ISOLATION AND MAINTENANCE OF AN L1-LIKE CULTURE FROM *FUSIFORMIS NECROPHORUS* (SYN. *BACT. FUNDULIFORME, BACTEROIDES FUNDULIFORMIS*)

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(With Plate 6)

In 1933 my attention was drawn for the first time to an organism of peculiar morphology, designated *Streptobacillus moniliformis*. It is well known as the cause of rat-bite fever in man and arthritic abscesses in mice. On a special boiled blood medium prepared from heart muscle infusion agar enriched by peptone and 30% of horse serum I isolated in 1934 from *Str. moniliformis* a culture which no longer contained any bacterial forms but was made up of soft bodies and granules. This new culture was called L1 and could be demonstrated in all strains of *Str. moniliformis* examined. I argued at that time that this new L1 organism was an independent microbe, presumably related to the pleuropneumonia-like organisms. As it was subsequently separated from various strains of *Str. moniliformis* of different origin I came to the conclusion that the bacterium and the L1 organism lived together in a close association of a symbiotic or perhaps parasitic nature (Klieneberger, 1935, 1936, 1938, 1940, 1942). The existence of the L1 organism and the peculiar morphology of cultures of *Str. moniliformis* was confirmed by various other workers, but my interpretation was challenged by all of them (Brown & Nunemaker, 1942; Dawson & Hobby, 1939; Dienes, 1939*a*, *b*, *c*, 1940*a*, *b*, 1941, 1942, 1943, 1944, 1945; Dienes & Smith, 1942, 1943, 1944; Heilman, 1941*a*, *b*; Ørskov, 1942). Dienes, in particular, published a number of papers relating to the *Str. moniliformis* phenomenon first described by myself. He was of opinion that the L1 component found in various bacteria, of which, among others, *Fusiformis necrophorus* had been studied most fully by him, is a stage in the life cycle of the bacterium and not an organism of its own. His interpretation was supported by two main facts: (1) Some L1 strains which seem morphologically pure and some L1 bodies revert into the bacillary parent organism. (2) The swelling up of bacteria and their transformation into L1 bodies can be followed up in some particularly suitable strains by microscopical studies. The main points supporting my conception of an association of two different microbes were the following: (1) The L1 strains derived from *Streptobacillus moniliformis* 12 and 13 years ago respectively have so far never reverted, either in my own or in other hands, during well over 550 consecutive passages. (2) L1 cultures differ greatly from bacteria, viz. they show the colony type, food requirements and morphology of pleuropneumonia-like organisms; they possess filterable units almost as small as elementary bodies of vaccinia virus; their protoplasm is extremely soft; they show no cell boundaries or cell walls comparable to those of bacteria; they flow very thinly in amorphous sheets over the agar surface while growing and increasing their substance; in liquid media they form small, drop-like elements or are drawn out into fine filamentous structures following environmental influences and no inherent formative principle of their own.

I have always been of the opinion that the isolation of a component which differed so much from the bacteria and its maintenance over periods of many years distinguished the L1 component of *Str. moniliformis* from all other variations of bacterial organisms so far described. It therefore would be of considerable interest, and might promote better understanding of the relationship between bacterium and L1 component, if further L1 cultures derived from other organisms than *Str. moniliformis* were established. Dienes describes that he obtained L1-like growth from various organisms such as *Fusiformis necrophorus* (already mentioned by Klieneberger in 1938 as a so-called symbiotic culture), *Escherichia coli*, *Haemophylus influenzae*, *Neisseria gonorrhoeae* and a few others, but he does not seem to have maintained a pure L1 culture, derived from any of these bacteria, over a long period. I should like to record briefly my experience with a new L1-like culture of non-*Streptobacillus moniliformis* origin.

In 1938, Wilson Smith and myself grew simultaneously but independently an organism resembling *Str. moniliformis* but distinct from it. It was obtained from cervical lymphadenitis in guinea-pigs. From it I separated an L1-like culture in

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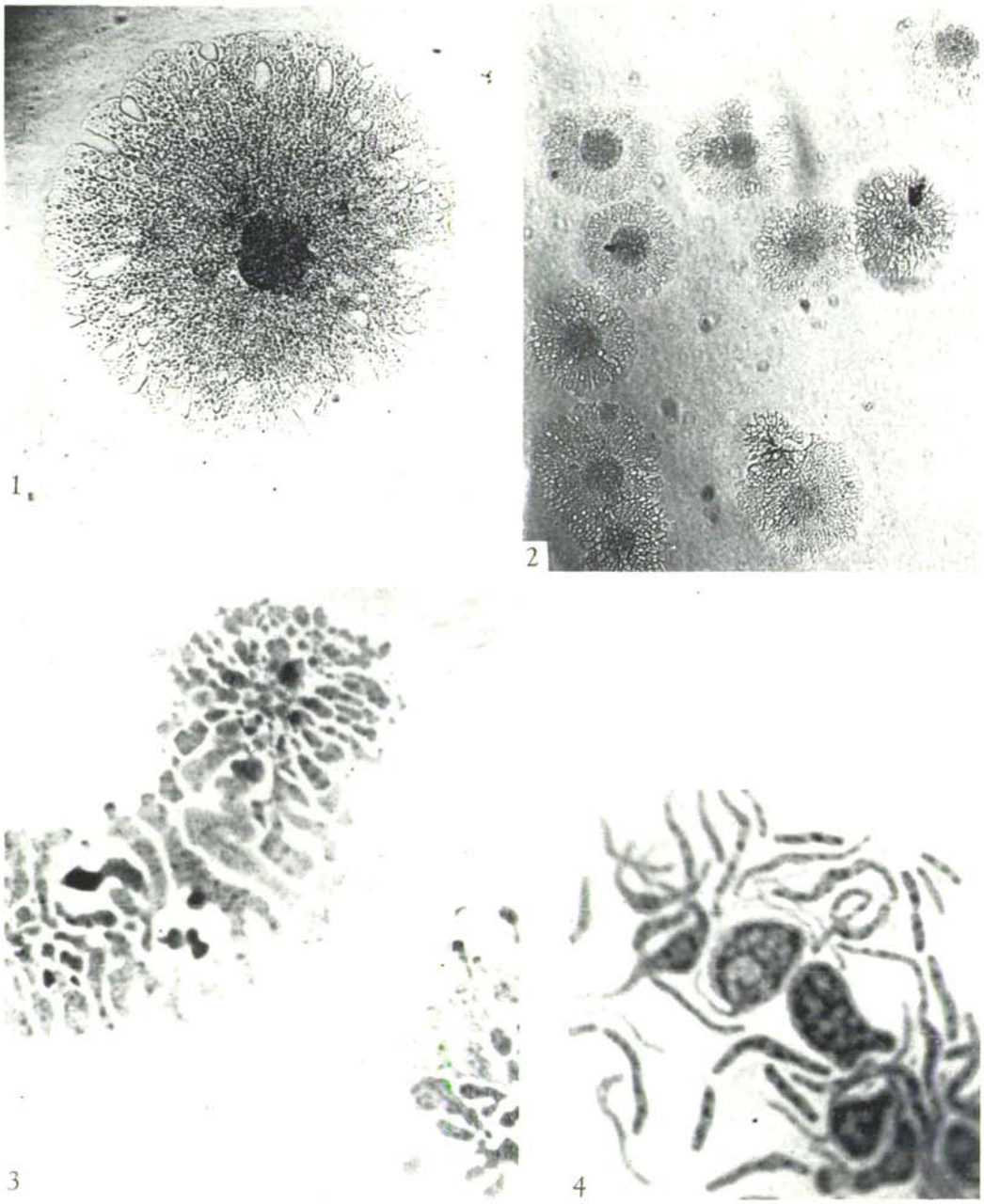
1939 which I was able to maintain in twenty consecutive passages; but I lost it at the outbreak of war without having an opportunity to re-establish it. By the kindness of Dr Dienes I obtained, early in 1946, one of his 'Bacteroides strains' (syn. *B. funduliforme*, *Fusiformis necrophorus*). This particular strain, '132', had been extensively studied by Dienes & Smith in the past. Using the methods devised for *Streptobacillus moniliformis* and also that used for the strain from cervical adenitis in guinea-pigs, I obtained plate cultures on which two kinds of colonies were to be seen, viz. flat unstructured ones of the well-known *Fusiformis necrophorus* type and small opaque ones showing a dark granular centre and a more transparent, beautifully structured peripheral part. Ten well-isolated, opaque colonies were picked, seven of which yielded pure L 1 growth while only three of them apparently still contained bacteria and reproduced the parent culture. Out of the seven pure L 1 growths two were selected without further purifications for consecutive passages. They are grown on the surface of small special medium plates, incubated anaerobically for 3–4 days and subcultured at half-weekly intervals by means of the transference of cut-out pieces of the culture. Both of them have undergone well over 100 passages up to date in the course of a year and they have not changed. The new L 1 strains from *F. necrophorus* so closely resemble L 1 from *Streptobacillus moniliformis* in regard to their colony appearance and morphological structure that they are indistinguishable (Pl. 6, figs. 1–3; compare with Klieneberger, 1938, Pl. XVII, fig. 1 and 1942, Pl. XI, figs. 25, 26 and 29 respectively). Both kinds of L 1 organisms grow well on the special media originally used for the culture of pleuropneumonia-like organisms but not on ordinary media. Both contain in their colonies large amounts of oily droplets which in the case of L 1 from *Str. moniliformis* have been identified as cholesterol droplets (Partridge & Klieneberger, 1941). Yet there is one significant difference between the two L 1 strains of different origin. L1-*Str. moniliformis*, like its parent culture, grows equally well under aerobic and anaerobic conditions, while L1-*Fusiformis necrophorus* grows under anaerobic conditions exclusively, again just as its parent culture. Repeated attempts to grow L 1-*F. necrophorus* under aerobic conditions—with and without the addition of CO_2—have completely failed. This difference can be regarded as in favour of Dienes's interpretation of the L 1 phenomenon. The fact that the two organisms resemble each other so closely while the two parent cultures are distinct in colony type and morphology, might, however, be interpreted in favour of the symbiosis assumption.

To watch the development of a *F. necrophorus* culture on solid medium is fascinating. When the cultures are kept in Brewer's medium enriched with horse serum, subcultured fairly heavily once a week, allowed to remain at 37° C. for only 8 hr. and then stored in the cold, they seem to keep very well in an unchanged condition and they grow vigorously when transplanted. In order to obtain a good development of L 1 elements from bacillary forms 0·5 c.c. from the growth in Brewer's medium was subcultured into an ordinary serum-broth tube (10 % horse serum). At the bottom of the tube the oxygen tension is such that—though the tube is incubated aerobically—fair growth develops overnight. Material from such a serum-broth culture transferred to serum-agar plates (special medium) easily produces surface growth under anaerobic conditions. Impressions taken from such plates after 4–6 hr. of incubation already show many bacteria with slight swellings containing well-stainable chromatinic granules, particularly inside the swellings. As incubation is continued the swellings increase and gradually grow into monster forms which often show delicate chromatinic structures embedded in their cytoplasm (Pl. 6, fig. 4). The chromatin sometimes shows a compact and in other instances a ring-like arrangement. Whatever the particular form, the structure of the swellings in the bacterial culture are similar to the structures of the elements building up the L 1 colonies. The recorded observations of the development of the big forms in *F. necrophorus* '132' which lends itself more readily to morphological studies than the slender *Streptobacillus moniliformis* fully confirm Dienes & Smith's description (1944, p. 128). Here the attention should also be drawn to a paper of Pokrowskaja who studied the development of plague bacilli in 1930 by means of the Feulgen reaction. From the description of the development of the very pleomorphic culture and the beautiful drawings it would appear that similar swellings with similar nuclear arrangements as in *Fusiformis necrophorus* occur in this organism.

With a new type of L 1 culture available some of the earlier experiments carried out with L 1 cultures from *Streptobacillus moniliformis* were now repeated. It was not possible to produce any swellings of aerobic or anaerobic bacteria such as *B. coli*, *B. proteus* or *Bact. tetanomorphus* by the addition of the pure L 1 culture. With the same conception of possible 'infection' in mind an attempt was made to induce young, mainly bacillary cultures of *Fusiformis necrophorus* to produce swollen forms by adding pure L 1 growth. In all experiments carried out in order to bring about what might have been interpreted as an infection the mixtures—consisting of bacillary cultures plus pure L 1 growth—behaved exactly like the controls which contained bacillary culture only. The presence of a large amount of L 1 material had therefore not exerted any influence on the bacteria.



Figs. 1—4

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SUMMARY AND DISCUSSION

The study of the *Fusiformis necrophorus* culture '132' showed that new pure L 1 cultures could be isolated from it, which have already been maintained for one year and undergone more than 100 passages without reverting to their original type. The new cultures resemble the old L 1 strains from *Streptobacillus moniliformis* in every respect except for their strict anaerobiosis. It has been observed that swellings of the bacteria develop into large bodies not distinguishable from the elements of which the pure L 1 culture consists.

In the light of the new observations on *Fusiformis necrophorus* which are in full agreement with those of Dienes & Smith it is still not possible to disprove the symbiosis theory, for the newly established L 1 cultures have not reverted so far. Secondly, though it has been seen that L 1 forms develop from swellings of bacteria, the possibility that a parasite might behave in a similar way cannot be excluded. On the other hand Dienes's opinion of the bacterial nature of the L 1 component cannot be disproved either. However, we know yet too little about the normal developmental cycle of bacteria to be able to decide whether the L 1 form is an independent entity or whether it may be a normal stage through which bacteria have to pass from time to time or whether it may represent a 'loss-mutant' which some bacteria might produce under not yet known conditions. If in the future the bacterial nature of the L1 could be convincingly proved we would be faced with the fact that bacteria can assume a form in which they grow as a thin shapeless slime containing nuclear structures arranged as bodies, rings or very finely dispersed particles—not the dumbbell pattern so characteristic for young bacteria of all kinds—and that further very small parts of the material (filterable granules?) are able to reproduce the L1 culture which at least can go on for many years without reverting into the bacterium from which it was derived.

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EXPLANATION OF PLATE 6

Fig. 1. Big single colony of L 1-*Fusiformis necrophorus* grown for 5 days. Magn. 1 : 200.
Fig. 2. Colonies of L 1-*Fusiformis necrophorus* grown for 2–3 days. Magn. 1 : 200.
Fig. 3. Young growth of a pure culture of L 1-*Fusiformis necrophorus*. Fixed with Bouin's solution through the agar, stained with Giemsa solution. Magn. 1 : 2000.
Fig. 4. *Fusiformis necrophorus* plated from serum broth on to serum agar. Incubated anaerobically for 18 hr. Fixed with osmic acid vapours, stained with Giemsa solution. Magn. 1 : 3500.

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