

STUDIES ON THE FILTRABILITY OF BACTERIUM GRANULOSIS

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The question of whether a filtrable virus plays any rôle in the etiology of trachoma has long been a subject of investigation.

As early as 1905, Pfeiffer and Kuhnt (1) unsuccessfully attempted to produce trachoma in man with filtrates of trachomatous materials. In 1907, Fermi and Repetto (2) inoculated twenty-three humans and two apes with filtrates derived from the conjunctival material of fifty fresh trachoma cases. They used Berkefeld filters which had been tested for permeability with *B. prodigiosus*, and limited the time of filtration to 5 minutes. None of those inoculated with filtrates became infected, but the controls, injected with the unfiltered material, showed conjunctival follicles which, after 2 months, had resisted cauterization treatment. In 1910, Hess and Römer (3) excised twenty culs-de-sac of trachomatous individuals and prepared a filtrate with which they inoculated three *cynocephalus* monkeys. With the unfiltered material, they injected three other monkeys. The animals receiving the filtrate showed no reaction after several months' observation; but those which had been given the unfiltered suspension developed granulation of their conjunctivae in 4 days. Hess and Römer concluded that trachoma was not caused by a filtrable virus. As quoted by Weiss (4), Bajardi, as well as Lindner, reported negative results from inoculation of trachomatous filtrates in monkeys.

Nicolle (5) and his coworkers, in 1913, filtered trachomatous tissue suspensions through a Berkefeld filter of the "most permeable type" (presumably Type V), especially reconstructed by them for small amounts of material. The candle was impervious to *Vibrio cholerae*. The filtrate was injected into the conjunctiva of a magot. As a control, another magot was similarly inoculated with the unfiltered material. After 43 days, the first animal showed what was considered to be "typical" trachoma. This condition disappeared after a few months. The control animal exhibited granulations on the tarsal conjunctiva in 20 days. A *Macacus rhesus* monkey, also serving as a control, failed to react to the inoculations. In a second experiment, Nicolle and his associates followed the methods of the first test, save that material from two trachomatous individuals was pooled and a chimpanzee was injected with the filtrate and a magot with the unfiltered material. The chimpanzee showed granulations of the conjunctiva in 14 days; the control magot in 10 days. 50 days after the inoculation of the chimpanzee, the maximum reaction was reached, but on the 80th day the lesions had disappeared.

Trapesontzewa (6), in 1930, prepared a filtrate of the pooled conjunctival material from sixteen trachomatous patients and inoculated three blind persons. The filtration was made through a filter which permitted small amounts of fluid to be used. During 6 months' observation none of the inoculated individuals developed lesions other than an initial, slight, transitory edema. In a second experiment, the pooled and filtered conjunctival material from twelve persons with trachoma was inoculated subconjunctivally into three other individuals, including the experimenter. The only effect was a slight transitory edema, lasting not more than 36 hours. Trapesontzewa repeated this experiment twice, using material from twenty-four trachoma patients, and obtained a similar effect in both instances. No mention was made of the infectivity of the unfiltered material.

Recent work has brought out the fact that several species of bacteria are capable of passing through filters of sorts which had been supposed to exclude them. Grinnell (7) demonstrated that one has but to alter the physical conditions of a suspension of an organism in order to affect its filtrability. Cuénod (8), one of Nicolle's coworkers in the filtration experiments of the etiological agent of trachoma, says: "That trachoma can be induced experimentally by a filtrate is not opposed to the possibility of a bacterium visible microscopically."

The positive evidence that trachoma is caused by a filtrable virus rests upon the two experiments of Nicolle and his associates, in which a filtrate was active in one animal in each test. Furthermore, the "virus" of these investigators is described as being rapidly destroyed by heat at 56°C., by desiccation, and by glycerol (in 8 days). Such sensitiveness is not usual with ultramicroscopic viruses.

The following questions are unsettled (*a*) whether the incitant of trachoma is filtrable; (*b*) whether *Bacterium granulosis*, which Noguchi (9) and others have shown to be associated with trachoma, is filtrable, and, if so, whether it is pathogenic in this state, and (*c*) whether, as Cuénod and Nataf (10) suggest, a filtrable virus may be adsorbed to *Bacterium granulosis*. Because of the present uncertainty with regard to these matters, investigators believing that trachoma is due to a filtrable virus despite the greater amount of evidence to the contrary, we have made filtration tests with trachomatous materials and with *Bacterium granulosis*.

Materials and Methods

Preparation of Human and Monkey Material.—There were available for study the tarssectomized tissues from eight patients with trachoma of from 2 to 11 years' duration.¹

¹ For the clinical material we are very grateful to Dr. Martin Cohen of New York City.

A suspension was made of the tissue from each case by grinding for a few minutes in a sterile mortar with 2 to 5 cc. of sterile physiological saline solution. (In Cases V and VI the suspensions were made in Dunham's peptone water medium instead of in saline.) Prior to filtration, 0.2 cc. of this material was inoculated subconjunctivally into one upper cul-de-sac of each control *Macacus rhesus* monkey, and a small amount of the suspension was set aside for cultivation of *Bacterium granulosis* after the manner of Noguchi (9). Of the filtrate, prepared as described below, 0.2 cc. was inoculated subconjunctivally into each test animal and all of the remainder was used for cultures which were made by adding from 2 to 5 drops of the filtrate to tubes of leptospira medium. The tubes were kept for at least 3 weeks before they were discarded as negative.

The conjunctivae of two *Macacus rhesus* monkeys with the disease induced by inoculation of human tissues or *granulosis* cultures were used for similar filtration experiments. The conjunctivae from the upper and lower lids of one eye of each were removed,² and prepared for inoculation and filtration in the same manner as were the human tissues.

Standardization of Filters.—Berkefeld V filters were used. The permeability of the filters was tested with cultures of *B. prodigiosus* both before and after the filtration proper. This was done as follows:

A suspension of *B. prodigiosus* of ground glass opacity was prepared by washing growths of the organism from agar slants with sterile physiological saline solution. About 5 cc. of this suspension were introduced into the filter, under a pressure difference of 25 to 35 cm. Hg. In no test did the filtration time exceed 3 minutes. The filtrate thus obtained was inoculated into tubes of plain broth, which were incubated at 37°C. and not discarded as negative until after at least 3 days' incubation. After cleaning and reesterilization, these filters were used for the experiment. Immediately afterwards they were changed to other flasks and the suspension of *B. prodigiosus* was again introduced. If, in either test, *B. prodigiosus* passed through the candle, the results of the experiment were thrown out. Almost one-half of the new Berkefeld V filters examined allowed the passage of the organisms.

Method for the Filtration of Bacterium granulosis.—Cultures of *Bacterium granulosis* from 4 to 7 days old, grown on slants of horse blood agar containing a mixture of carbohydrates (11), were employed. To each tube, 8 cc. of sterile physiological saline solution were added, the culture was scraped from the surface of the agar, and the tube shaken vigorously until a suspension of ground glass opacity was obtained. 5 cc. of this suspension were removed and put into a Berkefeld V

² Full ether anesthesia was used for all operative procedures.

filter, standardized as described above. The filtration was aided by a pressure difference of 35 to 40 cm. Hg and required no more than 3 minutes, usually about 45 seconds. The filtrate was cultured in tubes of leptospira medium, not more than 1 cc. being added to each tube. These cultures were incubated at 28°C. and examined daily. Before being discarded as negative, they were observed for at least 3 weeks.

Tests for a Filtrable Agent of Trachoma

Human Trachoma.—With the material from eight cases of human trachoma, seven tests were made, the suspensions from the first two cases being pooled.

Cases I and II.—Suspensions from two cases of human trachoma were pooled and filtered. The filtrate was inoculated into three *Macacus rhesus* monkeys. One of these showed a transitory, slight follicular reaction; another had a mild follicular conjunctivitis, which had disappeared in 12 weeks, and a third showed no effect. Two control animals, inoculated with unfiltered material, were used. One of these was not affected. The other showed a mild, transitory type of reaction, with a few follicles in both eyes. *Bacterium granulosis* was recovered from the unfiltered suspension. The cultures of the filtrate were negative.

Case III.—Three monkeys were inoculated with the filtrate. None of them was affected. Tarsectomy was performed on one animal and the material was cultured for *Bacterium granulosis*. The organism was not recovered.

Two control animals, inoculated with unfiltered tissue suspension, were used. One of them showed after 8 weeks a slight follicular reaction which had cleared up in 12 weeks. The other was unaffected. *Bacterium granulosis* was not recovered from the original suspension, nor was any organism recovered from the filtrate.

Case IV.—Three monkeys were inoculated with the filtrate. All remained negative. The material derived from one of them was cultured for *Bacterium granulosis* but the organism was not recovered. Of two control animals, one developed the characteristic experimental disease; the other showed a transitory, mild follicular reaction. *Bacterium granulosis* was recovered from the human case and also from the more severely infected control monkey. The culture of the filtrate was negative.

Case V.—The filtrate was tested in three monkeys. Two of them remained negative. The other animal exhibited in 2 weeks characteristic trachomatous conjunctivitis, which in 8 weeks had largely subsided. *Bacterium granulosis* was not recovered from this animal. Of two control animals, one showed no reaction; the other showed in 8 weeks a few follicles in the inoculated eye, which have persisted. *Bacterium granulosis* was not recovered from the human case. The filtrate culture was negative.

Case VI.—Two animals received the filtrate. Both remained negative. Two

control animals developed experimental trachoma. *Bacterium granulosis* was recovered from the human case and from the one control monkey which was examined. The filtrate culture remained negative.

Case VII.—Two monkeys were inoculated with the filtrate. Neither of them was affected. Of two controls, one remained negative; the other developed a persistent, mild follicular reaction. *Bacterium granulosis* was not recovered from this animal. Culture of the human tissue yielded *Bacterium granulosis*. The filtrate culture was negative.

A decision as to the positive or negative character of the results was rendered difficult by the mild follicular type of reaction which some of the animals exhibited. This condition either cleared within 2 to 6 weeks, or persisted for over 12 weeks. The exact significance of this transitory type of follicular conjunctivitis is not yet clear; it may possibly have some relation to the experimental disease. Inasmuch as true experimental trachoma is progressive and persistent, only those reactions which endured for long periods of time could, on the basis of our present knowledge, be considered positive (Table I).

Monkey Trachoma.—Two *Macacus rhesus* monkeys (AA and BB) which showed the characteristic experimental disease as result of inoculation with *Bacterium granulosis* were used for this group of tests. Conjunctival tissue was removed from the animals and prepared and filtered in the same manner as with human tissue.

The filtrate from *Macacus rhesus* AA was injected into two monkeys. Neither developed granular conjunctivitis. One control animal inoculated with unfiltered tissue suspension developed experimental trachoma, and the other control showed a persistent, although mild, follicular conjunctivitis. Culture of the conjunctiva of the latter animal revealed no *Bacterium granulosis*, but that from *Rhesus* AA yielded the organism. The culture of the filtrate remained sterile.

The conjunctival suspension from *Rhesus* BB was inoculated into two monkeys. Of these, one developed characteristic trachomatous conjunctivitis, and from its conjunctiva *Bacterium granulosis* was isolated. The filtrate was inoculated into three monkeys, none of which showed conjunctival lesions. The filtrate failed to yield cultures of *Bacterium granulosis* (Table II).

A summary of the results of experiments on filtration of tissue suspensions of human and experimental trachoma reveals that only one of twenty monkeys inoculated with filtrates yielded a positive reaction, while nine of sixteen animals inoculated with unfiltered material showed characteristic trachomatous conjunctivitis. That all

TABLE I
Filtration Experiments with Human Tissue

Case	<i>B. granulosis</i> culture from human case	Unfiltered suspension			Filtered suspension				
		Monkey a	Monkey b	<i>B. granulosis</i> culture from monkey's conjunctiva*	Culture of filtrate	Monkey c	Monkey d	Monkey e	<i>B. granulosis</i> culture from monkey's conjunctiva
I and II	Positive (A. P.)	Negative	Negative		Negative	Negative	Negative	Negative	Negative
III	Negative	Positive	Negative		Negative	Negative	Negative	Negative	Negative
IV	Positive	Negative	Positive	Positive Monkey b	Negative	Negative	Negative	Negative	Negative
V	Negative	Positive	Negative	Negative	Negative	Negative	Positive	Negative	Negative
VI	Positive	Positive	Positive	Positive Monkey b	Negative	Negative	Negative	Negative	Negative
VII	Positive	Negative	Positive	Negative Monkey b	Negative	Negative	Negative	Negative	Negative

* Blank spaces indicate that no test was made.

TABLE II
Filtration Experiments with Monkey Tissue

Case	<i>B. granulosis</i> culture from case	Unfiltered suspension			Filtered suspension				
		Monkey a	Monkey b	<i>B. granulosis</i> culture from inoculated monkey conjunctiva	Culture of filtrate	Monkey c	Monkey d	Monkey e	<i>B. granulosis</i> culture from inoculated monkey conjunctiva
AA	Positive	Positive	Positive	Negative Monkey a	Negative	Negative	Negative	Negative	Negative
BB	Positive	Positive	Negative	Positive Monkey a	Negative	Negative	Negative	Negative	Negative

Macacus rhesus monkeys do not develop trachoma after subconjunctival injections of trachomatous tissue suspensions has already been pointed out by us (12) and by others. The significance of the single positive result with a filtrate remains to be determined.

Filtrability of Bacterium granulosis

In an attempt to determine whether or not *Bacterium granulosis* was capable of passing through tested Berkefeld V filters, thirty-one filtration tests were performed on ten strains of the organism. One

TABLE III
Filtration of Bacterium granulosis

Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid		Strain	Berkefeld filter	Suspension fluid	
		Normal saline	Hormone broth			Normal saline	Hormone broth			Normal saline	Hormone broth			Normal saline	Hormone broth
Alb. No. 1	V	-	+	EL	V	-		L-9	V	-		L-6	V	+	
Alb. No. 1	V	+	+	HD	V	-		L-5	V	-		L-6	V	+	
MC	V	-	+	HD	V	-		L-5	V	+		L-6	V	+	
MC	V	-	+	HD	V	-		Ariz. No. 3	V	-		L-6	N	-	
AP	V	-		HD	V	-		L-6	V	+	+				
AP	V	-		510	V	+		L-6	V	+	+				
EL	V	-		510	V	+		L-6	V	+					

culture was derived from one of the cases (I) mentioned above. Another was obtained from a monkey which had been inoculated with tissue from a human case not included in this series, which also yielded *Bacterium granulosis*. The remainder were old strains that had been in our possession for from 1 to 3 years.

Two tests each were made on three of the strains (including that from Case I) and no growth occurred in the filtrate. In the case of the two other strains, each passed through the filters once in two trials. Two more strains failed to come through in one test for each. The culture derived from the monkey was filtrable both times in two tests. One strain (L-6) passed through the filters in each of six trials. The experiments were repeated, using the last strain mentioned,

together with one that had filtered once out of two tests, and one that had twice failed to pass the filters. Hormone broth was used for suspension instead of normal saline solution, since it has been found by Ward and Tang (13) to favor the filtrability of herpes and vaccine viruses; and by Grinnell (7) to facilitate the filtration of *Bacillus prodigiosus*. Two tests were made on each strain, and in all six trials the organisms were recovered from all tubes which had been inoculated with the filtrate. The strain which consistently passed the Berkefeld V filters was tested through an N filter but was not then found to be filtrable (Table III).

Less than half of the inoculated leptospira tubes of the "filtrable" strains showed a growth, in some instances only one tube of ten. Hence one may infer that very few organisms were present in the filtrate.

We have found, therefore, that four out of ten strains of *Bacterium granulosis* were filtrable through Berkefeld V candles. Only two of these strains filtered consistently, and it is evident that even in these cultures the organisms were present in the filtrate in small numbers.

Action of Filtrates of Bacterium granulosis

It has not been found possible up to the present time to pass a pathogenic strain of *Bacterium granulosis* through tested filters.

Filtrates of pathogenic strains of the organism, bacteriologically sterile, were inoculated into two monkeys, neither of which developed conjunctivitis. In these tests five control animals, inoculated with unfiltered suspensions of growths, developed the characteristic experimental disease. In another similar experiment, two monkeys injected with filtrates of *granulosis* cultures were likewise unaffected.

DISCUSSION AND SUMMARY

The evidence hitherto reported concerning the filtration of trachomatous material, and inoculation of man and monkeys with the filtrates points to the conclusion that the incitant of trachoma is not, as a rule, filtrable. Our findings confirm this view and indicate further that no virus causing the disease is adsorbed to *Bacterium granulosis*. On the other hand, *Bacterium granulosis* itself in heavy suspensions is irregularly filtrable through Berkefeld V candles, like some other bacteria (14), but it is present in the filtrates in only small numbers. When suspensions were used of trachomatous human and monkey tissues, which contain much fewer organisms than do actual cultures, *Bacterium granulosis* was never recovered from the filtrates.

The conception that trachoma is a disease caused by an ultra-microscopic virus is based on (a) the positive results of filtration in two animals, as reported by Nicolle and his coworkers, and (b) the presence of so called "inclusion bodies" in some of the cells of the lesions. One can state definitely that the evidence is now greatly against the filtrability of the etiological agent of trachoma. Furthermore, filtrability does not in itself suffice for the classification of an agent as an ultra-microscopic virus. Concerning (b), a vast literature has accumulated which indicates that the "inclusion bodies" of trachoma are not specific for the disease and that the bodies themselves may be bacterial in origin (15). We have not as yet found bodies of the kind characteristic of many filtrable viruses in the tissues of man or of monkeys with the experimental disease.

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