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Carrion's Disease II. Behavior of Bartonella Bacilliformis in the Developing Chick Embryo.

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Following the suggestion made by Goodpasture[1] numerous reports have appeared which have established the use of the developing chick embryo for the experimental study of numerous bacterial infections. We are reporting results obtained with the experimental infection of this host with *Bartonella bacilliformis* both in regard to the cultivation of this organism and to the course of the experimental infection so far as it has been observed.

Experimental. As a source of the infection 4 different strains of *Bartonella bacilliformis* were used. They were maintained in Noguchi's leptospira medium, cystine blood agar and in glycerine blood agar.

In the course of the experimental work it was found that blood-glucose-cystine agar as devised by Francis for the cultivation of *B. tularense* provided an excellent medium for the rapid growth of this organism. The only modification in the preparation of this medium which was introduced was to omit heating the flask of prepared medium for 2 hr. at 60°C.

Ordinary blood agar to which 1% glycerine was added also proved to be a very favorable medium for growth. In this medium rapid proliferation occurs within 48 to 72 hours at room temperature, 25-28°C. The organism grows as a thin, almost imperceptible film over the surface of the agar. Smears stained by Giemsa's method reveal the organisms in great abundance. These media have been found to be much more satisfactory to handle and provide for a much more rapid and luxurious growth than does Noguchi's leptospira medium.

Chick embryos of 8- and 12-day incubation were used. The chorio-allantois, the allantoic fluid, the amniotic fluid and the yolk sac were investigated as to their relative suitableness for the proliferation of this organism. The embryos were inoculated by the cover-slip technic as described by Goodpasture and Buddingh.[2] In-

1 Goodpasture, E. W., *Southern M. J.*, 1933, **26**, 418.

2 Goodpasture, E. W., and Buddingh, G. John, *Am. J. Hyg.*, 1935, **21**, 319.

cubation was carried out at 37°C and at room temperature, 25-28°C.

The course of the infection was followed by examining smears stained by Giemsa's method made from the inoculated areas at daily intervals and was further controlled by inoculation of cystine blood agar, or blood glycerine agar as well as in plain infusion broth.

The lesions developing in the chorio-allantois were fixed in Zenker's-acetic acid fluid, embedded in paraffin and sections were stained with hematoxylin and eosin and also by Giemsa's method.

It was found that room temperature (25-28°C) was more favorable for the rapid proliferation of the organism than 37°C. The lower temperature has the disadvantage in that there is a higher mortality of the embryos and secondary contamination occurs more frequently.

The best growth of the organism was obtained in the chorio-allantoic fluid. This was readily inoculated by inserting a needle through the chorio-allantois and introducing the inoculum by means of a syringe. In this medium the organism is found to proliferate with great rapidity. Twenty-four hours after inoculation stained smears made by withdrawing a drop of the fluid with a capillary pipette reveal numerous bacillary forms diffusely dispersed but showing a marked tendency toward clumping which appears to begin about a nidus of cellular debris or fibrin strands. At 72 hours the allantoic fluid is grossly turbid. Great numbers of organisms are present in smears. They appear as small bacillary forms, usually closely clumped together in large masses. A few polymorphonuclears, monocytes and red blood cells are also present. The organisms have been found to continue proliferation as long as 6 days following inoculation. Transfers to the chorio-allantoic fluid were usually made at 72 hours. In this manner the organism has been maintained through 6 successive passages in this medium.

It was found that chorio-allantoic fluid aspirated from the embryo by means of a sterile capillary pipette and transferred to test tubes also provided an excellent medium for cultivating this organism. Growth is not quite as rapid as *in vivo,* but the *in vitro* cultures show large numbers of organisms at 48 hours and beyond. Transfers to fresh tubes of chorio-allantoic fluid can be successfully made.

Growth of the micro-organism is also obtained following inoculation of the yolk sac, as introduced by Cox[3] for the rickettsiae. Smears show the organisms in abundance in 48 to 72 hours. A few organisms can occasionally be found within the cells of the yolk membrane, but the preponderance of the growth takes place within the yolk fluids. Thus far inoculation of the amniotic fluid has not met with any considerable success.

The chorio-allantois was infected by simply dropping a small amount of the suspended organisms onto the surface of the membrane and also in many instances by producing slight injury to the membrane by light scarification with the point of the needle at the time of inoculation. The course of the infection was followed by preparing stained smears of scrapings from the surface of the membrane.

During the first 48 hours following introduction of the inoculum without scarification there is a marked inflammatory response which at first is composed chiefly of polymorphonuclear leukocytes but later is predominantly made up of large mononuclears. In the earlier stages the micro-organisms are present as free clumps but later they are mostly found within the cytoplasm of the mononuclear cells. This cytologic picture persists in smears until the 5th or 6th day, after which the parasite is no longer demonstrable. Grossly, only slight thickening by edema and clouding of the inoculated area are observed during the first 4 days. These changes then rapidly disappear.

When slight mechanical injury to the membrane is added at the time of inoculation the infection presents a different appearance. There is a rapidly developing edema during the first 24 hours. Numerous small round depressions appear on the surface of the inoculated area. These have a thickened edematous border. At 48 hours the rim of these shallow craters develops numerous small pinpoint to pinhead sized granulations, many of which have vesicular centers. These gradually develop into firm white opaque nodules which attain their maximum intensity between 72 and 96 hours. These granular proliferations are also often observed in the bottom of the depressions. After the 4th day they gradually disappear so that by the 7th day only a thin linear depression is present on the surface of the membrane. The granular lesion is also often found dispersed over the surface of the membrane in areas where no crater-like depressions are present.

Smears from scrapings of these lesions show the same cytological picture as described above. The number of micro-organisms present, however, is much greater.

Attempts to transfer the infection in series by inoculation of the ground lesions were successful only for one passage. Further passages than the second failed to produce any signs of infection. Inoculations of emulsion of the lesions of the 2nd passage on suitable artificial medium were positive up to the 4th day following inoculation.

In embryos inoculated on the chorio-allantois in which no organ-

isms could be demonstrated in scrapings from the membrane the organism could always be demonstrated in smears from the allantoic fluid and also by culture of the fluid on suitable artificial medium.

A limited number of membranal lesions were examnied histologically. The reaction is limited chiefly to the mesodermal layer. There is considerable edema, and numerous foci of hemorrhage are scattered throughout. The ectodermal epithelium is generally well preserved except at such points where there has been mechanical injury. In these areas there is active regeneration of the epithelium at the edges of the destroyed ectoderm.

The lesion in the mesoderm is predominantly perivascular consisting of proliferated fibroblasts, mononuclears and an occasional polymorphonuclear. In areas of such foci, adjacent to the ectoderm, there is necrosis consisting of hyalinized cellular debris, and numerous leukocytes. Surrounding these necrotic foci is a prominent border of large mononuclear cells and numerous multinucleated giant cells of the foreign body type. Within the mononuclears in these foci typical Bartonella are found. They are occasionally found within the cytoplasm of the giant cells. These focal cellular proliferations correspond to the granular lesions observed in the gross. These granulomatous lesions in the mesoderm are richly supplied with newly formed capillaries.

Although all the essential characteristics of the human Carrionic cutaneous granuloma are not present in this experimental

FIG. 1.
Gross appearance of 4-day lesion in the chorio-allantois produced by *B. bacilliformis.*

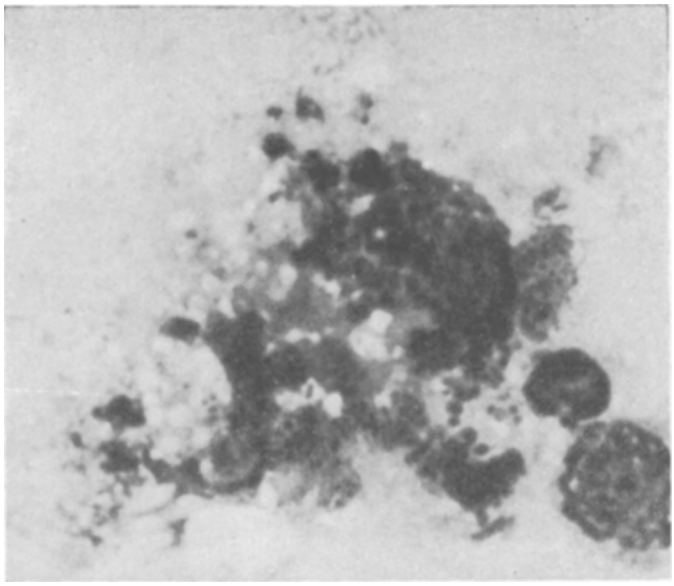

FIG. 2.
Giemsa stained smear preparation from 4-day lesion in the chorio-allantois. × 1800.

lesion its formation is very similar especially in regard to its granulomatous angioblastic features and also in respect to the presence of the etiological agent.

The presence of giant cells is rarely observed in the human lesion and these are interpreted as vascular buds. In the membrane they probably represent a foreign body reaction in response to the presence of the inoculum.

Discussion. Pinkerton and Weinman[4] are of the opinion that *B. bacilliformis* is a facultative intracellular parasite which will proliferate extra and intracellularly in media containing growing or surviving cells *in vitro* in contradistinction to the rickettsiae which are more strictly intracellular parasites. Cox,[3] on the other hand, has stressed the extracellular growth of rickettsiae which he has successfully cultivated in the yolk sac of the developing hen's egg. An analogous circumstance has been observed with *B. bacilliformis* in relation to its growth in the fluid and the membrane of the yolk sac.

Summary. The observations reported here indicate that *Bartonella bacilliformis* is readily cultivated within the allantoic fluid of the developing chick maintained at room temperature, 25-28°C.

[3] Cox, R. H., *Public Health Rep.*, 1938, **53**, 2241.

[4] Pinkerton, H., and Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 587.

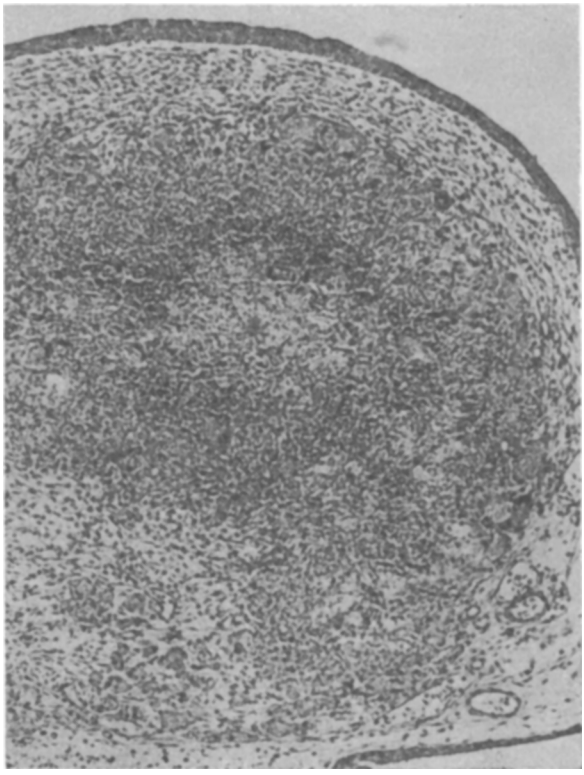

FIG. 3.
Microscopic appearance of 4-day lesion in the chorio-allantois. Hematoxylin and eosin. × 120.

The growth of *B. bacilliformis* in this environment is much more rapid and abundant than in any of the suitable artificial media. Furthermore, the allantoic fluid of the embryo is a suitable medium for the growth *in vitro* of this micro-organism.

The pleomorphism of this organism is again confirmed and the observations here reported are in accord with the interpretation of Pinkerton and Weinman[4] who regard the various morphological aspects of the organism as expressions of pleomorphism rather than as representing stages in a progressive life cycle.

The chorio-allantois thus far has proven to be unsuitable for the transfer in series of *B. bacilliformis*. However, the first passage direct from artificial medium with this pathogen induces the development of a granulomatous lesion the histological features of which bear a close resemblance to that of the human cutaneous infection (verruca) and in which the presence of the etiological agent can be demonstrated.