Serological Reactions of L Type Cultures Isolated from Proteus.*† (18213)

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Freshly isolated Proteus cultures when transferred to soft horse serum agar plates containing high concentrations of penicillin usually produce L type colonies abundantly(1). These correspond in appearance and morphology to the Ll isolated from *Streptobacillus moniliformis* by Klieneberger. On appropriate media penicillin induces a similar transformation in various species of Gram negative and Gram positive bacilli(2). However, the development of L type colonies has been observed under varying conditions in several species in the absence of penicillin(3). Two types of colonies develop on penicillin plates inoculated with Proteus. One type grows to a larger size, does not require animal serum for development and reproduces the usual bacilli when transferred to penicillin-free media. These colonies have been designated as 3B L type colonies. On soft horse serum agar plates containing penicillin, many tiny colonies also develop among the 3B colonies. These colonies can be subcultured only on media containing animal serum and have been designated as 3A L type colonies. These colonies also reproduce the usual bacillary forms of Proteus on penicillin-free media. In contrast to the large 3B colonies, this requires at least 24 hours and may be delayed for several weeks if the cultures have been propagated for a long period of time in the presence of penicillin. There is no essential difference in morphology between the organisms in the 3A and the 3B L type colonies. When the 3B colonies are transferred to soft horse serum agar containing penicillin, abundant small 3A colonies develop, indicating the close relationship between the 3A and 3B L type colonies. The conditions under which 3A and 3B L type colonies develop, their similarity to the Ll of *Streptobacillus moniliformis* and their properties have been described elsewhere(1). The consistent reappearance of the usual bacillary forms of Proteus from these colonies, even after long cultivation on penicillin plates, leaves no doubt that the L type colonies are produced by Proteus growing in a changed form. This conclusion is supported also by the observation that the L type colonies grow out from the bacilli. The bacilli, as in *Streptobacillus monoliformis* and Bacteroides, first swell into large bodies from which the L forms grow. Further evidence for the identity of the bacillary and L forms is their serological similarity. Data concerning this will be presented in this paper.

The serological study of the L type cultures was undertaken primarily to obtain additional evidence of their genetic relationship to the bacilli. A more thorough study of the antigenic structure of the L forms may provide information as to the nature of the L transformation of bacteria and would therefore be of great value. The technical difficulties involved made it necessary to restrict the present work to the original purpose.

Cultures of the small 3A L type colonies were used for the serological studies. These cultures are easier to keep growing than the large 3B L type colonies. Furthermore, the differences between the 3A and the bacillary cultures are more marked than between the 3B and the bacillary cultures. Thus, serological similarity between the 3A L type cultures and the usual bacillary cultures is more significant. The 3A cultures when they are adapted to the medium grow abundantly on

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

† This is publication No. 116 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

1. Dienes, L., *J. Bact.*, 1949, v57, 529.

2. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 30.

3. Dienes, L., Weinberger, H. J. and Madoff, S., *J. Bact.*, 1950, v59, 755.

TABLE I. Agglutination Reactions of *Proteus bacilli* and of Their L Forms.

Rabbit antisera	Antigens: #3 Bac 1:40	80	160	320	640	1280	2560	5120	#3 L 1:20	40	80	160	320	640
3 Bac	3+	3+	3+	3+	3+	+	0	0	+	2+	3+	3+	2+	+
3 L	3+	2+	2+	2+	+	±	±	0	3+	3+	2+	+	0	0
14 Bac	0	0	0	0	0	0	0	0	0	±	0	0	0	0
14 L	+	+	±	0	0	0	0	0	0	0	0	0	0	0
52 Bac	0	0	0	0	0	0	0	0	0	0	0	0	0	0
52 L	2+	+	0	0	0	0	0	0	0	0	0	0	0	0
	#14 Bac								#14 L					
3 Bac	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
3 L	3+	3+	3+	3+	2+	2+	2+	0	0	0	0	0	0	0
14 Bac	3+	3+	3+	3+	2+	+	0	—	2+	2+	2+	+	0	0
14 L	3+	3+	2+	2+	2+	2+	2+	—	+	±	0	0	0	0
52 Bac	3+	3+	2+	+	0	0	0	0	0	0	0	0	0	0
52 L	+	+	±	0	0	0	0	—	0	0	0	0	0	0
	#52 Bac								#52 L					
3 Bac	2+	±	0	0	0	0	—	—	0	0	0	0	0	0
3 L	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14 Bac	3+	3+	3+	+	0	0	—	—	0	0	0	0	0	0
14 L	±	0	0	0	0	0	0	0	0	0	0	0	0	0
52 Bac	3+	3+	3+	2+	2+	+	—	—	0*	0*	0	0	0	0
52 L	±	0	0	0	0	0	0	0	3+	3+	3+	3+	2+	+

* Slight agglutination with 0 serum (obtained with boiled cultures).

serum agar plates. In 5 to 7 days they produce a thick firm film in heavily inoculated areas. The individual colonies are hard and rubbery, 1-2 mm in size. The culture is firmly embedded in the medium and cannot be separated from it. For the inoculation of the rabbits the L cultures were grown on soft agar containing rabbit serum and 1600 units of penicillin per ml. The surface of the agar containing the cultures was scraped off, emulsified in saline and injected intraperitoneally. Three L type cultures isolated from our Proteus strains 3, 14 and 52 were used for this purpose. These strains had been used previously for other studies. Other rabbits were treated by intravenous injection of the bacilli, some with fresh living organisms and some with organisms killed by boiling. The rabbits were injected on 2 consecutive days of each week for 6 weeks. The agglutination reactions of the bacillary antigen with the sera obtained after 4 injections of the living cultures are recorded in Table I. The strain specificity of these sera was more marked than that of sera obtained after further inoculation. With the L antigens stronger reactions were obtained both with the bacillary and L sera after further treatment of the rabbits and the reactions with these sera are recorded in the table.

The most successful method to obtain an emulsion of the L cultures for the agglutination test was the following: Squares of agar with the well-grown cultures were cut out and inverted with the colonies downward in a petri dish containing 1-2 ml of salt solution. By rubbing the agar blocks on the dish a portion of the colonies was emulsified. After sedimentation of the larger particles, the emulsion was stable and gave specific agglutination reactions. The emulsion did not consist of single organisms but of small clumps of granules and of large forms of various sizes. Compared with the bacillary antigens the agglutination titre of the L antigens remained low and agglutination occurred in the form of loose flocci. In positive tests the turbidity of the L antigens markedly increased with higher concentrations of the sera. In these respects the reaction resembled the precipitin test. A clear filtrate of the emulsion and the extract of the agar medium on which the L type culture was grown gave a strong precipitin test with specific sera. The L forms of

Salmonella gave the same type of agglutination as the Proteus L, although no precipitation was obtained with extracts of Salmonella L type cultures(4).

The differences in the reactions of the 3 strains were marked both *in vivo* and *in vitro*. The bacillary and L antigens of Strain 3 reacted well with the antisera obtained with both and reacted only slightly or not at all with any of the other sera. In previous experiments this strain had presented the most marked strain antagonism against other Proteus strains. The 3 L serum agglutinated the bacillary antigen of strain 14 but not the L emulsion. In strain 14 also the common reactions between the bacillary and L antigens and antisera were marked. The 14 L antigen was agglutinated only by the strain specific sera; the bacillary antigen was agglutinated somewhat by all sera. In strain 52 there was hardly any common reaction between the bacilli and the L forms; the L sera agglutinated the bacillary antigens of strains 3 and 14 slightly more than their own. The Proteus bacillary and L antigens were tested with antisera against *Salmonella typhosa* and *S. typhimurium* and the Salmonella bacillary and L antigens were tested with Proteus antisera. No overlapping of reactions was observed between the L forms of Proteus and Salmonella.

The L forms of Proteus apparently show a marked strain specificity in the agglutination test. In 2 strains they reacted only with sera produced by themselves and by the corresponding bacilli. However, the sera produced by the L forms have a wider range and react more often with bacillary antigens of nonspecific strains. This difference between the *in vivo* and *in vitro* specificity may be only an apparent one, a consequence of the low sensitivity of the L cultures in the agglutination test.

The serological reactions of the L forms of *Streptobacillus moniliformis, Salmonella typhosa* and *Salmonella typhimurium* have been studied and found to be very similar to those of the corresponding bacilli(5,6). The results with Proteus strains 3 and 14 correspond to these observations, but strain 52 is apparently an exception. The bacteria regained from the L forms were examined and found to be unchanged in their serological properties. In some experiments L forms isolated from different strains were grown in mixed culture; no change was observed in the serological properties of the bacilli regained from these cultures.

Agglutinin absorption tests were conducted with bacillary and L sera of strains 3 and 52. In each case both bacillary and L emulsions were used for absorption of the antibodies. Three cc of the serum diluted 1:10 was mixed with a loopful of bacillary mass taken from an agar culture and then placed in the refrigerator for 1 hour. The serum was then centrifugalized and the supernatant was again mixed with a loopful of culture mass and placed in the refrigerator for 1 hour. After centrifugalization the supernatant was removed and was used for the agglutination test. The absorption of the sera with the L cultures was performed in a similar manner. For each absorption half of the culture scraped from a well-grown agar plate was mixed with the serum. The amount of L culture added to the serum was probably sufficient but it could not be emulsified as well as the bacilli.

The low titre of the sera against the L antigen makes the interpretation of the results somewhat uncertain. The 52 sera which had not shown any common reaction between the bacilli and L forms were not noticeably weakened by absorption with the heterologous antigen. The bacillary emulsion of strain 3 absorbed the agglutinins both from the bacillary and L sera. The L emulsion absorbed the agglutinins from the L sera against both the bacillary and L antigens but did not weaken the reaction of the bacilli with the bacillary sera. According to these limited observations the bacillary and L forms of strain 52 both contain antigens which are not active in the other either in the agglutination test *in vitro* or in the immunization of the rab-

4. Weinberger, H. J., Madoff, S., and Dienes, L., *J. Bact.*, 1950, v59, 765.

5. Heilman, F. R., *J. Infect. Dis.*, 1941, v69, 32.

6. Dawson, M. H., and Hobby, G. L., Third International Congress for Microbiology, *Abstracts of Communications*, 1939, p. 21.

TABLE II. Agglutinin Absorption Tests with Bacillary and L Immune Sera (Proteus Strain 3)

Rabbit antisera	Bacillary antigen 1:20	1:40	1:80	1:160	1:320	L antigen 1:20	1:40	1:80	1:160	1:320
				Agglutination Before Absorption.						
3 Bac	+	2+	3+	3+	2+*	+	2+	3+	3+	2+
3 L	3+	3+	3+	2+	2+†	3+	3+	3+	2+	0
				Agglutination after absorption with bacilli						
3 Bac	0	±	±	0	0	+	±	±	0	0
3 L	0	0	0	0	0	0	0	0	0	0
				Agglutination after absorption with L						
3 Bac	2+	3+	2+	2+	2+	±	±	±	0	0
3 L	±	0	0	0	0	0	0	0	0	0

* The titer of this serum was 1:5120.
† " " " " " " 1:2560.

bits. The L forms of strain 3 which gave a marked common reaction with the bacilli seem to lack certain antigens which are present in the bacilli. The agglutinin absorption experiment conducted with Proteus strain 3 is recorded in Table II.

Summary. The serological properties of 3 Proteus strains and the L forms isolated from them have been studied. The L antigens were agglutinated both by the bacillary and L immune rabbit sera in a relatively low titre and agglutination occurred in large loose flocci. Clear filtrates of L emulsions gave a good precipitation. The reactions of 2 L type cultures were strain specific. A marked common reaction was present between the bacillary and L forms of 2 strains both in the production of antibodies and in the *in vitro* tests. There was only a slight overlapping in the reactions of the L forms and of the bacilli in the third strain. The similarity of the serological reactions of the bacillary and L forms of 2 Proteus strains is further evidence for the genetical identity of the L forms and the bacilli. The nature of the serological dissimilarity between the bacillary and L forms of one strain, apparent both in *in vitro* tests and in antibody production, can not be interpreted at present.

Received August 18, 1950. P.S.E.B.M., 1950. v75.

Isolation of L Type Cultures from Clostridia.*† (18214)

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Under certain conditions many Gram negative bacilli grow in very small soft pleomorphic forms which are usually designated as pleuropneumonia-like or L forms. The development of similar forms has been observed in the cultures of a few large aerobic Gram positive bacilli probably belonging to the genus Bacillus(1). The isolation of L forms from 2 strains of Clostridium is the subject of this paper. The most efficient method for the isolation of L forms is exposure of the bacilli to penicillin on agar plates. Many Gram negative bacilli produce L type colonies abundantly if they are inoculated on soft horse serum agar plates containing either 400 or 1600 units of penicillin

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

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1. Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 30.