THE PATHOGENESIS OF TUBERCULOSIS IN MICE INFECTED INTRAVENOUSLY WITH HUMAN TUBERCLE BACILLI; THE USE OF MICE IN CHEMOTHERAPEUTIC TESTS.

G. T. STEWART.

Imperial Chemical Industries Limited, Biological Laboratories, Hexagon House, Manchester, 9.

Received for publication November 21, 1949.

IT is clear from the literature, reviewed recently by Raleigh and Youmans (1948*a*), that white mice can be easily infected with mammalian and avian varieties of *M. tuberculosis* by several routes. The type of disease produced varies according to the variety, virulence and dose of the bacillus, and the route of inoculation. There are major discrepancies, however, between the observations of different workers, especially with regard to the virulence of the organism, the immune-response of the mouse and the exact histogenesis of the lesions.

Discrepancies arising from variations in dose appear to have been largely overcome in recent work by the use of weighed inocula. Variations in virulence of different strains of mammalian tubercle bacilli are still imperfectly understood. Thus Schwabacher and Wilson (1937) found no difference in mouse-virulence between certain human and bovine strains, whereas Stamatin and Stamatin (1939) found the bovine strain to be about ten times as virulent as the human strain. Little or no evidence is available about differences in behaviour between different members and different doses of any one (so-called) strain. Alterations in virulence may well arise in respect of ages of the cultures, medium of growth, species of mouse (Gerstle and Thomas, 1941) and other factors; it is obvious that the term virulence cannot at present be assigned an absolute scientific value, and it is therefore important, especially in chemotherapeutic tests with murine tuberculosis, for each investigator to recognize any variations in the experimental infection employed.

The researches of Schwabacher and Wilson (1937) showed that murine tuberculosis was essentially a progressive disease. Inocula of 10^6 to 10^8 mammalian bacilli, injected intravenously into mice, caused death with specific lesions in 4 weeks to 4 months; smaller inocula, 10^2 to 10^5 bacilli, induced a chronic but still progressive disease. For the induction of a still more chronic infection these workers recommended the use of avirulent strains, or raising the resistance of the mice by preliminary vaccination. They conceded, nevertheless, that neither procedure gave a disease strictly comparable to tuberculosis in man.

The use of intravenously-infected mice in the experimental chemotherapy of tuberculosis has been described by Martin (1946) and Raleigh and Youmans (1948*b*). Martin assessed the activity of a drug by prolongation of survival-time in treated groups of mice. This single criterion was criticized by Raleigh and Youmans, who made a detailed study of the histopathological evolution of the

experimental disease and formulated from this a more complex system of assessment.

The present investigation was made to define the pathogenesis of the standard infection used in these laboratories, to study some of the variable factors described above, and to correlate the pathological findings with weight and mortality. A scheme of histopathological assessment, less complex than that of Raleigh and Youmans, was devised for application to chemotherapeutic tests.

METHODS.

Infection in mice.

Groups of 20 to 30 white mice were inoculated intravenously with known weights of *M. tuberculosis*, harvested from 17-day cultures grown on Lowenstein's medium. The standard infection, used routinely in these laboratories, is induced by injecting 0·75 mg. Strain 905 in 0·1 c.c. distilled water, as described by Martin (1946). For purposes of comparison, infections with different doses of this and other human strains of *M. tuberculosis* were also studied, as described below.

Assessment of results.

The mice were weighed at weekly intervals, or oftener, and deaths were recorded daily. Each dead animal was examined for the presence of specific lesions. Tissues for histological examination were taken as follows from :—

(i) Mice killed at random, in groups of at least three, from the various infected groups at stated intervals after inoculation.

(ii) Selected mice which were more severely ill than other members of the group.

(iii) Mice which had recently died, and in which post-mortem decomposition was not advanced.

TABLE I.—*Pathological Assessment of Lung Changes in Mice Infected with* M. tuberculosis.

Score.	Macroscopic changes.	Histology.	*M. tuberculosis.*
0	Normal	Normal or slightly congested	Absent.
1	Congested (all degrees) or oedematous (frothy). No obvious nodules.	Congested. Alveolar haemorrhages. Increase in mononuclears and polynuclears. Thickening of septa	Scanty intracellular.
2	A few grey, pin-head nodules. Remainder of lung normal or congested	Focal proliferative lesions, affecting 10–25 per cent of section. No necrosis	Solitary, or 4–5 clumped in macrophages of proliferative foci.
3	Large yellow nodules and consolidation throughout lungs	Necrotic lesions affecting 25–50 per cent of section ; *or* proliferative lesions affecting >50 per cent of section ; *or* consolidation, with alveolar breakdown, affecting entire section	Small clumps of 6–12 throughout lung.
4	—	Necrotic lesions affecting > 50 per cent of section	Masses.

The lung of each mouse is assigned a score for each of the three columns in the table; the total scores for each mouse are then added, and divided by the number of mice examined, to give an index of the severity of the lesions in mice of that group.

The lungs from these mice were removed *en bloc*; the macroscopic changes in the lungs and other organs were noted, and representative portions transferred to Zenker-acetic for histological sections. Staining was by Ziehl-Neelsen and haematoxylin-eosin.

Weight and mortality data for each type of infection were derived from independent groups, in which none of the mice were sacrificed. Dead mice were examined for the presence of tuberculous lesions, and non-specific deaths were excluded from the records.

The gross and microscopic changes in the lungs were assessed according to the scheme shown in Table I.

RESULTS.

Infection with virulent human tubercle bacilli.

Inoculum of 0·75 mg. Strain 905.

In a typical experiment, intravenous inoculation of 0·75 mg. Strain 905 caused loss of weight and death of all the mice in the group between 20 and 33

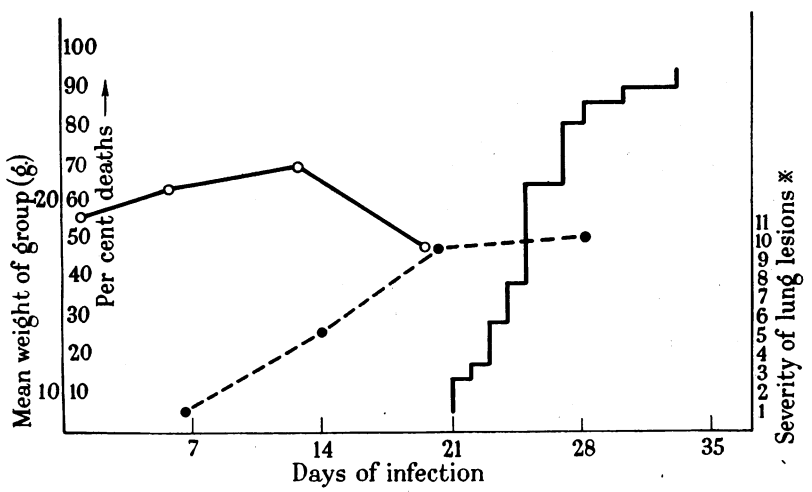

FIG. 1.—Course of infection in mice inoculated with 0·75 mg. *M. tuberculosis*, Strain 905.

Weight o——o

Deaths % ⌐

Lesions ●——●

* Mean value scored as in Table I.

Lung	Gross changes	Normal or congested	Grey nodules, congestion, œdema	Yellow nodules, consolidation.
	Histology	Diffuse cellular infiltration	Proliferative foci	Necrotic lesions.
	Bacteria	Scanty, intracellular	Numerous small clumps, intracellular	Masses.
	Liver	Normal	Small cellular foci	Cellular or necrotic foci.
	Spleen	,,	Normal	Enlarged lympho-reticular proliferation.
	Days	0–7	7–14	14–33.

days, the mean survival time being 24·1 days (S.D. 5·1). One non-specific death occurred before 20 days; the remainder of the mice died with nodular lesions in the lungs, containing innumerable acid-fast bacilli. The mice gained weight during the first 14 days of the infection; thereafter loss of weight and illness developed rapidly (Fig. 1).

Lungs, examined at 4 hours and 24 hours after inoculation, appeared slightly congested; isolated or small clumps of acid-fast bacilli were readily found. Microscopically the septa showed diffuse infiltration with mononuclear and polymorphonuclear cells. Between the 2nd and 7th day after inoculation acid-fast bacilli were difficult to find, and no further pathological changes were observed in lung sections. Between the 7th and 14th days minute grey nodules could be discerned on the lung-surfaces, with a variable degree of congestion, cyanosis or oedematous enlargement of the lobes. Histologically, the alveolar septa were thickened by vascular congestion and mononuclear infiltration; in places the mononuclear cells were aggregated into foci, infiltrating or replacing many alveolar compartments and forming the earliest nodules. These foci were essentially proliferative, with no areas of necrosis. Single, or 6 to 12 clumped acid-fast bacilli could be seen within the mononuclears; frequently swollen or fragmented clusters of brown, acid-fast and eosinophilic material were observed intracellularly, and presumably represented abnormal or partially-digested bacilli. These proliferative foci were usually perivascular in distribution, and not, at this stage, extensive; the remainder of the lung tissue showed small haemorrhages, septal thickening and acidophilic exudate into the alveoli (Fig. 2).

No definite lesions were observed in other organs during the first 14 days of the infection. Thereafter, minute grey foci were observed in the liver; these corresponded histologically to cellular areas containing a few intracellular acid-fast bacilli. Splenomegaly, due to lymphoid and mononuclear proliferation, also occurred after 14 days, but acid-fact bacilli were excessively rare.

Between the 14th and 18th days the proliferative lesions expanded and the lungs became studded with yellow or greyish nodules, the intervening areas being cyanosed and consolidated. Histologically the nodules were composed of dense aggregates in the alveoli of mono- and polynuclear cells, and acid-fast bacilli; the alveolar septa between these cellular foci were either destroyed, or persisted only as thready remnants (Fig. 3 and 5).

Until about the 18th day the necrotic element in the histological picture was limited, and the bulk of the lesion was formed by confluent cellular foci affecting up to half of the area of the section. The remainder of the lung showed diffuse cellular infiltration, septal thickening and alveolar exudates. The lesions contained innumerable clumps of acid-fast bacilli, and isolated bacilli were present throughout the lung. After the 18th day, the lesions became largely necrotic with widespread alveolar breakdown and rapid multiplication of the bacteria, forming massive acid-fast blocks visible to the naked-eye in stained specimens (Fig. 4 and 6).

Liver lesions and splenomegaly were conspicuous after the 21st day; the former consisted of cellular and necrotic foci containing scanty acid-fast bacilli, but the splenic enlargement was attributable to general cellular hyperplasia, without localized lesions. Minute cellular foci were noted in some kidney sections, but there was no evidence of widespread extra-pulmonary tuberculosis.

Mononuclears were the predominant cells in the lung lesions. In the early

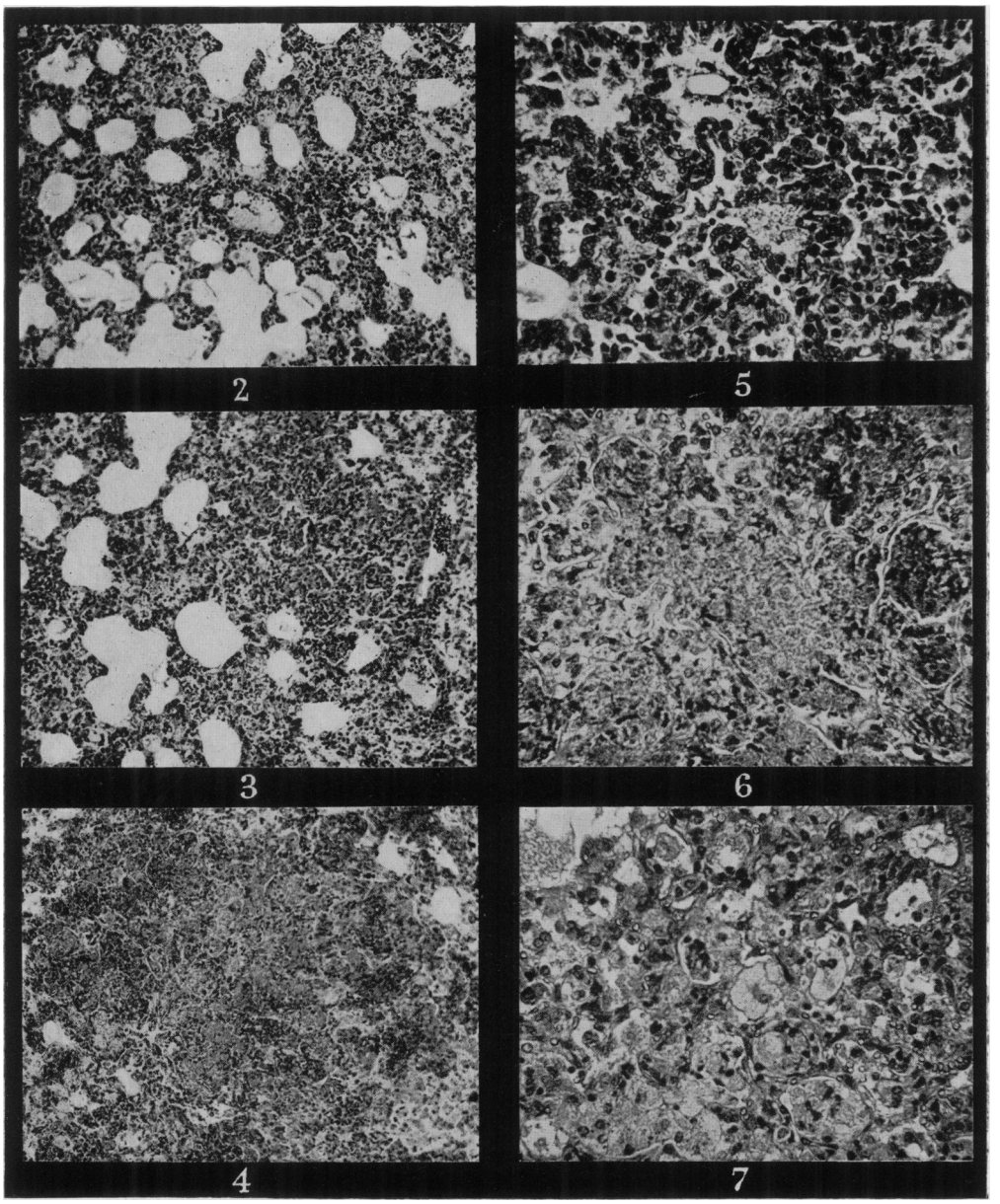

FIG. 2–6.—Lungs of mice infected with 0·75 mg. *M. tuberculosis* Strain 905.
FIG. 2.—7th day of infection. Infiltration of septa and alveoli by inflammatory cells. × 90.
FIG. 3 and 5.—14th day of infection. Proliferative lesions. × 90 and × 250.
FIG. 4 and 6.—21st day of infection. Necrotic lesions. × 90 and × 250.
FIG. 7.—Lung of mouse infected with 0·75 mg. *M. tuberculosis* Strain M, 60th day of infection. Foam-cell reaction in alveoli. × 250.

stages (microscopic focal lesions) these cells were large and epithelioid in type; the cytoplasm was sometimes foamy, or it might contain bacilli showing normal or altered staining reactions, as described above. As the infection advanced, small mononuclears and lymphocytes became more numerous. Polymorphonuclear cells were noted mainly in the earliest stage of interstitial inflammation and, at the other extreme, around the necrotic areas. Giant cells and fibroblasts were absent throughout; no true tubercles were formed; fibrosis and cavitation did not occur.

Inoculum of 0·02 mg. Strain 905.

Intravenous inoculation with this dose caused illness and death of all the mice in the group between 25 and 100 days. Fifty per cent of the group died between the 25th and 66th days (mean 33·5). On average, the mice gained weight until the 30th day of infection and thereafter declined, though a few mice surviving for long periods continued to gain weight.

During the first 14 days isolated bacilli could be found in the lungs, but no specific morbid reaction occurred. At 14 days small proliferative foci appeared and the bacilli multiplied. Large proliferative foci and early necrotic changes were evident in some mice at 21 days. With this infection survival-time varied considerably. Mice dying early (21 days) showed necrotic lesions, whereas those surviving for longer than 35 days seemed able to arrest the disease at the stage of cell-proliferation, and even gained weight in the later stages of the infection.

Inoculum of 0·0004–0·01 mg. Strain 905.

The survival time of mice lengthened and the severity of the lesions decreased as the infecting dose of *M. tuberculosis* was lowered (Table II). With doses of 0·01 mg. and below, the lesions observed at 70 days were mainly or entirely proliferative. The infection was, nevertheless, progressive and unremitting. A conspicuous "foam-cell" reaction was often observed in the pulmonary alveoli (Fig. 7). Splenomegaly, without focal lesions, was again observed in the later stages of infection, as with inocula of 0·75 mg. and 0·02 mg., but lesions were minimal or absent in the other viscera.

TABLE II.—*Effect of Different Inocula of* M. tuberculosis *Strain* 905 *in Mice.*

Inoculum. (mg.).	Specific deaths (days). Minimum.	Maximum.	Severity of lesions at (days).*
0·0004	> 100	—	4·3 (70)
0·002	70–100	—	6·2 (70)
0·01	< 70	—	8·2 (70)
0·02	21	100	10 (35)
0·75	17	33	11 (21)

* Mean of 5 mice, assessed as in Table I.

Infection with tubercle bacilli of low virulence.

When mice were inoculated intravenously with 0·75 mg. of Strain M, deaths with specific lesions began to occur 27 days after infection; the mean survival time was 45·9 days (S.D. 14·9), and one mouse survived for 102 days. The pathological changes followed the pattern of slow evolution described above for small

inocula of virulent organisms. Ultimately there was massive pulmonary involvement, but the lesions, even at a later stage, were characterized by proliferative rather than necrotic changes; the alveoli contained many foam cells; acid-fast bacilli were numerous, but did not form massive clumps; many bacilli showed altered staining reactions. No lesions were detected in organs other than the lung.

Sections were also examined from mice infected with 1 mg. or more of other avirulent strains. The rate of evolution of the lesions varied, but there was no evidence of any real difference in histological pattern or of the development of chronic, fibro-caseous lesions.

From the above findings it is obvious that a lowering of the inoculum or a fall in virulence of *M. tuberculosis* var. *hominis* is associated, in infected mice, with the substitution of proliferative for necrotic lesions, a foam-cell reaction in the alveoli, and longer survival; the infection is nevertheless progressive and unremitting in character.

Effect of vaccination.

Three groups of mice were inoculated intravenously with 0·0004, 0·002 and 0·01 mg. respectively of *M. tuberculosis* Strain 905. Thirty days later half the mice in each group were re-infected with 0·75 mg., the remainder being retained as controls. Mice from each of the 6 groups were killed at 70 days. Sections of lungs from the reinfected mice showed that the necrotic lesions characteristic of primary infection with 0·75 mg. were absent; in all groups the lesions were purely proliferative and, on histological evidence, the maximum protection was conferred by the smallest vaccination dose (Table III). The secondary infection was still, however, progressive and unremitting in its course.

TABLE III.—*Severity of Lesions in Mice Infected with* M. tuberculosis *Strain* 905. *Effect of Vaccination.*

Inoculum in mg.		Severity of lesions at (days).
Primary.	Reinfection.	
0·0004	—	4·3 (70)
0·0004	0·75	7·5 (70)
0·002	—	6·2 (70)
0·002	0·75	8·2 (70)
0·01	—	8·2 (70)
0·01	0·75	10 (70)
—	0·75	11 (21)

DISCUSSION.

The results show that the course of experimental tuberculosis and character of the lesions in mice are directly dependent upon the dose and virulence of the infecting strain of the bacillus. A large dose (0·75 mg.) of virulent bacilli causes death with necrotic lesions in about 25 days; smaller doses (0·02 mg. or less) permit longer survival, and cause proliferative rather than necrotic lesions. The pattern of evolution of the lesions conforms to that described and analysed in detail by Raleigh and Youmans (1948*b*).

It is possible, therefore, in terms of survival-time and duration of illness in infected groups of mice to define an " acute " disease produced by a large inoculum (0·75 mg.), and an apparently " chronic " disease produced by smaller inocula. Pathologically this difference is associated with necrotic lesions in the former and proliferative lesions in the latter type of infection. In both types, however, the disease is progressive, and there appears to be no final immunity or natural remission.

Large inocula (0·75 mg. or more) of various strains of *M. tuberculosis* of low virulence produce a disease comparable in every respect to that produced by smaller inocula (0·02 mg. or less) of the virulent Strain 905. Again, this apparently " chronic " disease differs from the " acute " only in its slower development, and in the prolongation of the proliferative stage of tissue-reaction. In both types, true caseation, fibrosis, cavitation and giant-cells are absent.

Vaccination with small inocula of living, virulent bacilli allows mice to resist reinfection for a longer period than unvaccinated controls. Such resistance is also associated with prolongation of the proliferative tissue-reaction, but it does not confer the power to arrest by fibrosis the development of the lesions or, in general terms, to cause remission of the disease. A small vaccinating dose is more effective than a larger dose. It seems likely, therefore, that the efficacy of vaccination in mice depends upon a balance between the resistance induced by the primary infection alone and the cumulative effect of the primary and secondary infections. It is of interest to note that, in mice, the primary infection is not even temporarily activated, and no allergic reactions are caused by reinfection. A similar observation was made by Pagel (1940), using mice infected intracutaneously.

In terms of the histogenesis of the disease, the resistance of mice to experimental tuberculosis is expressed firstly by an apparent decrease in the number of acid-fast bacilli in the lungs. This decrease is associated with interstitial infiltration by mono- and polymorphonuclear cells, within which bacilli with altered staining reactions may be observed. The infection is then quiescent for one or more weeks, depending upon the number and " virulence " of the bacilli in the original inoculum. Bacterial multiplication and proliferation of mononuclear cells then become apparent, and continue without remission until death occurs with necrosis or massive consolidation of pulmonary tissue. During the quiescent or early proliferative stages of the infection mice gain weight and look healthy. Loss of weight and decline in health are evident only when there is necrosis or massive consolidation in the lungs. Even with virulent infections no extensive lesions are observed in organs other than the lungs.

These general findings must be borne in mind when mice are used in the experimental chemotherapy of tuberculosis. Infection is always progressive, so that no false results need be expected from natural remission. With small inocula or avirulent organisms, however, there is some variation in the rate of progress of the disease, as instanced by the early deaths (necrotic lesions) and later deaths (proliferative lesions) in mice infected with 0·02 mg. Strain 905. Obviously, in a treated group, chemotherapeutic response to a non-toxic substance will be to some extent enhanced by the proportion of mice naturally destined to develop proliferative lesions.

Raleigh and Youmans (1948) noted a similar variation when mice were infected with 0·1 mg. human tubercle bacilli ; it is clearly of importance for each

investigator in chemotherapeutic work to establish the pathological course and define the variations of the experimental infection employed. In the experiments reported above, variation is minimal in the " acute " infection induced by 0·75 mg. Strain 905, and this infection should therefore provide an exacting test of therapeutic substances.

During the first 14 days of the " acute " infection morbid changes are minimal and bacilli are scanty and isolated. Intervention at this stage would presumably favour a drug rather than the bacillus. Treatment thereafter, when lesions are established, obviously decreases the likelihood of chemotherapeutic response and, with large inocula which overpower the resistance of the mouse, it may be impossible to intervene before a proportion of the mice have already developed lethal lesions. Delayed treatment may therefore be better demonstrated with " chronic " (e.g. 0·02 mg.) infections and, under these conditions, might reasonably simulate the treatment of established tuberculous lesions.

On the basis of their histopathological studies, Raleigh and Youmans (1948) formulated a system for assessing chemotherapeutic activity in experimental tuberculosis. Essentially, the " score " of each mouse is proportional to the amount of lung-tissue involved in the reaction to the infection, together with the number of bacteria and degree of involvement of liver, spleen and kidney. This system is undoubtedly carefully constructed and comprehensive, but in practice it is laborious, and it refers only to one specific infection (0·1 mg. *M. tuberculosis* Strain H. 37 RV).

The chief difficulty in devising or manipulating any such system is to know where to draw the line histologically between the immune and morbid components of the tissue reaction in infected lungs. According to Raleigh and Youmans the pathological unit should be " a more or less finite lesion, rather than general changes, such as congestion and oedema." Unfortunately, even a finite lesion may be purely proliferative in character ; and, in prolonged experimental infections (e.g. with small inocula, avirulent bacilli or as a result of chemotherapeutic suppression), the pathological changes may be diffuse and finite lesions absent.

Further difficulties arise over the changes in the spleen and kidney. Such lesions are usually proliferative with virulent strains and minimal or absent with avirulent strains. In the spleen the changes are completely diffuse, and it is by no means certain that the resulting splenomegaly is necessarily an indicator of the severity of the infection.

The alternative scheme of pathological assessment (Table I) used in our studies is based upon the following general principles :

(*a*) The number of bacteria present, and necrotic lesions in the lung, can both be regarded as absolute indicators of the advance of infection.

(*b*) Proliferative and exudative processes represent defensive reactions, and assume a morbid role only when 50 to 75 per cent of the lung tissue is replaced by them, whether as finite lesions or diffusely ; prior to this stage a limited score can be assigned to these processes to indicate the potential development of severe infection.

(*c*) Lesions occurring outside the lung are minimal or absent in " chronic " infections, and do not contribute significantly to the morbidity of the acute infection. Such lesions are, therefore, disregarded.

As in many other fields of experimental chemotherapy, it is important in anti-tuberculosis research to define the limitations as well as the assets of the

tests employed. Experimental tuberculosis in the mouse is a progressive disease, dissimilar in its course and morbid pattern to tuberculosis in man or cattle. It can, nevertheless, be readily standardized, and its progress at various stages can be easily assessed. With these reservations the experimental infection of mice with human strains of *M. tuberculosis* provides a reasonable basis for chemotherapeutic screening, though not necessarily for the final identification of compounds likely to be useful against tuberculosis in man.

SUMMARY.

Mice inoculated intravenously with virulent and avirulent human tubercle bacilli develop a progressive pulmonary infection.

0·75 mg. of virulent bacilli causes deaths with necrotic lesions in three weeks. Smaller doses of virulent bacilli (or large doses of less virulent bacilli) permit longer survival, and give proliferative rather than necrotic lesions.

Previous vaccination with living tubercle bacilli makes mice more resistant to reinfection. This resistance is expressed by the formation of proliferative lesions, and longer survival, but not by fibro-caseous lesions or remission of the disease.

In its pathological features, murine tuberculosis is dissimilar to human tuberculosis. Standardized murine infections may, nevertheless, lend themselves to " screening " tests in experimental chemotherapy. A scheme for pathological assessment of the experimental infection is presented.

REFERENCES.

GERSTLE, B., AND THOMAS, R. M.—(1941) *Yale J. Biol. Med.*, **13**, 679.
MARTIN, A. R.—(1946) *J. Path. Bact.*, **58**, 580.
PAGEL, W.—(1940) *Amer. Rev. Tuberc.*, **42**, 58.
RALEIGH, G. W., AND YOUMANS, G. P.—(1948*a*) *J. infect. Dis.*, **82**, 197.—(1948*b*) *Ibid.*, **82**, 205.
SCHWABACHER, H., AND WILSON, G. S.—(1937) *Tubercle, Lond.*, **18**, 442.
STAMATIN, N., AND STAMATIN, L.—(1939) *Ann. Inst. Pasteur*, **63**, 269.