EFFECT OF MACROCYCLON IN ACUTE AND CHRONIC PULMONARY TUBERCULOUS INFECTION IN MICE AS SHOWN BY VIABLE AND TOTAL BACTERIAL COUNTS

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ANTITUBERCULOUS drugs are generally assessed by their ability to prolong life or reduce lesions in animals with acute tuberculosis. The trends of the viable populations of tubercle bacilli in the lungs and spleens of mice have also been made the basis of assessment of various drugs, a chronic tuberculous infection being used (McCune and Tompsett, 1956 ; McCune, Tompsett and McDermott, 1956). We have extended this technique by following in parallel the viable and the total (stainable) bacillary population in the lungs of mice with acute and chronic tuberculous infection. The object of such dual counts was to distinguish a bacteriostatic from a bactericidal action.

The method of dual counts has been used previously for untreated infected mice, but fell into disrepute since the total count was often inexplicably less than the viable (see Gray and Jennings, 1955). However, Fenner (1951) showed that, with careful examination of suspensions of tubercle bacilli from tween 80-containing culture medium, the number of stained organisms was not significantly different from the viable count. Affleck and Gray (1957) had similar results with counts from lungs of mice with slowly progressive tuberculous infection. We have also found close correspondence betwen total and viable counts from mouse lungs in untreated acute and chronic infections. Apparently the method has not previously been used for analysing the effect of drugs on tuberculous infections.

Macrocyclon is a surface-active agent with suppressive effect on acute experimental tuberculous infections in mice and guinea-pigs, but without antituberculous effect *in vitro* (Cornforth, Hart, Rees and Stock, 1951 ; Cornforth, Hart, Nicholls, Rees and Stock, 1955 ; Rees, 1953, 1958). The dual method of population counts seemed a promising method for analysing the effect of this unusual drug *in vivo*. It seemed of particular interest to study the effect, if any, of macrocyclon in chronic murine tuberculosis, an infection resembling chronic pulmonary tuberculosis in man.

Our approach to the problem of macrocyclon is complementary to that of Niffenegger and Youmans (1960).

MATERIALS AND METHODS

Acute infection.—Female mice (18–20 g.) of the albino P strain were infected intravenously with 0·1 mg. wet-weight bacilli (containing about 4×10^7 viable units) of the human virulent strain of *Mycobacterium tuberculosis* H37Rv, prepared by grinding a fully-grown culture (2–3 weeks old) obtained from the surface of Proskauer and Beck's liquid medium. Under these conditions the median survival time is usually 18–21 days, with all dead by 30–35 days and showing gross pulmonary tuberculosis.

Chronic infection.—As above, but the infecting dose was reduced to 0·02 mg. wet-weight bacilli. Only about 30 per cent of the mice died from tuberculosis within the first 8 weeks (80 per cent of these deaths, in fact, occurred by the 5th week). The majority of the remaining mice survived in an apparently healthy state for many weeks with an established and predominantly pulmonary infection. Mice killed during this stable phase showed discrete and proliferative-type lesions in the lungs. During the stable phase a few mice die of respiratory insufficiency (with obvious cyanosis) due to cellular infiltration and oedema of the lungs and not due to progression of the infection with an increase in the number of tubercle bacilli (Sever and Youmans, 1957). This fairly stable infection is similar to the one used for studying the effect of cortisone and of suramin on chronic mouse tuberculosis (Hart and Rees, 1950 ; Rees and Hart, 1956) produced with an infecting dose of only 0·002 mg. bacilli. A larger infecting dose was chosen for the present study in order to obtain an adequate number of tubercle bacilli in the lung for accurate counting.

Macrocyclon treatment.—Macrocyclon, a polyethylene glycol ether of a *p-tert*-octylphenol-formaldehyde cyclic tetramer (with average 12½ ethylene oxide units per phenolic group), was autoclaved as a 12·5 per cent solution in 0·45 per cent saline, and 25 mg. in 0·2 ml. injected subcutaneously twice weekly.

For the acute infections the treatment was started 2 days after the infection. For the chronic infections it was started 60 days after the infection.

Preparation of lung homogenates.—At appropriate intervals 3–4 mice were taken at random from apparently healthy members of the treated and/or untreated group, in the case of chronic infections ; and from the fittest members of the control group and from any of the macrocyclon group (because they were all fit), in the case of acute infections. They were killed by exposure to coal-gas, and under sterile conditions the chest wall was opened and both lungs removed. The lungs from each mouse were homogenized individually under aseptic conditions by hand with pestle and mortar, finally suspending each lung in 10 ml. 0·01 M phosphate buffer pH 7·0.

Number of viable units of tubercle bacilli in lungs.—Viable counts were made by a modification of the method of Miles and Misra (1938). Suitable dilutions (in 0·1 per cent albumin-saline) of each homogenate were made within an hour, and 0·02 ml. drops were inoculated on plates of solid nutrient medium. The medium was oleic acid-albumin agar (Dubos and Middlebrook, 1947), modified according to Fenner, Martin and Pierce (1949) and (in the method of incorporating the glucose) to Yegian and Budd (1951). Counts were made after incubating the plates at 37° for 14 days in plastic bags.

Total number of acid-fast bacillary units in lungs.—Total bacillary counts (of stainable acid-fast bacilli) were made concurrently with the viable counts on each homogenate of mouse lung by a modification of the method described by Hobby, Hanks, Donikan and Backerman (1954). It is the method used in these laboratories for counting *Mycobacterium lepraemurium*, referred to by Rees and Wong (1958) but not previously described in detail. An 0·003 ml. drop of suitable dilutions (in 0·1 per cent albumin–1·6 per cent formaldehyde-water) of each lung homogenate was spread over an 8 mm. diameter circle marked out on a glass slide (4 circles per slide). Spread-smears were prepared in quadruplicate from each dilution. The albumin/formalin diluting fluid acted as both a protein-filming and a fixative agent. The spread-smears were finally fixed on the slide by drying on a lamp housing for 5 min., exposing to formalin fumes for 5 min., and reheating, to drive off the formalin, for a further 5 min. The smears were stained by the Ziehl-Neelsen method, modified to avoid loss of bacilli : carbol fuchsin for 2 min.; gentle washing in water and decolorizing and counter-staining in one process, using a mixture of 4 per cent sulphuric acid in 0·2 per cent methylene blue for 1 min.

Stained bacilli were counted in 16 microscope fields per smear, using a 2 mm. oil immersion objective and × 6 binocular eyepieces. The fields were 1 mm. apart, 8 along each of 2 diameters, set at right angles. Four spread-smears were counted from each dilution. The average number of bacilli/microscope field was determined, and the number of bacilli/lung calculated by the use of calibration factors for the optical system, the area of the smear and the dilution factor. The spread-smears were almost cell-free and the bacilli either single or in small groups. Since a " viable unit " count is based on the assumption that a colony can originate from a single bacillus or group of bacilli, the total bacillary counts in this study were brought into line by counting each stained bacillus or group of bacilli as one unit—giving the " total acid-fast bacillary unit count."

All counts were expressed as $\log_{10}$ viable, or total acid-fast bacillary, units/organ. Simple averages of these logarithms were used for the charts.

RESULTS

Effect of macrocyclon in acute tuberculosis

Thirty-six mice were infected and randomized into 2 equal groups, untreated and macrocyclon-treated (see p. 00). Treatment with macrocyclon was started on day 2 and was continued twice weekly until day 26. Mice were killed on days

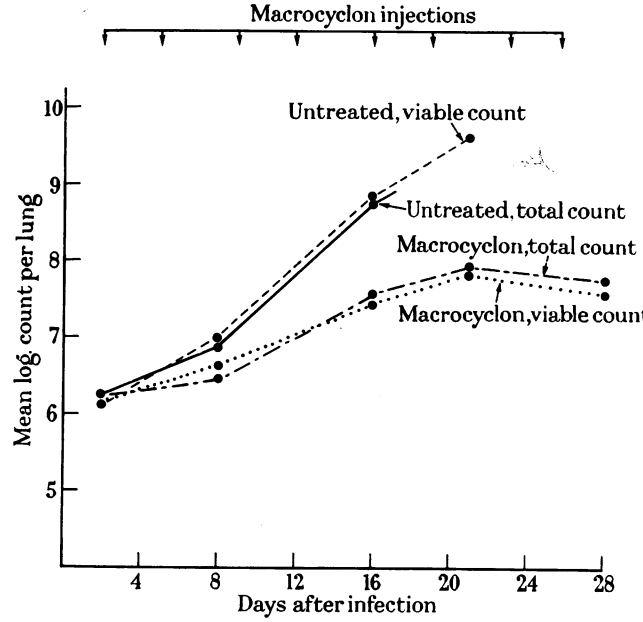

FIG. 1.—Influence of macrocyclon on viable and total populations of *Myco. tuberculosis* in mouse lungs during an acute infection (0·1 mg. H37Rv).

There were too many bacilli in the homogenate from untreated mice on day 21 for an accurate total log count (> 9·2).

2, 8, 16, 21 and 28. On day 2, 4 mice were killed from the untreated group, and on day 28, 4 from the treated group ; while on the intermediate days 3 mice were killed from each group. The mice killed from the untreated group were fit except for 2 slightly sick and 3 very sick, on days 16 and 21, respectively. No untreated mice were alive on day 28. The individual and average viable and total bacillary units/lung at each time of sacrifice, expressed as $\log_{10}$, are given in Table I, and the average lung counts are also shown in Fig. 1.

Table I shows that the total count in each lung from the untreated mice was very similar to the viable count ; in 5 of the 10 paired figures the total counts slightly exceeded the viable, while in 5 they were slightly less. It may be concluded that most of the bacterial population was viable. Fig. 1 shows a continuous rise in both the viable and the total counts from the untreated mice, indicating a multiplying population.

TABLE I.—*Viable and Total Counts of Bacilli in the Lungs of Untreated and Macrocyclon-treated Mice in Acute Tuberculosis*

	Untreated					Macrocyclon-treated*				
Day	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)
2	1	6·04	6·16	6·08	6·25	—	—	—	—	—
	2	6·40		6·08		—	—	—	—	—
	3	6·15		6·38		—	—	—	—	—
	4	6·04		6·47		—	—	—	—	—
8	1	6·90	7·03	6·95	6·90	1	6·57	6·61	6·25	6·49
	2	7·10		6·89		2	6·73		6·55	
	3	7·08		6·85		3	6·54		6·68	
16	1	8·34	8·87	8·37	8·80	1	7·83	7·46	7·92	7·59
	2	9·15		9·02		2	7·59		7·87	
	3	9·13		9·01		3	6·95		6·98	
21	1	9·61	9·61	>9·2	>9·2†	1	7·95	7·87	7·97	7·92
	2	9·65		>9·2		2	8·15		8·08	
	3	9·57		>9·2		3	7·51		7·70	
28‡	—	—	—	—	—	1	7·18	7·59	7·35	7·80
	—	—	—	—	—	2	8·06		8·23	
	—	—	—	—	—	3	7·95		8·19	
	—	—	—	—	—	4	7·15		7·42	

* Treatment, subcutaneously twice weekly, started day 2 and continued to day 26 (inclusive).
† Too many bacilli in spread-smears for accurate count.
‡ All untreated mice died with acute tuberculosis before day 28.

The divergence between the counts from the macrocyclon-treated and the untreated mice (Fig. 1) indicates a definite, but not powerful, antibacterial effect, which became more effective after day 8 and even more so after day 16. The closeness of the curves for total and viable counts in the treated group indicates that macrocyclon had little or no killing effect on the tubercle bacilli, but that its action was bacteriostatic. The latter conclusion is supported by the paired individual counts (Table I), which were very similar to one another ; in 10 out of 13 the total count slightly exceeded the viable, while in 3 it was slightly less. Similar results were obtained in a second, duplicate, experiment.

Effect of macrocyclon in chronic tuberculosis

Fifty surviving mice from an original stock of 90 infected (33 per cent having died from tuberculosis) were randomized into 2 groups on day 60, untreated (27 mice) and macrocyclon-treated (23 mice). Treatment with macrocyclon was started on day 60 and continued twice weekly until day 131, representing a total of 21 doses. Mice were killed on days 60, 88, 111 and 134. On day 60, 4 mice were killed from the untreated group ; on days 88 and 111, 3 mice and on day 134, 4 mice were killed from both the untreated and the treated groups. Only fit mice were killed, and these were picked each time at random from the 2 groups. It was not considered necessary to withhold treatment just before killing, in order to avoid carry-over of macrocyclon in the lung homogenates, since there is no evidence that the drug is antituberculous *in vitro*. The individual and average viable

and total bacillary units/lung at the time of death, expressed as $\log_{10}$, are given in Table II, and the average lung counts are also shown in Fig. 2.

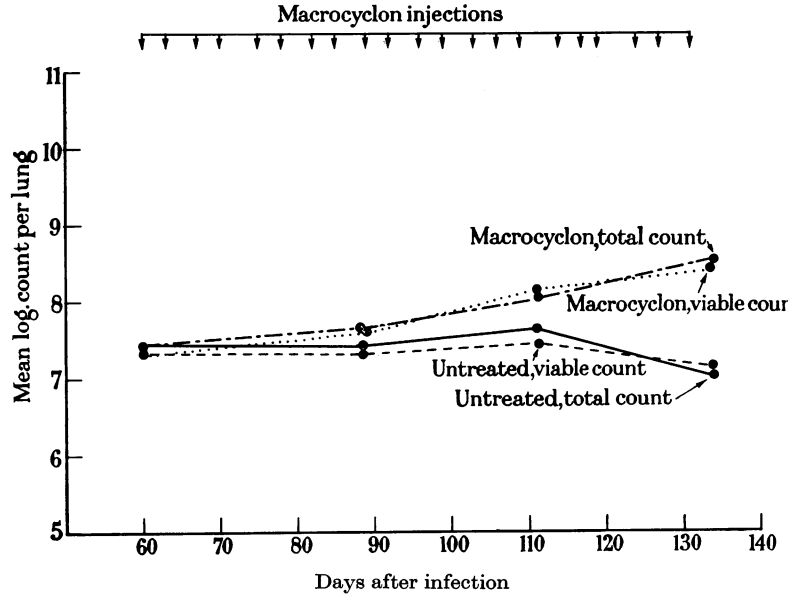

FIG. 2.—Influence of macrocyclon on viable and total populations of *Myco. tuberculosis* in mouse lungs during a chronic infection (0·02 mg. H37Rv).

TABLE II.—*Viable and Total Counts of Bacili in the Lungs of Untreated and Macrocyclon-treated Mice in Chronic Tuberculosis*

	Untreated					Macrocyclon-treated*				
Day	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)
60	1	6·93		6·89		—	—	—	—	—
	2	7·57	7·32	7·77	7·42	—	—	—	—	—
	3	7·59		7·83		—	—	—	—	—
	4	7·18		7·18		—	—	—	—	—
88	1	7·27		7·42		1	8·04		8·05	
	2	6·88	7·32	6·98	7·44	2	7·67	7·66	7·64	7·69
	3	7·80		7·91		3	7·26		7·38	
111	1	8·15		8·18		1	8·38		8·21	
	2	7·22	7·46	7·43	7·65	2	7·04	8·15	7·12	8·05
	3	7·02		7·34		3	9·03		8·83	
134	1	6·67		6·73		1	9·22		9·28	
	2	8·53		8·38		2	8·57		8·69	
	3	6·79	7·16	6·73	7·06	3	7·53	8·51	7·44	8·54
	4	6·66		6·38		4	8·70		8·75	

* Treatment, subcutaneously twice weekly, started day 60 and continued to day 131 (inclusive).

Table II shows that the total count in each lung from the untreated mice was very similar to, though usually slightly greater than, the viable count ; in 9 of the 14 paired figures the total counts slightly exceeded the viable, while in 5 they were slightly less. It may be concluded that there was no build-up of dead bacilli in the lungs, most of the bacterial population in this chronic tuberculous infection being viable. This table, as well as Fig. 2, shows that both viable and total counts from the untreated mice remained remarkably constant throughout the period of 74 days, indicating a steady population.

Fig. 2 also shows that, unlike the results obtained in acute tuberculosis, macrocyclon had no antibacterial effect in this chronic infection, even when treatment was continued throughout a period of 10 weeks. Bearing in mind that, without treatment, the bacterial population was steady, this finding is not inconsistent with the bacteriostatic action of macrocyclon, observed in the acute infection.

Although the results show that macrocyclon had no antibacterial effect in chronic tuberculosis, there was a tendency in this experiment for the bacillary population actually to increase in the drug-treated animals (Fig. 2). However, in a similar, though shorter, experiment, which confirmed the lack of antibacterial effect of the drug, there was no increase in the bacillary population of the treated animals above that of the untreated.

DISCUSSION

Macrocyclon is remarkable in showing no direct antibacterial effect *in vitro*. Its antituberculous effect is presumed to be due to interaction with the host defence mechanisms—possibly within macrophages, which it undoubtedly enters (Lovelock and Rees, 1955 ; Cornforth, Potts and Rees, unpublished). It is this drug-host interaction, resulting in an indirect antibacterial effect, that has been analysed in the present experiments.

The antibacterial effect of macrocyclon in acute murine tuberculosis is attributable, from the closeness of the total and viable counts, to a bacteriostatic rather than bactericidal action. In the chronic infection, on the other hand, the drug was not antibacterial. Since the population of the untreated controls in the latter infection was steady throughout the experiment, and the viable and total counts remained close to one another, it may be inferred that, unless dead organisms were being removed, the majority of the bacilli were not multiplying and were therefore in a comparatively static or resting phase. The contrast between the effectiveness of macrocyclon *in vivo* against a freely multiplying population, as found in the acute infection, and its ineffectiveness against a non-multiplying population, represented by the chronic infection, is analogous to similar contrasts *in vitro* with a number of other drugs (Singh and Mitchison, 1955 ; Mitchison and Selkon, 1956 ; Hobby and Lenert, 1957).

The ineffectiveness of macrocyclon in the chronic murine infection is strikingly different from that of the very powerful combination, pyrazinamide-isoniazid. This has been shown to be markedly antibacterial in this type of mouse infection (McCune, Tompsett and McDermott, 1956) ; moreover, the lung viable count falls rapidly below the total count (Rees and Hart, unpublished).

In the preceding paper Niffenegger and Youmans (1960) also report an antituberculous effect of macrocyclon in acute murine infection, using viable population counts. The early trends of these counts following infection in treated and

untreated mice were somewhat different from those observed in our experiments, and they discuss these differences. Macrocyclon was shown by these authors to be active even when administered prophylactically 28 days before an acute infection. We obtained similar prophylactic effects in acute tuberculosis, judged by survival. When a single dose of macrocyclon was given 1, 2 or 3 weeks before an acute infection, it was as effective as a dose given one day after ; but the effect was less at 4 weeks and unobtainable at 5 weeks before the infection (Rees, unpublished). Niffenegger and Youmans suggest that macrocyclon may affect the host immune response in a similar manner to BCG. If this be so, the inactivity of macrocyclon in our experiments on chronic murine tuberculosis might result from the immune responses being already fully stimulated by the established infection itself. This explanation would be an alternative to that suggested above (ineffectiveness of the drug against a resting population).

Recently a pilot clinical trial of macrocyclon has been carried out in patients with advanced chronic pulmonary tuberculosis (Boyd, Stewart, Somner, Crofton and Rees, 1959) ; but the drug was ineffective, although this type of chronic disease can respond to other forms of chemotherapy. It is obviously tempting to suggest chronicity of the infection as the common causative factor for the ineffectiveness of macrocyclon in chronic disease in man and mouse. The tubercle bacilli in both instances would generally be either in a resting phase or multiplying slowly, compared with the rapid rate of multiplication in acute murine infections. Macrocyclon can, however, have a therapeutic effect in man, as has been shown by Dr. J. A. McFadzean in a pilot trial of the drug in an infection with another mycobacterial species—leprosy.

SUMMARY

The effect of macrocyclon, a surface-active agent that is antituberculous *in vivo* but not *in vitro*, was studied in tuberculous mice by dual bacterial counts—viable and total (stainable)—in lung homogenates. Two types of experimental infection were used : acute and fatal produced by a large dose of bacilli, and chronic, following a smaller dose. The drug was given subcutaneously twice weekly, from 2 until 26 days after the acute infection and from 60 until 131 days after the chronic infection.

In the acute infection the total counts from the lungs of untreated mice were very similar to the viable counts throughout, indicating that most of the bacterial population was viable ; and the continuous rise in both counts showed that the population was multiplying. The counts from the treated mice diverged from these counts, indicating a definite though not powerful antibacterial effect, which was more manifest after the first week and even more so after a fortnight. From the close similarity of the total and viable counts in the treated group it was concluded that macrocylon's action was bacteriostatic rather than bactericidal.

In the chronic infection the two counts from the untreated mice were also very similar, indicating that here too most of the bacterial population was viable, without a build-up of dead bacilli in the lungs ; but (unlike the acute infection) both counts remained constant throughout the observation period, indicating a steady population. The trend of the counts from the treated mice showed no evidence of any antibacterial effect by macrocyclon. It was considered that, unless dead organisms were being removed from the lungs of the untreated

animals, the majority of the bacilli were not multiplying and that in this resting phase they may not have been susceptible to the drug's action.

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