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THE PRESENT STATUS OF LABORATORY RESEARCH IN TRACHOMA

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SYNOPSIS

The present status of laboratory research in trachoma is summarized. The principal factors which have retarded research on this disease have been the lack of a suitable experimental animal and the failure of tissue-culture methods to provide serial cultures of the virus. Some avenues of investigation for the future are suggested.

Laboratory research in trachoma has lagged behind research in many other virus diseases for a number of reasons. Chief among these are the lack of a suitable experimental animal and the failure of the disease to respond serologically with any strength. The failure of the virus to grow readily in tissue culture has of course greatly hindered the study of its biological properties. Laboratory research is further hindered by the fact that the disease is disappearing, or has disappeared, from many of the countries of Europe and America whose laboratories are particularly well equipped for the study of virus diseases. In addition to these influences, the fact that the sulfonamides and broad- and medium-spectrum antibiotics are therapeutically effective in trachoma has served to lessen interest in fundamental trachoma research.

Nature of the Virus

There is now general agreement that the agent of trachoma can be seen in epithelial scrapings from the disease in the form of elementary and initial bodies which, together with a carbohydrate matrix (Rice [77]), make up the inclusion bodies of Halberstaedter & Prowazek.[39] There is still, however, some uncertainty as to the proper classification of the agent. It has many of the properties of the viruses, including filterability, inclusion-body formation, and cytotropism; in other respects it resembles the rickettsiae, as, for example, in its staining reactions and its susceptibility to sulfonamides and antibiotics.

Tentatively, the agents of both trachoma and inclusion conjunctivitis have been placed in the psittacosis-lymphogranuloma-venereum family of viruses which has recently been given the name " Chlamydozoaceae ". Under the generic term " *Chlamydozoon* ", the agent of trachoma has been designated *Chlamydozoon trachomatis*, and the agent of inclusion conjunctivitis, *Chlamydozoon oculogenitale*. This classification, proposed by Moshkovsky and accepted by Rake for inclusion in Bergey's *Manual of Determinative Bacteriology*,[76] is currently under study and subject to revision.

Pathology of Trachoma

Many excellent studies on the pathology of trachoma have been reported in the literature. Wilson [100] recognized as the first pathological sign the appearance of Halberstaedter-Prowazek inclusion bodies in the cytoplasm of conjunctival and corneal epithelial cells, and a number of observers have found inclusions during the incubation period of experimental trachoma in human volunteers. The lymphoid follicle, which is the characteristic feature of trachoma, is histologically similar to the follicles of non-trachomatous disease, but differs in its predilection for the upper tarsus and upper fornix, and in the degenerative changes that characterize it and lead to scar formation. These degenerative changes are reflected in the large number of macrophages, or Leber cells, filled with engulfed cytoplasmic and nuclear debris, that are seen in follicular expressions. The attempt on the part of some trachomatologists (Badir [5]) to define the trachoma follicle as a specific granuloma comparable to the tubercle of tuberculosis seems to me to be unjustified.

The consensus of opinion is that follicles are invariably present in the active stages of trachoma even if sometimes obscured clinically by papillary hypertrophy. The infiltrating cells of the papillary hypertrophy are in large part plasma cells, and it is these cells, along with a lesser number of lymphocytes, which form the subepithelial infiltrate between follicles that characterizes all cases of active trachoma. The neutrophilic leucocyte, so prominent in the exudate of early and acute cases, is seen characteristically in sections in the epithelium, along with the lymphocyte, in the process of passing into the exudate. The plasma cell, which has its own significance when seen in smears and scrapings, as will be discussed later, does not seem to share this ability to migrate through the intact epithelium.

There now seems to be general agreement that trachoma, at least in its initial phases, is a so-called " epitheliosis ", with the epitheliotropic virus limited to the corneal and conjunctival epithelium. Although the majority of trachoma investigators agree that the virus remains limited to the epithelium throughout the activity of the disease, some still claim that a subepithelial, invasive stage exists (Busacca;[22] Cuenod & Nataf;[29, 30, 31] Chams, Mohsenine & Armine;[24] Badir [5]).

In view of the controversy on this point, I have recently reviewed my collection of histopathological slides of trachoma. This collection consists principally of slides of Egyptian trachoma given me by Dr R. P. Wilson, but also includes material from trachoma in Japan given me by Dr Y. Mitsui, material from trachoma in Tunisia, and material from trachoma in the white and Indian populations of the USA. The results of this review confirm my previous conclusion, shared by Mitsui,[53, 65] Wilson,[101] and others, that the inclusion bodies of trachoma do not occur in cells of the subepithelial tissues. I feel that there is no microscopic evidence of invasion of any cells other than epithelial cells, and that the so-called " rickettsia " described by Busacca,[22] and the " rickettsioid bodies " described by Cuenod & Nataf,[29, 30, 31] are non-specific. Judging by the negative results of inoculations of infective material through the skin of the lids into the subepithelial tissues of human volunteers and monkeys, by such observers as Michail & Vancea,[52] Thygeson & Richards,[92] and Y. Mitsui (personal communication), it seems very unlikely that the virus ever proliferates in tissues other than epithelium. This conclusion is supported by similar results in experiments I have made on the related disease, inclusion conjunctivitis, in which the localization of the virus is also strictly epithelial. Only the work of Chams, Mohsenine & Armine,[24] who claim to have produced infection by subepithelial inoculation, stands in opposition. To settle the point beyond all question, further studies are indicated.

The other features of the pathology of trachoma are uncontroversial. The recent study by Badir, Wilson & Maxwell Lyons,[6] which amplifies Wilson's earlier work,[100] outlines the essential pathological features of the conjunctival disease, including the cicatrization and pseudo-gland formation, the papillary hypertrophy, the initial hypertrophy and subsequent atrophy of the epithelium, and such late complications as hyaline degeneration.

Somewhat less is known of the pathology of the trachomatous limbus and cornea because of the difficulty of obtaining biopsy or autopsy specimens. The studies of Busacca,[23] however, have contributed greatly to our knowledge of the pathology of the limbal follicle and its cicatricial remains, known as Herbert's pits, as well as to our understanding of such other corneal changes as the characteristic trachomatous epithelial keratitis.

Some difference of opinion may still exist as to the nature of the ptosis that is so characteristic a feature of the trachoma facies. It must be due in part to increased weight of the lid from cellular infiltration, but there is definite evidence that in certain cases it is also due, at least in part, to cellular infiltration of Müller's muscle.

Dacryocystitis is a common complication of trachoma but its nature is not entirely clear (Charamis,[25] Djacos [32]). Follicles have been demonstrated in tissues from excised sacs, but since they may also occur in non-trachomatous dacryocystitis, their significance must be regarded as questionable.

Mitsui's demonstration of inclusion bodies in the epithelial cells of excised sacs (personal communication) is more convincing evidence of an etiological relationship.

The exfoliative cytology of trachomatous epithelial scrapings and expressed follicular material has recently been explored. That diagnostic information can be derived from the study of such material has been affirmed by such authors as Taborisky,[84] Thygeson,[89] Sezer,[79] Agarwal & Saxena,[1] and Kimura & Thygeson.[47] Taborisky has reported the epithelial changes as the most significant, while the other authors seem to have given more weight to the changes in the follicles that reflect the necrotizing propensities of the disease. These changes will be discussed in detail in the following section.

Laboratory Diagnosis of Trachoma

Diagnosis by the demonstration of inclusion bodies

The recent controversy over the status of the Poleff stain [72] has emphasized the need for a re-evaluation of our standards for the identification of trachoma inclusion bodies. The elementary body inclusion, or mature inclusion, would seem to be the type least likely to be confused with nonspecific material. Perhaps the best criterion for its identification would be a preliminary demonstration of the carbohydrate matrix in iodine-stained scrapings, followed by identification of the component elementary bodies after restaining with Giemsa (Thygeson [86]). Other stains, including the Castañeda stain, the Lindner contrast stain, and the Macchiavello stain, have not been widely useful in trachoma work, and enough evidence [81] has now accumulated to indicate that the Poleff stain is not reliable and should be abandoned.

Up to the present time it has not been possible to distinguish the inclusion bodies of trachoma from those of inclusion conjunctivitis, but Braley [19] found the different tissue affinities and localizations of the two agents highly satisfactory for differential diagnosis. He found that the inclusion bodies of trachoma were always more numerous in scrapings from the upper fornix or upper tarsus than from the lower tarsus or lower fornix, and that the reverse was true of the inclusion bodies of inclusion conjunctivitis. I have been able to confirm these findings fully. This test is of course of value only in cases in which inclusions are reasonably abundant.

Unfortunately the diagnostic value of the inclusion bodies has been limited by their scarcity in the chronic and cicatricial stages of the disease. They are usually not difficult to find in early trachoma and in acute stages of the disease that have not been induced by secondary bacterial infection. Various provocative tests have been applied in attempts to increase the number of inclusions in mild cases, and, although some success has been

attained with cortisone and other steroids,[a, 33, 88] and with copper sulfate, silver nitrate, other cauterizing agents, and mechanical manipulation, the results have been inconsistent.

There is a definite correlation between the number of inclusions and the amount of conjunctival exudate. Inclusion-body studies[87] have shown very definitely, moreover, that acute trachoma is an entity not necessarily dependent on secondary bacterial infection as Morax[59] believed it to be. The current controversy, precipitated by Mitsui,[54] as to whether or not the onset of trachoma is always acute, must be settled with the assistance of the laboratory. Experimental human inoculations of material from trachoma have almost invariably induced a disease with acute onset and abundant inclusions in which the number of inclusions has varied directly with the severity of the disease. Accidental infections have also usually begun acutely. Of the considerable number of cases of pure trachoma that I have been able to follow from their inception, however, some have started insidiously, others acutely. That acute symptoms in trachoma, at onset or otherwise, can also be the result of secondary bacterial infection has been confirmed in the laboratory, and it must be recognized that secondary viral infection, as with epidemic keratoconjunctivitis virus or herpes simplex virus, for example, can also be responsible for acute symptoms.

In the related disease, inclusion conjunctivitis, diagnosis can often be established in the early acute stage by the finding of myriads of free elementary and initial bodies in secretion preparations, or in the more satisfactory preparations of epithelial scrapings. In trachoma, free bodies have been seen only in early or acute cases. In certain of these, however, they have been present in enormous numbers, together with extruded intact inclusions in which the elementary bodies were still held together by the matrix.

The literature is replete with reports of studies in which non-specific bodies have been mistaken for trachoma inclusions by untrained observers. These pseudo-inclusions have been considered in an article by Braley.[20] Mast cells have been one source of confusion, particularly in biopsy material, and have accounted, I believe, for the few claims of finding inclusions in subepithelial tissue. Workers using the Poleff stain in particular have tended to mistake mast cells for inclusions. Pseudo-inclusions consisting of extruded nuclear material, engulfed nuclear debris, pigment granules, and other cell granules seen in degenerating cells or in goblet cells have also caused confusion. In tissue cultures of conjunctival epithelium, collections of cell granules localized to one pole of the cell are sometimes confusing when seen under low power, but become readily differentiable under oil immersion. The claims made in reports by inexperienced workers must be analysed critically. Unless photographs have been taken at high magnification, the illustrations accompanying such reports must be regarded as

[a] Unpublished working document WHO/Trachoma/18

inconclusive. An experienced worker, on the other hand, particularly if he has worked with other members of the psittacosis-lymphogranuloma group, can usually differentiate trachoma inclusions from non-specific bodies without difficulty.

Electron microscope studies of trachoma virus that has been purified by differential centrifugation have been made by a number of Japanese workers, including Sugita & Sugita (cited by Hagino & Hamada [38]), Hagino & Hamada,[38] Fujiyama,[34] Tsutsui,[95] Ito & Sasaki,[40] Mitsui, Tsutsui & Tanaka,[56] and Arakawa (cited by Tsutsui & Takeda [97]). Similar studies by Barski, Grom & Croissant [7] of thin sections of trachomatous epithelium have been discussed in a report by Tsutsui & Takeda.[97] None of these workers has had any difficulty in demonstrating the elementary bodies of trachoma by these techniques.

In a recent study of trachoma virus in section, Mitsui & Suzuki [55] made an important contribution. They compared the morphology of the virus in inclusion bodies by light microscopy and electron microscopy, and found that a fully developed " elementary body inclusion " when examined under low magnification by the electron microscope looked just as it did under the light microscope. Under higher magnification, however, certain structural differences appeared. The elementary bodies were round or elliptical but their internal composition was unhomogeneous. Typically each elementary body consisted of a central core of high density, with a cortex and surrounding membrane of low density. A few atypical dense forms without surrounding cortex were also seen. The diameter of the solid central core measured from 200 mμ to 350 mμ, and the diameter of the membranous sphere from 400 mμ to 500 mμ. Apparently only the central core stains with Giemsa and it is thus the only element visible under the light microscope.

The authors found it more difficult to identify initial body forms but described them as varying in size from 400 mμ to 800 mμ and as being variously granular, reticular, or alveolar. There appeared to be a gradual transition from large initial bodies, through small initial bodies, to typical elementary bodies. The authors noted an amorphous substance, sometimes polygonal in structure, associated with the virus particles, and suggest that the particles may be produced by a condensation of this substance.

While electron microscopy has great theoretical interest in trachoma, it has as yet no practical diagnostic value. It has supported the findings of light microscopy, however, particularly with respect to the localization of the virus in the epithelium and the absence of any virus-like particles in the subepithelial tissues.

Diagnosis on the basis of cytological findings

It is well recognized that the finding of inclusion bodies cannot serve to differentiate trachoma from inclusion conjunctivitis. Since the inclusions

are morphologically identical, their relative distribution, and the cytology of epithelial scrapings and expressed follicular material must be studied before a diagnosis can be established. As mentioned above, the epithelial changes alone have been regarded as diagnostic by Taborisky,[84] whereas Thygeson,[89] and Kimura & Thygeson,[47] have found the most characteristic changes to be those in expressed follicular material in which the signs of necrosis differentiate the trachoma follicle from all non-trachomatous follicles. These necrotic changes include cytoplasmic debris, cells with bare nuclei, and numerous macrophages which act as scavengers to take up broken-down cellular material.

Thygeson [89] has considered the plasma cell diagnostically important when seen in exudate smears or epithelial scrapings. Plasma cells seem to be incapable of passing an intact epithelial barrier in any number and are therefore seen only rarely in epithelial scrapings or exudate smears from non-trachomatous follicular disease. In trachoma, on the other hand, they are seen more frequently, especially in epithelial scrapings, probably as a result of an impaired epithelial barrier, particularly over the degenerating follicles.

In a number of instances I have been able to make a diagnosis of trachoma solely on the basis of these various cytological changes, all of which can also be seen in punch biopsies of the trachomatous conjunctiva, as demonstrated by Badir.[5] It can thus be stated categorically that under certain circumstances a trained observer can make a reliable microscopic diagnosis of trachoma, even when inclusions cannot be found.

Diagnosis by the inoculation of monkeys or apes

Although experimental trachoma in monkeys and apes differs markedly from the human disease in that neither cicatrization nor pannus develops, the trachomatous nature of the experimental disease was established by Nicolle, Cuenod & Blaisot [63] and by Bland [17] on the basis of its successful transmission to the human conjunctiva. It has not been possible, however, to differentiate unequivocally between experimental trachoma and experimental inclusion conjunctivitis in the primates, although one important difference does seem to exist: experimental trachoma in the *Macacus rhesus* and in various species of baboons has been uniformly milder and longer-lasting than experimental inclusion conjunctivitis. The inclusions, moreover, have been demonstrated with relative ease in material from inclusion conjunctivitis in these animals, but have not been found at all in experimental trachoma in the same species. It should be pointed out here that the first trachoma inclusion body was identified by Halberstaedter & Prowazek [39] in experimental trachoma in an ape. There can be no doubt, therefore, that inclusions do appear in the experimental disease in this animal, but in the very mild experimental trachoma of monkeys and baboons, their number must be so small as to evade detection in routine scrapings.

Limiting factors in the use of monkeys in trachoma experimentation have been: (1) the lack of uniform susceptibility; (2) the occasional occurrence of a spontaneous folliculosis, which must be differentiated from experimental trachoma; (3) the failure of the experimental disease to produce pannus or major scarring, so important in the diagnosis of human trachoma; and (4) the lack of a satisfactory microscopic method of diagnosis.

In a study of experimental trachoma of the magot (*Macacus sylvanus*), Blanc, Pages & Martin [14] noted minor cicatrization but felt it had no diagnostic value. The importance of spontaneous folliculosis has been stressed particularly by Wilson, Stewart & Bland,[16] all working at the Giza Memorial Laboratory in Cairo, and in 1944 Bland [15] enumerated the precautions which must be taken in monkey experiments. It should be noted, however, that trachoma investigators in other parts of the world have only rarely encountered spontaneous folliculosis in monkey colonies. In a recent study of experimental trachoma and inclusion conjunctivitis, Thygeson & Crocker [90] reconsidered this problem and concluded that experimental trachoma and spontaneous folliculosis could be distinguished on the basis of clinical observation and the microscopic examination of epithelial scrapings and biopsy material. The spontaneous folliculosis they observed was unaccompanied by any leucocytic reaction, but the animals with experimental trachoma developed exudate and biopsy changes resembling those of early human trachoma. These authors concluded that monkeys have a limited use in trachoma work and that results must be controlled by human inoculation experiments. In no event can monkey inoculations be regarded as of value in the routine diagnosis of suspected trachoma. By way of contrast, the inoculation of baboons was found useful by Thygeson & Stone [93] in the detection of inclusion conjunctivitis virus in urethritis.

Present Status of Trachoma Virus Cultivation Studies

In spite of a number of claims concerning the cultivation of trachoma virus on the developing chick embryo or in tissue culture, no strain of the virus is available for study at the time of writing. In view of the many failures reported in the past by competent early investigators (Julianelle; [43] J. O. W. Bland (personal communication) and others) it is evident that trachoma virus is uncommonly fastidious in its *in vitro* growth requirements. There can be little doubt, however, in view of the recent advances in tissue-culture techniques that have permitted the cultivation of such resistant viruses as those of poliomyelitis, herpes zoster, and varicella, that a laboratory strain of trachoma virus will eventually become available.

Proof of cultivation will certainly require rigidly-documented human inoculation experiments. It would be helpful, indeed, if the WHO Expert

Committee on Trachoma would set up criteria for such proof. These might well include the following:

(1) Demonstration of Halberstaedter-Prowazek inclusion bodies in cultivated cells in series.

(2) Production with the cultivated virus of clinical experimental trachoma in monkeys or apes, with transmission of the experimental disease in series to monkeys or man.

(3) Production with the cultivated virus of typical trachoma with pannus in human volunteers, with demonstration of Halberstaedter-Prowazek inclusion bodies during the incubation period and initial phase of the clinical disease.

(4) Maintenance of a strain of the virus that can be tested in other laboratories.

Cultivation on the developing chick embryo

Numerous workers have attempted to cultivate trachoma virus on the developing chick embryo. Among them may be mentioned Pandit et al.,[68] John & Hamburger,[41] Vancea,[98] Babbar & Shukla,[3] Burnet, Cuenod & Nataf,[21] Gallardo, Ibáñez & Aldave,[35] Hagino & Hamada,[38] Poleff,[71] Macchiavello,[51] Stewart & Badir,[83] and Sezer.[80] Of special interest is Macchiavello's claim to have successfully inoculated a human volunteer with the filtrate of an emulsion of the yolk sac in which he had grown the virus. He found that after cultivation in the egg, the agent would pass Seitz EK and Berkefeld V filters. This was followed in 1950 by the report of Stewart & Badir,[83] from the Giza Memorial Ophthalmic Laboratory, that they had performed an experiment in which the disease was transferred first to monkeys and then to the yolk sac of an egg, and that from the infected yolk sac they had induced a chain of cultures alternating between monkeys and eggs through seven generations. The periods spent in the eggs by the virus during the three egg generations were 8, 8, and 6 days respectively. The last monkey reacted with follicles in 23 days.

Bietti[13] has recently reported extensive attempts at cultivation on the yolk sacs of a large number of eggs. For the inoculum he used scrapings from numerous active cases. He noted free elementary-body-like granules in the yolk sacs but no inclusions. Hirst's haemagglutination test showed a low titre. Since no human or monkey eyes were inoculated, however, no conclusions could be drawn.

Poleff[71] claimed to have effected cultivation in the yolk sac of the chick embryo, and Sezer[80] claimed to have produced typical virus-type focal lesions on the chorio-allantoic membrane; in a specimen he sent to our laboratory we were unable to find inclusion bodies in Giemsa-stained sections.

Over a number of years I have made numerous inoculations of the chorio-allantois and yolk sac with trachomatous materials without obtaining any evidence of proliferation, or even of preservation of the virus in the egg tissues. In view of the epitheliotropic nature of the virus, it would seem unlikely that the yolk sac would support growth. All claims of cultivation in the chick embryo must in any event be examined critically. Certainly the chorio-allantois tends to react non-specifically so that little significance can be attached to the development of a lesion *per se*. Even if temporary cultivation has been obtained—and this must be regarded as doubtful—it has certainly not been in quantity or in series.

Cultivation in tissue culture

All claims of the cultivation of trachoma virus in tissue culture must also be subjected to the most careful scrutiny. The claim of Poleff [70] can be cited as an example. This observer inoculated cultures of human corneal tissue with a suspension of conjunctival scrapings from a case of trachoma showing numerous inclusions. After ten days' incubation the culture was said to be pathogenic for a blind human eye and it was claimed that inclusions developed in the tissue-culture cells. In the absence of serial cultures, however, this experiment proved only that the virus survived for a period of ten days in the culture medium.

A number of Japanese workers have claimed success with tissue culture. Mitsui [53] demonstrated inclusions in a culture that was virulent for four human volunteers but was unable to maintain the virus in successive cultures. In a recent personal communication he stated that he had been unable to obtain growth on HeLa cell cultures. Sugiura & Tsutsui (cited by Poleff [73]) employed Maitland cultures with chick-embryo tissues. They claimed passage through 12 generations, but adequate controls were lacking.

The problem of cultivation is now under intensive study in a number of laboratories throughout the world. Among these may be mentioned the Institut Pasteur, Tunis (Nataf), the University of Rome (Bietti), the Lister Institute of Preventive Medicine, London (Collier), the Department of Ophthalmology, Kumamoto University, Japan (Mitsui), the Harvard School of Public Health, Boston, Mass. (Snyder, Murray & Chang), and the University of California School of Medicine, San Francisco, Calif. (Thygeson, Crocker & Jawetz). At the time of writing, successful cultivation has not been effected at any of these laboratories in spite of the use of a variety of techniques and a variety of cell strains.

Present Status of Serological Studies

All the accumulated evidence indicates that trachoma in man confers little or no immunity. The recent experiments of Tsutsui [96] suggest that the severity of induced human trachoma may be slightly modified by repeated

experimental inoculations. The resistance of monkeys to the disease does not seem to have been increased by recovery from experimental infection, and in my experience no modification of experimental trachoma in the baboon or rhesus monkey could be detected after repeated infections.

In 1939 Julianelle [42] reported that neither the serum from trachoma patients, nor sera from infected or recovered monkeys, exerted any neutralizing effect on the virus. He also noted that the sera of rabbits receiving intravenous injections of emulsions of conjunctival scrapings from active trachoma cases contained no antiviral substances. His conclusion was that trachoma virus was an impotent antigen.

In 1942 Rake, Shaffer & Thygeson [75] reported that sera from trachoma and inclusion conjunctivitis gave weakly positive results to complement-fixation tests with a group-specific antigen derived from lymphogranuloma-venereum virus cultivated in the yolk sac of the developing chick embryo. In the same year Macchiavello [51] reported that, of 14 sera from cases of trachoma, 3 contained neutralizing antibodies for lymphogranuloma-venereum virus. In 1951 Bietti and Sanna (cited by Bietti [11]) reported a series of complement-fixation tests on trachomatous patients. When they used psittacosis antigen, the tests were positive in 3 out of 18 cases; with lymphogranuloma antigen they were positive in only 2 out of 43 cases. In a second series they used a trachoma antigen derived from epithelial scrapings from active cases for testing against sera from trachoma, psittacosis, and lymphogranuloma venereum. With this material they obtained a significant percentage of positive results and concluded that in trachoma a moderate production of antibodies for the psittacosis-lymphogranuloma group occurs.

The same conclusion could be drawn from the report made by Kornblueth, Feigenbaum & Bernkopf [48] at the International Congress of Ophthalmology in September 1954. These workers found complement-fixing antibodies against lygranum antigen (Squibb) in the sera of 24% of 104 trachomatous patients. Of 41 with active trachoma in both eyes, 20 (50%) were serologically positive; of 56 with inactive trachoma, only 5 (9%) were positive. These results, too, would seem to indicate that trachoma virus stimulates weak complement-fixing antibodies common to the psittacosis-lymphogranuloma group.

In 1955 Babudieri, Bietti & Pannarale [4] presented a report entitled " Further contributions on the existence of antigenic affinities between the agent of trachoma and the agents of the psittacosis-lymphogranuloma venereum group ". In this paper they recorded the development of complement-fixing bodies in the sera of patients with trachoma, lymphogranuloma venereum, and ornithosis, when scrapings from trachoma patients were used as antigen. The sera of the trachoma patients gave titres up to 1 : 32, while the sera of the ornithosis and lymphogranuloma patients reached 1 : 16. With ornithosis antigen, reactions with ornithosis and lym-

phogranuloma sera reached titres of 1 : 128, whereas the maximum titres of trachoma sera in this instance were only 1 : 8.

In 1956 Giroud & Renoux [36] reported the finding of antibodies for the psittacosis group in the blood of patients with active trachoma. They also claimed that antibodies could be demonstrated in extracts of trachomatous conjunctiva.

If trachoma-specific antibodies are eventually to be demonstrated, it is clear that further exploration will be necessary. Bedson [8] found that absorption of the serum of a case of psittacosis by the group antigen leaves the specific antibody which can then be detected in a complement-fixation test made with fresh unheated virus as antigen. The application of this procedure to trachoma should be attempted, although the low titres obtained in trachoma would be a limiting factor.

In addition to the standard complement-fixation and neutralization tests, a number of new serological techniques have been introduced into virology. Their full use in connexion with trachoma, however, must await the development of a suitable trachoma antigen derived from tissue culture. Three of these techniques (the haemagglutination-inhibition test, the test for antitoxins, and the fluorescent antibody technique and its modifications) will be discussed briefly.

Haemagglutination-inhibition test

Bietti's [11] experiments with Hirst's haemagglutination test suggest that trachoma virus, like the viruses of influenza and Newcastle disease of fowl, may be capable of producing haemagglutination of chicken red blood cells. The agglutination was of low titre, but further studies would seem to be warranted in spite of the fact that haemagglutination has not been demonstrated for psittacosis, lymphogranuloma, or other members of the group. If it should prove to be reliable in trachoma, tests for antihaemagglutinins in trachomatous sera would then be in order.

Antitoxin test

A number of Chlamydozoaceae, including the viruses of psittacosis and lymphogranuloma venereum, produce soluble toxins that are capable of inducing antitoxins in experimental animals. From clinical observation there is every reason to suppose that trachoma virus, located in the conjunctival and corneal epithelium, produces a soluble toxin capable of inducing in the subepithelial tissues the follicular hypertrophy and degenerative changes so characteristic of the disease. The experiments of Mitsui et al.[57] would seem to confirm the existence of such a toxin in trachoma. When large quantities of virus from culture become available, tests for specific antitoxins can be performed. Such antitoxins have already been demon-

strated in human sera from cases of psittacosis and lymphogranuloma venereum.[74]

Fluorescent antibody technique

Coons and his co-workers [26, 27] have employed fluorescein-labelled immune serum as a specific stain to visualize virus antigen directly within infected cells. This method has been employed successfully for the observation of multiplication in tissues of viruses too small to be seen with the ordinary light microscope, such as mumps virus. Its application to trachoma must necessarily await cultivation of the virus in quantities necessary to produce immune serum in experimental animals; sera from trachoma patients, as judged by complement-fixation studies, has too little antibody to be of value. If suitable immune serum can be obtained in the future, this technique might have diagnostic value. It is conceivable that epithelial scrapings from trachoma, stained with fluorescein-labelled immune serum, might prove superior for inclusion-body detection to scrapings stained by present methods, and might even serve to differentiate trachoma from inclusion conjunctivitis.

More recently Weller & Coons [102] advanced an alternative technique for the demonstration of virus propagated *in vitro* which they consider useful for the detection of viruses that do not manifest overt cytopathogenicity in tissue culture. By means of this technique, focal lesions in tissue cultures of material from varicella and herpes zoster were stained by fluorescein-conjugated antiserum. The application of this method to trachoma research would seem to depend upon obtaining high-titre antiserum from severe cases of the disease.

Present Knowledge of the Properties of Trachoma Virus

Much has been written on the biological properties of trachoma virus but the claims in the literature are as conflicting as they are numerous and the published data must be interpreted with extreme caution. This is due in part to the fact that most of the laboratory work on trachoma has been done by ophthalmologists, the majority of whom have had little or no virological training. Unreliable data have stemmed also from the difficulties inherent in trachoma research owing to the lack of a suitable experimental animal and the failure of the virus to proliferate in tissue culture. In defence of the laboratory research carried out by ophthalmologists, may I say that major mistakes have also been made in trachoma research conducted by prominent microbiologists. Among these may be mentioned the incrimination of a bacterium (*B. granulosis*) as the cause of the disease by Noguchi,[64] and the interpretation of the inclusion bodies of trachoma as non-specific formations by Wolbach [103] and by Park & Williams.[69]

Particle size

The particle size of trachoma virus has been satisfactorily determined by light microscopy. The smallest form, the elementary body, has a uniform diameter of 0.25 μ when stained by Giemsa; the larger forms, the coccobacillary initial bodies, vary up to 1.0 μ or more in greatest diameter. It has not been possible to use the ultracentrifuge or graded collodion filters to obtain measurements because of the impossibility of procuring the virus in quantity, but electron microscope findings (Mitsui et al.[55]) have fully confirmed light microscope findings as far as the elementary bodies are concerned. The difficulties encountered in the electron microscopy of the less dense initial bodies have already been described. The problems involved in the electron microscopy of the similar bodies of another of the Chlamydozoaceae have been reported by Crocker & Bennett [28] in their paper on the virus of meningo-pneumonitis.

The existence of smaller forms of trachoma virus has been postulated (Bietti [13]) because of failure to demonstrate inclusion bodies in experimental trachoma of monkeys, in mild forms of the human disease, and in inadequately treated cases in which the inclusions temporarily disappear, only to reappear with relapse of the clinical disease. It is my opinion that if smaller forms of the virus existed, positive filtrates would not have been so difficult to obtain; and, more significantly, that a variation in the size of the elementary bodies (which are in fact remarkably uniform in size and shape) would be expected. The only support for the " smaller form " hypothesis has come from the electron microscope study of Tsutsui & Takeda,[97] who reported finding particles as small as 50 mμ. Other investigators have failed to confirm this claim.

General bacteriological experience with many cultivable bacteria has shown that micro-organisms in small numbers may be missed in smears but uncovered by means of sensitive culture methods. The necessity for obtaining repeated negative smears in gonorrhoeal urethritis and pulmonary tuberculosis is a case in point. Until sensitive culture methods for detecting trachoma virus are devised, the best working hypothesis to explain negative findings in low-grade trachoma is that the elementary bodies are too few in number to be detected in a single microscopic examination.

Filterability

Trachoma filtration studies up to 1935 were well summarized by Van Rooyen & Rhodes,[99] and up to 1950 by Bedson et al.[9] As would be expected in view of the large size of the virus and its low titre in chronic trachoma, the results of filtration experiments have in general been negative. As shown originally by Nicolle, Cuenod & Blaisot,[63] and subsequently by Thygeson, Proctor & Richards,[91] however, positive filtrations are possible if the two factors of virus concentration and adsorption losses in the filter are ade-

quately controlled. The reports of the filtration experiments of Julianelle, Morris & Harrison,[45] of Stewart,[82] and more recently of Poleff (cited by Bietti [13]) and Macchiavello,[51] illuminate the difficulties which have to be overcome in order to obtain positive filtrations.

Ability to survive

There is a paucity of data on the period of survival of the virus outside the body. There is agreement, however, that it is destroyed rapidly at room temperature and by desiccation, and that it will survive freezing for at least several weeks. Nicolle's original studies [63] suggesting that glycerol had a preservative effect might be further explored; the results could have an important bearing on the air-mail transmission of trachomatous materials to distant laboratories.

Staining properties

Trachoma virus has the staining properties of the other members of the Chlamydozoaceae, which, unlike the typical large viruses such as vaccinia virus, exhibit an intracellular cycle of morphological variation from the relatively large, bipolar-staining bodies (the initial bodies of Lindner[49]) to the smaller, more numerous elementary bodies. With ordinary blood stains like Giemsa, the elementary bodies of all the Chlamydozoaceae stain a reddish blue, and the initial bodies a pure blue.

The elementary bodies of trachoma and inclusion conjunctivitis, as well as of the other Chlamydozoaceae, differ from all other virus elementary bodies in staining with the Castañeda and Macchiavello stains that are used to stain rickettsiae. With the Castañeda stain, the elementary bodies stain blue against a red background, and with the Macchiavello stain, they stain red against a blue background. With the Lindner contrast stain, the basophilic initial bodies stain blue against a red background, and the more acidophilic elementary bodies stain red.

It is of interest that the initial bodies are Gram-negative and can be recognized occasionally in Gram-stained preparations from acute cases. Because of their bipolar staining they have often been mistaken for diplococci.

Other biochemical properties

Although our knowledge of the biochemistry of certain viruses, e.g., the bacteriophages, is now considerable, very little is known as yet of the biochemistry of trachoma virus. Full biochemical analysis of this virus must of necessity await the preparation of purified elementary-body suspensions from tissue culture. Nevertheless, some information on the chemical nature of the virus and its inclusion body has been gained from their reactions to biological stains and from electron microscope studies.

The work of Rice,[77] confirmed by Thygeson,[86] indicated that the essential component of the sticky matrix of the trachoma inclusion was a carbohydrate with the properties of glycogen. The absence of a protein component in the matrix seemed to be indicated by its failure to stain with ordinary biological dyes. The inclusion itself is Feulgen-positive,[37] indicating the presence of desoxyribonucleic acid (DNA). A chemical difference between the denser elementary body and the less dense initial body is clearly indicated by the marked tinctorial difference between the two forms in the virus multiplication cycle. The dissolution of the virus by bile suggests a possible chemical similarity to the pneumococcus.

The fact that the staining properties of the elementary and initial bodies of trachoma, like the staining properties of the other Chlamydozoaceae, are the same as those of the rickettsiae suggests that the trachoma bodies may have the same complex biochemical nature as these other forms.

Reactions to chemical and physical agents and to temperature

Trachoma virus is inactivated by the commonly used disinfectants and is destroyed by bile. The sulfonamides do not seem to exert any direct virucidal effect on it (Julianelle & Smith [46]). A 4% solution of cocaine seems to have a deleterious effect, but nothing is known of the action of other anaesthetics.

Data on the effect of temperature on the virus have been summarized by Van Rooyen & Rhodes.[99] The virus would appear to be heat-sensitive, being inactivated by temperatures as low as 45°-50°C for 15 minutes.

Response to centrifugation

According to Julianelle & Harrison,[44] centrifugation for 30 minutes at 5000 revolutions per minute sediments the virus. Thygeson [85] was able to obtain elementary-body suspensions of reasonable purity by differential centrifugation of material from cases with abundant inclusions. Thygeson, Proctor & Richards [91] demonstrated elementary bodies in the bacteria-free filtrate used for a successful human inoculation.

Transmissibility to apes, monkeys, and other animals

There is general agreement that experimental trachoma can be induced in monkeys and apes but that the induced disease resembles human follicular conjunctivitis in being self-limited and uncomplicated by pannus or cicatrization. There is agreement also that apes are more susceptible to trachoma virus than monkeys.

There is unfortunately no satisfactory criterion for the diagnosis of experimental trachoma. A presumptive diagnosis can be made, however, if a follicular conjunctivis of insidious onset develops after an incubation

period of 7-14 days in animals that were known to be free from " spontaneous folliculosis " prior to inoculation. Except in the apes, inclusion bodies have not been demonstrated in the experimental disease.

The consensus of opinion is that none of the ordinary laboratory animals is susceptible to trachoma virus. There has been no confirmation of Arakawa's [2] claim that it can be established in the mouse, or of the claim of Cuenod & Nataf (cited by Nataf [61]) that it multiplies in the louse.

Status of Various Special Studies on Trachoma

Experimental chemotherapy

Experimental trachoma in monkeys and apes is unsatisfactory for controlled chemotherapeutic research. At the moment little is known of the mode of action of the sulfonamides and antibiotics against trachoma, but available evidence suggests that it is the intracellular multiplication of the virus that is prevented. It can be supposed that enzyme systems necessary for reproduction are interrupted, and that in the normal process of desquamation the superficial, virus-containing cells are cast off. Evidence of degenerative changes in the component virus particles of the trachoma inclusion have been noted by Bietti [12] and others. In my experience inclusions have rarely if ever been demonstrated beyond the third day of treatment in properly treated cases, and I have a number of examples of cases in which apparent " arrests " were achieved in treatment times of from five to seven days. Such " cures " are of course exceptional.

Healing can also be obtained in trachoma by measures other than the use of the sulfonamides and antibiotics. These include repeated and regular applications of such caustics as copper sulfate and silver nitrate, repeated massage, and conjunctival scrapings. The effect of all these measures is to desquamate the superficial epithelial cells and replace them with young, vigorous cells. It is well established that trachoma inclusions are not to be found in basal cells, and rarely even in the deeper cells of the conjunctiva and cornea. They occur predominantly in the superficial cells and it can be theorized that the curative effect of these procedures lies principally in the replacement of susceptible cells by insusceptible cells.

Laboratory findings bearing on epidemiology

In countries where trachoma is endemic, the common mode of spread of the disease, i.e., from mother to child, can usually be determined without difficulty. In countries where trachoma is rare, however, as in the USA, patients with early trachoma are occasionally encountered whose contact with known cases of the disease cannot be traced. I have seen a number of cases with acute onset in which incubation periods of from five to seven

days could reasonably be postulated, but in which careful epidemiological studies have failed to uncover contacts. Such failure would seem to support the theory of the existence of trachoma carriers, or the theory of the existence of a genito-urinary trachoma comparable to inclusion cervicitis and urethritis. No evidence of either of these possibilities has come to light, however. Bodian's [18] claim that he found healthy carriers of trachoma inclusions in the Fiji Islands is, I feel sure, erroneous. In my opinion Bodian confused melanin granules in epithelial cells with inclusion bodies.

It is well known that apparently healed cases of trachoma may relapse. The possibility of reinfection must be considered in such cases, but there is also good reason to believe that the disease may smoulder in a state of low activity for many months, or even years, before healing spontaneously. If follicles are present in such cases, cytological study of their contents may be useful in determining activity. The cortisone provocative test (Nataf, Maurin & Dupland [62]) may also be useful in differentiating low degrees of activity from complete healing.

Secondary bacterial infection

Secondary bacterial infection in trachoma has been the subject of many reports [67] and it is agreed that secondary invaders may profoundly modify an underlying trachoma. The detection and identification of such invaders is therefore of great importance. Adequate microscopic and culture methods are available for the common secondary contaminants, including the pyogenic cocci and bacilli of the genera *Haemophilus*, *Neisseria*, and *Moraxella*. An effort should be made, however, to develop simple media for use in the field to detect such bacteria as *Neisseria* and *Moraxella*, which are fastidious in their growth requirements. Field studies could be designed to compare the reliability of diagnosis by exudate smear and epithelial scraping with the reliability of diagnosis by culture methods.

Secondary viral infection

Recent studies on poliomyelitis and the respiratory viral diseases indicate that multiple viral infections are not uncommon. How often trachoma may be involved in such mixed infections is not known, but it is known that herpes simplex virus and the virus of epidemic keratoconjunctivitis can infect a trachomatous patient secondarily. Many years ago Lindner [50] found that simultaneous infection with trachoma and inclusion conjunctivitis was possible experimentally. This should be further explored in animals to see if experimental trachoma can be modified significantly by superadded inclusion-conjunctivitis virus infection.

In a recent study of trachoma material from Saudi Arabia, Murray et al.[60] isolated 13 viruses from 200 cases of conjunctivitis presumed to be trachoma. One of the 13 was a Coxsackie virus, group B-1, and the remain-

ing 12 were adenoviruses of various types. In the University of California studies on trachoma among the American Indians, no viruses have as yet been recovered from trachoma material. It seems clear that the conjunctiva, unlike the pharynx and intestinal tract, has little tendency to support latent virus infection, but further tissue-culture experiments, paralleled by serological studies, are very much indicated.

Associated allergy

The association of vernal catarrh with trachoma is common in the Middle East and in tropical countries. A conjunctival eosinophilia is regularly associated with such an allergy, and since eosinophilia does not occur normally in smears from trachoma, it would seem that this finding would be *prima facie* evidence of an associated allergy. Further work is needed, however, to establish the minimum number of eosinophils that are to be considered diagnostically significant.

Among the American Indians, bacterial allergy in the form of phlyctenular conjunctivitis is often associated with trachoma, and on rare occasions phlyctenular pannus has complicated the differential diagnosis. There is a great need for the development of a diagnostic test for phlyctenulosis for use in cases without active limbal phlyctenules.

Blood studies

The literature contains a number of reports of studies on blood changes in trachoma. They were summarized by Nataf [61] in 1952 and by Bietti [11] in 1953. It is of interest that Licheri (cited by Bietti [11]) determined the vitamin-A level of the blood in a series of trachomatous patients and found it within normal limits, and that Moncino [58] found the sedimentation rate of trachomatous individuals normal except in cases complicated by corneal ulcers and acute dacryocystitis. In view of the localized character of the disease, it would seem logical to assume that blood changes specifically related to trachoma would be rare.

Possibility of developing a vaccine

The studies of Julianelle [42] dramatized the fact that trachoma virus is antigenically ineffectual. The localized nature of the infection, the limitation of the virus to the conjunctival and corneal epithelium, and the mildness of the disease in its chronic stage, all militate against the production of antibodies in the natural disease. The literature contains numerous reports of experiments in which conjunctival scrapings or follicular material have been introduced parenterally with questionable modification of the disease. These negative results should not, however, be regarded as an indication that a vaccine from tissue-culture virus would be ineffective. That the virus might have important immunizing properties when introduced parenterally

in large quantities from tissue culture is a distinct possibility. Unfortunately its investigation must await the growth of trachoma virus in tissue culture in quantity.

Skin tests

Although there is little evidence to indicate that skin sensitization occurs in trachoma, the possibility should be further explored when suitable antigens from tissue culture become available. It has been established that trachoma cases are Frei-negative. The results of such testing as has been done with trachoma antigen derived from conjunctival scrapings or follicular expressions from active cases (Tricoire [94]) must be considered equivocal, although it should be mentioned that Sedan [78] reported positive reactions in 61% of 150 persons with florid trachoma. Belot,[10] however, found that 43% of normal subjects reacted positively.

Provocative tests

Mechanical abrasion of the conjunctiva and a number of provocative agents, including steroid hormones, jequirity, copper sulfate and other caustics, have occasionally produced reactivation of trachoma with reappearance of inclusion bodies. In a few trials staphylococcus toxin has also effected reactivation. This subject deserves further exploration, not only by reason of its importance in diagnosis but as a means of determining cure. Reactivation of trachoma can be recognized earliest by microscopic examination of epithelial scrapings; the clinical signs develop later.

The cortisone effect, first noted by Ormsby and associates [66] in subsiding inclusion conjunctivitis, has been explored particularly by Nataf and Freyche[a, 33] and by Thygeson,[88] and may prove to have practical value. Thygeson found that activated cases were more sensitive to chemotherapy than cases of low activity, and thus required shorter treatment times. Further exploration of this effect, particularly with the more active steroids, seems definitely indicated.

Relation of trachoma virus (Chlamydozoon trachomatis) *to inclusion conjunctivitis virus* (Chlamydozoon oculogenitale)

The clinical studies of many observers have shown clearly that trachoma and inclusion conjunctivitis are distinct diseases. Repeated observations have established inclusion conjunctivitis as a benign, self-limited disease, never complicated by scarring or pannus, and related epidemiologically to a benign genito-urinary disease. Trachoma, on the other hand, is (1) a cicatrizing disease that produces scars even in the rare mild cases which heal spontaneously, and (2) invariably a keratoconjunctivitis with corneal

[a] Unpublished working document WHO/Trachoma/18

involvement that can always be demonstrated by biomicroscopic examination, even in the initial phase. The similarity of the inclusion bodies, however, has led to recurrent claims, particularly by Japanese workers (Y. Mitsui, personal communication), that the two diseases are in fact one and the same. Such confusion could occur only in countries where trachoma is common in infancy. In the USA, where trachoma in the white population has become almost extinct, inclusion conjunctivitis of the newborn is common and there is no case on record of trachoma developing from it. Moreover, in spite of the morphological identity of the inclusions of the two viruses, and of their component elementary and initial bodies, the following differences obtain:

(1) As reported by Braley,[19] and fully confirmed by the present writer, the inclusions of inclusion conjunctivitis are invariably more numerous in scrapings from the conjunctiva of the lower lid than from the conjunctiva of the upper lid, while in trachoma the reverse is true. This corresponds exactly to the clinical finding that trachoma involves the upper half of the conjunctival sac more than the lower half, and that inclusion conjunctivitis involves the lower half more than the upper.

(2) In trachoma I have been able to demonstrate inclusions consistently in scrapings from the upper limbus but have never been able to demonstrate them in scrapings from this area in inclusion conjunctivitis.

(3) Inclusions have been found consistently in experimental inclusion conjunctivitis in monkeys,[90] even in the relatively insusceptible *M. rhesus*, but have never been found in experimental monkey trachoma, except in the severe disease produced in apes.

It is my considered opinion that these laboratory findings fully confirm the clinical findings and serve thus to establish the two diseases as separate entities.

On the other hand, the similarities between them are such that information gained from one can often be applied to the other. The benign nature of inclusion conjunctivitis, and the short treatment time required for its cure permit human inoculation experiments which cannot be performed with trachoma.

RÉSUMÉ

L'état actuel des recherches de laboratoire sur le trachome est décrit dans cet article. L'auteur y rappelle d'abord la nature du virus, sa classification provisoire dans le groupe des virus de la psittacose-lymphogranulomatose vénérienne, récemment désignés sous le nom de Chlamydozoaccae *(Chlamydozoon trachomatis)*. Quant à la pathologie, on s'accorde à considérer le trachome, au moins dans sa phase initiale, comme une épithéliose, le virus épithéliotrope étant localisé dans l'épithélium cornéen et conjonctival. Certains auteurs estiment pourtant qu'il existe un stade sous-épithélial. La présence et la valeur diagnostique des divers caractères pathologiques est décrite et discutée.

Le diagnostic de laboratoire peut se faire par démonstration des inclusions épithéliales, avec lesquelles des inclusions non spécifiques ont été souvent confondues; par l'étude au microscope électronique; par l'examen des diverses modifications cytologiques (dans certaines circonstances un chercheur entraîné peut poser un diagnostic microscopique du trachome fondé sur l'examen cytologique, même en l'absence d'inclusions); par inoculation aux singes (l'emploi de cette méthode est limité par les différences de sensibilité individuelle de ces animaux, la présence d'une folliculose spontanée, l'absence de pannus cornéen et de cicatrices caractéristiques, le manque d'une méthode de diagnostic microscopique satisfaisante).

De nombreux laboratoires dans le monde sont à la recherche d'une méthode de culture du virus du trachome sur embryon de poulet ou sur tissu. A l'heure présente, aucun essai dans ce sens n'a donné de résultat positif, malgré l'emploi de techniques diverses et de lignées cellulaires variées. Les diverses méthodes mises à l'étude sont décrites.

Le trachome ne paraît conférer à l'homme aucune immunité. Il en est de même chez le singe, qui, guéri d'une infection expérimentale, n'est pas pour autant résistant à une nouvelle infection par le virus du trachome. D'après certains résultats, il semble que ce dernier stimule faiblement la production d'anticorps fixateurs du complément, communs au groupe psittacose-lymphogranulomatose vénérienne. Divers autres tests sérologiques ont été mis à l'étude (inhibition de l'hémagglutination, antitoxines, techniques de détection des anticorps par la fluorescéine). On ne pourra appliquer ces techniques avec fruit tant que l'on ne disposera pas de cultures de virus du trachome. Les corps élémentaires colorés au Giemsa ont un diamètre de 0,25μ, les corps initiaux de 1,0μ. On a peu de données au sujet de la survie du virus hors de l'organisme. Il est rapidement détruit par dessiccation et exposition à la température ambiante; il semble résister à la congélation pendant plusieurs semaines.

Les propriétés tinctoriales et le microscope électronique donnent quelques indications sur la composition biochimique des corps élémentaires et initiaux et suggèrent une parenté avec les rickettsies.

L'auteur passe en revue ensuite diverses questions en relation avec le trachome: la chimiothérapie expérimentale, l'influence des infections bactériennes secondaires, les allergies associées, les chances éventuelles de la vaccination (qui ne peut être envisagée tant que l'on ne peut pas cultiver le virus), les relations et les différences entre le virus du trachome et la conjonctivite à inclusions.

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