AN ETIOLOGIC CONSIDERATION OF DONOVANIA GRANULOMATIS CULTIVATED FROM GRANULOMA INGUINALE (THREE CASES) IN EMBRYONIC YOLK*

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Plates 1 and 2

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The identification of the etiologic agent of granuloma inguinale has remained an unsolved problem since Donovan in 1905 described peculiar intracellular parasites as being consistently present in smears from the ulcerated lesions of this disease (1). Donovan himself was puzzled as to the exact nature of these intracellular bodies that bear his name, but considered them to be protozoa. Donovan bodies are now generally accepted as being pathognomonic for granuloma inguinale and evidence has accumulated to support the hypothesis of their etiologic function (Figs. 1, 2). However, in spite of the efforts of many investigators, no microorganism acceptable as the etiologic agent of granuloma inguinale has been isolated and cultivated outside the human host.

Numerous investigators have cultivated various bacteria to which they attached etiologic significance (2–7). There has been no convincing consistency in the types of bacteria isolated by various workers beyond the fact that a majority of them seem to have been related to the Friedländer group. All the bacteria appear to have grown well on ordinary culture media; differential identification was based on variable cultural characteristics. A few attempts have been made to attach an etiologic significance to these bacteria by serological means (8, 9), but no experimental lesion comparable to that of granuloma inguinale has been induced by any of them.

The best experimental investigations directed toward a solution of the problem of etiology of granuloma inguinale led to conclusions that the Donovan body is the etiologic agent and that it has not as yet been cultivated outside the body of the human host (10). Recent experiments supporting these conclusions deserve some detailed consideration.

In 1931 DeMonbreun and Goodpasture aspirated material from two unruptured lesions in a negro man with other ulcerated lesions of granuloma inguinale. This material was especially rich in Donovan bodies and entirely free from contaminating microorganisms. From this inoculum the authors failed to cultivate the Donovan organism on any of a wide variety of media. Scrapings from ulcerated lesions, rich in Donovan bodies and with only slight contamination, failed to induce infection in guinea pigs, rabbits, kittens, dogs, and rats. An infection was established in monkeys by

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repeated injections of this especially good inoculum but the lesions healed without ulceration. These authors observed that the Donovan microorganism appeared to remain viable in uncontaminated human tissue for a considerable length of time. They concluded that the Donovan body is the etiologic agent of granuloma inguinale and judged that none of the microorganisms which had been reported as cultivated from human lesions was responsible for the disease (11).

More recently Dienst, Greenblatt, and Sanderson (12), and Greenblatt, Dienst, Pund, and Torpin (13) have recognized unulcerated lesions in human patients which they called pseudobubos. From such lesions they, like DeMonbreun and Goodpasture, were able to aspirate material rich in Donovan bodies and entirely free from recognizable or cultivable contaminants. This material appeared to contain a pure culture of Donovan bodies. Neither contaminants nor Donovan microorganisms were cultivable on various artificial media; nor was this inoculum infectious for ordinary experimental animals, including the chorioallantois of chick embryos. They were able, however, to reproduce in four human volunteers experimental lesions acceptable both clinically and microscopically as granuloma inguinale. This series of experiments justifies the authors' conclusions that the Donovan body is the etiologic agent of granuloma inguinale, that it is not related to the Friedländer-*aerogenes* group of bacteria, and that it had not as yet been cultivated outside the human body. Carter, Jones, and Thomas (14) recognized Donovan bodies in pure culture in pseudobubos and likewise failed to cultivate them on ordinary culture media, in ordinary laboratory animals, or on the chorioallantois of chick embryos. They agreed with Greenblatt and his associates that the Donovan body is not cultivable on a wide variety of culture media known to be useful for the cultivation of certain fastidious microorganisms and that ordinary laboratory animals including the chorioallantois of chick embryos are not susceptible to infection by it.

In a previous report one of us described very briefly the successful cultivation in the yolk of living chick embryos of a microorganism having all the morphological characteristics of Donovan bodies (15). In the present paper we describe in greater detail our continued observations. The same microorganism has been isolated and cultivated from two additional cases of granuloma inguinale. It has been cultivated in the yolk of chick embryos inoculated with human tissue free from contamination but the cultured strains have not grown on any of a variety of ordinary culture media nor are they infectious for mice, dogs, chickens, rabbits, and monkeys. It does not grow on the chorioallantois of chick embryos. It appears, then, that the yolk of the intact, living chick embryo is a suitable and a very favorable medium for the cultivation of Donovan bodies. From this medium we are able to obtain the Donovan microorganism in quantity satisfactory for skin tests. We are also able to obtain a precipitable mucoid substance from infected yolk which gives a specific precipitation response with patients' serums and likewise elicits positive complement fixation reactions. The remainder of this report will describe in some detail the technique of isolation and cultivation of what we may call the Donovan microorganism. We will describe its variable morphology and its behavior

in embryonic yolk. An accompanying paper will describe something of its immunologic relations to patient's serum and skin reactions following intracutaneous injection of the bacterial cells and their products.

Technique of Isolation and Culture

The 3 cases from which the Donovan microorganism was cultivated were carefully selected. Successful cultivation of it by the technique to be described depends primarily upon its isolation from contaminants in fragments of infected human tissue, and subsequently upon its inoculation into the yolk of developing chick embryos.

Isolation of Strain I, Oct. 26, 1942.—The first strain was isolated from a case of granuloma inguinale whose ulcerated lesion, after removal of superficial exudate by swabbing with saline-soaked gauze sponges, yielded scrapings of tissue unusually rich in Donovan bodies and with minimal bacterial contamination, as seen in smears stained by Wright's method.

Part of the lesion was again thoroughly swabbed with sterile saline. A small piece of tissue was clipped from the granulating surface with sharp scissors and placed between folds of moist gauze. With sterile spear-point dissecting needles part of the tissue was fragmented into bits 1 c. mm. or less in size. One or two fragments were smeared over the surface of 6 cystine agar slants. Bits of tissue were left on the slants which were incubated at 37°C.

The remaining fragments were ground with a pestle, suspended in saline, and injected in progressive and arbitrary dilutions into the yolk of twenty-four 9-day-old chick embryos. The last embryo inoculated received extremely small amounts of the original tissue. A blood agar slant inoculated with 3 to 4 drops of the final dilution showed bacterial contamination.

All embryos were killed within 24 hours by an overwhelming growth of various bacterial contaminants. After 48 hours' incubation 4 cystine agar slants showed 1 to 4 staphylococcus colonies. Two were apparently free from contaminants. Smears from the latter stained by Gram's method showed small groups of Gram-negative bipolar rods resembling the non-encapsulated bacilli always associated with Donovan bodies in smears from human lesions. Wright's stain of a similar smear failed to demonstrate encapsulated microorganisms but confirmed the likeness of these forms to those of unencapsulated Donovan microorganisms. After 96 hours' incubation neither slant gave evidence of bacterial contamination and additional smears showed the continued presence of Gram-negative bacilli occurring in small groups in and about dead cells. They appeared to be viable by the quality of their staining reaction but one could not judge either grossly or microscopically that they were growing. Each apparently uncontaminated slant was washed with 3 cc. of saline; the washings were pooled and 0.5 to 1 cc. was inoculated into the yolk of six 8-day-old embryos. Subcultures from the original cystine agar slants to other cystine agar slants failed to give the slightest evidence of growth. On the 3rd day 2 embryos were dead without evidence of bacterial growth. Smears from the yolk of live embryos did not show evidence of bacterial growth.

On the 8th day smears from the yolk of the 4 living embryos stained with Wright's and Gram's stains revealed the presence in abundance of both encapsulated and non-encapsulated Gram-negative microorganisms indistinguishable from Donovan bodies and from those pleomorphic Gram-negative non-encapsulated forms always present in smears from lesions of granuloma inguinale (Fig. 4).

Infected yolk was drawn from each embryo. Parts of yolk sacs were ground, suspended in saline, and used as inoculum for various media and for embryos. Some yolk sac tissue was fixed in Zenker's fluid for histological sections. Additional 8-day-old embryos were inoculated with 0.5 cc. of infected yolk or suspended ground yolk sac.

As far as we could determine the original yolk sac cultures were pure. Nothing grew on any artificial culture medium and the microorganism was easily and readily maintained by serial transfer in yolk of developing embryos. Each yolk of the 2nd passage of the microorganism gave a positive culture within 6 to 8 days. Most of the forms observed in smears were encapsulated. This strain has been maintained for a year and a half by uninterrupted yolk to yolk transfer. In its encapsulated form its likeness to the Donovan body is precise. Its morphological variation from the encapsulated form is entirely consistent with other forms found in the human lesion (Figs. 7, 13).

Analysis of this technical procedure indicates that the Donovan microorganism remained viable in pieces of human tissue for 4 days at 37°C. during which interval contaminated pieces of tissue were separated from uncontaminated ones by culture on agar slants. An uncontaminated culture of Donovan bodies in bits of human tissue grew in the yolk of living chick embryos and has been easily maintained in serial culture. This technical principle has been applied successfully in 2 other instances of granuloma inguinale.

Isolation of Strain II, Feb. 12, 1943.—Six months later smears from the lesions of another patient with granuloma inguinale indicated the availability of tissue rich enough in Donovan bodies and free enough from contaminants to justify a second attempt to isolate and cultivate the Donovan microorganism.

Tissue was obtained from this second case as from the first. Small fragments of the biopsy specimen were teased out and each fragment was washed twice in separate portions of saline. Bits of tissue were smeared over and left on each of 4 cystine agar slants and two 10 per cent blood agar slants. Two blood agar plates were quadrated, and a single piece of tissue was smeared over and left on each quadrant.

Three other small pieces of tissue ground separately to avoid a spread of contaminants, were suspended in separate lots of saline and injected in progressive and arbitrary dilutions into the yolks of twenty-four 5-day-old embryos. As in the first case, the last dilutions of this ground tissue contained only very small amounts of infected tissue. A few drops of the last dilutions inoculated on blood agar plates revealed the presence of contaminants.

From the 2nd through the 6th day after inoculation embryos were found dead each day with an overgrowth of contaminants. The chief contaminant was a slowly growing Gram-positive diphtheroid. Its slow growth accounted for the survival of some infected embryos even to the 6th day. The same contaminant appeared on the cystine slants in 3 to 4 days and on all blood media in 5 to 6 days. Two embryos were alive on the 7th day without evidence of contamination. Not a single piece of tissue on any solid medium appeared free from a Gram-positive contaminant. Isolation of an uncontaminated bit of tissue on solid culture medium failed in this instance. However smears on the 8th day from yolk of one of the remaining living embryos inoculated with a high dilution of the fresh human tissue showed a very few Donovan bodies without apparent contamination. In this culture no unencapsulated forms were seen. All forms appeared as typical Donovan bodies. Bacillary bodies containing one or two small condensations of chromatin were surrounded by a very definite, compact, stainable capsule. One can assume from this experiment that an inoculation of only a very few Donovan bodies without contamination will yield a successful culture in embryonic yolk.

Cultures of the infected yolk on blood plates showed no bacterial growth. Subcultures in the yolk of 6-day-old embryos showed evidence of slow growth in 5 days. On the 11th day after inoculation the yolks contained a good growth of both encapsulated and unencapsulated Donovan microorganisms. Smears of yolk sacs showed both intracellular and extracellular Donovan bodies. Serial transfer to embryonic yolk has maintained this strain for over a year. In the early passages it did not grow as vigorously as the first strain.

Isolation of Strain III.—Not until Apr. 28, 1944, did a third patient appear whose lesions seemed suitable for attempts to cultivate the Donovan microorganism. Smears from the lesion were characterized by large numbers of Donovan bodies and scarcely any evidence of secondary contamination. A granulating surface of the lesion was swabbed thoroughly with sterile saline and dried with a gauze swab. A small piece of tissue was clipped from the surface with sharp scissors.

The tissue was kept moist with saline. Bits of tissue about 1 mm. square were teased out. Dissecting needles were flamed between the manipulations of each bit of tissue. Six or eight of these pin-head sized pieces of tissue were placed without smearing at isolated points on a blood agar plate. A total of 24 bits of tissue was thus put onto 4 blood plates. In addition a single bit of tissue was put onto the surface of each of 12 blood agar slants.

Larger pieces of tissue were minced, rather than ground, in a mortar, suspended in saline, and injected in progressive dilutions into the yolk of twelve 7-day-old embryos and twelve 4-day-old embryos. Inoculations with this tissue were made as soon as possible, *i. e.* within 2 hours after the biopsy was taken, in order to isolate fragments before significant multiplication of contaminants occurred.

Most embryos were dead at 48 hours because of the growth of contaminants. Smears from the yolk of 2 embryos on the 7th day showed a positive culture of Donovan bodies without contamination.

Forty-eight hours after inoculation 24 of the 36 bits of tissue on blood agar were obliterated by the growth of large staphylococcus colonies from and over them. Twelve pieces of tissue appeared free from bacterial contamination.

Smears from 1 piece of apparently uncontaminated tissue stained with Wright's and Gram's stains showed the presence of Gram-negative, unencapsulated bacilli like the Donovan organism. There was no gross or microscopic evidence that they were growing on blood medium.

Two kinds of inoculations were made with these small fragments of apparently uncontaminated tissue. A small slit was made in the yolk sac of six 7-day-old embryos and a single bit of tissue was washed from a sterile capillary glass rod into each yolk.

Several bits of tissue were pooled in the concavity of a hanging drop slide, ground with a sterile glass rod, drawn into a syringe with saline, and injected into the yolks of other 7-day embryos. Two such lots of tissue were ground and suspended separately. Eighteen embryos were inoculated with these suspensions.

Of these 24 embryos inoculated with tissue taken from blood agar none showed contamination from the inoculum (several membranes became secondarily contaminated during the period of examination). At least 9 embryos from both types of inoculum showed pure cultures of Donovan bodies. This strain like the second one grew very slowly for the first several serial passages.

A second procedure designed to improve the technique of isolation was tried on this third patient. A part of the granulated lesion on the lower quadrant of the abdomen was kept continuously moist with 5 per cent sulfanilamide solution for 4 days. A second biopsy was taken and treated exactly as the first. Thirty bits of tissue were put on blood plates and 18 embryos were inoculated with a suspension of finely minced tissue.

Twenty-four hours later not a single piece of tissue appeared to be contaminated and only 1 embryo was dead. This 24-hour-picture of apparent freedom from contamination as a result of chemotherapy was misleading. Subsequent inoculations into embryonic yolk revealed contamination to the same degree as in the biopsy taken before the application of the drug. Contaminants also appeared later in yolk of embryos inoculated with the fresh tissue. The contaminants grew feebly. They did not grow in the tissue on the plates and grew so slowly in the yolk of embryos that eventually we saw Donovan bodies appearing in yolk in the presence of feebly multiplying contaminants. This was a picture not previously encountered. Heretofore we had observed that contaminated embryos died before the appearance

of Donovan organisms or that uncontaminated yolks yielded pure cultures of the latter. We did not attempt to isolate the Donovan organism from contaminated yolk. A number of embryos infected with Donovan microorganisms in pure culture survived, but not as many as from the series inoculated with tissue from the first biopsy.

COMMENT

These three experiments show that the Donovan microorganism remains viable on ordinary blood agar for as long as 4 days at 37°C. This fact affords the opportunity to isolate bits of uncontaminated human tissue for inoculation into the yolk of incubating chick embryos which proved to be a favorable medium for its cultivation. It is also evident that an uncontaminated inoculum can be obtained by dilution. The technique used for isolating the microorganism from the first biopsy of the third case gave the most satisfactory results.

We have attempted cultivation of the Donovan microorganism by this technique from only these 3 cases of granuloma inguinale and we succeeded in each instance. This leads us to emphasize our opinion that successful application of the technique depends upon the selection of clinical cases whose lesions contain so few contaminants that fragments of infected tissue can be isolated free of them, or Donovan bodies be separated by dilution.

Morphology and Cultural Characteristics

The expression "Donovan body" describes a morphological entity composed of a single small nubby condensation or of bipolar condensations of chromatin within a bacillary body surrounded by a well defined capsule. The capsule is clearly demonstrated by Wright's stain (Fig. 3). It appears as a definitely limited, dense, pinkish material surrounding a blue bacillary body with darker chromatin condensations. Although the intracellular Donovan body is pathognomonic for granuloma inguinale there are always conspicuously associated with it in the human lesion groups of small unencapsulated bacilli, somewhat variable in their morphology, that are recognized by students of the disease as a form of the Donovan microorganism. In all of its forms the microorganism is Gram-negative.

In the cultures of the Donovan microorganism in yolk of living embryos all morphological entities observed in the human lesion occur. In the first few serial cultures the encapsulated form was indistinguishable from the Donovan body in human tissue (Fig. 7). As it became adapted to the medium the capsular portion became relatively larger and stained so that it appeared less compact (Fig. 8). The unencapsulated forms, stained with Wright's method, appear as small and rather delicately tinted bacilli (Fig. 13). A recognizable thin cell wall encloses a very pale central protoplasm that terminates in bipolar condensations of chromatin. Very often the central portion bulges a little and one lateral wall stains a little more deeply. The term "safety-pin" form applied to similar structures in the human lesion is appropriately descriptive (Figs. 10, 13).

In other instances the pale central portion elongates and a very long bipolar bacillus appears. As in the smear from human tissue the bacillary forms often occur in small groups of six or eight.

Such an attempt at description fails to express the degree of morphological variation that this microorganism exhibits. Certain variations peculiar to its growth in this unnatural host will be described later.

If one single cultural feature of the Donovan microorganism seems more important than another it is the fact that it has never appeared to grow either in the gross or microscopically on any ordinary artificial culture medium. Yolk cultures were tested as routine for contamination on blood agar plates and the Donovan microorganism has never appeared to grow on this medium. This one fact makes this microorganism a particular one and differentiates it very strikingly from all other bacteria that have been cultured by various people from lesions of granuloma inguinale. It separates it from known members of the Friedländer and *aerogenes* groups of bacteria.

The first yolk generation of strain I failed to grow on 10 per cent blood agar, blood broth, plain infusion broth, potato-dextrose agar (pH 5.6), and anaerobic broth and meat. Subcultures to cystine chocolate agar failed to grow. Similar cultures of the 2nd, 18th, and 50th passages were likewise negative.

Yolk especially rich in Donovan bodies (16th and 18th passages) was injected intraperitoneally into mice, intradermally and subcutaneously into *M. rhesus* monkeys, and subcutaneously into dogs. The microorganisms expressed no pathogenicity for any of these animals. From a small area of induration at the site of injection in 1 monkey encapsulated forms were recovered 4 days after inoculation but there was no progression of the lesion and these forms were judged to be survivors of the inoculum. The microorganism did not grow on the chorioallantois of chick embryos, nor was it pathogenic for rabbits when inoculated into the anterior chamber of the eye. The second and third strains have been continuously tested for growth and contamination on blood agar. More extensive attempts to grow them on artificial media and to test their pathogenicity for laboratory animals have not been made.

The Donovan microorganism has exhibited variations when grown *in vivo* in yolk of different aged embryos, and it has been possible to cultivate it *in vitro* on modified yolk medium.

Characteristics of Yolk Cultures in Vivo

The consistency of normal incubating chick yolk varies as incubation proceeds. During the 1st week its thick consistency is changed to a pale yellow material of watery character. Ten to 15 cc. of such thin yolk are found immediately underneath the developing embryo. From the 11th or 12th day of incubation to the time of hatching the yolk becomes progressively concentrated and thick.

The first embryos in which positive cultures of Donovan microorganisms were discovered were 17 days old, having been inoculated 8 days previously. The fairly thick yolk contained a very heavy culture of both encapsulated and unencapsulated forms. As has been stated, serial cultivation in older embryos by means of inoculation of 9, 10, or 11 day old embryos, tends to produce a culture of the microorganism that is almost wholly unencapsulated (Fig. 5). In thick yolk the bacilli tend to occur in small groups. The chromatic material of bacilli in these compact groups becomes particularly prominent, and the delicate structure of the individual bacillus is obscured. On the other hand the same culture yielded completely encapsulated forms when propagated repeatedly in the thin yolk of 4- to 6-day-old embryos (Fig. 6). The thin yolk of young embryos seems to be the most favorable environment for its growth, but noticeable changes in the character of the Donovan body occurred during continued culture in it. As stated previously, the size of the capsule increased. Between the 8th and the 12th serial passages very large, loosely constructed capsules appeared. Between the 15th and the 18th passages the infected yolk became noticeably sticky. This character increased to about the 25th passage when almost the whole yolk, during 4 to 5 days' incubation, became very sticky and mucoid. During this interval the large capsule of the Donovan body appeared to become easily fragmented and to dissolve into the surrounding medium (Fig. 9). Finally the whole sticky mass seemed to become a blob of capsular material in which very small, delicately staining bacilli were growing. Smears stained by Wright's method showed a fragmented background that took a pink stain of the same quality as capsular material. The embryos of these later passages have died at an earlier interval than the first. Whether this was due to increased virulence for the embryo, to an increased inoculum, or to less resistance by younger embryos, we have not determined. The bacillus has never been observed to invade the embryo and seems to be one of low virulence. One might speculate that the growth of the parasite may so alter the yolk as to interfere with the nourishment of the embryo.

In comparison with the first strain there has been no essential difference in the behavior or appearance of the second strain. Both the second and third strains grew less vigorously in the initial passages. The second strain has caused the same sticky character of the yolk to appear on continued passage in young embryos. The third strain has not been cultured long enough to develop that character. None of the three has grown on blood medium. Tests for growth on other media and for pathogenicity in animals have not been carried out. Attempts to culture the Donovan microorganism in yolk from incubated fertile eggs *in vitro* have met with some success.

Hens' eggs, unincubated, fertile, and infertile were inoculated in the yolk with Donovan microorganisms from strain I, and incubated. The microorganism did not grow in the yolk of the unfertilized eggs even after 4 serial passages.

As embryos developed in the fertilized eggs the Donovan microorganism grew as usual. This experiment was clear cut and qualitative in its result. It indicated the appearance of something that is necessary for the growth and metabolic activity of the Donovan microorganism in the yolk of developing chick embryos not present in infertile yolk.

YOLK CULTURES IN VITRO

Yolk was withdrawn from embryos of 4 or 5 days' incubation, put into test tubes, and inoculated. The microorganism did not appear to grow in this medium at first but similar cultures to which viable embryonic chick heart was added did grow. Serial passage in this medium was successful. The 5th serial culture in incubated yolk with heart, transferred to yolk without heart, grew. Serial passages in both these media *in vitro* have been maintained for a year. Yolk with heart seemed to be a better medium than yolk with kidney. In yolk-heart medium the Donovan body is typically reproduced. Yolk *in vitro* in these cultures becomes sticky also; the metabolism of the bacillus seems to be slower and the morphology and integrity of the capsule more easily maintained. Certain other morphological pictures have been found in *in vitro* cultures that point toward a slower metabolism. The actual division of the encapsulated microorganism proceeds slowly, consequently different stages of division of the Donovan body could be clearly observed. The encapsulated bacillus divided by binary fission. Pinching in the center of the capsule and a pulling-apart resulted in 2 new Donovan bodies fully encapsulated from the beginning. In yolk without heart actual separation of two new Donovan bodies seemed to proceed so slowly that they often appeared as two bodies held together by a thin thread of material staining like the capsule 6 or 8 times as long as the Donovan bodies themselves (Figs. 11, 12). We can judge that encapsulation is not a phase in an obligate cycle of the Donovan microorganism but is the reflection of a particular environment. A picture seen occasionally in *in vitro* cultures was that of long chains of thin unencapsulated bacilli very delicately stained. These *in vitro* vultures establish the fact that the Donovan organism is not an obligate intracellular parasite.

In attempts to devise a medium less complicated than incubated egg yolk, to isolate the essential nutrient factor, and to get a culture of the microorganism as free of yolk proteins as possible, a number of media varying from whole incubated yolk were tried. Yolk from incubated fertile eggs sealed with vaseline supported growth but was not a better medium. The same was true of yolk cultures incubated in an atmosphere of increased carbon dioxide tension.

Similar yolk diluted half and nine-tenths with saline supported serial cultures feebly. The supernatant portion of yolk from 5- to 7-day embryos centrifuged at 1200 to 1500 R.P.M. for 15 to 20 minutes proved to be unsatisfactory as a culture medium.

Amniotic fluid from chick embryos supported growth *in vitro* for 4 serial passages. The microorganism grew in a very small form in this medium; it often appeared in delicate chains. The 5th transfer failed to grow. Amniotic fluid plus a small amount of yolk and amniotic fluid plus embryonic heart did not prove to be favorable combinations.

Embryonic yolk in Petri dishes was heated in an Arnold sterilizer just long enough to coagulate it. The Donovan microorganism failed to grow on the surface of this solid medium. Yolk does not coagulate at 60°C., and after being heated in a water bath at that temperature for 30 minutes it supported serial culture of the Donovan microorganism. This experiment indicated a heat stability to the conditions necessary for growth.

When embryonic yolk is added to melted infusion agar, in a proportion of 50 per cent, a yolk-agar slant may be made in which a small amount of fluid is squeezed out and settles at the bottom of the slant. A culture of the Donovan microorganism has been maintained in the fluid on such a slant through 25 passages during the past 8 months. In this medium the microorganism has never developed a morphological capsule but it seems to secrete a thick mucoid capsular substance in which the bacillus appears as in a matrix. The bacillary forms are small and stain delicately with Wright's stain. This thick mucoid material appears largely at the bottom of the slant. Occasionally and quite unpredictably a blob of the material with its incorporated bacilli will assume or maintain the form of a colony on the slant. The consistency of such a "colony" and its appearance in stained smears are peculiar and very different from any ordinary bacterial colony. The relative bulk of bacteria to the mass seems very small. If a "colony" is allowed to become old and dry on a slant it appears to have become a hollow shell under which there is nothing.

Although the work we have done toward isolating an antigen has been largely with *in vivo* cultures, the yolk-agar culture would seem to offer a source of antigen freer from yolk protein than other cultures. A precipitable substance is present in the yolk slant culture and a more thorough investigation of it seems warranted.

One experiment testing the viability of the Donovan microorganism under different storage conditions was carried out. Infected yolk of the 10th passage of strain I was drawn from the embryo and stored in sealed test tubes at 5°C., 25°C., and at 37°C. After 17 days stored yolk was diluted with saline and injected in 0.5 cc. amounts into the yolk of 6-day-old embryos. That stored at 25°C. grew out promptly at 72 hours; that stored at 5°C. and at 37°C. grew more slowly, but all embryos showed a good growth at the end of a week. Similar tests for survival made at the end of 33 days showed that the microorganism had survived only at 25°C. On the whole the Donovan microorganism is a fairly rugged one. Regular stock cultures consisting of infected yolk withdrawn from embryos have been kept at room temperature.

Histology of the Yolk Sac Infected with the Donovan Microorganism

In the earlier generations of all 3 strains of the Donovan microorganism infection of epithelial cells of the yolk sac is a conspicuous feature. Intracellular epithelial infection can be demonstrated by spreading minute fragments of the membrane on a slide, drying, and staining by Wright's method. Both encapsulated and non-encapsulated bacillary forms are revealed (Fig. 4).

In addition to smears microscopic sections were studied at intervals from successive generations. The membranes, including some yolk, were fixed in Zenker's fluid and stained by Wright's method or with hematoxylin and eosin. The Donovan microorganisms take a rather faint hematoxylin stain in these preparations, but are intensely blue following Wright's stain. We have not yet developed a method for staining the capsules. Within infected epithelial cells the Donovan microorganisms are arranged for the most part about an oval or spherical clear cyst-like cytoplasmic space, very much like those that occur in the characteristic infected mononuclear cells of the human lesion (16). They appear to cause little injury to these cells, although there are occasional foci of exudate lying in the folds of mucosa. These are composed of poly- and mononuclear leucocytes, both of which contain the microorganism. Rarely a large phagocyte filled with bacillary forms is found beneath the epithelial layer in the vascular supporting tissue.

In the adjacent yolk one finds numerous microscopic colonies of the bacilli usually likewise arranged peripherally about a clear spherical space.

It is evident from these microscopic preparations that the microorganism grows readily in the yolk in an extracellular position, but it also gains entrance into and grows well within the cytoplasm of the lining endodermal epithelium. It can therefore be considered a facultative intracellular parasite. The infectious agent has not been found in the tissues of embryos outside the yolk sac.

DISCUSSION

Our interpretation of the results of these studies is that the Donovan microorganism of granuloma inguinale has been cultivated from 3 cases of this disease in pure strain in the yolk and yolk sac of developing chick embryos inoculated with uncontaminated material from human lesions. Reproduction of all the characteristic forms of the microorganism demonstrable in smears from the human lesions, including the pathognomonic "Donovan body" has been observed in the yolk cultures. Thus far the yolk of developing embryos has been found necessary to initiate the cultures, for the incubated yolk of infertile eggs did not support growth when inoculated with an established strain. The microorganism grew *in vitro* only on modified yolk from developing embryos. This proves that special conditions, existing so far as is at present known only in human tissues and in developing embryos, are necessary for its multiplication. Some factor, perhaps furnished by living cells, provides the required

conditions. This substance or condition resisted heat of 60°C. for 30 minutes, but yolk coagulated at 80–90°C. did not permit growth.

Special, and as yet undefined, conditions also seem to govern the production of capsules by the microorganism, and even in the yolk of a single embryo both encapsulated and unencapsulated forms appear. The yolk of the earlier stages of embryonic development (4 to 8 days) supports growth better than that of later stages (12 to 14 days).

Conditions suitable for growth of the Donovan microorganism exist likewise apparently within the cytoplasm of the endodermal epithelial cells of the yolk sac, and in that of large mononuclear cells of the human lesion. It would therefore seem probable that, so far as the pathogenic capacity of this infectious agent is concerned, it is dependent upon enzymic or other properties of certain cells of its living hosts. A determination of the nature of the necessary conditions for its growth would possibly throw light upon the important problem of obligatory parasitic agents, and we hope to follow this line of investigation further.

Our experiments indicate that one or more antigenic components of the agent have specific effects upon the infected human host (17). One of these appears to be derived from the bodies of the microorganisms and renders the skin of the host especially sensitive to an injection of a suspension of the bacilli. Other evidence was elicited by the use of a capsular substance which when mixed with serums of active cases of the disease caused precipitation and complement fixation.

In our judgment these specific immunological reactions offer strong support to the assumption, justifiable on morphological and cultural grounds, that the microorganism cultivated in pure strain from 3 cases of active granuloma inguinale is indeed the true etiological agent of the disease.

The objection might be raised that secondary bacterial infection, such as by strains of the Friedländer group, might have been responsible for the serum antibodies demonstrably present in the blood of patients with active granuloma inguinale.

No member of the Friedländer group appeared in cultures from the lesions of the 3 cases from which our microorganism was derived. On the other hand adult chickens that received injections of pure cultures of the "Donovan microorganisms" developed precipitating antibodies responding to our "capsular" material quite like those present in the serum from cases of the active human disease. The chickens also responded to an intracutaneous injection of "bacillary antigen" in a manner analogous to human patients. It is evident therefore that the microorganism with which we are dealing is capable of inducing both cutaneous sensitivity to the bacterial cells and the production of serum antibodies that respond to the "capsular" substance in a manner identical with those present in the blood of patients.

Thus all the evidence we have obtained, namely morphological, cultural, and immunological, leads to the conclusion that the cultured "Donovan microorganism" described by us is the etiological agent of granuloma inguinale.

In order to identify the cultured microorganism with the Donovan body and other forms of a parasite characteristic of the lesion of granuloma inguinale, it seems pertinent and advisable to classify the agent which we regard as the etiological factor of this disease, and in doing so we would desire to associate it with the name of Donovan and with the "bodies" which he discovered that have become recognized as the pathognomonic feature of the lesion when they are found incorporated within the cytoplasm of large mononuclear cells present in the granulating tissue.

From a morphological and cultural point of view we conclude that the Donovan microorganism is bacterial and bacillary, and that an encapsulated state of the bacillus constitutes the so called "Donovan body;" that the bacillus is Gram-negative and non-motile; that it requires especial conditions for its growth, which at present are restricted to the environment of human cutaneous tissue, the yolk, yolk sac, and amniotic fluid of developing chick embryos. It is not cultivable on any of the usual artificial media which we have used, and it is not pathogenic for the laboratory animals that we have inoculated with it including *M. rhesus* monkeys. The microorganism elaborates antigenic substances that can induce a hypersensitive state in the skin of human patients infected with it and in chickens inoculated with pure cultures. Its components or products also cause antibodies to appear in the serum of immunized chickens which give precipitation in the presence of a "capsular" substance from yolk cultures. This "capsular" substance likewise causes precipitation and complement fixation with serum from patients with granuloma inguinale. We would therefore propose the creation of a new genus of pathogenic bacteria with the strain cultivated by Anderson as the type. For this genus we propose the name *Donovania* and for the specific type the name *Donovania granulomatis.*

Inasmuch as the Donovan microorganism both in the human and yolk sac infections appears to be a facultative intracellular parasite interesting problems concerning its growth requirements are presented. Our experiments indicate that only the yolk from developing chick embryos possesses the required factor or conditions. The use of the incubating egg thus affords an excellent means for investigating this problem which may have an important bearing upon the general problem of the host-parasite relationship in similar rigidly restricted types of parasitism.

The demonstrated reproduction of the Donovan microorganism in embryonic yolk both *in vivo* and *in vitro* likewise offers an opportunity for chemotherapeutic studies which would be particularly appropriate and significant because of the known therapeutic effectiveness of antimony compounds in the treatment of granuloma inguinale. If our conception of the bacterial nature and

etiologic function of this microorganism is correct it would seem that granuloma inguinale is in fact the first bacterial disease for which chemotherapy proved to be effective. Such an assumption was made as early as 1924 by Johns and Gage (10).

SUMMARY

1. A microorganism identical with that originally described by Anderson has been cultivated in pure strain from two additional cases of granuloma inguinale by means of inoculating the yolk of chick embryos with uncontaminated human tissue containing Donovan bodies.

2. The morphological and cultural characteristics of the three isolated strains are described and discussed.

3. This microorganism has been cultivated *in vitro* only in media containing embryonic yolk. It failed to grow on any of a variety of artificial media.

4. It has not proved to be pathogenic for common experimental animals.

5. The Donovan body is reproduced in the epithelial cells of the yolk sac and in the yolk. The microorganism evidently reproduces both extracellularly and intracellularly.

6. The microorganism produces in culture antigens that elicit immune reactions in the skin and serum of granuloma inguinale patients.

7. This microorganism is judged to be a bacterium and the etiological agent of granuloma inguinale.

8. It is proposed that these strains of the bacterium be the type of a new genus, *Donovania*, in recognition of Donovan's original description of the pathognomonic bodies of granuloma inguinale; and that the specific name *granulomatis* be applied to designate its relationship to the characteristic lesion of the disease.

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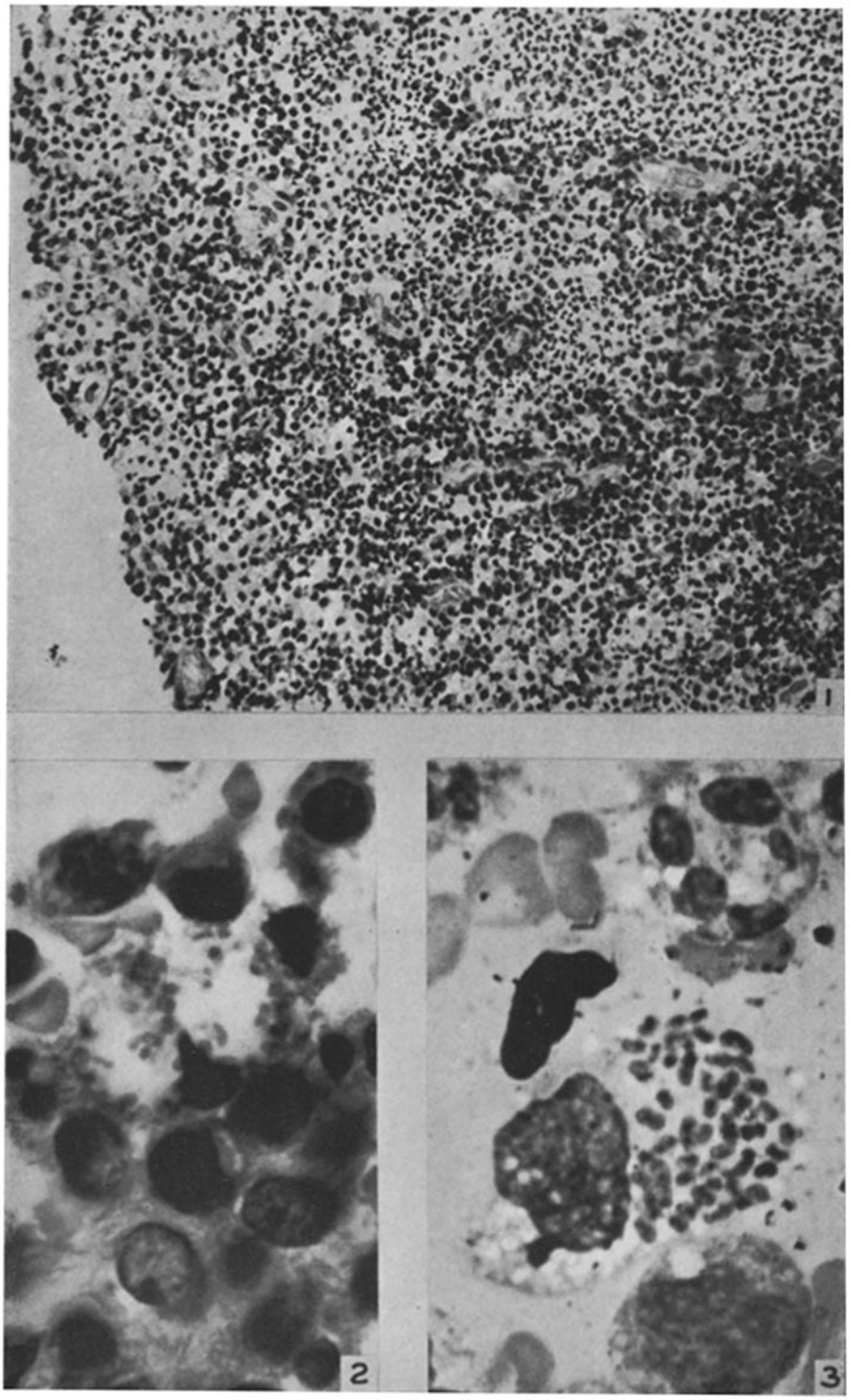
EXPLANATION OF PLATES

PLATE 1

FIG. 1. Granulation tissue from lesion of granuloma inguinale showing minute foci of pustulation. Hematoxylin and eosin. × 225.

FIG. 2. Mononuclear cell with intracellular Donovan bodies in 2 vacuoles. Histological section. Human biopsy. Hematoxylin and eosin. × 2000.

FIG. 3. Smear from same human lesion. Monunuclear cell with intracellular Donovan bodies. Wright's stain. × 2000.



(Anderson *et al.*: *Donovania granulomatis* from granuloma inguinale)

PLATE 2

FIG. 4 Smear of yolk sac epithelium of chick embryo stained by Wright's method. Many intracellular Donovan bodies. Arrows to intracellular encapsulated bodies entirely comparable to forms in human cell, Fig. 3. × 2000.

FIG. 5. Extracellular unencapsulated Donovan microorganisms. Smear from yolk of 17 day embryo with a 6 day infection. Wright's stain. × 2000.

FIG. 6. Extracellular encapsulated Donovan microorganisms comparable to intracellular Donovan bodies of human cells. Smear of yolk from 13 day embryo with 6 day infection. Wright's stain. × 2000.

FIG. 7. Both encapsulated and unencapsulated Donovan microorganisms in smear of yolk from 11 day embryo with 5 day infection. Wright's stain. × 2000.

FIG. 8. Encapsulated Donovan microorganisms in smear of yolk from 11 day embryo with 6 day infection. Capsule larger and more delicately constructed but still intact. Wright's stain. × 2000.

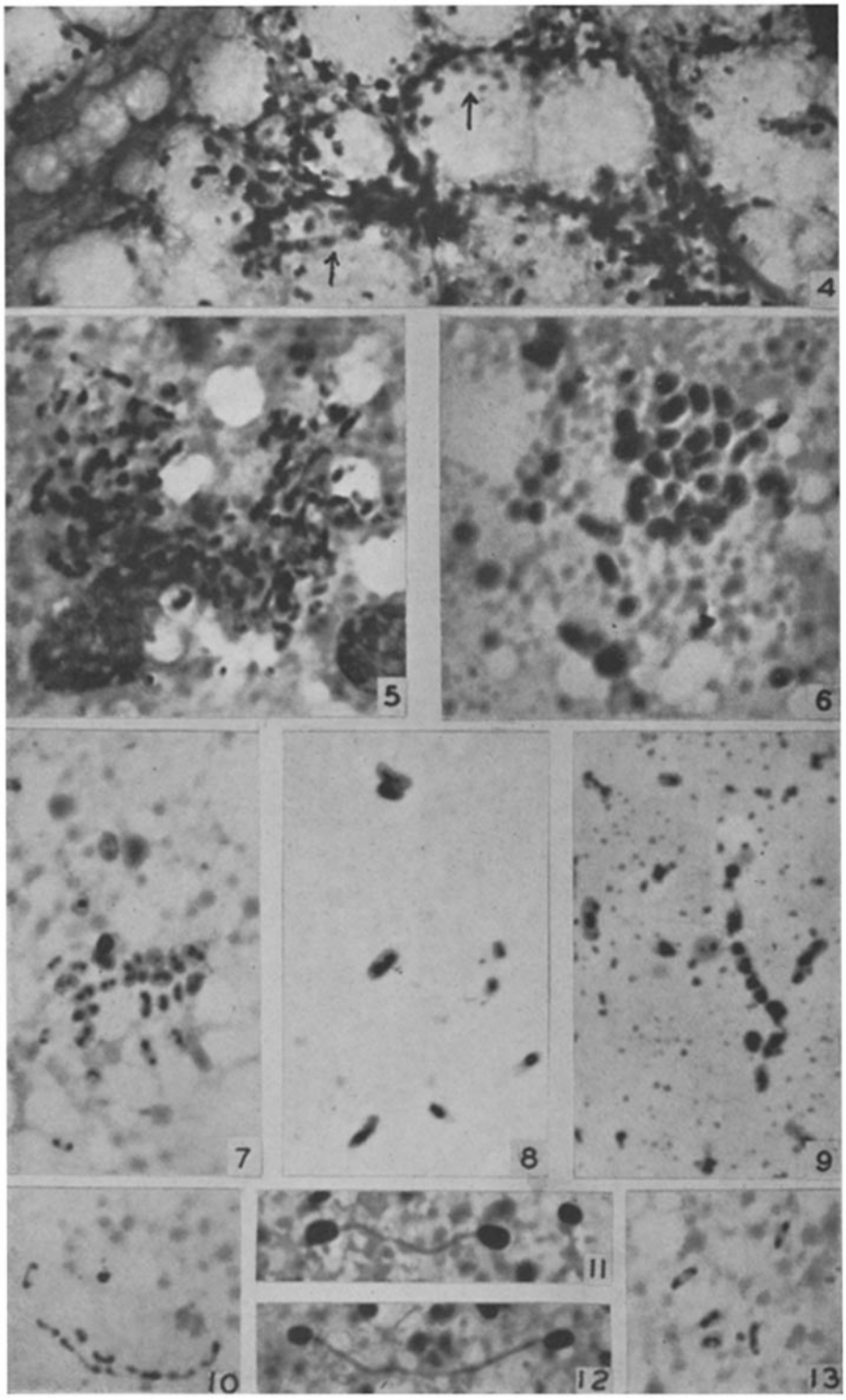
FIG. 9. Donovan microorganisms, encapsulated and unencapsulated. Ten day embryo, 6 day infection showing tendency of capsule of later generations to fragment or dissolve into surrounding yolk. Many of the particles in the background stain with the quality of capsular material. Wright's stain. × 2000.

FIG. 10. Unencapsulated Donovan microorganisms from infected chick yolk. "Safety-pin" form and chains. Wright's stain. × 2000.

FIG. 11. Slowly dividing encapsulated Donovan microorganisms in smear from embryonic yolk *in vitro* without heart. Long thin strand of capsular material joining 2 new forms. Wright's stain. × 2000.

FIG. 12. Same as Fig. 11.

FIG. 13. Unencapsulated Donovan microorganisms in infected yolk. Bipolar and "safety-pin" forms. Wright's stain. × 2000.



(Anderson *et al.*: *Donovania granulomatis* from granuloma inguinale)