Identification of bacteria from blood and joint fluids of human subjects as *Bacillus licheniformis*

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In previous papers, the occurrence of an L-form of a Gram-positive bacterium resembling *Listeria*, in the blood and joint fluids of arthritic, and the blood of normal persons, was reported (Pease, 1967, 1969, 1970a, b). Although similar organisms have been described from this source in the past, a satisfactory bacteriological description has been lacking, in most cases, and the purpose of this paper is to provide such a description. The organism is, in fact, identifiable as the L-form of *Bacillus licheniformis*, which has previously been found in association with arthritic conditions of poultry (Roberts, 1964) and probably of man (Benedek, 1955).

Methods

ORGANISMS

Seventy strains of bacteria were examined, representing those isolated from 170 out of 176 cases of rheumatoid arthritis, by the techniques described below. For comparison, three type strains of *Listeria monocytogenes* (NCTC 5214, 5348, 10357); one each of *Bacillus licheniformis* (NCTC 10341) and *B. licheniformis* var. *lentus* (NCTC 4824) were examined. The synovial fluid isolates of Duthie, Stewart, Alexander, and Dayhoff (1967), and a strain of the bacterium isolated by Alexander-Jackson (1954) were also investigated. Twelve newly isolated strains of *B. licheniformis*, from this laboratory and elsewhere, were examined physiologically for comparison; and all 21 types species of *Bacillus* (excl. *B. anthracis*) available from the NCTC, were compared serologically.

CULTURE

The original cultures were made by adding 2 ml. serum, whole blood, or joint fluid to 15 ml. of Brewer's medium in a McCartney screw-capped bottle, and incubating at 37°C. until slight visible turbidity could be detected with the naked eye, which was usually after 2 to 6 weeks. Subcultures were made on nutrient agar (Oxoid Blood-agar base No. 2, or BBL Trypticase Soy). Numerous cultural controls against contamination were included. These are listed in Pease (1969). The growth was concentrated by centrifugation at 2,500 r.p.m., and resuspended in 1 ml. sterile distilled water for examination. For phase-contrast microscopy, a few drops were mounted between slide and coverslip and sealed at the edges with beeswax. These were examined with a Wild $\times 50$ phase-contrast lens and

condenser. In some cases, the serum or fluid was examined directly by these means, without prior culturing.

Physiological tests were performed on the bacterial phase, mainly according to the methods of Cowan and Steel (1965), all at 37°C. and with the following details or modifications. In the starch-hydrolysis test, Lugol's iodine was used, and in the Indole test, Kovac's reagent. For the gelatin liquefaction test, Cowan and Steel's Method 2 was used. Slopes inoculated from heavy growth on nutrient agar were used for the citrate utilization test. Slopes of ammonium salt sugars, sterilized by filtration, were used in the tests for acid from carbohydrates. In the casein hydrolysis test, Oxoid Skim Milk powder (10 per cent.) was used in place of skim milk, and sterilization was at 121°C. for 5 min.

ANTIGENIC ANALYSIS

The organisms were grown on BBL Trypticase Soy agar plates for 18 hrs. Antigens for gel-diffusion tests were prepared against all the strains listed, from heavy, heat-killed, washed suspensions of bacteria in 0.2 per cent. (v/v) formol saline, and ultrasonically disintegrated.

Antisera were prepared in rabbits against all three strains of *Listeria monocytogenes* and four strains of new isolates, by giving two subcutaneous injections of 1 ml. of ultrasonically disintegrated antigen plus 1 ml. of Freund's adjuvant (Bacto) at an interval of 14 days. After a further 2 weeks, a series of subcutaneous injections of 1 ml. of the antigen alone was given on ten occasions over 3 weeks. This procedure was usually sufficient to produce antisera capable of giving rise to strong lines of precipitation in gel-diffusion tests set up against homologous antigens.

Results

Primary cultures in fluid medium were clearly visible after 2 to 6 weeks' incubation, previous to which, no sign of growth appeared. Typically, they showed as a turbidity of the medium, that was readily seen by phase-contrast microscopy to consist of budding spheroplasts, as described by Pease (1969). When these cultures were subcultured on nutrient agar, the bacillary phase could be obtained from any L-phase culture that showed turbidity at about 6 weeks' incubation. A loopful of fluid culture, plated on solid medium, produced a profuse growth of very

small, pale, translucent colonies, composed of asporogenous, Gram-positive, occasionally acid-fast rods, morphologically resembling *Listeria* (with which, in view of their antigenic reactions, *see below*, they had provisionally been identified). On sub-culture, over a period of several weeks, these cultures gradually assumed the very characteristic appearance of *Bacillus licheniformis* colonies.

The earlier cultures seldom or never bore visible endospores, but latterly these became common, as the typical morphology was progressively restored.

PHYSIOLOGY

The results of physiological tests on fifteen of the new, human isolates and the twelve saprophytes are shown in the Table, by comparison with type *Bacillus licheniformis* and Alexander-Jackson's isolate. Most of the human isolates and all the saprophytes were identical with the type strain, or varied only slightly, by the criteria employed, but two of the former were weak fermenters, and rather resembled the inactive, *B. lentus*, variant.

ANTIGENIC ANALYSIS

Antigens prepared from all the strains of *Listeria* and *Bacillus* listed under 'Methods', the isolates of Duthie and others (1967), the organism of Alexander-Jackson, and seventy new human isolates were tested by the gel-diffusion precipitin reaction against the sera prepared from three strains of *Listeria* and four strains of new isolates. Tests were performed with these antigens and sera in all possible combinations. All seventy antigens from new isolates, Alexander-Jackson's organism, and *B. licheniformis* var. *lentus* reacted positively with the antisera against new isolates, giving strong multiple lines of precipitation. The other NCTC species of *Bacillus* gave occasional, weak, and variable lines. The isolates of Duthie and others (1967) appeared to be serologically unrelated.

The antiserum produced against *Listeria* strain 5214 gave a strong reaction against all seventy new isolates. The remaining two *Listeria* antisera did not produce lines of precipitation against the new isolates, but the presence of common antigens was detectable in all these by the continuity of lines when their antigens were placed in cups, alternating with the antigens of the new isolates, surrounding *Listeria* (5214) serum.

Discussion

The evidence, in this paper, suggests that the blood of many human beings is infected with L-forms of *Bacillus licheniformis*, which can be isolated in fluid culture (but only after very prolonged incubation) in the form of spheroplasts. This is surprising at first encounter, but not without support from other sources. This organism is transformed, after sub-culture on solid medium into a Gram-positive rod, resembling *Listeria* or a 'diphtheroid', or even an acid-fast bacillus, with all of which it seems to have been confused in the past. My previous tentative identification of this asporogenous phase of culture as 'Listeria-like' (Pease, 1967) was based both on morphology and on antigenic analysis, and it has since been shown that there is a true antigenic relationship between *L. monocytogenes* and *B. licheniformis* that is not caused by the non-specific Rantz antigen (Pease, Nicholls, and Stuart, 1972). Also, *B. licheniformis*, which is very small for a *Bacillus* species (c. 1 µ in diameter), exists in two morphologically distinct phases in ordinary pure cultures, and in one of these the bacilli are asporogenous (Bisset and Street, 1973). This leaves little doubt that the organisms now isolated, while bearing some resemblance to *Listeria*, are identifiable as *B. licheniformis* or variants of it, on morphological, biochemical, and serological grounds. The same

Table Physiological tests

Characteristics	Arthritis isolates															Alexander-Jackson's isolates	Four strains isolated from vicinity	Eight strains isolated from laboratory	Type species	
	71	11	11	90	115	54	62	16	72	161	11	58	20	7	107				B. licheniformis	B. lentus
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth in glucose broth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose (acid)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose (acid)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol (acid)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

explanation undoubtedly applies to the well-known organism of Alexander-Jackson (1954; cf. also Livingston and Alexander-Jackson, 1970), which is described as passing from an L-form through a weakly acid-fast stage to a sporogenous stage, and which has now been shown to be identifiable with my own isolates.

Gram-positive sporing bacilli have been widely reported in the literature as occurring in the blood and tissues of healthy and diseased mammals (see Pease, 1967, 1970b for references), but of particular interest in this instance is the work of Roberts (1964), who isolated a bacterial L-form from outbreaks of synovitis in fowls, and identified it as *Bacillus licheniformis*, and also that of Benedek (1955), whose isolates, which he believed to be associated with human arthritis, appear from this description and photographs to be *B. licheniformis*. Since *B. licheniformis* is an unusually stable species of this variable genus, in its physiology (Bisset, personal communication), it is of interest to note that these, apparently parasitic, strains included several variant forms, whereas the saprophytic isolates, including those from this laboratory, were identical with the type strain in their reactions. It is also of interest that, serologically, the strongest reaction of the new, human isolates, was against the *lentus* variant. This suggests that they are, at least, not recent contaminants, but have developed separately from the normal, saprophytic strains of the species.

Even without the unusually long period of time that these L-form isolates require to establish themselves in primary culture, the bacteria are peculiarly liable to be overlooked. Such isolations are habitually treated with great reserve by bacteriologists, who are conditioned to disregard as contaminants any

bacteria that show an unexpected ability to produce spores. Thus, it can be regarded as certain that, even if they were given time to develop, the majority of previous isolations have tended to be discarded, so soon as the bacteria had begun to revert to the stage at which they were recognizable as sporing bacilli. Since, however, there does exist a certain amount of independent evidence that *B. licheniformis* can be isolated from human beings, these observations should be repeated, as widely as possible, in order to evaluate the significance of the findings.

Summary

The bacterial L-forms, previously described as isolable from the blood and joint fluids of arthritic subjects, and also occurring in healthy persons, are identified, by morphology, physiology, and antigenic analysis as *Bacillus licheniformis*. Some strains are identical with the type cultures, and with new saprophytic isolates; some vary slightly in their physiological reactions, and may resemble the inactive *lentus* variant, with which both physiological types are more closely allied serologically.

The original isolates were usually in the L-phase and grew very slowly. On subculture they passed through a stage morphologically and antigenically resembling *Listeria*, and eventually reverted to recognizable sporogenous *B. licheniformis*: the process takes several weeks to complete.

This organism has been claimed by previous workers to be associated with arthritic conditions, but it is emphasized that further study is required.

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