STUDIES ON EXPERIMENTAL TUBERCULOSIS IN MICE.

THE SUSCEPTIBILITY OF MICE TO INOCULATION WITH TUBERCLE BACILLI[1].

By C. H. BROWNING and R. GULBRANSEN.

(*From the Pathological Department of the University and Western Infirmary, Glasgow.*)

(With 2 Charts.)

Koch (1884) originally tested the pathogenic action of cultures of tubercle bacilli when inoculated into white mice, subcutaneously or intraperitoneally, or when administered by inhalation. When the animals were killed from 4 to 16 weeks later the majority of those inoculated subcutaneously showed no lesions. In those inoculated intraperitoneally numerous tubercles were found in the lungs and the organisms were very abundant in the much enlarged spleen. Inhalation of cultures led to the appearance of numerous tubercles situated in the lungs and confined to that organ except for scanty nodules in the spleen. The conclusion reached by Koch was that mice (and also rats) cannot resist inoculation with large quantities of pure cultures, but that as compared with guinea-pigs and rabbits the former animals are much less susceptible to tuberculosis, since the individual tubercles develop much more slowly and the disease does not spread so rapidly. Straus (1895) had more success than Koch in producing infections in mice by subcutaneous inoculation. Straus pointed out also, that when mice which had been inoculated with tubercle bacilli died after a period of weeks or months, although no obvious tubercles were seen in the viscera, the spleen was enlarged and in common with other organs contained abundant tubercle bacilli. In fact the number of organisms found in the lesions in tuberculous affections of mice can only be compared with the richness of leprosy bacilli in lepra nodules in the human subject. Straus used cultures of avian as well as human type. Römer's (1903) extensive work appears to have been to a great extent overlooked; he emphasised that infection is produced with greater certainty by intraperitoneal than by subcutaneous inoculation. Römer also compared the effects of inoculation with cultures of the different types; his results in this connection will be referred to later. Weber and Bofinger (1904) produced infection in mice by feeding with cultures of avian and mammalian types. The results of subsequent observers, *e.g.* Fraenkel and Baumann (1906), Cobbett (1907), Griffith (1907), Boquet and Nègre (1921), have in general confirmed previous work. Tubercu-

[1] This work was done with the support of the Medical Research Council.

losis in mice has, however, been comparatively little investigated, apparently on account of the impression that these animals are too highly resistant to infection. Such relatively insusceptible subjects, however, appear to be in certain respects more suitable than guinea-pigs or rabbits for investigating the effects of therapeutic agents on account especially of the chronicity of the infection. Thus, in mice, as in the human subject, the disease often extends over a considerable portion of the average lifetime of the individual. An essential control for therapeutic investigations is a knowledge of the natural course of tuberculosis in the animal species concerned, and our observations in this connection have now been in progress for over eight years[1].

As regards the possibility of spontaneous tuberculous infection being a complicating factor, it should be noted that tuberculosis is very rare as a natural disease among tame mice. According to de Jong (1903) a spontaneous infection met with in a white mouse was due to tubercle bacilli of avian type. Weber and Bofinger (1904) reported also that a grey mouse kept along with white mice which had been fed with cultures of avian type, acquired the infection. In the examination of many thousand mice used for various purposes we have not seen lesions suggestive of tuberculosis acquired by natural infection. In this connection it is noteworthy that the occurrence of tuberculosis in any species of animals under natural conditions cannot be measured by their susceptibility to inoculation, since guinea-pigs which are preeminently susceptible to tuberculosis as a result of any form of parenteral inoculation, only rarely develop the infection spontaneously even when kept in cages along with infected animals—see Remlinger (1923, 1924).

Virulence.

The subject of virulence of an infective agent is one of extreme complexity. Two aspects of virulence especially obtrude themselves—(*a*) the capacity for producing disease-effects and (*b*) the capacity for flourishing in the tissues. These properties are not necessarily associated; thus there are infections which seem to produce little or no disease-effects although the organisms multiply abundantly in the blood, *e.g. Trypanosoma lewisi* in rats and *Spirochaeta laverani* in mice. On the other hand, chronic tuberculosis in man, and, even more strikingly, late syphilis, are examples of infections in which massive lesions may result from the presence of only scanty organisms. In the case of tuberculosis in mice an outstanding feature, noticed by the earliest observers, is the enormous numbers of tubercle bacilli which are present in the lesions and also in the spleen (this organ being either enlarged or normal in size). Accordingly, there is good ground for Römer's conclusion that mice are remarkably insusceptible to the toxic products of tubercle bacilli. In agreement with this is the fact that tuberculous mice are apparently unaffected by enormous doses of tuberculin as was first demonstrated by Römer, who showed that the

[1] A summary of a part of this work was communicated to the Tuberculosis Society of Scotland in 1922 (*Edinburgh Med. Journ.* (1923), xxx. 96).

intraperitoneal injection of a dose equivalent to ten times the minimum lethal dose for a tuberculous guinea-pig caused no obvious local or general reaction. We (1919) found also, in confirmation of this, that in mice, which had been inoculated some months previously with cultures of tubercle bacilli, a subcutaneous injection of 0·1 c.c. undiluted old tuberculin had no harmful effect; examination of these animals a week later showed in every case fairly massive abdominal lesions which contained abundant tubercle bacilli.

The effect of cultures of bovine and human types upon mice.

It is now generally accepted that tubercle bacilli recovered from infections in mammals may be divided into two groups on the basis of microscopic and cultural characters, as well as on account of their selective pathogenic action for certain animals—the rabbit and the calf being those suited for their differentiation under experimental conditions. Römer examined the action in mice of four human and five bovine strains, some of which had been recently recovered from the tissues; he concluded that one human strain alone approached the bovine type in virulence. This culture had been passed through a goat and then, before it would grow satisfactorily on culture media, had to be passed through several guinea-pigs. Several strains of each type were tested by Trommsdorff (1909) and Peters (1912), the inoculations being made intravenously, the route by which tubercle bacilli exert greater virulence than when injected subcutaneously or intraperitoneally—Fraenkel and Baumann (1906). The conclusion reached was that the human strains were much less virulent than the bovine ones. In our experiments thirteen strains were employed. Twelve of these were obtained from children[1], seven being of human and five of bovine type, and all of them had been recovered at post-mortem examination, except two of human type (from sputum and hip-joint disease respectively). The material containing tubercle bacilli had been injected into rabbits and after the death of the animals cultures were made upon horse heart digest egg medium (Wilson, 1920). The primary cultures from the rabbits or the first subcultures from these were used for inoculating mice. One strain of bovine type was recovered by the use of antiformin from meat previously kept frozen for some months; the primary culture was used in this case. Masses of tubercle bacilli from the surface of solid cultures were emulsified in saline and a volume of 0·5 c.c., corresponding to about 0·75 milligramme of moist bacilli, was injected intraperitoneally, three animals being inoculated as a rule with each culture. The animals were examined at death and smears from lungs, spleen, liver, kidney and heart blood were stained for acid-fast organisms and also by Gram's method. The results are seen in Table I. It is clear that in general the animals infected with bovine strains have survived for a shorter time than those infected with the human strains. Also, the only failures to produce infection occurred in the case of human strains (Nos. II and IV). On the other hand, no constant difference exists

[1] We are indebted to Professor G. Haswell Wilson for these cultures.

Table I.

A. *Human type.*

Strain designation	Age of culture in weeks	Period of survival of mice in days	Average survival in days	Presence of tuberculous lesions to the naked eye	Presence of tubercle bacilli
II	15	(3) 243+*	243+	−	−
IV	15	127, 163, 174	155	−	−
VI	15·5	70, 95, 127	98	+	+
VII	15·5	57, 104, 120, 122, 215	124	+	+
XIII	8	70, 114, 132	105	− +	− +
XIV	15	29, 36, 78	48	+	+
XXIX	7	8, 12	10	+ −	+

B. *Bovine type.*

M	15	32, 35, 35	34	+	+
VIII	15·5	58, 87, 89	78	+	+
XI	15·5	36, 38, 55	43	+	+
XV	14	24, 26, 37	29	+	+
XVI	13·5	25, 28, 30	28	+	+
LV	5	7, 7, 10	8	+	+

* The animals were chloroformed at the end of the period shown.

between the duration of infections with the two types. With one strain of each type (Nos. XXIX and LV) very rapidly fatal infections were produced. It will appear later that such a high grade of virulence is to be regarded, not as a constant property of given strains, but rather as a phase. Differences in the age of the cultures used for inoculation (5 to 15 weeks) are clearly not responsible for the differences in behaviour of the various cultures. Thus the culture of Strain XIII, aged 8 weeks, caused a relatively chronic course, whereas, as noted above, that of No. XXIX, aged 7 weeks, caused extremely

acute infections. The periods of survival of animals inoculated with the same culture sometimes varied to only a very limited extent; but, on the other hand, great differences occurred, *e.g.* 57 to 215 days in the case of Strain VII. It is to be noted that the weight of the animals at the time of inoculation was not a determining factor as regards duration of the infection. Thus the animals inoculated with Strain VII weighed in the order shown 15·8, 15·3, 15·2, 17·7 and 21·3 grammes respectively; again, those inoculated with Strain VIII weighed respectively 21·3, 15·9 and 18·8 grammes.

There is a tendency in tuberculous infection of mice, as of other animals, for mixed infections to occur and frequently abdominal nodules examined at death showed a few other organisms, especially Gram-positive cocci, along with great numbers of tubercle bacilli. But in the case of the extremely acute infections there was no evidence that superadded infection was responsible for the death of the animals (this subject is discussed further on p. 331).

The effect of varying dosage of the inoculum.

In the case of certain species of highly virulent and pathogenic organisms it has frequently been stated that an acute and rapidly fatal infection has been produced by the injection of one or two bacilli; but this is probably an altogether exceptional circumstance. It is likely that, in general, other things being equal, an important influence on the course of an infection may be attributable to the number of the organisms originally introduced into the tissues. Thus we have found (1923) that when mice receive a series of graded doses of moderately virulent pneumococci a point is reached below which smaller numbers of organisms fail to cause acutely fatal septicaemia, but a proportion of the inoculated animals, although remaining apparently well, become the subjects of chronic infection. Where passage of infective material

Table II.

Effect of varying the amount of inoculum.

The dose of organisms (from a culture on egg medium) was injected intraperitoneally into mice in a volume of 0·5 c.c.

A. *Strain* M (*bovine*)—*culture* 14 *weeks old.*

Dose in mgm.	Ratio of doses	Periods of survival in days		
		No. 1	No. 2	No. 3
0·75	1	25	32	47
0·075	1/10	41	53	84
0·015	1/50	69	87	144
0·005	1/150	41	89	123

B. *Strain XV* (*bovine*)—*culture* 7 *weeks old.*

0·75	1	45	49	51
0·375	1/2	39	44	71
0·25	1/3	38	63	65
0·15	1/5	76	91	124
0·075	1/10	64	72	110

Every animal was found to have lesions either in the abdomen or the lungs or in both situations and tubercle bacilli were demonstrated in every instance.

is carried out directly from animal to animal this factor of dosage may play an important part. Accordingly, the effects of a wide range of doses of tubercle bacilli in mice were ascertained in order to control experiments on direct passage. The results of experiments with two strains of considerable, but not excessive virulence, both of bovine type, are shown in Table II. A certain effect of alteration in dosage is apparent, those receiving the higher doses tending to die earlier. But with the range of doses employed, which is considerable, being 150 to 1 in series A, it is evident that the individual factor of the host plays a much more important part than the dose, since the shortest and the longest survivals with the lowest dose are equal to and three times the longest survival with the highest dose; further, the longest survival of all occurred in the case of an animal inoculated with the second lowest dose. This range of doses exceeded greatly any variation likely to occur in passing infective material from animal to animal according to the procedures adopted.

The effect of direct passage of tuberculous infection from mouse to mouse.

It is generally accepted that, in the case of tuberculosis of the lungs in adults, the infection is conveyed from one person to another. In other words, the infection is propagated by "passage." One of the strongest facts in favour of the occurrence of this mode of spread of the infection, at least in a proportion of cases, is the observation that certain infants which have been exclusively breast-fed, die within the first few months from general tuberculosis of the lungs and other organs due to bacilli of the human type. In such cases opportunity for inoculation with bovine tubercle bacilli seems to be lacking. Also, in view of the tenacity with which tubercle bacilli retain their type-characters, neither time nor any other factor is likely to have conduced to a transformation of a bovine strain into one with human characters in the bodies of these children. It appeared, therefore, that the effect of direct passage of tubercle bacilli through a series of animals merited investigation. The effect of passage upon the virulence of pathogenic organisms, in view of Pasteur's classical work, is probably held to be in most cases one of exaltation for the species of host concerned. It must be remembered, however, that the outstanding results obtained by Pasteur as regards exaltation of virulence were secured with rabies, a virus still of unknown character. Recently the view has been clearly expressed by Nicolle and Césari (1924) that exaltation of virulence is by no means constantly or even frequently to be achieved by passage in the case of the ordinary bacteria, and our own observations on pneumococcal infections in mice (1923) are in agreement with this opinion. Tuberculous infection in mice appeared well suited for a study of this question, and it was decided to examine the effects of direct inoculation from animal to animal without subjecting the organisms to intervening periods of growth in artificial culture media. The observations recorded above on the relatively slight effects of marked differences in the dosage of the inocula within the

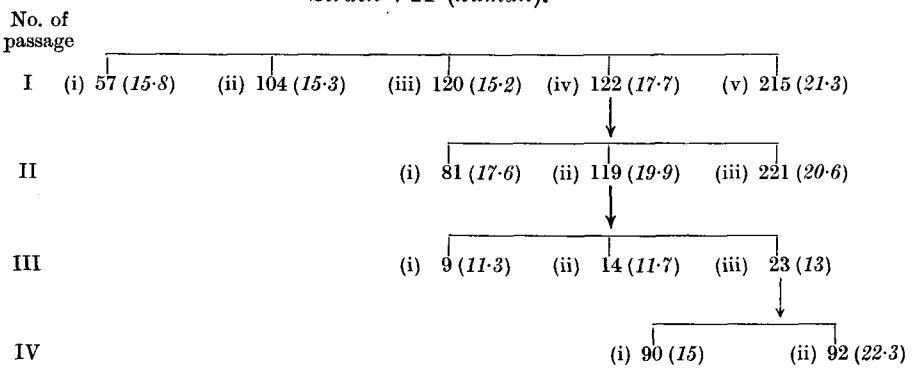
limits investigated, permitted the conclusion that in such passage experiments the results would depend almost wholly on two factors, namely the virulence of the organism and the resistance of the individual host, and that therefore justifiable conclusions regarding these two factors might be obtained by comparing the outcome in different passages. Accordingly, the effects of passage have been examined with eight strains, the number of passages varying from three to eight, and the animals employed numbering over one hundred and fifty. It is unnecessary to cite all the results in detail, since the following two charts illustrate well the general findings, which may be summarised as follows.. (1) The virulence of the infection has repeatedly appeared to undergo an increase in the course of passage, *e.g.* compare the rapid progress of the infection in passage III of Strain VII (human) with that in the preceding passages (see Chart I); but there has never been a maintenance of such increased virulence (see passage IV of the same series). This fact is important also, as evidence that the short duration of life in the preceding passage was not due to intercurrent mixed infection with some other virulent organism. Had such a superadded infection been the cause of death (in passage III, see Chart I), it is highly unlikely that in the next passage the animals would have lived for three months. Strain *M* (bovine) also illustrates the fluctuation of virulence in successive passages (Chart II, passage IV, as contrasted with the preceding passages). As may be seen, the weights of the individual animals at the time of inoculation had no constant or significant effect upon the duration of the disease. (2) There is evidence that the individual host plays an important part in modifying the virulence of the tubercle bacilli. Thus in the case of Strain VII all three animals inoculated from mouse (ii) of passage II showed a virulent infection, although the disease in this animal had a protracted course. On the other hand, the mice inoculated from animal (iii) of passage III, which itself lived for a relatively short time, acquired a comparatively chronic infection. Observations of this nature have been repeatedly made. Similar conclusions as to the effect of the individual host on the virulence of tubercle bacilli have been reached by Theobald Smith (1917) and by Borrel, Boez and de Coulon (1923). (3) At the same time, it must be remembered that the individual characters of the animals have a great share in determining the chronicity of the infection which they acquire. This is clearly shown in passage I of Strain VII, where the periods of survival ranged from 57 to 215 days.

Maintenance of virulence in cultures.

In contrast to the variable results obtained on passage were the relatively constant effects which repeatedly followed from inoculations with cultures. Thus, to take an example, in the case of the bovine strain (*M*)—Chart II—a culture made from tuberculous nodules attached to the spleen of mouse (ii) of passage I was grown at 37° C. for 10 weeks and was then employed to inoculate three mice (see Series (*a*) in Chart II), which all died in 12 days

Chart I.

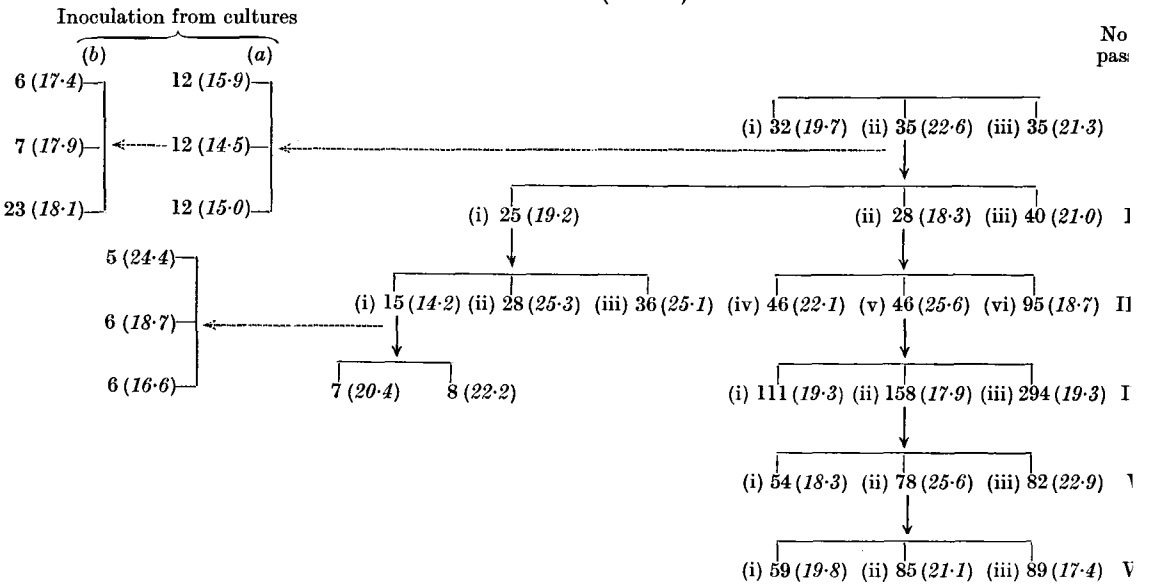
Strain VII (human).



The animals indicated on the same horizontal line in Charts I and II were all inoculated with an equal amount of material from the same source. The figures indicate the number of days of survival after inoculation. The italicised figures in brackets give the weights of the animals in grammes at the time of inoculation. The arrows point away from the animals used as the source of material for inoculating the succeeding series. All the animals showed lesions which contained tubercle bacilli.

Chart II

Strain M (*bovine*).



The arrows with broken lines indicate that the material was cultured and the culture was used for inoculation. Thus the series of animals inoculated with cultures are represented in vertical lines.

https://doi.org/10.1017/S0022172400017435 Published online by Cambridge University Press

with tuberculous peritonitis and also with tubercle bacilli in the lungs. A culture was made from a tuberculous nodule in the mesentery of one of these animals, and in the course of the following eleven months it was subcultured three times. Three mice inoculated with the last subculture (grown for 10 weeks at 37° C.), as shown in series (*b*), Chart II, died in 6, 7, and 23 days respectively. In the first two of these animals the lesions were mainly peritoneal, but in the third the lungs showed numerous large nodules. The peritoneal contents of the mice which lived 6 and 7 days showed after death a mixed infection in addition to tubercle bacilli, but in smears from the abdominal cavity and the various organs of the third mouse only tubercle bacilli were seen.

Two strains (bovine *M* and human VI) have now been used for over five years. They have been preserved on egg medium at 37° C. continuously, being subcultured at intervals of about 10 to 15 weeks. Numerous series of mice have been inoculated from these cultures throughout those years, and the general result is that no progressive alteration in virulence has been detected. On the other hand, considerable variations in the periods of survival of animals of successive series inoculated with the same strain have been observed. In view of what has been already stated, these differences can scarcely be attributed to differences in dosage of the inocula, therefore it is difficult to escape the conclusion that the virulence of the cultures has undergone spontaneous alterations from time to time.

MIXED INFECTIONS.

It has been commonly found at autopsy on mice which had been inoculated with tubercle bacilli that other organisms were also present in the tissues and no doubt, in some cases at least, this is not merely an invasion after death. It is noteworthy, however, that no acute epidemic infection attacked the inoculated animals and of those which died acutely—within one to several weeks—the majority showed no signs of mixed infection. Thus there is evidence of the occurrence at times of an acutely fatal infection with *B. tuberculosis*. This is further supported by the fact already mentioned that direct inoculation from the tissues of such animals as died acutely did not, as a rule, cause acutely fatal results. The latter event would, on the contrary, have been anticipated had a virulent mixed infection been responsible for the rapid course.

SUMMARY.

(1) Mice can be readily infected with many strains of *B. tuberculosis*, both of human and bovine type, by intraperitoneal inoculation with a dose of 0·75 milligramme of moist culture. But, with a fairly virulent culture of bovine type, doses of 0·005 milligramme produced infections of almost the same duration as the larger doses.

(2) The investigation of seven cultures of human and six of bovine type showed that in general the animals infected with the bovine strains survived

for a shorter time than those infected with the human strains. Also, several human cultures failed to produce infection, whereas no failure occurred with the bovine cultures. But no constant difference was found between the duration of infections with the two types of bacilli. Thus rapidly fatal infections were produced by certain cultures of both types.

(3) The period of survival of infected animals depends partly on the particular culture used for inoculation, and partly on the individual resistance of the animal and, in the case of nearly full-grown mice, is independent of their weight at the time of inoculation.

(4) The effect of repeated passage of tuberculous material from mouse to mouse without interposing cultivation of the bacilli on artificial media was investigated with eight strains, the series of passages numbering from three to eight. Increase of virulence was repeatedly observed, but such increase was never maintained in subsequent passages. The evidence indicates that the individual host plays an important part in modifying the virulence of the organisms for other members of the same species.

(5) As regards maintenance of virulence, comparatively constant results were obtained with different subcultures of the same strain. But repeated tests over a period exceeding five years have given indications that the virulence of a strain may become altered spontaneously from time to time.

REFERENCES.

Boquet and Nègre (1921). *Ann. Inst. Pasteur*, xxxv. 142.

Borrel, Boez and de Coulon (1923). *Ibid.* xxxvii. 1012.

Browning and Gulbransen (1919). *J. Path. and Bact.* xxiii. 128.

—— —— (1923). *Ibid.* xxvi. 121, 428.

Cobbett (1907). *Royal Commission on Tuberculosis, 2nd Interim Report*, Part ii, Appendix ii. 1200; *Ibid.* iii. 187.

Fraenkel and Baumann (1906). *Zeitschr. f. Hyg.* liv. 247.

Griffith (1907). *Royal Commission on Tuberculosis, 2nd Interim Report*, Part ii, Appendix, i. 20, 125.

de Jong (1903). Cited by Weber and Bofinger.

Koch (1884). *Mit. a. d. Kais. Ges. Amt.* Berlin (1884), ii. 1.

Nicolle and César (1924). *Ann. Inst. Pasteur*, xxxviii. 73.

Peters (1912). *Centralbl. f. Bakt.* I Orig. lxii. 1.

Remlinger (1923). *Ann. Inst. Pasteur*, xxxvii. 686.

—— (1924). *C. R. Soc. Biol.* xc. 1222.

Römer (1903). Behrings *Beiträge z. exper. Therap.* Marburg, Heft vi. 1.

Smith, Theobald (1917). *J. Amer. Med. Ass.* lxviii. 669, 764.

Straus (1895). *La tuberculose et son Bacille.* Paris.

Trommsdorff (1909). *Arb. a. d. Kais. Ges. Amt.* xxxii. 568.

Weber and Bofinger (1904). *Tuberkulose Arb. a. d. Kais. Ges. Amt.* Heft i. 83.

Wilson (1920). *Brit. Med. J.* i. 146.

(*MS. received for publication* 27. v. 1926.—Ed.)