THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.*

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PLATES 18 TO 20.

Realizing that in ordinary bacterial culture media as usually employed the conditions are far from ideal, and at best a lifeless inactive medium in contradistinction to the active living medium presented by the human or animal body, I was led, in the fall of 1913, to undertake a series of experiments with tissue cultures according to the now well known method of Harrison as modified by Burrows (1). These actively growing cell cultures were used as media on which to attempt cultivation of various pathogenic organisms, and some interesting and suggestive results were obtained which are reported here as opening up what seems to be a promising vista for more detailed observations along the same lines. In these growing cultures the cells are constantly splitting the protein bodies of the plasma to form amino-acids which even bacteria with no proteolytic enzymes can easily handle, and also here we have the opportunity, hitherto lacking, of observing from day to day, directly under the microscope, the interaction of bacteria and tissue cells in normal homogenous or heterogenous plasma, in plasma from immunized animals, and in artificial media which may be varied at will.

TECHNIQUE.

In most of the studies here reported chick embryo cultures were employed, especially heart tissue and splenic tissue from seven to fourteen day embryos. The former gives two indications of bacterial action, the rate and extent of cell multiplication and the rate and force of pulsations or the arrest of the same; while in splenic cultures connective tissue growth and leucocytic growth and migra-

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Bacterial inoculations may be made on solidified cultures at varying intervals after making, into diluted plasma at or before the time of tissue planting, or as controls into plasma without tissue. With each series of cultures sterile controls of normal tissue should always be made, as conditions vary with each planting and results are valueless without comparisons under identical conditions of tissue, plasma, temperature, etc. In all inoculations, except those on solidified cultures twenty-four hours or more after planting, the natural bactericidal action of the plasma must be taken into consideration.

The organisms studied so far have been *Micrococcus aureus* and *Bacterium diphtheriticum*, both pathogenic to chickens and to man, a Hoffman type *Bacterium pseudodiphtheriticum* for comparative purposes, *Bacillus typhosus*, pathogenic to man but not to chickens, *Bacillus coli verus*, usually non-pathogenic and normal to both man and chickens, and *Bacillus prodigiosus*, a non-pathogenic air organism. The diphtheria and typhoid organisms gave the most interesting and characteristic results, but the actions of all organisms used were instructive when comparisons were made. Cultures were made in series and observed daily, and specimens were fixed and stained for permanent preparations at regular intervals. With diphtheria, observations on the action of toxin and antitoxin on tissue cultures were also made. In judging results it must be remembered that one is dealing with very minute fragments of tissue, and to approach normal conditions, bacterial inoculations should be very light. In most series two classes of cultures were made, small cover-glass hanging block preparations with one or two drops of diluted plasma and one to four tissue fragments, and larger cultures on the under surface of lids of glass boxes, the culture containing five to ten drops of diluted plasma and numerous tissue fragments. Where plasma inoculations are made the best plan is to inoculate the diluting fluid, sterile water, or Ringer's saline solution, before adding it to the plasma, and if light inoculations are desired two or more dilution tubes in series should be used, as in plating on agar. A dilution of one part water or saline to two parts plasma gives good results for routine work. In all instances agar slants were made from inoculated plasma tubes to control the inoculations.
BACILLUS PRODIGIOSUS AND MICROCOCCUS AUREUS.

As the two extremes of action, Bacillus prodigiosus and Micrococcus aureus may be taken. Prodigiosus failed to develop colonies in chicken plasma without tissue, and in most cases also in the presence of tissue. Thiele and Embledon (2) claim that when inoculated from a hypertonic medium prodigiosus becomes pathogenic owing to the protective action of the saline preventing early bacteriolysis, and in confirmation of this fact it was found that when prodigiosus was grown in hypertonic bouillon (3 per cent. sodium chloride) it would invariably develop a few colonies in tissue cultures. In contrast with prodigiosus, Micrococcus aureus invariably grew very freely and vigorously under all conditions in such cultures with or without tissue, and with all tissues used. With most tissues, while the aureus colonies were abundant throughout the culture, they were larger and more numerous near the tissue fragments and entirely prevented or very much inhibited tissue growth. With splenic cultures, however, and to a less degree with embryonal liver tissue cultures, this was not the case. If inoculations were heavy the colonies clustering around such tissue fragments were fewer and much smaller, while with moderate and light inoculations these tissues were surrounded with clear bacteria-free halos almost coextensive with the areas of migration of the lymphatic cell elements. This contrast between heart and splenic tissue action is well shown in figure 1 where fragments of both tissues have been planted in the same infected culture.

BACILLUS TYPHOSUS AND BACILLUS COLI VERUS.

Bacillus typhosus and Bacillus coli verus, closely related to each other in morphology and cultural characteristics, showed marked contrasts in tissue cultures, more sharply marked than in ordinary laboratory differential tests for the same. Bacillus typhosus never in a single instance in ninety tests showed any development in plasma cultures, with or without tissue, when inoculated from isotonic or from hypertonic media. That this was due to strong bactericidal action of the chicken plasma for Bacillus typhosus was shown by the following results: when freshly solidified cultures or six hour old
cultures were inoculated with this organism, by means of a platinum needle dipped in a heavy suspension, no bacterial growth occurred; but if the cultures were first incubated for twenty-four hours and then inoculated there was usually a light spreading growth; and if incubated for forty-eight hours before inoculation there was always a heavy growth, the incubation having destroyed the complement at least, if not the amboceptor, in the plasma which had prevented bacterial development. That the bacteria were actually killed was shown by lack of growth on agar slants from such cultures after incubation. Typhosus growth developing on such incubated cultures did not interfere with tissue cell growth or pulsation of heart tissue for at least forty-eight hours, or until the tissue cells were heavily coated with the encroaching bacteria, which had a tendency, in old cultures especially, to cling to the new cells like vines to a decaying tree, giving the picture in stained cultures referred to by the author as "bacterial trees," as seen in figure 2. This same appearance was seen with one other organism, a spore-forming rod of the subtilis type developing as an accidental contamination. In one instance only was it possible to overcome the bactericidal action of fresh plasma on Bacillus typhosus; here the moist tissue fragments were covered with a heavy suspension of active typhoid organisms several minutes before the plasma was added, thus giving an extremely heavy dose of organisms in proportion to plasma. In contrast with the failure of typhoid bacilli to develop in tissue cultures, Bacillus coli verus seems to be entirely uninfluenced by any bactericidal properties of chicken plasma and to grow freely in vigorous colonies generally scattered throughout the plasma and, if the inoculation is heavy, to have a decidedly inhibitory action on tissue cell growth. Growth was also vigorous in clear plasma without tissue fragments. The clear halos seen around splenic fragments with Micrococcus aureus were absent with coli verus, the colonies developing up to and on the splenic tissue as vigorously as elsewhere.

Bacillus diphtheriticum and Bacillus pseudo-diphtheriticum.

The most interesting results of bacterial infections of tissue cultures were those obtained with Bacterium diphtheriticum in contrast
to those with *Bacterium pseudodiphtheriticum*. With heavy inoculations both organisms develop freely, with or without tissue fragments, but when tissue is present by far the most vigorous colonies are seen immediately surrounding the tissue fragments. With diphtheria bacilli in such cases the tissue makes no growth and heart fragments do not pulsate, but with pseudodiphtheria bacilli, even with heavy inoculations, there is some new cell growth, and heart fragments may at times pulsate when laden with colonies. With moderate or light infections, however, there is a far more marked contrast. Pseudodiphtheria bacilli in light inoculations only occasionally develop and then only an individual colony survives here and there through the plasma, this happening more often in the smaller cover-glass preparations than in the box cultures. If inoculated from hypertonic bouillon, however, it usually developed more freely, as did also *prodigiosus*. Diphtheria bacilli in light or moderate inoculations showed a striking and peculiar development, the colonies appearing in numbers clustered around the individual tissue fragments, as in figure 3, and never in the clear plasma away from tissue, or in plasma controls with no tissue. In box cultures containing numerous tissue fragments each fragment resembled a planet with numerous satellite colonies around it. With splenic tissue the colonies clustered around the outer limits of the zone of lymphatic cell migration, leaving a clear zone next to the original tissue which resembled a sun, the center of a microscopic solar system (figure 4). The bacilli, ordinarily destroyed, if not in too great numbers, by the bactericidal action of the plasma, seem to be afforded some protection or stimulation by some cell secretion or product of cell metabolism which enables them to overcome the bactericidal influence of plasma. This fact may explain why clinically diphtheria is a local infection and rarely, if ever, a bacteremia, except in pre-agonal states where the plasma has lost its bactericidal properties. The clear, bacteria-free halo was lacking around a culture of splenic tissue from a seven day chick embryo, but at this time the spleen has little, if any, lymphatic elements and shows a pure connective tissue type of growth. That the failure of bacilli to grow in plasma without tissue was due to a bacteriolysis was shown by comparing the results of cultures made from the same batch of plasma one
and four days after its inoculation with diphtheria organisms, in the former case there being numerous colonies clustered around and through the heart tissue fragments, while in the latter there was only an occasional colony developed. As a general rule, heart cultures infected with diphtheria bacilli pulsed less vigorously and ceased pulsation much earlier than sterile controls in the same series, and when solidified cultures, after incubation, were inoculated pulsedation usually ceased within twenty-four hours after the bacterial growth had reached the tissue fragment. With moderately heavy plasma inoculations the tissues at first showed very slight evidence of new growth, as in figure 3, but after twenty-four to forty-eight hours, when the cells seem to have produced antitoxin enough to overcome the toxic action of the colonies, some new cell growth begins to take place. Previous addition of diphtheria antitoxin to the plasma did not influence bacterial growth, but it did prevent deleterious action of the bacilli on the tissue cells. An interesting confirmation of the close relation between true diphtheria bacilli and pseudodiphtheria bacilli was seen when pseudodiphtheria inoculations were made into plasma containing diphtheria toxin. In these cases pseudodiphtheria organisms behaved just as did true diphtheria in ordinary cultures, the colonies developing regularly and always clustering around the tissue fragments.

**SPLENIC EXTRACT.**

To endeavor to determine if the clear halos around splenic cultures seen in *aureus* and *diphtheriae* inoculations were due entirely to phagocytosis of bacteria by lymphatic cells, extracts of embryonal splenic pulp were prepared according to the method used by Carrel (3) in his work on growth stimulation, and these extracts, after storage for ten days or more on ice, were dropped on solidifying cultures of tissue with bacteria. In every instance the area covered by the splenic extract remained free, or nearly free, from colonies, while the remainder of the culture was filled with them. Embryonal liver, also rich in lymphatic elements, had a similar action to that of spleen, though not to so marked a degree.
DIPHTHERIA TOXIN.

In addition to the above experiments with bacterial inoculations a series of experiments was carried out to show the action of diphtheria toxin and antitoxin on cell growth. The toxin was added in varying amounts to the plasma before culturing, the dosage usually being calculated for the amount of plasma, not of tissue which was subject to more variation, in proportion to toxin, from the standard of the L. + dose of toxin employed for a 250 gram guinea pig; 0.5 of a cubic centimeter of plasma, the amount used for one box culture, containing 1/500 or N/500 of the L. + dose. In general, chick tissues seem to have a much higher resistance to toxin than do guinea pigs, for one L. + dose had little or no effect on cultures. Larger doses, however, had an increasing inhibitory action and if sufficiently large the growth and pulsation of heart tissue were prevented, as were growth and migration of lymphatic elements from splenic tissue. Moderately heavy doses, not sufficient to kill tissue, arrested growth for twenty-four to forty-eight hours, after which time some new cells began to develop. Growth of nervous tissue was arrested by decidedly smaller doses than was that of any other tissues. New cells developing from cultures with large doses of toxin were few in number and showed early advanced degeneration, heavy accumulation of fat droplets, blunted processes, and dense inactive or fragmented nuclei (figure 5). Cultures from the same series with the same dose of toxin but with also a corresponding dose of antitoxin showed fairly vigorous growth with many normal or nearly normal cells (figure 6).

SUMMARY.

This report gives an outline of the results of observations on over 1,100 tissue cultures made during the fall, winter, and spring of 1913 to 1914. The work has been resumed in the fall of 1914 and will be continued along the same and allied lines, confirming the above results with other strains of the same organisms and with other bacteria and bacterial products. Tests should be made with tissue and plasma from other animals, and the known pathogenicity of the organism for the animal and tissue used should always be
borne in mind. We hope to be able to grow on these cultures some of the more strictly parasitic bacteria not developing on ordinary media, as other strict parasites have been grown by other workers, viz., poliomyelitis virus by Levaditi (4), vaccinia by Steinhardt, Israeli, and Lambert (5), and rabies by Moon (6). By comparative studies with various types of cells and various natural and artificial media, clearer ideas as to the exact part of cell plasma in antibody production, by elaborating on the methods of Carrel and Ingebrigtsen (7), Lüdke (8), Przygode (9), and others, should be possible.

After this work was begun a reference was found to the use of some pathogenic bacteria in tissue cultures by Pheiier and Lentz (10), but no publication of the results of these observations has been observed.

The results here reported may be summarized as follows:

**Bactericidal Action of Chicken Plasma.**—On Bacillus typhosus, very strong—never grows in plasma alone; on Bacillus prodigiosus, very strong—never grows in plasma alone; on Bacterium pseudodiphtheriticum, strong—slight growth in cover-glass preparations; on Bacterium diphtheriticum, moderately strong; on Bacillus coli verus, slight; on Micrococcus aureus, very slight or none.

A few pseudodiphtheria bacilli and more diphtheria bacilli survived in plasma stored in the cold for four days. The presence of growing tissue overcomes the bactericidal influence of plasma on diphtheria bacilli and in some instances on pseudodiphtheria bacilli.

*Bacterium diphtheriticum* grows in plasma without tissue only if inoculations are very heavy; and very heavy inoculations of all organisms will probably overcome the bactericidal action of plasma, as it is undoubtedly a quantitative reaction. The bactericidal influence of plasma is overcome by exposure to incubator temperature for twenty-four to forty-eight hours. *Bacterium diphtheriticum* in light or moderate inoculations grows in tissue cultures only in clusters around the tissue fragments, and never in plasma away from tissue. The growth of this organism has a decided inhibitory influence on tissue activity and growth, especially marked with nervous tissue, but this action may be overcome by the addition of antitoxin to the plasma. Cultures inhibited by diphtheria growth
have a tendency to resume growth later, probably due to antitoxin production.

*Bacterium pseudodiphtheriticum* is distinctly less active in tissue cultures than is *Bacterium diphtheriticum* and never develops in plasma without tissue. The presence of diphtheria toxin in tissue cultures causes this organism to behave as does *Bacterium diphtheriticum*. Without toxin it has little or no direct influence on tissue growth except in massive doses.

*Bacillus prodigiosus* fails to develop, as a rule, in tissue cultures except where inoculated from hypertonic media, and then it has no decided influence on tissue growth.

*Micrococcus aureus* grows freely in these cultures with or without tissue, and inhibits tissue growth markedly, except as noted with splenic tissue.

*Bacillus coli verus* always grows freely with or without tissue fragments and is uninfluenced by splenic tissue growth. In heavy inoculations it lessens tissue growth.

*Bacillus typhosus*, except with extremely heavy inoculations, fails absolutely to grow in these cultures with or without plasma, unless the bactericidal action of the plasma has been destroyed by incubation. When this is the case it develops freely with especial affinity for the tissue cells either for support or nourishment. It appears to have no toxic action on the tissue cells. Note the sharp differentiation between typhoid and *coli verus* organisms.

Diphtheria toxin has a quantitatively inhibiting action on all tissue growth and on heart tissue pulsations, the action being greatest on nervous tissue and least on heart tissue growth. Tissues affected by toxin tend to recovery if not killed. Antitoxin counteracts the action of toxin.

Splenic tissue has little or no effect on the growth of *Bacillus coli verus*, but has a decided bactericidal action on *Bacterium diphtheriticum* and *Micrococcus aureus*, probably due to lymphatic cells and cell products, as seen by the area of cell migration coinciding with the bacteria-free area, by the similar action of splenic extract on cultures, and by the failure of such action in cultures of very early splenic tissue showing no lymphatic cells.
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CONCLUSIONS.

We have in tissue cultures in vitro a promising addition to our methods of bacteriological study. The reaction of bacteria to tissue cultures would seem to be more or less parallel to the pathogenicity of the organism for the animal supplying the tissue.

This method promises a reliable means of differentiation between some pathogenic and non-pathogenic organisms of the same species. It will probably shed more light on the protective action of tissue cells and cell products against bacterial action, as is seen with splenic cultures.

It will probably explain more clearly the action of pathogenic bacteria and the definite relation they bear to the tissues involved and to the blood plasma, as is seen from the peculiar behavior of *Bacterium diphtheriticum* in tissue cultures.

BIBLIOGRAPHY.


EXPLANATION OF PLATES.

PLATE 18.

**Fig. 1.** Twenty-four hour culture from twelve day chick embryo heart and splenic tissue in plasma inoculated with *Micrococcus aureus* before planting. Notice the limited tissue growth and general distribution of bacterial colonies, except in the clear halo around the splenic fragment. Magnified 30 diameters. H = heart fragment. S = splenic fragment. B = bacterial colonies.

**Fig. 2.** Seven day culture from eleven day chick embryo heart tissue in plasma. This culture was inoculated with *Bacillus typhosus* on the fifth day of incubation and shows new cell growth heavily invested with bacilli ("bacillary trees") with few or no bacilli between the cells. Magnified 220 diameters. H = heart fragment. C = new cell growth. T = bacteria-laden cells.
Smyth: Reactions between Bacteria and Animal Tissues.

FIG. 1.

FIG. 2.

(Smyth: Reactions between Bacteria and Animal Tissues.)
Fig. 3.
(Smyth: Reactions between Bacteria and Animal Tissues.)

Fig. 4.
(Smyth: Reactions between Bacteria and Animal Tissues.)
Henry Field Smyth.

PLATE 19.

Fig. 3. Twenty-four day culture from nine day chick embryo heart tissue in plasma inoculated with Bacterium diphtheriticum before planting. Notice the diphtheria colonies clustered around the tissue, with very little new tissue cell growth. Magnified 53 diameters. H = heart fragment. B = bacterial colonies.

Fig. 4. Two day culture from thirteen day chick embryo splenic tissue in plasma inoculated with Bacterium diphtheriticum before planting. Notice the large halo around the splenic tissue free from diphtheria colonies. The magnification is too small to show new tissue cells in this area. Magnified 12 diameters. S = splenic fragment. B = bacterial colonies.

PLATE 20.

Fig. 5. Four day culture from seven day chick embryo heart tissue in plasma plus diphtheria toxin. Note the limited growth of highly inoculated cells. With higher magnification these cells show degenerated fragmenting nuclei. Magnified 53 diameters. H = heart fragment. C = new cell growth.

Fig. 6. Four day culture from seven day chick embryo heart tissue in plasma plus diphtheria toxin and antitoxin. (Same toxin dosage as in figure 5.) Note the nearly normal new growth of healthy cells. Magnified 53 diameters. H = heart fragment. C = new cell growth.