STUDIES ON THE VIRULENCE OF TUBERCLE BACILLI

THE RELATIONSHIP OF THE PHYSIOLOGICAL STATE OF THE ORGANISMS TO THEIR PATHOGENICITY

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It has been reported (1) that a petroleum ether–soluble material can be extracted from virulent tubercle bacilli. Since it was obtained only from "cord-forming" organisms (2) it was called "cord factor." After being subjected to extraction with petroleum ether, the bacteria were still able to grow normally in suitable culture media. This fact suggested that the cord factor was located at the surface of the bacterial cell and that its removal did not disturb any essential structures of the cell. On the other hand, the importance of the bacterial surface in the establishment of an infection was emphasized by the fact that the isolated cord factor exerts some of the effects of intact virulent organisms, while the behavior of virulent bacteria after removal of the cord factor was in some respects more characteristic of avirulent than of virulent organisms. It is noteworthy that young cultures yielded considerably more cord factor than older ones.

These findings were consistent with the previous work of Middlebrook, Dubos, and Pierce (2) calling attention to the cord formation by virulent tubercle bacilli and correlating this phenomenon with properties of the bacterial surface concerned in the virulence of tubercle bacilli. Further evidence for differences in the surface structure of virulent and non-virulent mycobacteria was brought out in another series of experiments (3). Virulent organisms were shown to be considerably less permeable to the dye methylene blue than non-cord-forming avirulent bacteria. After extraction with petroleum ether, however, they became equally permeable. Apparently the petroleum ether extracts a surface material which is responsible for the low permeability of virulent strains of tubercle bacilli to even small molecules like methylene blue. Moreover, when cord-forming cultures were allowed to age *in vitro*, the cells became increasingly permeable to methylene blue; the greater the virulence of a strain, the longer its organisms remained relatively impermeable to the dye.

This observation parallels the small yield of cord factor obtained from older cultures, on one hand, and the fact that avirulent bacteria which did not

contain any measurable amount of cord factor were completely permeable to methylene blue, on the other.

Our earlier observations had already suggested that the cord factor was in some way concerned in the virulence of tubercle bacilli. If this was true, young cultures possessing more cord factor, should also be more virulent for susceptible animals than older cultures of the same strain.

The experiments reported in the present paper seem to bear out this working hypothesis.

Materials and Methods

Bacteria.—Three strains of tubercle bacilli were used in these experiments: The human strain H37Rv, obtained from the collection of Dr. R. J. Dubos; a human strain Jamaica No. 22, received from Dr. J. Freund; and a bovine strain Vallée, originally obtained from the Pasteur Institute in Paris and propagated on egg media for at least 20 years at the Institute for Hygiene of the Universtiy of Basel, Switzerland, from which it was brought to this laboratory in 1948.

The bacteria were cultured in 125 ml. Erlenmeyer flasks in 30 ml. of Tween-albumin liquid medium (4) containing 0.05 per cent Tween 80, and harvested after incubation periods of different lengths, as stated later. The flasks were inoculated with 0.3 ml. of a 1 week old culture and incubated at 37°C.

Animals.—For most infection experiments male mice of the CFI albino strain, 12 to 20 gm. in weight, were obtained from Carworth Farms, New City, New York. Other strains used were the C57 black (Carworth Farms) and the Swiss albino strain from Tumblebrook Farms, Brant Lake, New York. The animals were fed pellets and water and caged in groups of 5. Cross-infections were minimized by constant irradiation with germicidal lamps in the animal room (5). All mice were weighed in groups of 5 twice a week. Dead mice were autopsied and the lungs, spleen, kidneys, and liver inspected for gross lesions under a binocular dissection microscope (magnification × 10). If histological section were required the organs were fixed in 10 per cent formalin and stained as indicated below.

Infection.—The mice were infected intravenously with 0.1 ml. of bacillary suspensions. The density and number of bacteria used are described later in the text.

Plate Counts.—The number of living bacteria was determined according to the method of Fenner, Martin, and Pierce (6). The oleic acid–albumin–agar plates were sealed with rubber bands and the colonies counted under a dissecting microscope (× 10) after 3 weeks' incubation.

Turbidity Measurements of Bacterial Suspensions.—The various bacterial suspensions were standardized to a given optical density by means of a Coleman junior spectrophotometer at a wave length of $\lambda = 550$ mμ. 10 × 75 mm. tubes were used. Further details are mentioned in the text.

Reliability of the Plate Count Method.—The interpretation of the animal inoculation experiments depends partly on a knowledge of the number of living cells injected. "Living" in this connection refers to the ability to give rise within 3 weeks to a typical bacterial colony on a solid oleic acid–albumin–agar medium. The validity of the plate count method has been challenged and its reliability questioned. Wohlfeil (7) has devoted a careful study to this question, and more recently the usefulness of the method has again been investigated by Fenner *et al.* (6), and Dubos *et al.* (8). These authors were especially interested in the method as applied to mycobacteria. Their work indicates that under proper conditions the method is applicable for counting organisms with the same limitations and within the same margin of error as with

other bacteria. With tubercle bacilli the most serious limiting factor lies in the technical difficulty of preparing suspensions in which the organisms are uniformly distributed.

As will be seen, the conclusions drawn from our animal inoculation experiments would hold even if two counts of a given bacterial suspension could differ from one another by as much as 1 logarithmic unit. According to all previous investigators (6, 8), and according to our own findings as well, this allowable margin is definitely beyond the limits of error of the method.

In comparing young and old cultures of the same strain of tubercle bacilli, however, two additional complicating factors have to be considered. First, the cultures differ in the degree of clumping; even in Tween media very young cultures are somewhat more aggregated than older ones. Second, the size of the individual bacterial cell is considerably larger in a young culture with long, thin rods, than in an older one in which the cells are short and usually thicker.

The plate count method compares bacterial units, not individual cells. It is ovbious, therefore, that an old culture would give rise to a higher number of colonies than a younger culture of equal density, provided all cells are viable. In these experiments the plate counts gave consistently lower figures for younger than for older cultures of equal optical density; only part of this difference could be accounted for by the degree of clumping (see Table I, later in the text). The remaining part might well be due to the differences in individual bacterial size. The present paper verifies, however, the expectation that younger cultures have a higher degree of virulence than older ones. Since all the counts indicated that rather less than more younger bacterial units were injected when suspensions of old and young cultures of equal optical density were compared, observed differences in the degree of virulence of young cultures could safely be attributed to an inherent quality of these bacteria, and not to a numerical factor, since the number of older bacteria per injected unit of volume was actually higher than with young cultures.

Fenner *et al.* have already mentioned the possibility that the degree of clumping of a bacterial suspension might not be without influence on the outcome of an infection experiment. In interpreting the results of the animal inoculation experiments with suspensions of young and old cultures of tubercle bacilli, this point had to be taken into condideration, and appropriate control experiments were performed. It could be seen (Table III) that as far as these experiments go, the pathogenicity of a culture was not markedly affected by its degree of clumping.

EXPERIMENTAL

Plate Counts of Bacterial Suspensions from 3 Day and 3 Week Old Cultures.— The findings of Fenner *et al.* (6) concerning the accuracy of the plate count method as applied to tubercle bacilli were confirmed. The number of colonies of serial dilution tubes did not deviate more than ± 10 per cent from the expected values.

3 day and 3 week old cultures were centrifuged for 3 minutes at 1000 R.P.M. to sediment coarse particles and larger clumps. The supernatants were brought to equal optical density by diluting the older cultures with fresh Tween-albumin medium. At a wave length of 550 mμ, the light transmission of these suspensions was 94 per cent.

Microscopic observation revealed a different degree of clumping in suspensions of the two age groups. In order to obtain quantitative information about the total number of bacteria represented in a given number of clumps, stained smears of suspensions of the two groups were made and the state of dispersion determined by counting the number of bacteria in each of 200 clumps and thus differentiating these clumps according to the number of bacteria they

contained. 12 counts of different suspensions of each age group were made and the average values obtained listed in Table I.

It is obvious that the younger cultures contained somewhat larger numbers of bacteria per given number of clumps than the older ones.

Accordingly, plate counts from suspensions of equal turbidity of 3 day and 3 week old cultures gave a higher colony count for the older than for the younger cultures (Table II). But the difference was larger than could be ac-

TABLE I

State of Dispersion of 3 Day and 3 Week Old Cultures of the H37Rv Strain of Tubercle Bacilli

(Average values of 12 determinations)

No. of bacteria per unit	3 day old culture	Approximate No. of bacteria per 100 units	3 wk. old culture	Approximate No. of bacteria per 100 units
	per cent		*per cent*	
1	40.5	40	26	26
2	13	26	28.5	47
3–5	14.5	58	24.5	78
5–10	15	120	10.5	79
10–30	17	340	10.5	210
Total.....	100.0	584	100.0	440

TABLE II

Plate Counts of 3 Day Old and 3 Week Old Cultures of the H37Rv Strain of Tubercle Bacilli

3 day old cultures		3 wk. old cultures	
Colony counts at 10^{-4} (0.1 cc.)	Standard error $\epsilon = \frac{\sigma}{\sqrt{n}}$	Colony counts at 10^{-4} (0.1 cc.)	Standard error $\epsilon = \frac{\sigma}{\sqrt{n}}$
19.66	2.42	121.76	6.51

counted for by the different degree of clumping alone. The larger size of the younger cells, together with the slightly higher degree of aggregation, is probably responsible for the discrepancy between the two pairs of values; *i.e.*, the colony number and the number of bacteria expected from the cellular distribution. At any rate, it seems permissible to conclude that in no case did the 3 day old cultures contain more bacteria than the 3 week old cultures standardized to equal optical density. If anything, they contained less. The standard dose used for inoculation purposes was 0.1 ml. of cultures prepared as the ones used for these counts. Thus, from the younger cultures approximately 6 times less bacterial units were injected into the experimental animals.

The Results of Infection with 3 Week Old Cultures.—Mice infected with bac-

teria from 3 week old cultures, diluted to the standard density of a 3 day old culture, did not show any sign of disease for about 2 weeks. During this time they gained weight and appeared normal. Later they began to lose weight, their fur became rough, and their activity was reduced. At time of death, they had generally lost 25 to 35 per cent of their body weight.

The following gross macroscopic changes could be seen at autopsy:—

Lungs.—The volume was considerably increased, the entire surface was covered with yellowish areas of 2 to 3 mm. in diameter. Very often these foci formed large confluent masses occasionally covering the surface of an entire lobe. A bloody pleural exudate was present in most cases. The mediastinal lymph nodes were enlarged and necrotic.

Spleen.—Enlarged, sometimes up to 3 to 5 times the normal size.

Liver.—Occasional necrotic areas, but only in a small proportion of the animals.

Kidneys.—The organs were larger than normal and the surface in most cases spotted with necrotic foci smaller than the lesions of the lungs.

Heart.—No macroscopic lesions.

The extent of the described lesions was more pronounced the longer the animals survived; *i.e.*, the lesions in the organs of the last mice of a group to die were more extensive than the lesions in animals with a shorter survival time.

Microscopically these lesions differed little from one organ to another. They consisted of inflammatory foci showing the characteristic cellular elements of this tissue reaction: polymorphonuclear leukocytes, monocytes, and lymphocytes. Epithelioid cells, giant cells, and tubercle formation were extremely rare. They occurred occasionally in the spleen or the kidneys, practically never in the lungs or other organs. Despite the extent of the lung lesions, the confluent inflammatory areas of this organ did not show any caseation. The alveolar walls remained recognizable throughout.

Thus in the mouse tuberculous lesions do not have the characteristic aspect of a specific lesion in human organs or in the rabbit. The tuberculous origin of the lesions, however, can easily be demonstrated by staining the sections for tubercle bacilli. At this late stage of the disease tubercle bacilli are rare in the spleen but extremely abundant in the lungs, the kidneys, and occasionally the liver. The congested alveoli of the lungs are actually filled with bacteria.

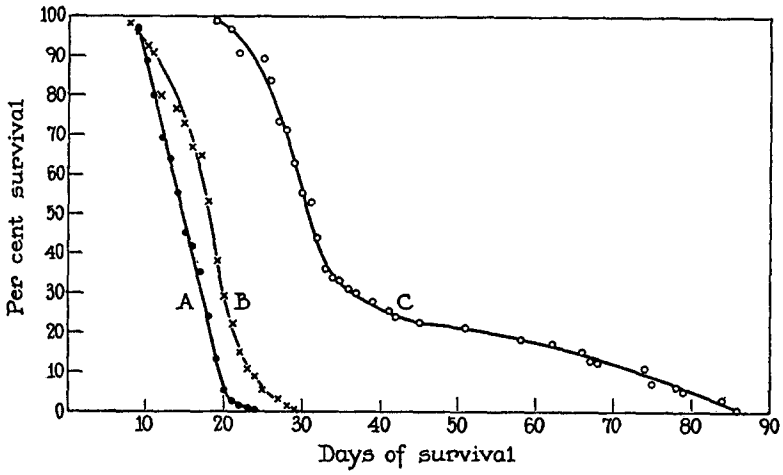
The survival time of the mice infected with the standard dose of a 3 week old culture follows a characteristic curve (Text-fig. 1, curve C). Some of the animals die within a relatively short period, and their survival time, if plotted against the percentage of survivors of the group, follows a logarithmic curve. Then the death rate becomes lower, the curve flattens out, and only after several weeks the rest of the group dies at a more accelerated rate again.

The survival curve takes its turn toward a more horizontal course 4 to 5 weeks after infection. The percentage of survival at that time depends on the total number of injected bacteria. The smaller the infective dose, the fewer mice have succumbed at this time. The general character of the curve, however, remains the same.

The Results of Infection with 3 Day Old Cultures.—To our knowledge, cultures of that age have not previously been used for animal inoculation. Before the

introduction of the new liquid media by Dubos and his collaborators, such young cultures were not readily available; but even since submerged cultures have become widely known, most workers have used fully grown 1 to 2 week old cultures for infection experiments.

If culture media are heavily inoculated in the way described (final dilution of the inoculum 1:100), good growth is present after 3 days (Table II). The average turbidity as determined by the method described was 94 per cent light transmission. Corrections were made by either concentrating or diluting cultures to this value.



TEXT-FIG. 1. Survival curves of CFI mice infected intravenously with tubercle bacilli, strain Vallée. Curve A, animals infected with the standard dose of bacteria from 3 day old cultures (195 mice). Curve B, animals infected with 10 times the standard dose of bacteria from 3 week old cultures (63 mice). Curve C, animals infected with the standard dose of bacteria from 3 week old cultures (123 mice).

The response of mice to infection with the standard dose of cultures of this age was entirely different from that described above for older cultures. The death rate follows a logarithmic curve throughout (Text-fig. 1, curve A). All animals died within a relatively short interval of time. After an initial gain of weight, they began to emaciate rapidly after less than 1 week; 2 weeks after infection 50 per cent of the mice were dead.

The findings at autopsy in these mice differed considerably from those described above. Whereas the most conspicuous lesions after infection with an old culture were found in the lungs, the first mice of a group dying from an infection with 3 day old cultures had the most striking changes in the heart.

The myocardium appeared covered with yellow spots about the size of a pinhead giving the organ a raspberry-like appearance. In contrast to the conspicuous heart lesions, none of

the other organs shows any macroscopic changes at this time, except for the enlarged spleen. Later, around 2 weeks after infection, small disseminated lesions appeared in the lungs, becoming more conspicuous the longer the animals survived. In contrast, the heart lesions became progressively less conspicuous in the last mice of a group to die. Macroscopically, the hearts of animals surviving the infection for 3 weeks or more appeared normal, the lungs were covered with small lesions of miliary type, and occasional foci could be seen in kidneys and liver.

Microscopically, the myocardium proves to be tightly interspersed with innumerable inflammatory foci varying in size. They consist chiefly of polymorphonuclear leukocytes, a small proportion of monocytes, and in hematoxylin-eosin–stained sections, a considerable mass of irregularly stained debris. Most foci are centered around small blood vessels. They spread within the connective tissue, but involve the myocardial fibers surprisingly little. The pericardium participates only occasionally in the process of inflammation, when a lesion is located close to the heart surface. Bacterial stains show that each one of the inflammatory areas is filled with a tremendous number of tubercle bacilli.The organisms, too, are located between the muscle fibrillae and only rarely seem to invade these directly. The masses of debris seen in hematoxylin-eosin stains consist most likely of tubercle bacilli (Figs. 1 to 4). While it was mentioned before that no macroscopic lesions could be found in the lungs at this time, microscopic sections revealed the presence of identical lesions scattered throughout the organ, forming a lobular pneumonia. They differed only by their smaller size from the lung lesions described in the first group. The alveolae were filled with bacteria whereas the bronchi at this stage were free from inflammation as well as from tubercle bacilli.

Blood-borne foci can also be found in sections of the kidneys, the liver, the spleen, and the bone marrow. In view of the extreme abundance of tubercle bacilli, it is not surprising that in this type of disease blood smears from the tail veins of mice were microscopically positive for tubercle bacilli for at least 2 weeks beginning at the 3rd day after infection. Bacteria in the blood were mostly intracellular.

Of a group which had no gross lesions in the heart those which died late still showed small cardiac foci containing tubercle bacilli upon microscopic inspection. Similar minimal myocardial lesions were occasionally found in the very first mice of a group which died after infection with 3 week old cultures.

The Effect of Age and Dosage of the Bacteria on the Results of Infection Experiments.—There was little difference between the findings in the earliest deaths of a group of mice infected with a 3 week old culture, and the longest survivors of mice infected with organisms from a 3 day old culture. Likewise, the survival curves of the two groups overlapped (Text-fig. 1, curves A and C).

In a 3 day old culture, probably all bacterial cells are in the stage of active multiplication. In a 3 week old culture which is essentially stationary, the majority of bacteria are in a resting stage, and only a very small proportion has recently divided and thus is "young" as in a 3 day old culture. By concentrating a 3 week old culture, however, the absolute number of "young" bacteria is increased and the effect of a concentrated infective dose might come close to the effect of infection with a 3 day old culture. It has to be kept in mind, though, that the resulting effect may be distorted by the overwhelming number of resting cells injected at the same time which cannot be separated from the "young" portion of a concentrated culture.

In a series of four experiments a total of 63 mice were infected with 10 times the amount of 3 week old bacteria previously used. The effect upon their survival time is seen in Text-fig. 1, curve B. The lesions, as well as the survival time, were of an intermediate character as far as the numerical ratio heart-lung lesions was concerned. It is instructive, though, that even 10 times as many bacteria from "old" cultures did not entirely equal the effect of "young" cultures.

The Independence of the Described Phenomena of the Strain of Mice or Bacteria Used in the Test.—All experiments reported above were performed with CFI mice and the Vallée strain of tubercle bacilli. The mice had an average weight of 12 to 14 gm. at the time of infection.

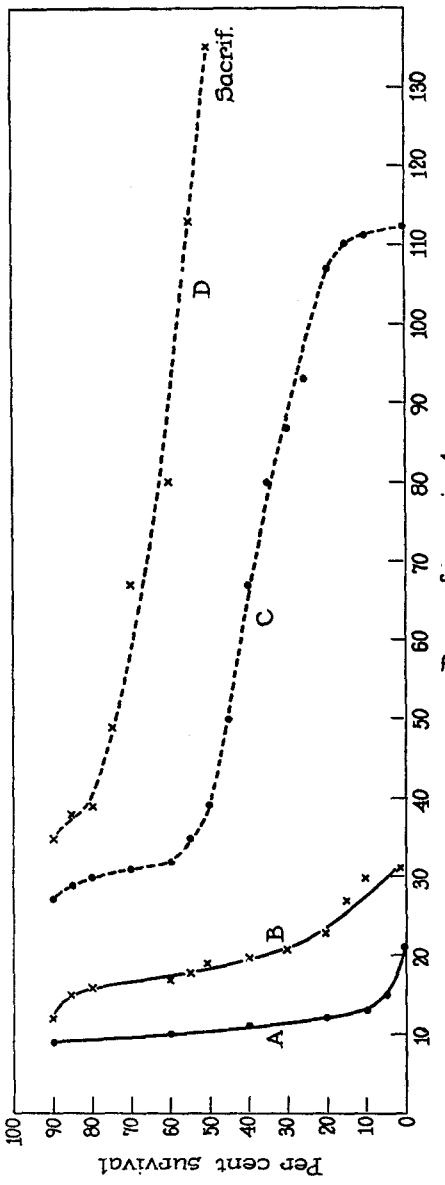
Control experiments with mice of the same strain, but of an average weight of 20 to 22 gm., gave completely identical results, with regard to the lesions produced as well as the survival time. Other groups were infected with organisms from 3 day old cultures of the H37Rv and the Jamaica No. 22 strains; and mice of the Swiss albino and the C57 strains were infected with Vallée and H37Rv cultures. All results were identical; none of the observations seemed to be specific for the strain of mice or the strain of bacteria as long as 3 day old cultures were used.

After infection with diluted 3 week old cultures, however, the survival curves were no longer identical. Here the well known differences in susceptibility of various strains of mice (9, 10) showed their influence; for example, within 135 days after infection only 10 out of 20 Swiss mice infected with the Jamaica No. 22 strain died of tuberculosis. Thus the difference in the response to infection with "old" and "young" bacteria was even more impressive in this case. Text-fig. 2 summarizes two experiments with mice of the Swiss albino strain, infected with H37Rv (curves A and C) and Jamaica No. 22 bacteria (curves B and D), each in two dilutions, 20 mice in each group.

The Influence of the Degree of Bacterial Aggregation upon Infection.—Fenner, Martin, and Pierce (6) have mentioned the possibility of a different animal response after infection with uniformly dispersed or clumped bacteria. Since "young" and "old" bacteria showed a slightly different degree of aggregation, the following experiment was performed:—

Bacteria of a 3 week old Vallée culture were diluted to equal density with a 3 day old culture of the same strain and divided into 2 portions. One was washed twice with culture medium (0.05 per cent Tween, 0.5 per cent albumin), the other with the same medium but without Tween. After the second washing (centrifugation at 5000 R.P.M. for 10 minutes each), the bacteria were resuspended in the original amount of culture medium. One portion was as dispersed as before, the one washed with Tween-free medium was clumpy. Two groups of 6 mice each were infected in the usual way with the two bacterial suspensions. The survival time of these animals is recorded in Table III.

The survival times of the mice in the 2 groups are not strikingly different



TEXT-FIG. 2. Survival time of 4 groups of 20 Swiss mice each. Curve A, animals infected with the standard dose of tubercle bacilli (strain H37Rv) from a 3 day old culture. Curve C, animals infected with a 1:15 dilution of the same suspension. Curve B, animals infected with the standard dose of tubercle bacilli (strain Jamaica No. 22) from a 3 day old culture. Curve D, animals infected with a 1:15 dilution of the same suspension.

and it does not seem that the characteristic effects obtained with young cultures are due to their higher degree of clumping.

Infection of Rabbits with Young Bacteria.—The experiments reported so far showed a type of infection which developed independently of the strain of mice as well as of the strain of bacteria. It was of interest to determine whether a similar acute disease could be produced in the rabbit with young bacteria of a human strain, to which the rabbit is known to exhibit only a moderate susceptibility.

TABLE III

The Survival Time of Two Groups of Mice, Infected with Diffuse and with Clumpy Suspensions of a 19 Day Old Culture of Tubercle Bacilli (Strain Vallée)

State of dispersion of the injected bacteria	No. of mice in the group	Length of survival after infection
		days
Diffuse	6	23;24;24;46;50;50
Clumpy	6	22;23;23;24;35;57

TABLE IV

The Survival Time of Rabbits Infected Intravenously with 4.5 Ml. of 3 Day Old Cultures of Different Strains of Mycobacteria

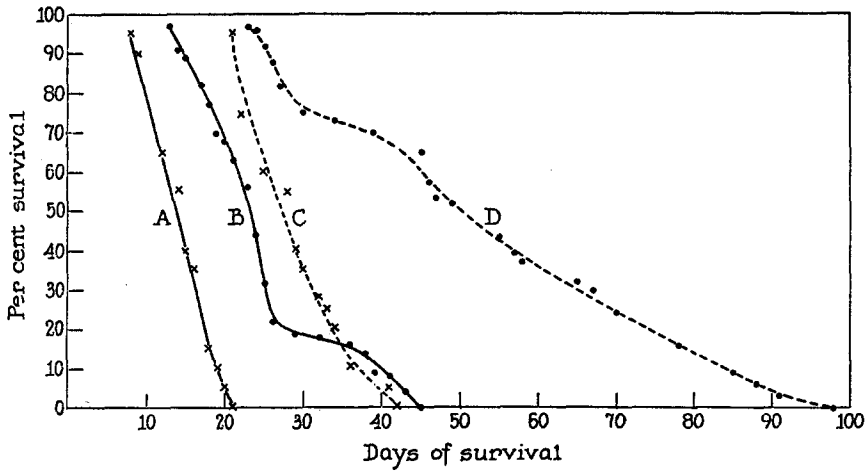
Average weight of the rabbits at the date of infection: 1900–2500 gm.

Strain of bacteria	Type	No. of rabbits	Length of survival
			days
H37Rv	Human	6	18;19;21;21;27;31
Jamaica No. 22	Human	1	22
Vallée	Bovine	3	13;15;24
Ravenel	Bovine	1	19

Rabbits were infected intravenously with 4.5 cc. of 3 day old cultures of different human and bovine strains. The results shown in Table IV reveal that all rabbits died within a short period of time, regardless of the type of mycobacteria used. At autopsy no gross lesions were found. The only conspicuous pathological change was a greatly enlarged, swollen spleen. Microscopically the lungs, spleen, liver, kidneys, and heart showed small inflammatory foci consisting chiefly of polymorphonuclear leukocytes, monocytes, and very few giant cells. Surprisingly enough, tubercle bacilli were extremely rare in these lesions; the spleen was the only organ in which any significant number of bacteria were found. This finding is in sharp contrast to that in mice, but it is in good agreement with earlier observations (11, 12).

Experiments with One Day Old Cultures.—Significant differences between

the virulence of 3 day and 3 week old cultures have been described above. 3 day old cultures were chosen as representative of "young" cultures because they yielded profuse growth under the conditions of the experiments. The results, however, were contradictory to the work of Nègre *et al.* (13–16), who claimed that the virulence of very young cultures of tubercle bacilli is less than that of fully grown cultures of the same strains. Since the French authors grew the organisms on the surface of Sauton medium, their findings are not directly comparable to ours. In an effort to reconcile Nègre's results with ours, some experiments with 24 to 36 hour cultures were performed since there are reasons



TEXT-FIG. 3. Survival curves of 4 groups of 20 CFI mice, each infected with different preparations of tubercle bacilli (strain Vallée). Curve A, animals infected with the standard dose of bacteria from a 3 day old culture. Curve C, animals infected with a 1:10 dilution of the same suspension. Curve B, animals infected with a concentrated suspension of bacteria from a 24 hour old culture. Curve D, animals infected with a 1:10 dilution of the same suspension.

to believe that tubercle bacilli grow faster in Tween-albumin than on Sauton media. While the lag phase of tubercle bacilli in a culture medium has never been accurately determined, gross observations showed that the inoculum used in these experiments had begun multiplying during the first 24 hours of incubation.

Tween-albumin cultures 24 to 36 hours old were concentrated by centrifugation until density equal to that of a 3 day old culture was reached. A concentration of about 60:1 was usually necessary. Groups of mice were injected with these bacteria, and the effect compared with the animal response after inoculation with 3 day old cultures. The results, summarized in Text-fig. 3, show that the average survival time of mice after infection with 1 day old cultures (Text-fig. 3, curve B) is longer than after inoculation with 3 day old cultures (Text-fig. 3, curve A) of the same strain. Even more striking is the response when

the infective doses were each reduced to one-tenth of the original size by diluting the bacterial suspensions with fresh culture medium (Text-fig. 3, curves C and D).

In view of the proposed working hypothesis, the lower virulence of very young cultures (consistent with Nègre's results) might correspond to a less abundant coating of these organisms with cord factor. While quantitative extractions of cord factor did not give satisfactory results with the available methods, some indirect evidence for this inference was obtained.

Staining Properties and Morphological Aspect of Bacteria Present in 1 Day Old Cultures.—It was a regular finding that 1 day cultures were considerably less acid-fast than bacteria from older cultures. Two types of organisms could be distinguished in young cultures: A small number of them showed the normal

TABLE V

The Inhibition of the Migration of Leukocytes from Guinea Pig Blood by Cultures of Different Age of the H37Rv Strain of Tubercle Bacilli

Age of culture	Extent of migration
days	
1	+++*
3	0
8	0
21	++*
31	+++*
Control without bacteria	++++

* The type of migration in these slides was different from the controls inasmuch as the leukocytes formed clumps around aggregates of bacteria.

staining properties and cell morphology of the strain, whereas most appeared as long, very slim, poorly acid-fast or almost blue-stained bacilli not showing the characteristic metachromatic polar endocellular granules of older organisms. It does not seem unsound to identify the first mentioned portion of the culture with the cells from the inoculum, and the second with younger organisms grown in the culture. Similar forms of poorly acid-fast, long rods are only occasionally found in older cultures.

It is not clear whether the "blue bacilli" mentioned by Hauduroy and others (17) belong to the same category.

The Effect of 1 Day, 1 Week, and 3 Week Old Cultures on the Migration of Leukocytes in Vitro.—It has been shown previously that the migration of leukocytes *in vitro* was inhibited following phagocytosis of virulent tubercle bacilli (18). These findings have been recently confirmed by Martin *et al.* (19). Since avirulent variants of tubercle bacilli did not have the same effect on migration of leukocytes, the possibility of a similar parallelism between virulence and

effect on leukocytic migration was tested with cultures of the same strain but of different age. The technique used was the modification described in detail by Martin *et al.* Table V summarizes the results. The values reported in this table are the average results of 11 to 16 determinations with bacteria of each age group. It can be seen that within the same strain of bacteria there are differences which, to some extent, parallel their virulence for mice.

Other experiments were designed to test whether very young bacteria would decolorize methylene blue in a Thunberg tube, as do non-virulent organisms, or virulent ones from old cultures. 1 day old cultures were therefore concentrated by centrifugation and tested according to the technique described before (3). Contrary to expectations, bacteria from 1 day old cultures did not decolorize methylene blue. This fact could not be reconciled with the rest of the observations.

DISCUSSION

This study was aimed at bringing additional support to a hypothesis based on a number of *in vitro* observations on the nature of the tubercle bacillus. According to this hypothesis, a cellular constituent of the bacillus which we called "cord factor" and which is soluble in petroleum ether, paraffin oil, and related products, is partly responsible for properties associated with the virulence of the organisms. Only indirect evidence for such a role of the cord factor has been found so far. The present experiments started from the assumption that tubercle bacilli containing more cord factor would be more virulent than others of the same strain containing less. From the yield of cord factor obtained after extracting the bacteria with petroleum ether, and from an increased surface permeability observed in aging bacteria (3), a higher degree of virulence was anticipated in young organisms.

When comparisons are made of the virulence of different strains of tubercle bacilli, variations in the animal response can be attributed to a number of variables both of the host and of the microorganisms (20). Animal variations can be eliminated by using the same strain of animals under entirely comparable conditions, but the bacteria of various age groups can still vary in a number of factors which, together or separately, affect the response. In the present study young and old cultures of the same bacterial strain were compared. One difference that we know to exist between such cultures is their varying contents of cord factor. Other measurable differences can be found such as cellular size and morphology (21), but no information relating these properties to the parasitic functions of the organisms is available. In view of our working hypothesis it was tempting to attribute differences in pathogenicity of young and old cultures to the varying amounts of cord factor they contain.

It was emphasized before (1) that while there were reasons to regard the cord factor as one essential element in the virulence of a strain of tubercle

bacilli, this substance is not the only determining factor. Other as yet unknown properties of the bacteria are undoubtedly of equal importance. This is clearly demonstrated by the fact that cord factor is also obtained from cord forming BCG strains which are not virulent for a normal host organism (1). Yet, while non-virulent, these mycobacteria do multiply in a normal animal and are able to cause progressive tuberculosis in silicotic guinea pigs (22).

Except when inoculating with a single cell, we are always confronted with average values in experimenting with bacterial cultures. This is particularly true with respect to the individual age of the single organisms in a culture flask. But it can be safely assumed that in a 3 day old culture the great majority of tubercle bacilli are younger than in a culture 3 weeks old, and differences between the effect of 3 day and 3 week old cultures may, therefore, be attributed to the average age of the organisms, provided equal numbers of living cells are compared. The quantitative methods for the evaluation of the number of living cells have already been discussed.

The differences in the pathogenic effects observed after infecting mice with young and old bacilli of the same strain were remarkable. Whereas the injection of old organisms resulted in the classical picture of mouse tuberculosis, the results obtained with young bacteria differed completely from previous experience. The mice died from an acute, septicemic type of disease, developing lesions in almost every organ. A tuberculous myocarditis seemed to be the most serious effect; it was most likely the immediate cause of death. The tuberculous origin of the lesions was revealed by the mass of tubercle bacilli they contained. Histologically, they bore the characteristics of fresh inflammatory foci without any of the productive or necrotic tissue reactions usually regarded as typical for tuberculosis. The distribution of the lesions clearly indicates that they were blood-borne. The extremely high number of organisms which have to be present in the blood stream in order that bacteria can be found microscopically indicates either multiplication of the bacteria in the blood, or a continuous invasion of the blood stream from the tissues. The fact that blood smears regularly showed tubercle bacilli from the 3rd day after infection on, *i.e.* long before the terminal stage of the disease was reached, at a time when the tissue lesions were still small and not suppurative, may be interpreted in favor of bacterial multiplication in the blood; but an unequivocal answer to this question cannot be given (23).

The question arises as to the factors responsible for the establishment of this acute type of tuberculous infection. According to the proposed working hypothesis, the large amount of cord factor present in young organisms would be related to the observed differences in the disease caused by young and by old bacteria. The function of the lipid coating could thereby be either protective against unfavorable environmental conditions in the host, or agressive, injuring the host tissues and thus creating favorable conditions for the fixation and

multiplication of the parasite. In addition, the abundant production of cord factor in young bacteria could in itself result in an intoxication of the host. A peculiar type of toxicity of the isolated cord factor was found earlier (1).

In 1888, Yersin (11) described an acute tuberculous infection which became known as the "Yersin type of tuberculosis." It is produced by injecting intravenously into rabbits 1 mg. or more of virulent tubercle bacilli of a recently isolated avian strain. The disease is characterized by its rapid progression, the rabbits dying within 2 to 3 weeks. At necropsy the macroscopic lesions are very inconspicuous. No gross tuberculous nodules are seen. A similar type of infection can also be produced with bovine type tubercle bacilli (24, 25), though the lesions there are somewhat larger and may form macroscopically recognizable nodules. It is important for the production of the Yersin type of tuberculosis that the bovine bacilli be highly virulent and grow in "smooth" colonies or that the avian tubercle bacilli be recently isolated from an acute case of avian tuberculosis.[1] This seemed to be of interest in connection with the problems dealt with here. On the classical culture media avian strains grow in a manner resembling human bacteria in fluid media containing Tween; *i.e.*, the growth is rapid and often diffuse. Thus it is most likely that fully grown avian cultures used for the production of Yersin type tuberculosis in rabbits were in most instances considerably younger cultures than the corresponding cultures of human bacteria. Since it is possible now, with the media developed by Dubos and his coworkers, to obtain rapid and diffuse growth of human strains, our views concerning the Yersin type of tuberculosis have to be revised. The experiments reported in this paper show that this type of infection can be produced equally well not only with bovine but also with human strains. It seems likely, therefore, that the production of Yersin type of tuberculosis depends not so much on the type of mycobacteria as on the physiological state of the culture, and that young diffuse cultures of either mammalian or avian type can produce this type of disease. Its characteristics are the rapid septicemic spread with metastatic tuberculous abscesses in numerous organs. The rabbits die before typical histologic lesions have developed.

In this respect, the response obtained in the mouse to massive infection with young virulent cultures is very similar. There, too, the dissemination is generalized throughout the body, and only mice surviving for a longer period of time

[1] This is the textbook definition of the Yersin type of tuberculosis (23, 26, 27). It is interesting to note that Yersin, in his original paper, described the culture he used as having been isolated from a tuberculous calf, then propagated in guinea pigs, and subcultured from a guinea pig spleen on glycerin agar. This culture, of bovine origin and pathogenic for guinea pigs, almost certainly was a bovine strain. Moreover, it seems to be of historical interest that avian tubercle bacilli were first described in 1889 by Rivolta (28), 1 year after Yersin's paper appeared. Furthermore, the so called Yersin type of tuberculosis in rabbits was first produced with avian bacteria only in 1891, 3 years after Yersin's original work, by Straus and Gamaleia (29).

show the lesions predominantly localized in the organ of predilection. The characteristic susceptibility of the different mice strains to tuberculous infection in general, as well as to the type of bacteria used, was of little, if any, importance for the severity of the acute disease produced with intravenous injections of young cultures.

It seems justifiable to conclude from these observations that an acute septicemic type of disease can be produced in mice as well as in rabbits by infecting the animals with young cultures of virulent strains. This disease, which is probably identical with the long known Yersin type of tuberculosis, is fatal within 1 to 3 weeks. It is not accompanied by any of the tissue responses occurring in allergic (tuberculin-positive) animals. Only when an animal survives the primary acute stage of the tuberculous infection and enters the second (allergic) phase of the disease, do all the characteristic histologic reactions occur and the specificity of types of bacteria as well as the characteristic susceptibility (or resistance) of the animals have their effect.

In earlier literature, the acute phase of the tuberculous infection has not been given much consideration, and the Yersin type of the disease is generally considered an artefact (25). A survey of publications shows, however, that the disease occurs in human pathology too, and is perhaps more frequent than suspected. It is known under the name of typhobacillosis of Landouzy, *sepsis tuberculosa acutissima*, generalized non-reactive tuberculosis, etc., (30). It is entirely different from the classical type of tuberculosis, and only the prepared pathologist will diagnose it postmortem, since it lacks all the histologic characteristics of tuberculosis and can be recognized only bacteriologically. But it is rare, under routine procedures, that tissue sections from autopsy material are stained for tubercle bacilli. A recent publication (31) suggests that acute tuberculous infections often pass unnoticed and are incorrectly diagnosed as agranulocytosis or similar blood diseases.

As to the unusual localization of tuberculous lesions in the heart muscle, there are reports of tuberculous myocarditis in cases of actue massive infections in children (32) or in adults in primary infections or miliary disseminations in terminal cases in which the tuberculin test had become negative (12, 33).

The two types of survival curves represented in these experiments indicate two entirely different phases of the same original disease—one is almost tempted to say two different diseases. Once an animal has survived the acute phase, it enters a second phase in which its reactivity toward the parasites changes completely. It has acquired means to localize the bacteria in certain organs. Though the animal finally succumbs, it can survive for a considerable time without conspicuous signs of illness.[2]

[2] This was illustrated by an instructive though unplanned experiment. Through carelessness a window in the animal house was left open overnight, and the temperature in the mouse room dropped close to the freezing point. During this night 43 mice died. Among over 600

Survival curves of a similar shape were published by Thomas (35). While it is not clear from his data whether the two types were caused by similar differences in the nature of the infecting microorganisms, the experiments reported in the present paper indicate that in the early phase of the disease the age and number of invading bacteria determine the fate of the animal, and only in the second phase do constitutional and acquired properties of the host have a chance to play their important part.

It is obvious that during the aging process of a culture the physiological state of the bacteria undergoes changes which are important enough to express themselves significantly in the response of an animal to infection. It cannot be ruled out, however, that the culture medium which is injected along with the bacteria plays a role too. While control experiments showed that filtrates of cultures of different age did not have any pathogenic effect, preliminary results indicate that washing bacteria thoroughly before they are injected might influence their pathogenicity and even accentuate the differences described (36).

All experiments reported here can easily be explained within the framework of the hypothesis presented in the introduction to this paper and are in agreement with the conclusions reached in the earlier phases of this work. It had been emphasized that the cord factor seemed to be an essential attribute of virulent tubercle bacilli without being the only factor responsible for their virulence. There is no reason now for modifying this view; the arguments enumerated earlier in favor of the role of the cord factor have been supported by a number of significant experimental facts. On the other hand, these experiments did not throw light on the question raised by the existence of mycobacteria which produce cord factor and yet are not virulent; *i.e.*, the attenuated strains of the BCG type. It has already been mentioned that these strains, while not causing progressive tuberculosis in a normal animal or man nevertheless multiply in a host organism and can cause fatal tuberculous infections in silicotic guinea pigs. They are equipped with some factors necessary for the establishment of a disease, but for unknown reasons they are virulent only under special conditions in which the host has already suffered an unspecific injury. The fact that we do not know yet the additional attribute which they lack for the manifestation of full virulence does not diminish the importance

mice housed in the room, 200 were normal and over 200 had been infected during the previous 2 weeks with highly virulent bacteria from young cultures, but none of these latter died; all the mice that died from the cold belonged to groups which had been inoculated 4 to 12 weeks earlier with tubercle bacilli from old cultures. Most of them had not shown conspicuous signs of illness, but the necropsies revealed extensive confluent lung lesions as commonly seen in mice dying spontaneously from chronic tuberculosis. The fact that only these mice died from the unspecific injury represented by the sudden cold indicates that at this chronic stage, the animals lived in an unstable equilibrium with their disease, easily disturbed by any unspecific impact from outside. The incident is given this interpretation in analogy to Dubos' suggestion for an explanation of tuberculosis mortality curves in European countries during the war (34).

of the role of the cord factor suggested by these experiments. While one could also speculate along other lines in interpreting these results, there is no immediate need for doing so. As long as the facts easily comply with the theory, it is proposed—for mere reasons of economy—to explain these experimental findings within the framework of the presented hypothesis.

SUMMARY AND CONCLUSIONS

On the basis of earlier observations dealing with the relation of a petroleum ether–soluble material (cord factor) obtained from young cultures of virulent tubercle bacilli to the pathogenicity of these organisms, it was expected that young cultures yielding more cord factor than older ones of the same strain would also be more virulent for susceptible animals. By infecting mice with equal numbers of bacteria from 3 day and 3 week old cultures, significant differences in the character of disease produced were observed. The mice infected with the younger cultures died of a rapid, septicemic infection with tuberculous lesions in many organs including the heart. A tuberculous myocarditis was probably the immediate cause of death. Mice infected with the older bacteria died of a chronic disease corresponding to the well known mouse tuberculosis. In these cases, the heart was completely free of lesions. No histologic tissue reactions typical of tuberculosis were seen in the animals dying from the acute type of the disease. A similar rapidly progressing infection was observed in rabbits infected with bacteria from young cultures. The symptoms corresponded to the ones seen in the disease known as the Yersin type of tuberculosis. It seems that the pathology of this latter can be produced with every type of pathogenic mycobacteria, human as well as bovine and avian, provided the cultures used are young. Thus it may be inferred that the acute type of tuberculosis is more frequent than commonly accepted both in experimental infection and in the naturally occurring disease. It is proposed to explain the mechanism of this acute infection within the framework of the cord factor hypothesis.

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EXPLANATION OF PLATE 24

The photographs were made by Mr. Julian Carlile.

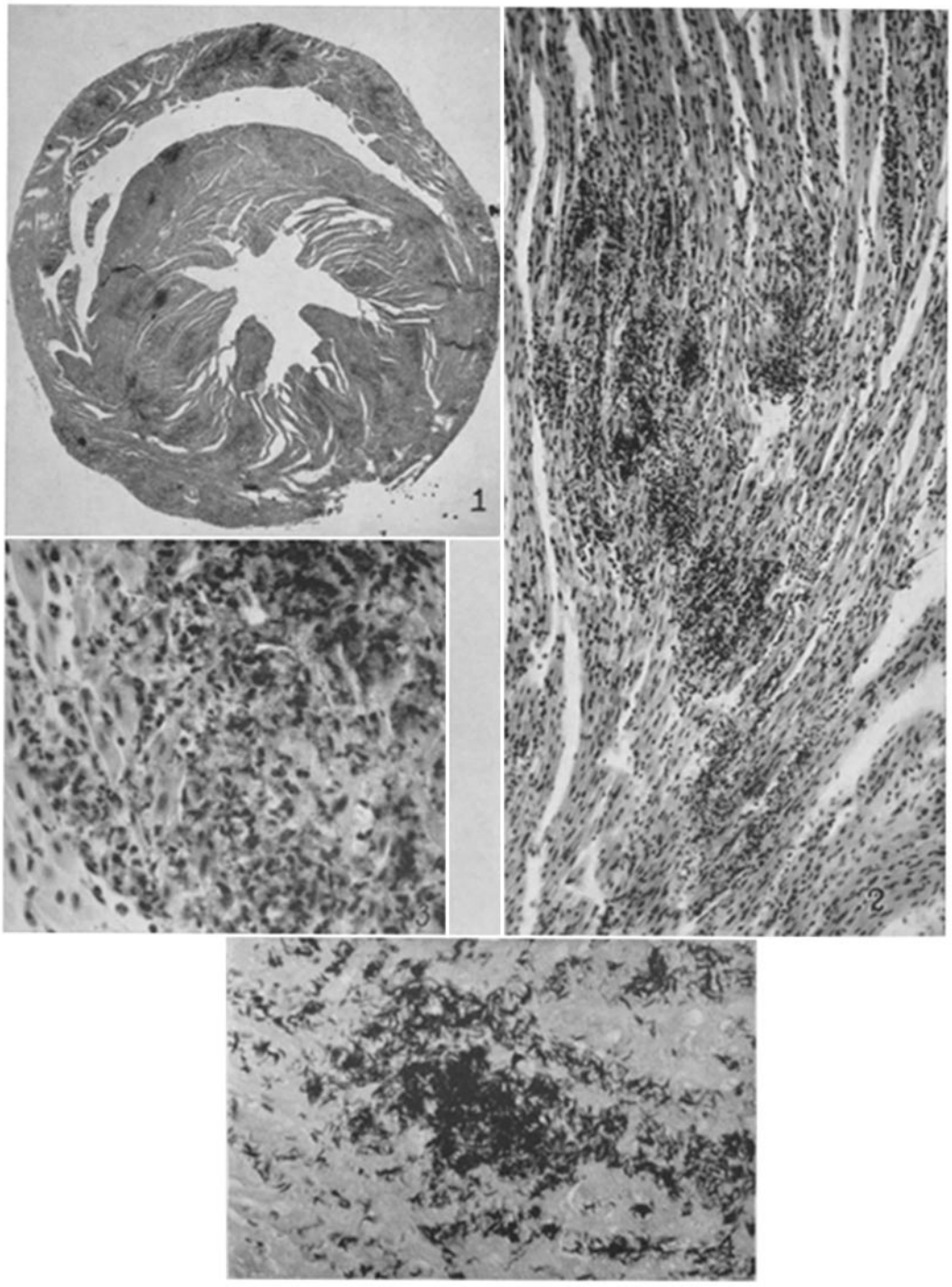
PLATE 24

FIG. 1. Cross-section of the heart of a mouse which died 11 days after infection with bacteria from a 3 day old culture of tubercle bacilli, strain Vallée. Random distribution of inflammatory foci throughout the myocardium. Hematoxylin-eosin stain. × 16.5.

FIG. 2. Photograph taken from the same section as Fig. 1. × 122.

FIG. 3. Photograph taken from the same section as Figs. 1 and 2. It shows the center of an inflammatory focus; the myocardium is infiltrated by leukocytes and poorly stained debris. × 328.

FIG. 4. Photograph taken from the same area as Fig. 3. Section stained according to Fite's method (37), showing the enormous masses of tubercle bacilli in the myocardium. × 305.



(Bloch: Virulence of tubercle bacilli)