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Attempts to Demonstrate a Surface Antigen of Staphylococci and Specific Phagocytosis.

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Lyons[1] has recently reported the results of his studies on antibacterial immunity to the staphylococcus. He states that young infusion-broth cultures of toxigenic and non-toxigenic strains of staphylococci are encapsulated; old infusion-cultures contain mostly non-encapsulated cocci; the capsule is not present in young serum-grown cultures; capsules are no longer demonstrable after agitation on a mechanical shaker, but they withstand heating to 100°C for 5 minutes; young (2-hour) broth cultures when exposed to defibrinated blood are only phagocyted in small numbers in 15 minutes, as compared to overnight cultures which are phagocyted in large numbers. In other words, the young encapsulated cocci are resistant to phagocytosis. Lyons indicates that a marked phagocytosis of a young culture is related to a specific factor present in serum. It should also be pointed out that he has reported type-specific agglutinins present in the serum of rabbits immunized with young cultures, whereas immunization with old cultures resulted in a preponderance of group-agglutinins. No swelling of the capsules *("Quellung")* could be demonstrated in young encapsulated organisms when added to this type-specific serum.

Because little is known concerning antibacterial immunity in patients with staphylococcal sepsis, it appeared highly important to extend the observations of Lyons as to whether young cultures of staphylococci isolated from patients possessed a capsule that could be demonstrated morphologically, and whether these young organisms resisted phagocytosis. In the present study, attempts were made to demonstrate an encapsulated phase of several strains of staphylococci. Quantitative phagocytic studies were also carried out with the use of human leukocytes and young and old cultures of staphylococci. Thirty strains of pathogenic staphylococci were used; 27 of which were isolated from patients, and 3 were supplied to us by Dr. A. T. Henrici of the Department of Bacteriology, University of Minnesota. Eleven non-pathogenic strains were also studied, 6 of which were sent to us by Dr. George H. Chapman

[1] Lyons, C. V., *Brit. J. Exp. Path.*, 1937, **18**, 411.

of New York, and 5 of which were grown from human urines.* Stock cultures of all the strains were maintained on veal-infusion agar-slants kept in a refrigerator. Transplants were made every 3 weeks without any demonstrable loss of, or change in, pathogenicity of the strains. Suspensions of the organisms were obtained by growth in veal-infusion broth containing 0.05% glucose, as recommended by Lyons. Capsule-stains were carried out as described below. Phagocytic tests were done with the bloods obtained from 8 patients with staphylococcal sepsis, 4 normal adults, and 3 infants.

We were unable to demonstrate encapsulated staphylococci in either young or old cultures with Hiss's capsule stain,[2] Muir's modified stain,[3] with the method of Lyons,[1] and with 15% colloidal silver solution. Lyons then recommended the following revised procedure.[4] Smears were prepared from young 2-hour broth cultures, and dried in the incubator, but never flamed. Carbol-fuchsin was prepared by dissolving 0.025 g of basic fuchsin† in 3 cc of dehydrated alcohol, and then adding 22 cc of 5% solution of phenol in distilled water. The solution, after shaking well, was filtered before using. The dried smear was covered with the carbol-fuchsin solution, which was allowed to remain for one to 3 minutes. The stain was decanted, and then covered for 10 seconds with an aqueous solution of 2% potassium hydroxide. This was decanted, the smear *blotted* dry, and then Lœffler's alkaline methylene blue was added for 10 seconds. *At no time was the smear rinsed in water.* With this technic, a deposition of the carbol-fuchsin around the cocci was observed. The usual appearance was a deep blue coccus surrounded by a deep pink material, which might be interpreted as an encapsulated coccus. However, this staining reaction was not found to be specific for young cultures alone. Broth cultures that had stood at room temperature for 72 hours showed a preponderance of "encapsulated" cocci. Young cultures subjected to mechanical agitation for 30 minutes likewise were "encapsulated." Young serum-grown cultures did not show a specific deposition of dye around the cocci because the whole

* The pathogenicity of all the strains used was determined by biological and serological methods to be described elsewhere. The non-pathogenic strains were killed in large numbers by normal human blood.

2 Zinsser, H., and Bayne-Jones, S., *Textbook of Bact.*, 7th ed. revised, 1937, 1026.

3 Muir, R., and Ritchie, J., *Manual of Bact.*, 6th edition, 1913, iii.

4 Lyons, C. V., personal communication.

† "Soloid" basic fuchsin obtained from Burroughs Wellcome and Co.

smear retained the stain, but when the same serum-grown organisms were separated from the serum by centrifuging, washed 3 times in sterile distilled water, and resuspended in sterile broth, a deposition of the carbol fuchsin around the coccus was readily demonstrated. Smears of animal charcoal suspended in infusion-broth revealed the same deposition of pink dye around the dark blue particles of charcoal. We may conclude from these observations that while the deposition of fuchsin in direct contact with the staphylococcus might possibly be interpreted as demonstrating capsular material, the stain is not specific for this material alone.

The next step was to determine whether there was any quantitative difference in the phagocytosis by human leukocytes of a young broth-culture of staphylococcus and an old culture of the same strain. If the young culture possessed an encapsulated phase, one would expect fewer cocci phagocyted when compared with an older culture that was non-encapsulated. It was believed *a priori* that regardless of whether or not the majority of cocci in young cultures were encapsulated, a larger number of organisms from old cultures would be phagocyted because the young culture would be in the "lag phase" of growth when metabolic activity was at its height, whereas the old culture would be in the "phase of decline" and would contain some dead or dying organisms.[5] In this study, an "old" culture was a 16- to 18-hour growth in infusion broth, while a "young" culture was prepared by seeding 0.2 cc of the "old" culture into 10 cc of infusion-broth, and incubating it at 37°C for 2 hours. Phagocytic tests were done with human defibrinated blood as follows: 0.25 cc of blood was added to each of 2 pyrex test tubes. To one tube 0.05 cc of the "young" culture was added and to the other 0.05 cc of the "old" culture. The tubes were sealed in a gas-oxygen flame and rotated in the incubator for 15 minutes. They were then opened, and smears were made and stained with Wright's stain. Fifty consecutive polymorphonuclear neutrophilic leukocytes were counted, and the total number of intracellular cocci in each leukocyte showing phagocytosis was noted.

The results from a large number of phagocytic experiments indicate that (1) the leukocytes of human defibrinated blood will phagocyte essentially the same number of cocci from "young" cultures as from "old" cultures. Occasionally, an isolated experiment showed fewer cocci phagocyted from the "young" culture,

[5] Topley, W. W. C., and Wilson, G. S., *The Principles of Bacteriology and Immunity*, 2nd edition, 1937, 67.

but such observations were inconstant. (2) While the total number of intracellular cocci is the same, it would appear that slightly fewer leukocytes entered into the phagocytosis of "young" cultures. (3) The bloods of patients with staphylococcal sepsis showed no greater degree of phagocytosis of "young" and "old" cultures of homologous strains than the bloods of normal adults and infants. (4) Pathogenic strains possessing potent hemolytic and lethal exotoxins were phagocyted as readily as pathogenic strains producing little or no exotoxins. (5) There was no appreciable difference in the phagocytosis of pathogenic strains as compared with non-pathogenic strains. In Table I are presented representative phagocytic studies of pathogenic staphylococci.

TABLE I.

Source of Blood	Strain‡	Old Culture†	Young Culture†
		%	%
1. Pt. "S" (osteomyelitis)*	"S"	479- 98	505- 92
2. Pt. "A" "	"A"	628-100	444- 98
3. Pt. "K" "	"K"	471-100	346- 85
4. Pt. "Sh" "	"Sh"	806-100	813- 88
5. Pt. "L" (bacterial endocarditis)	"Co"	385- 85	231- 80
6. Pt. "Fr" (carbuncle)	"Co"	322- 94	418- 96
7. Pt. "O" (bacteremia)	"Co"	699-100	757- 98
8. Normal adult*	"S"	522-100	618- 90
9. " "	"A"	634- 98	547- 90
10. " "	"K"	581- 98	604- 85
11. Normal infant (3 mo.)	J13	408- 86	463- 74
12. " " "	"S"	743- 96	576- 84
13. " " (6 mo.)	J13	562- 96	630- 80
14. " " "	"S"	710- 98	531- 80

* Tests were done with the bloods of 1, 2, 3, 4 at the same time as 8, 9, 10.

† Total number of intracellular cocci in 50 consecutive leukocytes with percentage of leukocytes showing phagocytosis.

‡ Source of Strains:

"S," "A," "K," "Sh" from patients with staphylococcal sepsis.

"Co"—Strain producing highly potent hemolytic and lethal exotoxin obtained from Dr. A. T. Henrici.

J13—Julianelle Type A organism obtained from Dr. A. T. Henrici.