STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF MONKEYS

II. Bacteriological Experiments

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Plates 57 to 59

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In the first paper of this series (1), we described the clinical appearance and histopathology of the inflammatory type (Type II) of spontaneous conjunctival folliculosis as it occurs in *Macacus rhesus* monkeys. It was shown that the incitant of the affection is apparently a specific infectious agent of microbic rather than ultramicroscopic type. In this paper we shall record the results of bacteriological studies on the disease.

Methods

The methods employed by Noguchi in his trachoma investigations (2) were closely followed. For cultivation tests, however, we depended on leptospira medium and horse blood agar plates and slants. Since the following modifications of Noguchi's original media not only facilitate the recovery of *Bacterium granulosis* (2) from affected tissues but also result in more profuse cultural growth, we employed them in the present study.

In preparing leptospira medium, 0.2 instead of 0.1 part of laked rabbit erythrocytes was added, and the horse blood agar was changed from its original composition to:

	cc.
Defibrinated horse blood	60
2 per cent nutrient agar, as employed by Noguchi	500
Mixture of carbohydrates (dextrose 10 per cent, sucrose 10 per cent, mannose 2.5 per cent, levulose 2.5 per cent, and inulin 2.5 per cent, sterilized by filtration through Berkefeld V or N candles)	25

It should be emphasized that both media should be freshly prepared; the agar slants should contain 0.5 to 1.5 cc. of condensation water and the horse blood and rabbit serum should be obtained a short time only before use.

Another change in the technique of Noguchi consisted in the inoculation of four tubes of leptospira medium, one with a fragment of conjunctival tissue and the other three with a suspension of ground tissue, undiluted and in dilutions of 1:10 and 1:100 respectively. Separate plates were seeded with undiluted, 1:10 and 1:100 dilutions of the tissue suspension. It was found advantageous to inoculate, in addition, one or two tubes containing about 5 cc. of hormone broth with the undiluted suspension of the tissue. After growth was obtained, plates were seeded with the material for the purpose of identification of the organisms.

We should state here that for bacterial cultivation conjunctival secretions, or material of expressed follicles, are not as suitable as ground tarsectomized tissue.

Results of Cultivation Tests

The conjunctival tissue derived from folliculosis monkeys yielded in cultures a variety of microorganisms. Among those most frequently recovered were diphtheroids, staphylococci, chromogenic Gram-negative bacilli, organisms of the *subtilis* group, *Sarcinae*, and moulds. Of these microorganisms, 24 different strains were injected subconjunctivally in 47 monkeys without specific effect. The conjunctival tissues of 6 of the inoculated animals were again cultured and yielded no special organisms. *Bacterium granulosis*, however, was not recovered from cases of spontaneous folliculosis in 50 monkeys studied especially with the object of isolating this bacterium. In general, the bacteria were of the same species as were found both in monkeys having granular conjunctivitis following inoculation of trachomatous materials, and in normal animals, although in the latter instances the organisms were present less frequently and regularly.

In addition to these innocuous varieties of bacteria, we have isolated and cultivated from spontaneous conjunctival folliculosis of *Macacus rhesus* monkeys a hitherto undescribed microorganism that is capable of reproducing a folliculosis disease in normal *rhesus* monkeys and chimpanzees indistinguishable from the spontaneous affection. It has been recovered thus far from seven of ten animals having folliculosis, and has not been isolated from thirty-two monkeys which were either normal, inoculated with the aforementioned indifferent bacteria, or affected with experimental trachomatous conjunctivitis. The tissues derived from five cases of human trachoma also failed to yield this organism.

Description of the Microorganism

The bacterium can be defined as a minute, Gram-negative, aerobic bacillus which is monotrichous, encapsulated, and actively motile. A detailed description of its morphological and growth characteristics follows.

Morphology.—The organisms occur as slender, tenuous bacilli, frequently discrete, less often in short chains or in parallel arrangement of two or three members, and least often as diplobacilli. They measure in width 0.2 or 0.3μ and in length from 0.8 to 1.2μ; the shorter forms are found in leptospira medium cultures, the longer ones in agar media. They have pointed ends and each organism is surrounded by a capsule which is somewhat wider than the somatic material itself. The capsular material is often demonstrable by ordinary staining methods. A single polar flagellum appears to be attached to the capsule but not to the bacterial body.[1] In rare instances the flagellum is double and infrequently its capsular attachment is lateral. The bacilli are actively motile; the motility being influenced neither by the age of the culture nor the particular medium used. They usually dart zigzag across a field but in some instances move in a pivotal or whirling manner, without forward progression.

Staining Reactions.—The organisms are invariably Gram-negative, whether in film preparations of cultures or in stained sections of affected tissues; they are not acid-fast. Spores or metachromatic granules are not detectable with Neisser's, toluidin blue, or Giemsa's stains. Infrequently, Loeffler's alkaline methylene blue solution stains certain parts of the bacterial protoplasm more deeply. In dark-field examinations, however, no spores or granules can be seen. (Figs. 1 to 9; Fig. 4 shows a characteristic appearance of ordinary Gram's stain film preparations,—a blurred background consisting of débris of capsular and flagellar material in which are embedded the bacterial bodies.)

Agar Plates.—The colonies appear on plain agar as small, circular, greyish, translucent growths. They are also smooth, convex, and slightly raised, tending toward confluence and having a sticky, or mucoid, consistence. Their appearance is similar on blood agar except that the colonies, more highly translucent and colorless in early growths, become greyish after 2 or 3 days (Fig. 10). We wish to emphasize that a positive culture of affected tissue shows at best only one, two, or three colonies of the microorganism scattered among a number of other miscellaneous growths (Fig. 11). Hence it is essential to use the same inoculum for several plate cultures.

Agar Slants.—The growth on this medium reveals a greyish white to white, moist, mucoid, heaped up, glistening appearance. When blood is added to the medium, the growth is more profuse (Figs. 12 and 13).

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[1] The apparent attachment of flagella to capsules was first shown by Churchman, J. W., and Emelianoff, N. V., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 996.

Leptospira Medium.—After 24 to 48 hours, one observes a homogeneous, dense, sharply defined layer, extending downwards about 0.5 cm. from the surface. Below this is a slight, nebulous, uniform opacity, about 1 cm. high (Fig. 14). During the ensuing 3 or 4 days this deep layer becomes more dense and thereafter slowly extends to the bottom of the tube.

Gelatin.—The colonies are more mucoid and raised than on agar but are alike in other respects. In gelatin stab cultures there is an arachnoid growth along the line of inoculation but no liquefaction.

Broth.—A uniform turbid growth occurs with a slight, greyish sediment and without pellicle formation.

Litmus Milk.—Unchanged.

Potato.—Surface cultures show spreading, abundant growths having a light tan color.

Indol.—Not produced.

Nitrates.—Not reduced.

Carbohydrate Reactions.—Acid, but no gas, is produced in Hiss' serum water cultures containing dextrose, levulose, mannose, galactose, xylose, arabinose, and rhamnose. When the acid production is considerable, coagulation of the medium occurs and a pellicle is formed on the surface. Only a small amount of acid is produced with dextrin. An occasional strain slightly acidifies media containing either saccharose, lactose, inulin, or mannitol. Raffinose, salicin, dulcitol, amygdalin, maltose, trehalose, sorbitol, and inositol media are unchanged.

Oxygen Requirements.—The organism is aerobic and facultatively anaerobic.

Other Properties.—There is no characteristic odor in cultures. Heating for 30 minutes at 56°C. kills the organism; the optimum temperature for growth is, however, 28–30°C. Cultures are bile-resistant.

Serological Reactions.—Rabbit antiserum is regularly obtained in an agglutination titer of at least 1:1,000 for all strains of the bacterium. Such sera are specific: agglutination was noted neither with ordinary bacteria nor with 54 cultures of Gram-negative bacilli found in the conjunctival secretions or tissues of man or monkey. Although we met with three strains of morphologically and culturally similar organisms that were clumped, one in 1:10, the second in 1:100, and the third in 1:1,000 dilution of the serum, they were dissimilar in carbohydrate reactions. We are not now prepared to say whether these bacteria showed non-specific reactions or whether they were variants of the bacterium. Conversely, six rabbit antisera prepared with individual strains of the indifferent, Gram-negative organisms described above failed to agglutinate the special bacterium. Moreover, specific rabbit antisera yielded no cross-agglutination between this organism and *Bacterium granulosis.*

We should mention in this place that thus far cultures of the organism have not been agglutinated by the serum of monkeys having spontaneous or experimentally induced folliculosis.

An examination of the characteristics of the microorganism reveals that it has a generic resemblance to *Bacterium granulosis* but differs from it in certain specific properties; namely, in motility, in manner of growth on agar or in leptospira medium, in distinctive carbohydrate reactions, and finally, in serological specificity. Hence to designate the organism we have named it tentatively *Bacterium simiae, n. sp.*[2]

Spontaneous Conjunctival Folliculosis in Chimpanzees

We have heretofore described the spontaneous follicular conjunctivitis as it occurs in *Macacus rhesus* monkeys. Further observations have shown that a similar disease exists among chimpanzees. In two lots, one containing eight and the other ten apes, the affection was present in two animals of the first group and in three of the second. We were able to study bacteriologically only three of the affected chimpanzees, and tarsectomized tissue was not available—only conjunctival scrapings and follicular contents. It has already been indicated that such material is inadequate for cultural purposes. However this may be, the simian organism was not recovered from the three apes, but as will be shown later, we have been more successful in implanting in chimpanzees, by means of cultures of the *rhesus* organism, a disease similar to that found in monkeys.

Pathogenicity Experiments

Subconjunctival Inoculation.[3]—*Macacus rhesus* monkeys and a chimpanzee were inoculated in the upper conjunctiva of one eye with each of the seven strains of the simian organisms that were cultivated from stock *rhesus* animals having spontaneous folliculosis.

The result of the inoculation was a counterpart of that following the injection of suspensions of tissue derived from monkeys having the disease, as described in detail in the preceding paper of this series (1).

There was, however, one difference to be noted. The immediate effects following injection of cultures consisted in more conspicuous inflammatory reaction of the skin and conjunctiva of the eyelid, as evidenced by more intense edema and swelling of the structures, ac-

[2] The generic name is based on the *Ehrenberg emend. Jensen*, classification of Bacteriaceae; the specific name is derived from *simia* (monkey or ape).

[3] Ether anesthesia was employed in all operations on animals.

companied by hemorrhage. This stage lasted for about a week or two, and was followed by the familiar, second period of inflammatory, progressive follicular conjunctivitis, during which time the uninoculated conjunctivae also became involved.

In the appended drawings is represented the clinical appearance of a *rhesus* monkey having spontaneous folliculosis (Fig. 20 *A*), showing also the characteristic follicles on the bulbar conjunctiva (Fig. 20 *B*). The figures can be used as a standard for comparison of these clinical appearances with those produced by inoculation of cultures (Fig. 20 *C*); the similarity of the natural and the induced disease is clearly shown.

The histopathology of experimental folliculosis following injection of the organism was also similar to that of the spontaneous disease, a description of which has already been given (1) (Figs. 15 to 19). Studies of stained sections of conjunctival tissue, derived from either the natural or the induced disease, revealed discrete or clumped, Gram-negative bacilli having morphological resemblances to *Bacterium granulosis* (Figs. 8 and 9).

The numerical results of the tests on pathogenicity of the seven cultures of *Bacterium simiae* recovered from stock monkeys having spontaneous folliculosis are tabulated.

Strain No.	No. of *rhesus* monkeys inoculated	No. of *rhesus* monkeys showing positive reactions
5A2	6	5
29B	2	2
97B	2	1
10A	2	1
50B	1	1
601	2	2
605	6	3
Totals..... 7	21	15

In addition, a chimpanzee that also received Strain 5A2 developed folliculosis indistinguishable from the spontaneous or the experimental disease of monkeys. Also, tissues taken from monkeys successfully inoculated with the cultures produced in the conjunctivae of fresh *rhesus* monkeys the characteristic follicular reaction.

Conjunctival Swabbing.—The method consists of applying to a cotton swab a suspension of the organism, prepared by adding 2 cc. of saline

solution to an agar slant growth, and then gently rubbing the material on the conjunctiva of one eye of a monkey. This was done daily for 6 or 7 consecutive days.

Four *rhesus* monkeys were swabbed in this manner. Two of them became affected, showing the first signs of the experimental disease 3 days after the last swabbing. 7 days later the untreated eye developed folliculosis.

To summarize, the seven strains of the simian organism recovered from stock *rhesus* monkeys having the spontaneous disease, in each instance induced characteristic folliculosis, positive reactions having been obtained after subconjunctival inoculation or swabbing, in seventeen of twenty-three *rhesus* monkeys and in a single chimpanzee.[4]

Recovery of Cultures from Experimental Folliculosis

We attempted to recover the organism from four *rhesus* monkeys and the ape which had developed characteristic experimental folliculosis as a result of inoculation of *Bacterium simiae*. Of these five attempts, three were successful: two cultures being derived from the affected *rhesus* monkeys, and one from the chimpanzee. The successful isolations were obtained from tissues 18 days to 11 weeks after inoculation.

The recovered microorganisms were again introduced into ten *rhesus* monkeys and three chimpanzees with these results:

Strain No.	Source	No. of animals inoculated	No. of animals showing positive reactions
57B	*Rhesus* monkey experimentally infected with culture	5 *rhesus* monkeys	4 monkeys
140B	*Rhesus* monkey experimentally infected with culture	2 *rhesus* monkeys 2 chimpanzees	1 monkey 2 chimpanzees
V	Chimpanzee experimentally infected with culture	3 *rhesus* monkeys 1 chimpanzee	2 monkeys 1 chimpanzee

[4] While the disease arising spontaneously (1) or following the inoculation of cultures of the microorganism is, as a rule, characterized by slow progression and by persistence, lasting usually throughout the life of the animal, we have observed an occasional monkey recover. We may therefore suppose that the resistance to inoculation as shown by some of the animals is due to their complete recovery and consequent immunity.

Thus the simian organism was recovered not only from stock *rhesus* monkeys having spontaneous folliculosis but also from monkeys and an ape experimentally infected with this bacterium. Indeed, these recovered strains were again proved to be specifically active in fresh *rhesus* monkeys and chimpanzees.

To sum up the total number of positive reactions obtained in monkeys and apes with cultures of the bacterium, irrespective of their source, that is whether recovered from stock *rhesus* monkeys with folliculosis or from animals experimentally infected by means of cultures, we find that of thirty-three monkeys and four chimpanzees inoculated, twenty-four of the former and all of the latter showed experimental follicular conjunctivitis.

Duration of Pathogenicity of Cultures.—One strain was found to be pathogenic for monkeys up to 355 days after its isolation; thereafter it proved to be inactive. The experimental disease was induced with another culture on the 21st day after its isolation but not on the 170th day. Hence it appears that the duration of pathogenicity of different strains maintained on artificial media can vary and with time lose the power to infect. Furthermore, infective action of the microorganism was observed in its first to tenth subplant in media, subcultures more remote than the second having been made at about a month's interval.

DISCUSSION AND CONCLUSIONS

In the bacteriological study here reported, we undertook an investigation of the flora associated with spontaneous conjunctival folliculosis. Following the plan of Noguchi (2), monkeys and chimpanzees were inoculated with the different organisms recovered from affected tissues. By this means, we disclosed among the bacteria a new species, *Bacterium simiae*, which was found to be specifically active, in that it induced follicular reactions in the conjunctiva apparently indistinguishable from the disease as it occurs in nature.

The specific action of the bacterium in animals is the more striking when it is compared with the innocuousness of other organisms isolated from cases of folliculosis, and also when considered in relation to the behavior of quarantined animals. While the disease arises spontaneously in stock animals, of some 300 normal *rhesus* monkeys—these being isolated in lots of ten to twenty and quarantined from 6 to

14 weeks—not one has as yet shown folliculosis. With the insignificant exception already mentioned (1), the experimental disease was produced only when the inoculum contained either folliculosis tissue or cultures of the simian organism.

Apart from these findings, the experimental results indicate that (*a*) the bacterium has thus far been recovered only from folliculosis cases and not from other forms of conjunctivitis nor from normal tissues; (*b*) the microorganism has been isolated not only from affected conjunctivae of stock monkeys but also from the tissue of animals—macaques and apes—experimentally infected with the bacterium, and (*c*) such recovered cultures have, in turn, been found to be specifically pathogenic in normal *rhesus* monkeys and chimpanzees.

We may therefore postulate from this experimental study that an intimate relation exists between *Bacterium simiae* and spontaneous conjunctival folliculosis of simians.

BIBLIOGRAPHY

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2. Noguchi, H., *J. Exp. Med.*, 1928, **48**, suppl. 2, 1–53.

EXPLANATION OF PLATES

PLATE 57

FIGS. 1 to 9. Morphological appearances of *Bacterium simiae.*

FIG. 1. Gram's stain. Leptospira medium culture. × 1,000.

FIG. 2. Gram's stain. Leptospira medium culture. × 1,500.

FIG. 3. Methylene blue stain. Film preparation from an agar plate; such preparations usually reveal longer forms than those seen in leptospira medium cultures. × 1,800.

In the above three figures incomplete or faint capsules can be observed, although ordinary stains have been employed.

FIG. 4. Gram's stain. Showing the background of capsular and flagellar débris—the usual, characteristic appearance in film preparations. × 1,000.

FIG. 5. Capsule and flagellum. Casares-Gil stain. × 2,000.

FIG. 6. Same. Depicting a longer and broader flagellum. × 1,800.

FIG. 7. Same. Illustrating two organisms, each with its separate capsule and flagellum. × 2,000.

FIG. 8. Eosin-methylene blue stain. Conjunctival tissue derived from monkey injected with a culture of the bacterium. At arrow-head can be seen discrete organisms. × 1,000.

FIG. 9. Eosin-methylene blue stain. Conjunctival tissue removed from a

monkey having spontaneous folliculosis. To be noted at arrow-head, a clump of the bacteria. × 1,500.

FIGS. 10 to 14. These figures represent cultural characteristics.

FIG. 10. 48 hour growth; colonies on blood agar plate. × 10.

FIG. 11. 48 hour growth; same medium; showing a mixed culture derived from a tissue suspension. Only two colonies of the bacterium are visible, indicated by arrow-heads. Natural size.

FIG. 12. 48 hour growth; plain agar slant. Natural size.

FIG. 13. 48 hour growth; blood agar slant. Natural size.

FIG. 14. 48 hour growth; leptospira medium. Natural size.

PLATE 58

FIG. 15. Section of conjunctiva of a monkey having spontaneous folliculosis. Hematoxylin and eosin stain. Three follicles, one of them deeply situated, and the destruction of the superficial epithelium, are shown. × 122.

FIG. 16. Section of same magnified ×1,000. The zonal lymphoid cell layer (*L*), within which is the core of macrophages (*M*), is represented.

FIG. 17. Section of conjunctiva of a monkey having experimental folliculosis induced by culture injection. Hematoxylin and eosin stain. To be noted is the similarity of lesions with those represented in Fig. 15. × 122.

FIG. 18. Section of conjunctiva of another monkey with experimental folliculosis following culture injection. Hematoxylin and eosin stain. A prodigious follicle is shown, which may have resulted from the confluence of two or three contiguous lesions. × 122.

FIG. 19. Same as Fig. 17, but magnified ×1,000. Here can be seen microscopic changes similar to those found in the spontaneous disease (Fig. 16).

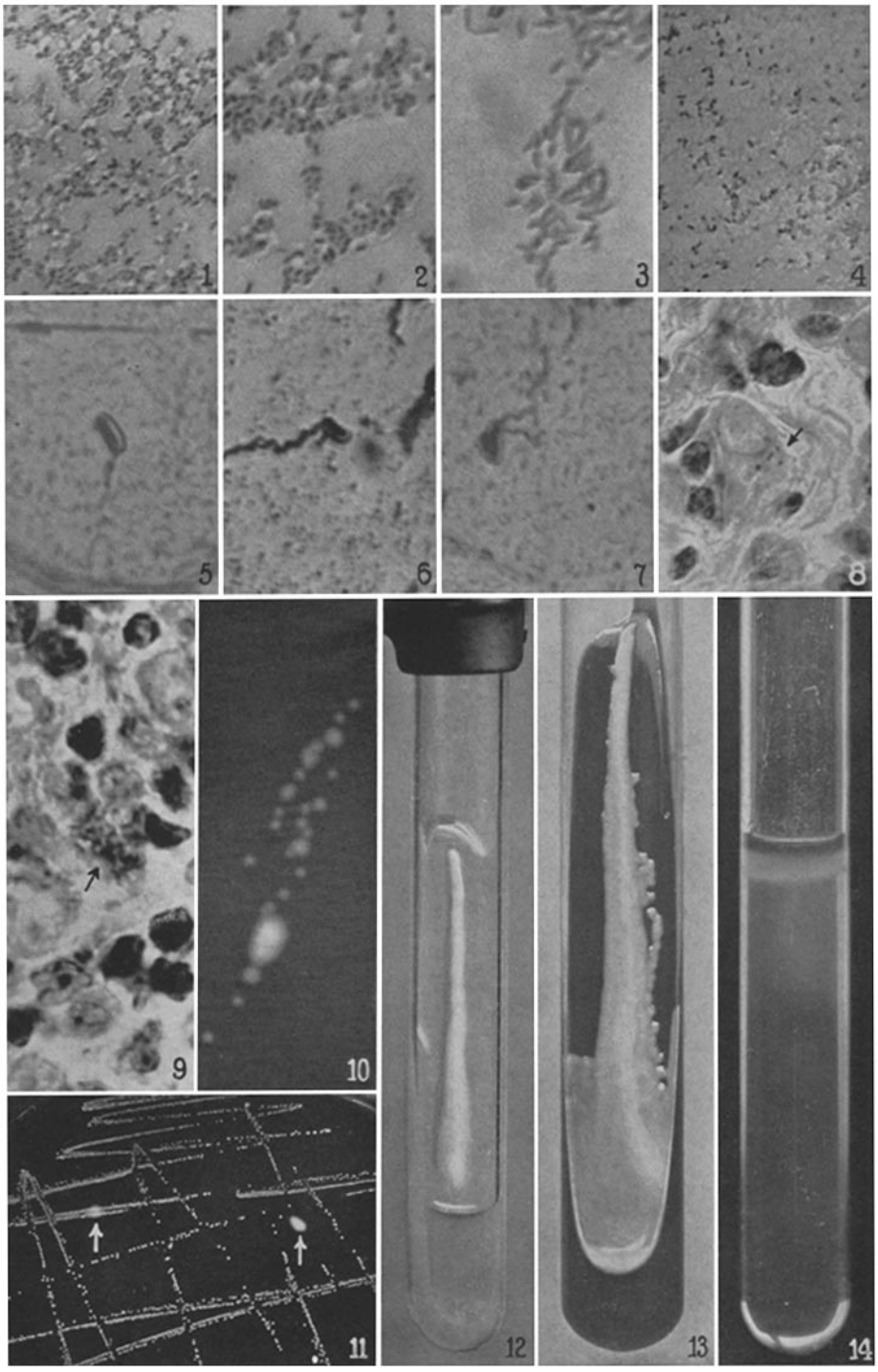
PLATE 59

FIG. 20. All drawings about twice natural size.

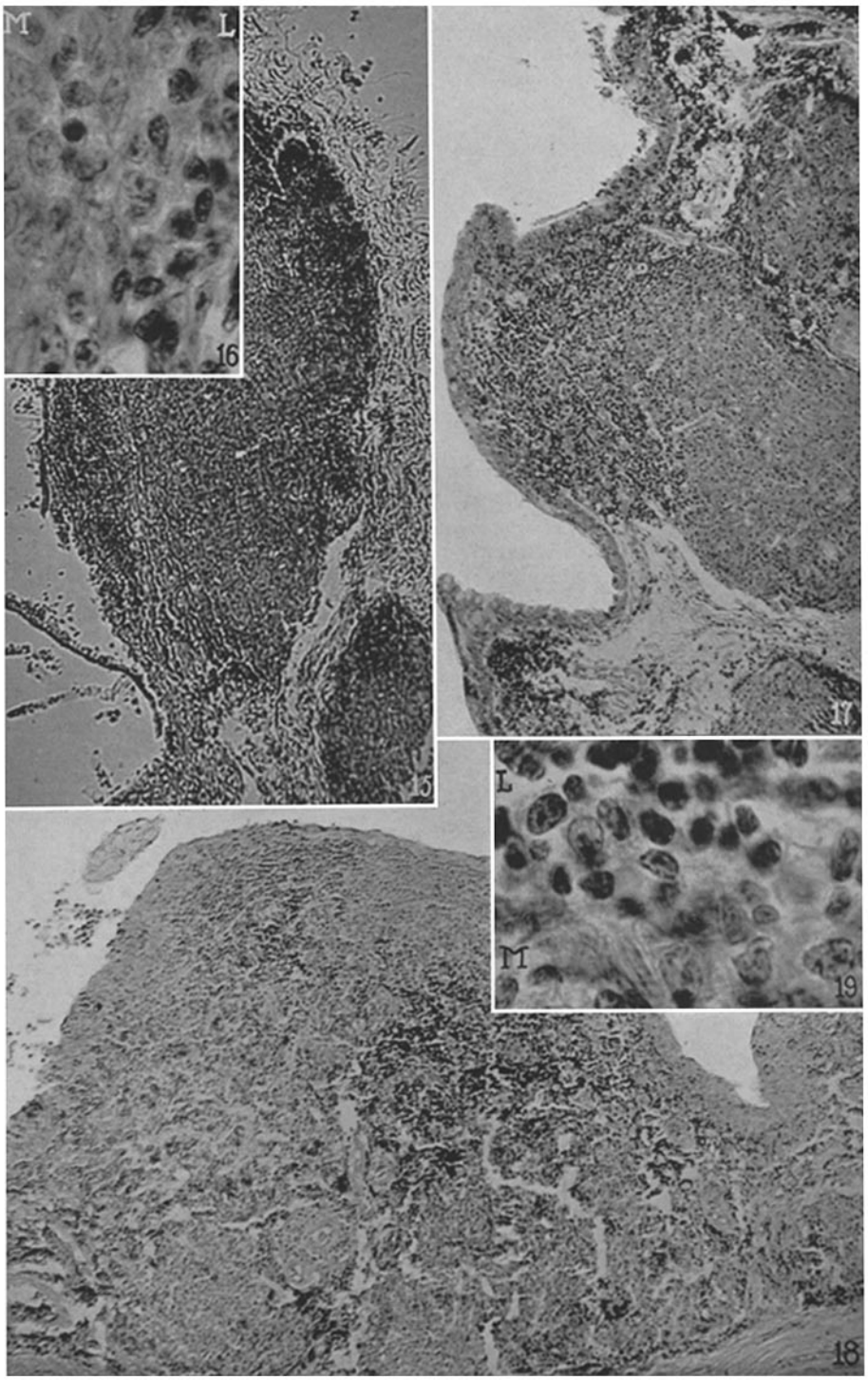
A. Eye of *M. rhesus* monkey having spontaneous folliculosis. Fully developed case, of over 5 months' duration. The chief features are the large discrete and massed succulent follicles, also seen over the tarsal plate, the edema and congestion, and the invisibility of vascular structures.

B. The bulbar conjunctiva of the same case. To be noted are the characteristic, discrete follicles.

C. Eye of *M. rhesus* monkey having experimental folliculosis induced by subconjunctival inoculation of the simian organism in the left upper lid. Fully developed reaction, 6 months after inoculation. There is no distinction between this clinical appearance and that of spontaneous folliculosis occurring in nature (*cf. A*). The bulbar conjunctiva of this animal revealed follicles similar to those shown in *B*.



Photographed by Louis Schmidt



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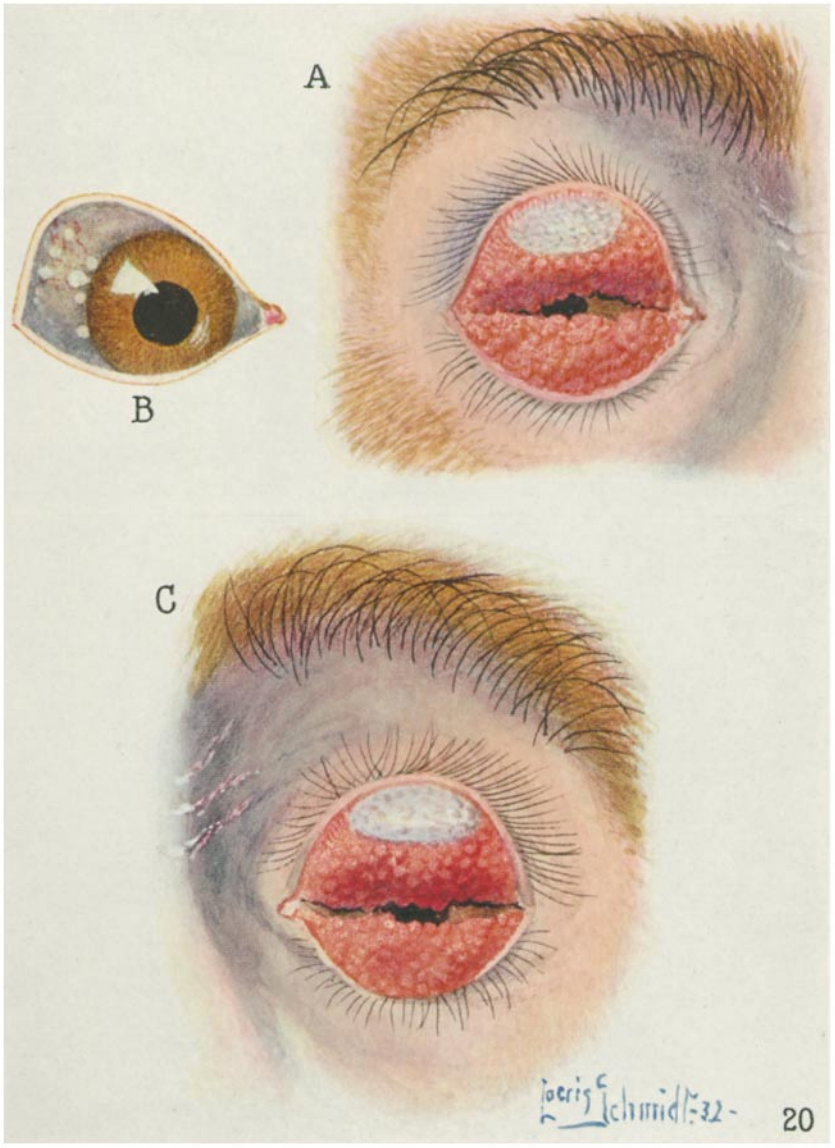
(Olitsky *et al.*: Conjunctival folliculosis. II)



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