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ORIGINAL ARTICLES

ISOLATION OF A LOCAL STRAIN OF THE VIRUS OF LYMPHOGRANULOMA VENEREUM IN THE YOLK SAC OF GROWING CHICK EMBRYO.

By

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Man is the natural host of the virus, M. lymphogranulomatis, causing the venereally transmitted disease "lymphogranuloma Venereum" (L. G. V.). Two types of artificial cultural techniques have been generally used in isolating this virus from human lesions for diagnostic and research purposes. One is inoculation in the yolk sac of embryonated chick eggs, and the other is intracerebrally in white mice. In this report is described, the materials and methods by which a local strain of the virus of L. G. V. has been primarily isolated and established successfully in artificial culture in embryonated chick yolk sac, in the laboratory of the Madras Institute of Venereology.

SOURCE OF ISOLATION OF THE VIRUS

The virus was isolated from a sample of pus aspirated from an inguinal "bubo" of a patient with clinically established lymphogranuloma venereum infection.

The patient was a man aged 22 years who attended the V. D. Clinic of the Madras Institute of Venereology, with a painful swelling in his right groin of 12 days duration. He gave a history of, sexual exposure and 18 days later of developing a painful lump in his right inguinal region, without his ever noticing any primary genital sore.

On examination, the swelling was unilateral in the right inguinal fold, adherent to and involving the right inguinal lymphnode, tender and fluctuant. There were no signs and symptoms of any other venereal or other infections. The V. D. R. L. test for syphilis was repeatedly non-reactive. The FREI skin test performed on the patient with an antigen prepared from pus from bubo of a case of human L. G. V. infection

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was reactive producing a indurated papule in the skin of the forearm 8 mm. & 8 mm. appearing and lasting beyond 48 hours.

The intact swelling in the groin was aspirated with a large bore needle and syringe, under strict asceptic precautions, providing about 5 ml. of thick frank pus. Direct smears of the pus on slides stained with the Gram and Casteneida stains, did not reveal any micro-organism resembling bacteria or virus, among or inside the several mononuclear and polymorpho-nuclear cells seen in the smears. The culture of the pus in nutrient agar and broth did not support the growth of any micro-organism.

The patient was treated with sulphonomide tablets, 2, three times a day, for 10 days, with subsidence of the bubo and relief of his symptoms.

THE TECHNIQUE OF ISOLATION OF THE VIRUS IN THE YOLK SAC OF GROWING CHICK EMBRYO

The specimen of pus received in a test tube, was immediately deeply frozen at minus 20°C, and kept over-night in deep freezing cabinet. Next day, the frozen pus was rapidly "thawed" by rolling the the test-tube between the warm surfaces of the palm of the hands. It was immediately frozen again at minus 20°C keeping at it for 2 hrs. and again thawed as before. This was carried out 3 times altogether in order to break up the cells in the pus containing possibly the "inclusion bodies of the virus of the L. G. V. Then the material was put in a cold centrifuge and spun lightly at about 2500 R. P. M. for 1 hour and about 1 ml. of clear supernatent was obtained. This was used as the source for the virus to inoculate in 0.3 ml. quantities into each of three, 6 day's old, Leghorn Chick eggs into the yolk sacs of its embryos. Three other eggs with growing Chick-embryos of the same number of days old, were kept as "controls" and all of them were incubated at 37°C. They were examined by "Candling" twice a day and all the 3 eggs inoculated with pus, were found moribund between the fourth and fifth day.

The yolk sac membranes of the dead and live embryos were harvested, under aseptic conditions. Small pieces of tissue of the size of 'pea', taken with a pair of small forceps from the most vascularised site of the yolk sac of dead embryos and freed of fat and moisture by tubbing against filter paper, was used to make impression smears of them on clean slides.

The Gram staining did not reveal any bacterial micro-organism microscopically under oil immersion lens but Casteneida stain showed "bodies" characteristic of the virus of L. G. V. blue stained and granular, in dense clusters, mostly free, but a few as "inclusions" inside yolk sac cells. The comparatively smaller bodies varying in size reaching up to half the diameter of a Staphylococcus, looked like the so-called "elementary bodies" and the larger bodies, "plaque-like" looked like the "initial bodies" of the virus of L. G. V. described in stained smears by earlier workers. They were all very few in numbers, at the primary isolation. The yolk sacs of the live-embryos similarly examined were free of these "bodies" in the stained smears. The culture of the yolk sac material in nutrient agar and broth showed them to be sterile, bacteriologically.

The primarily infected yolk sac membrane from each egg was ground separately under sterile conditions in mortor with pestle, in about 1.5 ml. of broth and to it 25 micrograms per ml. of streptomycin was added, as a further precaution against bacterial contamination. This was used in 0.3 ml. quantities to inoculate subsequate batches of embryonated eggs, in further transfers. The infected embryos died regularly between the third and fifth day and yolk sac membranes were more heavily infected at each subsequent passage. To date after several sub-cultures, the virus has been well adapted and established in the yolk sac of developing Chick embryo. In between transfers and growth in eggs, a batch of 6 white mice was inoculated intracerebrally with 0.1 ml. quantities of infected yolk sac material suspended in broth. 3 of 6 of the mice died obviously, of signs and symptoms of "meningo-encephalitis" by the 4th day of infection. Impression smears of their asceptically harvested brains revealed the characteristic intra-cellular and extra-cellular elementary and initial bodies of the virus of L. G. V. in smears stained with Casteneida's stain, indicating specific infection in mice. However mice inoculated intraperitoneally with 0.5 ml. of the infected yolk sac suspension did not produce any signs of infection or death of the mice. From the infected mouse brains the virus has been transferred back and established easily in the yolk sacs of Chick embryo.

"Lygranum" type of "Frie antigen" for skin test for L. G. V. was prepared from this local strain of the virus as follows:

The Yolk sac membranes heavily infected with the virus were washed free of yolk, in sterile normal saline, and pooled and under asceptic conditions, ground in a porcelain mortar, with sterile glass powder. A 10 percent suspension of it, by weight was made in 0.85 percent sodium chloride solution and was spun at 2500 R. P. M. for one hour in a cold centrifuge. The supernatent free from tissues was collected into special sterile centrifuge-tubes, and recentrifuged at 12000 R. P. M. for 2 hours in the cold centrifuge. The supernatent was pipetted off and the deposit was resuspended in half the original volume of sterile 0.85 percent sodium chloride containing 0.1 percent formalin and 0.3% phenol. This formed the skin test antigen. The same process was carried out simultaneously on normal yolk sac membranes harvested from the same days old uninfected eggs from the same batch as the infected, eggs, and used as "control antigen" for checking the possible "skin hypersensitivity" of some persons to egg contents. Sterility tests were carried on the test and control antigens and skin tests were conducted regarding the "sensitive" and "specific" potency, on cases known to be infected with L. G. V. virus and apparently normal persons respectively.

The "test antigen" was inoculated intradermally on one forearm and the "normal yolk sac antigen" on the other forearm, in 0.1 ml. quantities using tuberculin syringe and needle and the cases under study were examined after 48 hours. The normal yolk sac antigen did not produce any skin reaction in 6 normals and in any of the 6 known cases of L. G. V. The test antigen in contrast produced reaction in the skin forming raised papules, with and without erythema, of not less than 6 mm. in diameter in all

known L. G. V. cases investigated and produced negative reactions, in apparently normal persons.

"Complement fixing" type of phenolysed and boiled suspension of the antigen in saline was also prepared from the yolk sac infected with the strain of the virus and in the trials it was found to fix complement specifically to a significantly high titre, in the sera of patients known to have L. G. V. infection and negative reaction in a few apparently normal sera.

Thus the identity of the strain of virus isolated primarily in the yolk sac of the developing chick embryo in this study locally, has been considered to be M. lymphogranulomatis the virus of Lymphogranuloma venereum. This local strain of the virus has by now been passed in chick embryo in about 26 sub-cultures and has been well adapted and established in this laboratory just as the "foreign J. H. strain of virus", imported from abroad.

COMMENTS

The etiologic agent in lymphogranuloma venereum namely MIYAGAWANELLA LYMPHOGRANULOMATIS belongs to the "basophilic group" of viruses. This group has biologic properties of a distinctive nature, and resembles in several respects, the "Rickettsiae", and may be regarded as being intermediate in position between the "Rickettsiae" and true "animal viruses".

All the members of this group of viruses have affinity for the "basophilic stain", large size of 250 to 400 millimicrons, life cycle of development with formation of large basophilic, plaque–like inclusions. They have, endotoxins, virulence for man, mice, rodents or birds, capability of growth artificially in the tissues of developing chick embryo, have heatstable, group specific, complement fixing antigen shared by several of its species, and also heat– labile, species specific antigens, and finally susceptibility to sulphonamides and antibiotics.

The other members of this basophilic group are, the virus of "Psittacosis", "Ornithosis" and the numerous "Pneumonitis" viruses referred to as "Psittacosis-lymphogranuloma" sub-group or the genus "Miyagawanella". The "Trachoma-Inclusion conjunctivitis" (TRIC) sub-group of viruses is closely related and morphologically resembles the above, and are included under the genus "Chlamydia".

In 1930 HELLERSTROM showed that the L. G. V. was a virus infection, by intracerebral transmission of the agent concerned in the monkey. In 1932 LEVADITI first infected the white mouse which has been since then the animal most frequently used for isolating the virus. The white mouse may sometimes be spontaneously infected with the pneumonitis virus which is a member of the L. G. V. Psittacosis group of viruses and may carry them in a latent state. Therefore, the etiological role of the virus isolated in the white mouse, from a clinically suspicious case of L. G. V. may not be considered as conclusively proved. This possibility does not seem to arise in this study of isolation of the virus in the chick embryo since spontaneous infection in the chick embryo with this group of viruses has not been described.

In 1944, SHAFFER and associates reported that the Chick-embryo is comparatively a more reliable host for adapting the virus of L. G. V. outside artificially. But the artificial infection in the mouse with the L. G. V. has been noted to present itself earlier than in the egg and may be an advantage in quicker diagnosis of the infection. Further, easier suscebtibility of the growing chick embryo to bacterial infection may be a defect in that method. However this may be eliminated by pre-treatment of the infective inoculum with streptomycin which is not viricidal to the L. G. V. virus.

It has been considered by some workrs in this field that it is difficult to recognize this virus with certainty by the microscope, and attempts at culture and transmission of it have failed often (BEDSON 1950 A). It has been our experience too in our past several attempts. But by the special technique developed and applied in this study it has been found that the isolation and maintenance of this virus in Chick-embryo yolk sac may not be so difficult after all. Rapid, repeated freezing and thawing apparently cause "cryolyis" of the infected cells and the "inclusion bodies" of the virus are released and made available in a more concentrated condition to infect primarily the yolk sac of the chick comparatively more easily than otherwise.

In fact, more recently, it has been possible to isolate yet another local strain of the virus, from another clinical case of L. G. V. more easily in the chick-embryo. It also is being adapted to the chick-embryo, by repeated sub-passages.

Since the M. lymphogranulomatis is antigenically related to the other members of Miyagawanella, and since all member species resemble each other morphologically and are stained similarly, the immunologic and serologic methods used may be considered too indirect to conclude definitely that this particular strain of the virus isolated in this study is M. lymphogranulomatis, the etiological agent of the infection in this specific case. However, the circumstantial evidence obtained in this study, of relevent clinical history of venereal source of the infection, characteristic signs and symptoms of L. G. V., the susceptibility clinically to sulphonamide therapy, and the fact that the virus did not infect the mouse intraperitoneally, in contrast to M. Psittacosis, points more or less to its correct identity as M. lymphogranulomatis and as the causative agent in that particular case. It has been noted that the endotoxins produced by members of this group are species specific since only homologous antisera are capable of neutralising. Neutralisation experiments using the specific endotoxins have not been carried out in this case.

In the confirmation of the clinical suspicion of L. G. V. infection, Frie Skin test is resorted to as a routine. In the Madras Institute of Venereology, Frie antigen is usually prepared locally in the laboratory from the L. G. V. virus of human bubo pus origin. Since the 'bubo pus' in specific quality and adequate quantity are not available easily any more, 'Lygranum' type of Frie antigen is being prepared from Chick-embryo yolk sac infected with J. H. strain of the virus imported from abroad. It may be logical to think that "lygranum type antigen" prepared from a local strain of the virus would be more specific and sensitive in the diagnosis of a local infection with it

in case possibly there are strains and strains of the virus of L. G. V. If so, the isolation and establisment of this local strain of the virus may be considered significant and worth recording. This strain of this virus is now available to all interested in this field.

SUMMARY

The isolation and adaption of a local strain of the virus of lymphogranuloma venereum, in the yolk sac of developing Chick-embryo is reported. The materials and methods developed in this study which seemed to have made primary isolation of virus in the Chick-embryo comparatively easy, and methods used to identify the virus have been described. The use of the skin and complement fixing test antigens prepared from local strain of the virus, possibly giving more specific and sensitive results in diagnosis of L. G. V. infection is stressed.

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REFERENCES

1. Bedson, S. P. (1950A) Brit. Jour. Ven. D. 26–177.
2. Hellestrom, S. and Wassen, E. (1930) Cr. Ville. Cong. Int. Dermat. Syph. Copenhagen. page 11–47.
3. Levaditi et al (1932) Ann. Inst. Pasteor. 48.27.
4. Shaffer et al (1944) J. Infect. Dis. 75. 109.