The Significance of Pleomorphism in *Mycobacterium tuberculosis* var. *hominis*

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SUMMARY: Under adverse conditions, *Mycobacterium tuberculosis* may autolyse, or surviving cells may divide into progressively shorter cells which may even appear to be coccal. We have not observed a complex 'life cycle', but the cells whose cytoplasm has been reduced show qualitative differences which we term 'regression'.

The significance of non-acid-fast forms, granules, beads, 'substance cyanophil' and spore-like bodies, observed in cultures of *Mycobacterium tuberculosis*, and in tubercular material, has been one of the major controversies of medical bacteriology during the past fifty years.

Our interest in this problem arose from frequent observation of acid-fast coccal forms in pus from tuberculous lesions of bone in which no bacilli could be found. We decided to investigate the morphological variability of human strains of *M. tuberculosis* under different conditions, and to see whether we could induce changes corresponding to any of the 'life cycles' previously described. Some of the conditions which we imposed on our cultures were chosen because they were said to be successful in inducing the L cycle of other bacteria.

MATERIALS AND METHODS

Phase-contrast microscopy. A Beck microscope with a 2 mm. objective and $\times 10$ eyepiece, and illuminated by a 6 V. Tenslite with an Ilford 404 green filter was used for intermittent observations, and two Cooke microscopes for the observation of microcultures over longer periods. The larger of these was described in this *Journal* (Lack, 1952). The other has a Newman-Waterfield warm chamber (Kaylene Ltd. London) which maintains a slide culture at 37°. Both these microscopes are fitted with Cooke phase-contrast systems and Ilford green filters.

Staining techniques. Ziehl-Neelsen, with and without counterstain, Much's and Gram's methods and 1% malachite green counterstained by 0.5% safranin. Klieneberger-Nobel's (1950) techniques of agar cut-out and osmic acid vapour fixation were used for cytological studies.

Microcultures. The majority were made on Dubos agar prepared by the addition of 1.5 g. New Zealand agar to 90 ml. Tween-Dubos medium, which was steamed for 1 hr., adjusted to pH 7.2, filtered, and autoclaved at 10 lb. pressure for 10 min. Bovine albumen solution was added at 55° to give a final concentration of 1/26.

Strains. H37Rv was used in most of this work, but a large number of strains has been freshly isolated from patients.

Culture media. Löwenstein, Dubos, and Bordet Gengou with 50 % human red cells, were used most frequently. In order that pleomorphism might be studied under various controlled conditions, the growth of H37Rv was also observed in the following media: Dubos, Dubos agar, glucose peptone water, glucose broth, 10 % glycerol in Dubos, yeast agar, 1 % urea in Dubos, 1.5 % glycine in Dubos. Cultures were made in urea-Dubos because granular forms were frequently observed in urine. Glycine was tried because it has been found to favour the production of L-forms in some other genera.

Strain H37Rv was also grown in the following biological fluids: serum from tuberculous and non-tuberculous patients; serum inactivated at 56° for 30 min.; pus digested with plasmin and deoxyribonuclease; synovial, pleural and amniotic fluids. The action of physical and chemical agents and of various antibiotics was also studied.

OBSERVATIONS

In eighty-eight experiments the growth of different strains was observed in a wide range of media. Only in media containing glycine or antibiotics did we observe the development of giant forms, and under no conditions did we observe a true life cycle in any way resembling that of fungi. Although the variations in pellicle production in liquid media were striking, and merit more attention, they were not studied further since they were not connected with morphological variation.

The Tables set out a summary of some of our observations representing the changes observed (i) in exhausted or deficient media (Table 1); (ii) when grown with different antibiotics (Table 2). These changes were observed mainly by means of phase contrast, often over long periods; if the photographs (Pls. 1-3) are studied before the Tables, terms such as typical bacilli (Pl. 2, fig. 5), autolysed rafts (Pl. 1, figs. 1, 2), degenerate bacilli (Pl. 1, fig. 3), short forms and 'diphtheroids' (Pl. 2, figs. 6*a*, *b*), and coccal forms (Pl. 1, fig. 4 and Pl. 2, fig. 7) will be better understood. Unless a stain is mentioned (z.n. = Ziehl-Neelsen, m.g.s. = malachite green safranin), the description is that of the phase-contrast preparation.

Multiplication. We saw each of the methods of multiplication of bacilli described by Hu (1936), and illustrated diagrammatically (Fig. 1*a*). Lateral budding was rare. Under some adverse conditions, surviving cells continued to divide in these ways, but as they did not grow so long, the daughter cells produced the coccal groupings that have been so frequently described (Fig. 1*b*). In very old cultures or under the action of some antibiotics, cells sometimes grew so little before they divided that they were already cocciform before they sent out a bud; these Hu described as 'sprouting granules'.

Autolysis. We watched the changes which occur when cells autolyse, and correlated the appearances seen by phase contrast with corresponding stained preparations. When individual cells were about to lyse, their nuclei became spherical and distended the cell wall (Pl. 1, fig. 3), giving the appearance of spores. When, on the other hand, the cells were in a cord or raft, autolysis began in one portion while cells elsewhere were still growing and dividing.

The outlines of the dead cells disappeared, the cytoplasm of adjacent cells fused and the nuclei became globular. The globules in this symplasm varied in size just as the nuclei varied in the cells before autolysis (Pl. 1, fig. 1). As the symplasm shrank and the nuclear globules were drawn closer together the mass had the appearance of a cluster of cocci (Pl. 1, fig. 2). These products of autolysis could persist for a very long time. Often they were embedded in

Fig. 1

long strands of extracellular material (slime, 'substance cyanophil'). If bacilli were still dividing close to an area of autolysis, they were likely, for reasons we give below, to be short or even coccoid in form, so that it might appear as though they had their origin from this symplasm. We never observed regeneration from cells which had undergone autolysis, either individually or *en masse*.

Though subcultures and observation of growth in microcultures were used to check viability, we found during the course of these observations that the malachite green safranin stain was quite a reliable indication of the state of a culture—viable bacilli staining green and dead or dying bacilli staining pink, though both might have stained red with the orthodox Ziehl-Neelsen stain. In this connexion we confirmed Nedelkovitch's observation that uninoculated egg media may contain acid-fast material which, after staining with Ziehl-Neelsen, can easily be mistaken for the smaller forms of *M. tuberculosis* (Nedelkovitch, 1950).

The biological fluids listed all appeared to retard growth with the exception of digested pus. Treatment of tuberculous pus with 'streptokinase-strepto-

Table 1. *Changes observed in H 37 Rv during prolonged culture under varying conditions*

Dubos + Tween 80 + albumin. 37° in dark for 12 months	Dubos + Tween 80 + albumin. 37° in dark for 12 months	0.1 % glucose broth at 37° in dark	10 % glycerol broth at 37° in the dark	Dubos + 1.5 % glycine at 37°
Cords of bacilli. Acid-fast with z.n. Mostly green but a few red with m.g.s. Show very little change in morphology after 12 months at room temperature.	Dubos + Tween 80 + albumin. 37° in dark for 12 months	At 1 week, bacilli, short rods and cocci.	At 6 weeks, bacilli growing in ropes.	1st day, typical bacilli. 4th day, club and filamentous forms.
	After 4 months, rafts of bacilli showing areas of autolysis as in Pl. 1, fig. 1.	z.n.: acid-fast bacilli, 'diphtheroids' and cocci.	z.n.: acid-fast bacilli with some short forms.	7th day, some autolysis.
	After 5 months, remnants of autolysed rafts, numerous short forms.	At 2 weeks, fewer bacilli, more short forms.	At 14 weeks, ropes of bacilli have now autolysed.	10th day, photographed Pl. 3, fig. 10.
	z.n.: swollen bacilli, acid-fast cocci.	At 1 month, rafts of bacilli now numerous.	Numerous short forms.	16th day, bacilli shaped like pears, drum sticks and parsnips.
	m.g.s.: red bacilli short green forms.	z.n.: numerous acid-fast bacilli. <i>Subcultured</i> on to Lowenstein's medium.	Now kept at room temperature in dark for 6 months—only short forms remain.	25th day, tadpole forms still acid-fast. <i>Subcultured</i> into Dubos without glycine. Short
	After 12 months, similar appearance. <i>Subcultured</i> into Dubos and digest broth gave a growth of typical acid-fast bacilli in 3 weeks.	Growth in 2 weeks.	<i>Subcultured</i> into Dubos and digest broth. Growth of typical acid-fast bacilli in 6 weeks.	bacilli appeared in subculture on 4th day and by 19th day had become numerous.
The rate of growth having been retarded by lowering the temperature, nutrition remained adequate for 12 months.	COMMENTS	COMMENTS	COMMENTS	COMMENTS
	Continuous culture at 37° has resulted in limitation of cell size by nutrients.	There was a rapid autolysis of bacillary forms, after which short forms lengthened. Medium may be temporarily enriched by autolytic products.	Limitation of cell size by nutrients with restoration to normal size after subculture.	Some interference with cell division by glycine.
	The viable (green) short forms grow to normal size after subculture.			

z.n. = Ziehl-Neelsen.

m.g.s. = Malachite green safranin.

Table 2. *Observations of H 37 R_o exposed to various inhibitory substances*

Dubos + streptomycin 0.6 µg./ml.	Dubos + <i>p</i> -aminosalicylic acid 0.6 g. % (w/v)	Dubos + streptomycin 0.6 µg./ml. + <i>p</i> -amino- salicylic acid 0.6 g. % (w/v)	1 % glucose peptone water + 20 units penicillin	1 % glucose peptone water
1st day: typical bacilli, some in rafts.	1st day: typical bacilli some in rafts.	1st day: normal bacilli.	1st day: normal bacilli.	1st day: clumps of bacilli.
4th day: pleomorphic bacilli, some giants, some very short. Occasional club- forms.	4th day: autolysis.	4th day: diphtheroid forms numerous.	4th day: irregular forms, some long with terminal swellings.	4th day: no change.
7th day: autolysis of auto- lysis, 'diphtheroid' forms, and cocci (Pl. 2, fig. 6 <i>a</i> , <i>b</i>). 23rd day: short acid-fast forms. Coccal forms still acid-fast. <i>Subculture</i> , no growth.	7th day: autolysis. Short forms.	7th day: autolysis of clumps. Marked pleomorphism.	9th day: long beaded bacilli, 'diphtheroid' and coccal forms.	9th day: no change.
15th day: products of auto- lysis, 'diphtheroid' forms, z.n.: short acid-fast bacilli and cocci (Pl. 2, fig. 6 <i>a</i> , <i>b</i>). 23rd day: short acid-fast forms. Coccal forms still acid-fast. <i>Subculture</i> , no growth.	11th day: short forms losing acid-fastness. Numerous short forms (Pl. 3, fig. 8). 15th day: bacillary forms have nearly disappeared. 23rd day: debris of autolysis. Numerous granules.	11th day: autolysis of clumps. ceeding 'diphtheroid' and coccal forms.	19th day: numerous 'diph- theroid' and coccal forms. z.n.: numerous acid-fast short forms (Pl. 2, fig. 7). m.g.s.: numerous green short forms and debris. <i>Sub- cultured</i> after 6 months; Dubos good growth after 3 weeks. Digest broth scanty growth after 3 weeks.	2 months: still numerous cords of bacilli, some pleo- morphism. <i>Subcultured</i> on to Löwen- stein produced heavy growth.

COMMENT ON ACTION OF STREPTOMYCIN AND *p*-A.S.A.

A number of cultures were observed, with varying concentrations of these substances, both singly and in combination. The results were so variable that presumably drug-resistant mutants were complicating the studies at lower concentrations. In the three reported here, no growth was obtained on subculture on the 35th day. In another culture of the same strain in Dubos with 0.8 µg. streptomycin/ml., some 'green' bacilli persisted throughout, and subculture on to Löwenstein's medium at the end of six weeks produced growth. Streptomycin may produce giant forms; both growth inhibitors produce autolysis and regression to a varying degree.

COMMENT ON GLUCOSE PEPTONE WATER AND PENICILLIN

These observations are recorded because the presence of penicillin, by comparison with the control glucose peptone culture, appears to have accelerated regression.

dornase' (Burroughs Wellcome and Co.) enhanced the growth of tubercle bacilli and permitted degraded forms to grow on Löwenstein medium that otherwise would not (Tanner, 1951).

We have begun the study of morphological changes that occur immediately after inoculation into animals. A fuller account of this will be reported later, but attention may be drawn to the photograph (Pl. 3, fig. 9) of the 'diphtheroid' type of bacilli recovered from a rabbit's peritoneal exudate 10 days after intraperitoneal inoculation of a suspension of living bacilli. The strain H37Rv was inoculated intraperitoneally into rabbits, guinea-pigs and rats, and their peritoneal exudates were examined at intervals. No fully developed acid-fast bacilli could be found in the deposits of these exudates in the early stages, but numerous short acid-fast rods and cocci, both free and in macrophages, were seen, and culture on Löwenstein gave a luxurious growth of tubercle bacilli.

Lastly, our findings are illustrated in one of the number of clinical infections which provoked this study. A young man noticed a painless swelling on his right knee following exercise. This was treated by diathermy for 2 months without benefit. After 3 months a biopsy of the synovial membrane of this joint was taken. Histological examination showed no inflammatory reaction and no evidence of tuberculosis. Staining by the Ziehl-Neelsen method showed cocco-diphtheroid forms in the synovial membrane and fluid (Pl. 3, fig. 11), and inoculation of this material into a guinea-pig produced tuberculosis with typical acid-fast bacilli in the guinea-pig spleen (Pl. 3, fig. 12).

DISCUSSION

The two conclusions that we have reached from these studies are (*a*) that the process and appearance of autolysis has confused many workers in the past and discounts much of what has been written about stages in a 'life cycle', and (*b*) that the progressive shortening of bacilli under some adverse conditions, a process which we propose to call 'regression' instead of the usual term 'degeneration', represents not merely a reduction in size of the organisms but qualitative changes that have important implications.

Grigoraki (1950) seems to have confused these two processes. In his account of the sexual phase of development, the ectoplasm of lysed bacilli fused to form an amorphous veil in which the nuclei were transformed into granules which fused to form a symplasm. He describes stalks growing out from the symplasm bearing large ovoid cells which on bursting release male and female cocciform gametes. Short rods are supposed to form by fusion of these gametes. It seems more likely that the fusion and lysis of cells and the release of the nuclei therefrom is a process of autolysis, and that the short rods are derived from bacilli which have escaped autolysis, though they may still be attached to, and appear to grow out from, a clump of bacilli which have lysed.

Pl. 1, figs. 1 and 2, illustrate how easily such a confusion may arise, particularly if the preparation had been fixed and stained instead of being observed by phase contrast. Pl. 1, fig. 1, shows the process of autolysis in a raft of bacilli. Autolysis is spreading from right to left in this picture but

'coccal' and 'diphtheroid' forms derived from dividing bacilli are appearing in the top left-hand quadrant.

When the symplasm of an autolysing raft has disappeared and the nuclei are clumped together, as we see in Pl. 1, fig. 2, the multiplication of a few surviving bacilli may well appear to result from the fusion of 'coccal forms'.

Schaefer, Marshak & Burkhart state that in the absence of antibiotics, exhaustion of the nitrogen source in the culture medium is an essential condition for the appearance of autolysis, and that both oxygen and a source of energy such as glucose are necessary (Schaefer, Marshak & Burkhart, 1949; Marshak & Schaefer, 1952). In the presence of streptomycin, penicillin and *p*-amino salicylic acid, autolysis always occurs; giant forms and monstrosities appear in the presence of streptomycin and of glycine.

The important issue in this discussion is the significance of the coccal forms. We wish to emphasize that there may be three kinds of 'coccal' forms in a stained preparation: (a) nuclear remnants from autolysed bacilli appear 'coccal'; (b) in stained preparations coccal forms may be produced by the detachment of growing tips of bacilli in the smearing of cultures (these may frequently be observed as round bodies close to the growing end of mature bacilli, and may be regarded as artefacts—Yegian & Porter, 1944); (c) the coccal forms which are of the greatest interest are those which are produced in large numbers under adverse conditions by the division of diphtheroid forms, and which stain green with the M.G.S. stain.

Bisset's (1949) view is that tubercle bacilli are multicellular, a single bacillus containing up to twelve or more cells. We agree that this is so when it is fragmenting, but in our studies of human strains this mode of multiplication is uncommon. Growing from the tip of a bacillus with subsequent snapping of the newly formed bacillus to a Λ is seen most frequently among isolated bacilli, whereas in clumps the added bacilli form cords. Under adverse conditions, fragmentation, transverse division and lateral budding have all been observed, and as the bacilli become progressively shorter, multiplication in these ways gives rise to 'coccal' groupings.

A statement that cells diminish in size as they divide in an inadequate medium may appear so trite as to merit no further consideration. That the reduction in nitrogen content may be very considerable is shown by the findings of Marshak & Schaefer (1952) who report that 'in the presence of adequate amounts of glucose in a medium deficient in a source of nitrogen, tubercle bacilli continue to proliferate', and that 'the cells of the second generation (fourth day) produced under these conditions have a nitrogen content per milligram of dry weight which is only approximately 40 % of that in the cells used as inoculum. Relatively little of the cellular nitrogen originally added is lost to the medium.' The weight of the culture was nearly doubled and the final density was 2.6 times the initial density. They did not report their observations on the morphology of these organisms, and their statement as it stands might be taken to imply a $2\frac{1}{2}$ -fold relative increase in non-nitrogenous cell constituents. However, we find a marked reduction in size of dividing cells not only with nitrogen starvation but also in the presence

of antibiotics and biological fluids, and from our few initial observations it appears that bacilli dividing inside macrophages also tend to become diphtheroid or coccal. These coccal forms are capable of growing into full-bodied bacilli when transferred to a suitable environment.

Vaudremer (1926) showed that tubercle bacilli, when grown on poor media, become non-acid-fast and pleomorphic and cease to produce tuberculin. On return to suitable media they recovered their acid-fastness. Pagel (1934) found in histological sections of tuberculous foci slender chromophilic bacilli in some lesions and short plump chromophobic bacilli or granules in others. The former produced more rapid development and larger tuberculous infiltrations in guinea-pigs than the latter. We also found a striking difference in the morphology of bacilli from tuberculous sinuses where the bacilli are large and well formed, and those from closed lesions in bone where the organisms are usually coccal. The former produced rapid development of tuberculosis in guinea-pigs, the latter slowly or not at all; since the use of streptomycin, organisms seen in tuberculosis pus from sinuses are now usually like those from bone, that is, short, poorly acid-fast and with low infectivity.

Brieger, Miles, Cosslett & Horne (1951) recovered granular forms of bovine tubercle from the spleens and lungs of infected rabbits and found these to be infective to guinea-pigs, though they would not grow on laboratory media. Kahn & Nonidez (1936) found that culture filtrates of H37 and of a bovine strain that had been through membrane filters of a pore volume from 0.5 to 0.1 μ caused tuberculosis in four out of five guinea-pigs, though inoculations on to Petroff's, Long's, Löwenstein's and glycerol agar medium were sterile. We have also encountered the anomaly of granular forms in infected material which would not grow on routine media but which infected guinea-pigs, though in this respect growth has occurred on Bordet Gengou's medium with 50 % human red cells added, when the other media have remained sterile.

We suggest that tubercle bacilli, carried by the blood from a primary or secondary focus, may lodge in areas in the skeletal system and in other sites in which the micro-environment is unfavourable for full development, and that there the organisms may regress and remain dormant until the environment is altered in their favour by injury, haemorrhage, metabolic changes or infection by other organisms.

We have found short diphtheroid and coccal forms staining green with the malachite-green safranin in which no bacilli or tubercles could be found in joint tissue biopsies, and this tissue has infected guinea-pigs with tuberculosis. The patients have invariably been tuberculin-positive (evidence of an antecedent infection), but showed no signs of an active tuberculosis until after trauma. In our view, this suggests that the organisms were in the joint tissues in a form that did not produce a characteristic reaction even in sensitized hosts until the change in their environment allowed them to grow into bacilli again. Once this has occurred the characteristic tissue reaction follows.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Autolysis within a raft of bacilli. H37Rv. 3-week Dubos culture. Phase contrast. $\times 4000$.
- Fig. 2. Autolysis at a later stage: the nuclei resemble a cluster of cocci. H37Rv in Dubos with streptomycin and *p*-A.S.A. Phase contrast. $\times 4000$.
- Fig. 3. H37Rv in Dubos. 2 months. Bacilli about to lyse.
- Fig. 4. H37Rv. 1 year in glucose peptone water—still viable. Short forms resembling cocci. Much's stain. $\times 2000$.

PLATE 2

- Fig. 5. H37Rv. 14-day Dubos culture—normal bacilli. z.N. stain. $\times 2000$.
- Fig. 6*a, b*. H37Rv. 15-day culture in Dubos plus 0.17 μ g. streptomycin/ml. Short forms predominate. z.N. stain. $\times 2000$.
- Fig. 7. H37Rv. 19-day culture in glucose peptone water plus 20 units penicillin/ml. Short forms and autolysis. z.N. stain. $\times 2000$.

PLATE 3

- Fig. 8. Short forms of H37Rv grown in Dubos with *p*-A.S.A. Phase contrast. $\times 4000$.
- Fig. 9. Short forms of H37Rv recovered from rabbit peritoneal exudate 10 days after intraperitoneal inoculation. Phase contrast. $\times 4000$.
- Fig. 10. H37Rv in Dubos plus 1.5 % glycine for 10 days. Abnormal nuclei. z.N. $\times 2000$.
- Fig. 11. Acid-fast cocco-diphtheroid forms in the synovial fluid of a patient in which no acid-fast bacilli were found. z.N. stain. $\times 3000$. Ilford micro 2 filter has been used to reduce the density of the surrounding cells.
- Fig. 12. Acid-fast bacilli in the spleen of a guinea pig injected with the fluid illustrated in Fig. 11. z.N. stain. $\times 3000$.

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C. H. LACK & F. TANNER—PLEOMORPHISM OF *M. TUBERCULOSIS*. PLATE 1

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C. H. LACK & F. TANNER—PLEOMORPHISM OF *M. TUBERCULOSIS*. PLATE 3