be stated that the method is of value in testing the pathogenic properties of *Mycobacteria*.

Recently Neiman and Woolpert (16) have employed the intracerebral route for inoculating tubercle bacilli into fetal and new-born guinea pigs. In comparing their results with ours, it is apparent that fetal or new-born animals are not more susceptible to tuberculosis by this route of inoculation than are older animals; indeed, the reverse may be true. It is interesting to note, however, that they also obtained an acute and fatal disease.

From our results it is evident that a dose of 0.000001 mg. (moist weight, or about 50 organisms) is near the end-point of virulence for at least three strains of mammalian tubercle bacilli, since a certain number of animals which receive this quantity of bacilli survive. Of
five such animals which recovered, only one exhibited a temperature reaction at any time during the disease. This animal had temperatures exceeding 104.0°F on 4 successive days, the highest being 105.2°F. None of the five exhibited symptoms. Four of the five were skin tested with old tuberculin 5 months after inoculation; two showed negative and two showed positive reactions, the individual which had shown a temperature rise giving a negative reaction. Since it is not possible by the methods at our disposal to make perfect suspensions of tubercle bacilli, it is probable that the number of organisms injected into these five animals was extremely small. With improved methods of preparing fine suspensions of the bacteria, such as that proposed by Corper and Cohn (17), it is possible that the end-point of virulence might be extended and that even greater uniformity of longevity might be obtained.

SUMMARY

Intracerebral inoculation of tubercle bacilli into normal guinea pigs induces acute meningoencephalitis with minor metastatic lesions. The disease is fatal in a relatively short time and is characterized by a rather typical succession of symptoms and a fairly characteristic temperature curve. The disease is produced by very small numbers of bacilli; and under standard conditions, survival time is so uniform as to make possible quantitative or titration experiments. Certain possible applications of the method are discussed.

BIBLIOGRAPHY

KENNETH C. SMITHBURN


EXPLANATION OF PLATES

PLATE 42

Fig. 1. Section of the left hemisphere of the brain of guinea pig R 4613 which received 0.1 mg. of the O'Donnell (human) strain of tubercle bacilli intracerebrally and died on the 16th day thereafter. Photograph shows the meninges over the occipital lobe with dura intact and extensive tuberculous exudate in the pia-arachnoid space. Note the lack of necrosis. Hematoxylin and eosin. ×105.

Fig. 2. Section of the right hemisphere of the brain of guinea pig R 4455; inoculated with 0.15 mg. bovine tubercle bacilli and died on the 11th day. The photograph shows an area adjacent to the lateral ventricle in which there was marked perivascular infiltration of the brain substance with tuberculous tissue consisting of monocytes and polymorphonuclear leucocytes. This illustrates the mode of local spread of the lesions. Hematoxylin and eosin. ×105.

Fig. 3. Section of the right hemisphere of the brain of guinea pig R 4457 which received 0.15 mg. of the Fox (human) strain intracerebrally and died on the 17th day. Photograph at the base of the frontal lobe showing tuberculous menigitis and perivascular lesions in the brain substance. Hematoxylin and eosin. ×70.

Fig. 4. Section of the left cerebellar hemisphere of guinea pig R 4587 which received 0.000001 mg. of the O'Donnell (human) strain intracerebrally and died on the 53rd day. The photograph shows a tuberculous lesion (arrow) in the granular layer of the cerebellar cortex. Many bacilli were seen in this lesion. Hematoxylin and eosin. ×105.

PLATE 43

Fig. 5. Section of the left cerebellar hemisphere of guinea pig R 4594 which received 0.00001 mg. of the human strain O'Donnell intracerebrally and died on the 25th day following. Photograph shows extensive tuberculous meningitis with perivascular extensions into the cerebellar cortex. Hematoxylin and eosin. ×70.

Fig. 6. Section of the left hemisphere of the brain of guinea pig R 4458 which received 0.15 mg. of the Fox human strain and died on the 19th day. The photo-
graph shows a vein in the meninges at the base of the brain, cut longitudinally
and occluded by tuberculous tissue. The cells marked E are the endothelial
cells. Tubercle bacilli in the area are indicated by arrows. Hematoxylin and
anilin-fuchsin. ×450.

Fig. 7. Section of the left hemisphere of the brain of guinea pig R 4612 which
was inoculated intracerebrally with 0.1 mg. of the O'Donnell strain (human) and
died on the 21st day following. The photograph shows a vein in the ventricle
cut longitudinally with the lumen filled with tuberculous tissue. Vascular endo-
thelial cells are indicated by E. A tubercle bacillus is clearly shown at the
point indicated by the arrow. Hematoxylin and anilin-fuchsin. ×450.

PLATE 44

Fig. 8. Section of the left brain of guinea pig R 4455 which received intracereb-
labelly 0.15 mg. of bovine tubercle bacilli, strain 36, and died on the 11th day.
Photograph of the meningeal exudate at the base of the brain showing numerous
tubercle bacilli, indicated by the arrows. Hematoxylin, anilin-fuchsin, and light
green. ×1050.

Fig. 9. Section of the right brain of guinea pig R 4457 which received 0.15
mg. of the human strain Fox and died on the 17th day. Meningeal tuberculous
exudate composed chiefly of mononuclear cells at the base of the brain, showing
numerous tubercle bacilli, indicated by the arrows. Hematoxylin, anilin-fuchsin,
and light green. ×1050.

Fig. 10. Section of the right brain of guinea pig R 4614 which received intra-
cerebrally 0.000001 mg. of the bovine strain 36 and died on the 31st day. The
number of bacilli present, indicated by arrows, shows that multiplication of the
organisms must have occurred. Hematoxylin, anilin-fuchsin, and light green.
×1050.

Fig. 11. Section of the right brain of guinea pig R 4589 which received 0.001
mg. of the human strain O'Donnell and died on the 21st day. Note presence of
bacilli within cells, especially in the multinucleated cells (M). Bacilli lying end-
to-end, as indicated by the arrows, are believed to indicate multiplication of the
bacteria. Hematoxylin, anilin-fuchsin, and light green. ×1000.