

THE RÔLE OF CLASMATOCYTES AND CONNECTIVE TISSUE CELLS IN NON-SPECIFIC LOCAL CUTANEOUS IMMUNITY TO STAPHYLOCOCCUS.

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PLATES 29 TO 32.

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In 1923, Besredka (1-4) stated that he could produce a specific generalized immunity in rabbits and guinea pigs to the subcutaneous or intracutaneous injection of staphylococcus and streptococcus by the injection of specific broth filtrates, or, by the local application to the skin of specific broth dressings.

The immunization was best produced in two ways: (1) by the intracutaneous injections over a small area of either (a) a sterile specific broth filtrate (the organism was grown 10-14 days in broth, filtered and the filtrate reinoculated with the same organism and grown again for 14 days and again filtered) or (b) of a vaccine made by heating a 24 hour broth culture of the specific organism at 60° for 30 minutes; (2) by the application for 24 hours over a limited skin area of compresses moistened by either the sterile specific broth filtrate or the heated killed broth vaccine.

The reaction to a subcutaneous injection of 1-2 cc. of a 24 hour broth culture in the control animal was a large sloughing ulcer, whereas in the protected animal, there was only a small localized abscess. Others (5) repeated these experiments with similar results. Gratia (6), however, obtained the same results with broth. By intracutaneous injection, Mallory and Marble (7) produced a local cutaneous immunity to staphylococcus in rabbits with sterile broth as well as with the broth filtrate. Miller (8) protected guinea pigs against staphylococcus with specific broth filtrates, vaccines, horse serum, broth and concentrated meat broth, all of these substances being injected intracutaneously or applied as compresses. The immunity was well localized, spreading but slightly beyond the area treated and in no sense considered specific. Since Besredka's work, clinicians have treated infections of various kinds with specific broth filtrates or vaccines in broth. Citron and Picard (9, 10) review the work done by others and claim to have had good

results themselves in the treatment of erysipelas, furunculosis, carbuncles and osteomyelitis. They used polyvalent broth filtrates and take for granted that the action is specific.

The aim of the following experiments was (1) to investigate the effect of local broth compresses, (2) to determine how constant this protection was and (3) to endeavor to throw some light on the mechanism of the protection.

Method.

Staphylococcus aureus (our laboratory number 35), isolated from a case of human osteomyelitis, was grown on agar for 48 hours and suspended in saline just before injections. In a few experiments, the staphylococcus was grown in broth. In any one experiment, the suspension from all the tubes, obtained by adding 2 cc. of normal saline to each tube, was pooled and a sample of this standardized roughly by centrifugation in a Hopkins tube for 5 minutes at 2000 revolutions per minute (odometer). It was found that a dose of .015-.06 cc. of bacteria suspended in 3 cc. of saline when injected subcutaneously in the abdominal wall or shoulder of a guinea pig produced, in over 66.1 per cent of the control animals, the characteristic sloughing lesion to be described later. The same dose was used in all the animals of any one experiment, and in all instances, the suspension was shaken immediately before the animals were injected. The control and "compressed" animals were injected alternately.

The organism was grown in broth for 10-14 days, filtered through a Berkefeld filter, the filtrate reinoculated for 14 days and refiltered to produce Besredka's filtrate.

Compresses were made of 6-8 layers of gauze, large enough to cover the abdomen and held in place by adhesive strips for 48 hours previous to the injection. They were kept moistened by frequent applications of the various substances used.

Compresses were the only method of protection used. All the experiments were on guinea pigs weighing from 210-250 gm. The broth used in poulticing was the usual laboratory beef extract broth (3 gm. Liebig's Beef Extract, 5 gm. sodium chloride, 10 gm. peptone (Fairchild's), 1000 cc. distilled water—boiled 1 hour, filtered, titrated to pH 7.6 and sterilized).

The Lesion.

The injection of the standard dose of bacteria subcutaneously in the abdominal wall caused death in 23 of the 121 control animals. 80 of the remaining 98 showed a diffuse cystic swelling covering the entire abdomen which appeared during the first 24 hours. The ani-

mals were sluggish and did not take nourishment. Redness, induration and pain were conspicuously absent at this time. During the next 48-72 hours, the edges of this swelling became firm, indurated, red, painful and warm to the touch. The skin in the center became discolored and frequently broke open exuding a serosanguineous material. In 5-7 days, the whole central area sloughed, leaving an ulcer, 2-3 cm. or more in diameter, with raised indurated edges and a granulating base. Healing took place in 3-4 weeks. This lesion we called 3 plus (+++).

Compresses of broth or filtrate applied to the shaved abdominal wall for 48 hours made the skin wrinkled, thick and slightly boggy. 24 hours after the usual subcutaneous injection of *Staphylococcus aureus*, there was a diffuse induration, red and painful, quite in contrast to the soft cystic swelling in the control animal. There was little general reaction as evidenced by activity or feeding. In most of the animals, a small localized swelling, $\frac{1}{4}$ - $1\frac{1}{2}$ cm. in diameter, appeared by the 3rd or 4th day. This eventually discharged thick creamy pus or healed by resolution. This lesion is called 1 plus (+). In many of these cases, the lesion was a minute nodule containing a drop of pus.

In some of the animals the abscess formed was much larger and ran longitudinally up and down the midline, with thick indurated edges. After several days, this broke down discharging creamy pus. This lesion is called 2 plus (++) .

To recapitulate, the large ulcerative lesion is called 3 plus (+++), the long abscess 2 plus (++) and the nodule or localized abscess 1 plus (+).

RESULTS.

Experiments show that ordinary meat broth is just as effective in altering the control reaction as the specific filtrate (Experiments 16a and 70). Furthermore, dry compresses and compresses moistened with water or saline give some protection, but it is not as complete as that given by broth (Experiments 19 and 64a).

The protection obtained was localized, for when animals were compressed with broth on the abdomen for 48 hours and then injected elsewhere in the shoulder region as in our experiments, the reaction

was as great in these animals as it was in the controls that had no compresses (Experiments 11a, 16b, 64b).

To determine if the protection afforded by broth was constant, a number of experiments were done (Nos. 18, 30, 34, 35, 38, 39, 41, 58, 66, 71). Of 70 controls, 9 died, while 51 of the remaining 61 showed large ulcerative lesions (3 plus, +++). Of 66 treated with broth prior to injection, 4 died; while of the remaining 62, only 2 showed a 3 plus (+++) lesion; 3, a 2 plus (++) lesion; and 57 had small localized abscesses. If all experiments with broth are totaled, of 121 controls, 23 (19.0 per cent) died and 80 (66.1 per cent) had 3 plus (+++) ulcerations. Among the 116 broth "compressed" animals, there were 7 deaths (6.0 per cent), while 98 (84.4 per cent) had localized abscesses.

To determine how long immunity lasted, animals were injected at various intervals after removal of the broth compresses. Although the experiments were not conclusive, there was evidence that the protection lasted more than 24 hours and less than 7 days.

It is evident from these experiments that guinea pigs can be protected in a fairly constant manner against the subcutaneous injection of *Staphylococcus aureus* by the local application of broth compresses.

In order to observe the histological changes accompanying the foregoing phenomena, control animals and animals which had been treated with broth compresses for 48 hours were killed at various intervals after bacterial injection and sections were taken of the abdominal wall. Animals previously injected with trypan blue intraperitoneally (once daily with 3 cc. of a 1 per cent solution for 4 days) were similarly treated. In all, 218 guinea pigs were used for histological study.

Sections included (1) normal abdominal wall, (2) abdominal wall after 48 hours of broth compressing, (3) the abdominal wall from control and "compressed" animals at varying intervals after bacterial injection (6-9 hours, 18-30 hours, 72 hours, 96 hours, 120 hours, 6 days and 10 days), (4) duplicate sections from animals previously injected with trypan blue.

The animals were killed by injecting formalin into the heart and the specimens obtained were washed and fixed in the usual manner before being imbedded in paraffin. All sections were stained with hematoxylin and eosin. In addition from the specimens vitally stained with trypan blue, one section was mounted unstained and one was faintly counterstained with carmine.

Normal Skin.

The normal skin of the guinea pig presented no unusual features. The epidermis was approximately 2-4 cells in thickness, the corium had the usual compact papillary and lower reticular layers. Of most interest for our purpose was the narrow reticular zone of subcutis contained between the hair follicles and the striated muscle layer. In this layer, were distinguished the two chief types of cells found in the connective tissue—the elongated connective tissue type with a large elliptical, compact, dark staining nucleus and a small amount of cytoplasm and the clasmatocyte with an irregular, indefinite outline, a large eccentric nucleus and cytoplasm filled with vacuoles of various sizes. The latter cells, the clasmatocytes, were often clustered at nodal points in the reticulum. They took up trypan blue in the vacuoles. A few dye-containing cells were scattered through the corium, but the majority were in the subcutis.

After Broth Compress for 48 Hours.

After an animal had been “compressed” with broth for 48 hours, the demonstrable changes were: (1) thickening of the epidermis, (2) edema of the subcutis, (3) a striking proliferation of clasmatocytes and connective tissue cells in the subcutis and (4) a moderate exudation of small mononuclear and polymorphonuclear leucocytes.

The epidermis was definitely thickened, comprising 4-8 cell layers. There was an edema of both corium and subcutis, most striking in the latter, for while in the normal animal it was only about one-fifth of the width of the corium, now it had become of equal breadth. All through the skin there was an increased number of cells most marked in the subcutis. While there was a moderate number of polymorphonuclears and small mononuclear leucocytes, there was, especially in the subcutis, a marked increase in the number and size of the clasmatocytes and the elongated connective tissue cells. The tissue macrophages were often of enormous size with a very definite outline that was more rounded in this than in the resting stage. A large vesicular nucleus rather poor in chromatin was pushed eccentrically by the rich vacuolization of the cytoplasm. Occasionally they contained two nuclei. There was no striking evidence of mitosis in any section. Trypan blue was taken up in large amounts by the tissue macrophages and segregated and concentrated in the vacuoles. Although the cells were most dense in the subcutis, a fairly even distribution through the corium might be taken as evidence of motility. The clasmatocytes were enmeshed in a whorl of connective tissue cells

which were also distinctly increased in number and size. There were many small mononuclear cells of various sizes and shapes, grading from the smallest with a compact, eccentric, often bean-shaped nucleus and clear cytoplasm to larger cells with a more vesiculated nucleus, whose cytoplasm often contained a few vacuoles. The blood vessels of the lower corium were dilated and contained polymorphonuclear leucocytes, mononuclear cells and red cells. The endothelium was swollen and showed slight signs of proliferation. Occasionally around the capillary was found a cluster of small mononuclear cells.

The reaction of the skin to a broth compress took place largely in the subcutis as an edema accompanied by a striking increase of clasmatocytes and connective tissue cells. There was also a distinct thickening of the epidermis.

6-9 Hours after Bacterial Injection.

(a) *Controls.*—The control animals presented a tremendous edema of all layers of the skin, extending down through the connective tissue muscle sheaths. The epidermis was thinned, the papillæ flattened and the hair follicles compressed. Polymorphonuclear leucocytes were scattered throughout the corium and subcutis in large numbers. They were filled with bacteria, but showed definite signs of degeneration, *i.e.*, haziness of cell outlines, pycnosis, and fragmentation of the nuclei. There were relatively few mononuclear cells, most of which were small. Clasmatocytes were scarcely to be found, a fact which was confirmed in the trypan blue specimens which showed that very few cells ingested the dye. Connective tissue cells were not increased in number. Scattered through the edema, were many extravasated red blood cells. At this stage there were very few extracellular bacteria.

(b) *Broth-Protected.*—In the animals treated with broth compresses, the histological picture was quite different. The epidermis was still more thickened, the papillæ were prominent and the hair follicles well preserved. Edema was present throughout, although not as marked as in the control animals. The cellular infiltration, however, was much denser, particularly in the subcutis. Polymorphonuclear leucocytes predominated. They were filled with bacteria, but showed no signs of degeneration as evidenced by staining reactions. Small mononuclear cells of the types previously described were present in

very large numbers. There were many clasmatoocytes scattered through and at the periphery of the exudate. The number of fibroblasts was increased so definitely as to suggest beginning organization even at this early stage in the lesion. In the trypan blue sections, the clasmatoocytes at the periphery of the lesion took up the dye in large amounts, while in the most central part of the exudate, these macrophages were often filled with bacteria and phagocyted blood cells, so that the dye previously ingested was scattered through the cell. The blood vessels were dilated and congested and the swollen endothelium showed signs of proliferation. The differences between the control and the broth animals were found chiefly in the subcutis. In the broth-protected animal there was (1) a proliferation of clasmatoocytes, (2) a proliferation of fibroblasts with beginning organization and (3) the preservation of the integrity of the polymorphonuclear leucocytes. There was also a marked thickening of the epidermis.

21-30 Hours after Bacterial Injection.

(a) *Controls.*—In the control animals, the epidermis showed signs of compression, often being lifted from the corium. The whole corium stained poorly and was disintegrating. The edema, still marked throughout the subcutis, had not increased, but the polymorphonuclears present showed further signs of degeneration. There was no attempt at organization. In the trypan blue sections, practically no cells took the dye. The epidermis, however, was stained diffusely, a sign of necrosis. There were few extracellular bacteria.

(b) *Broth-Protected.*—In the broth-prepared animal, the epidermis was still further increased in thickness. The edema was decreased, but the cell exudation was richer in the subcutis. A definite zone of proliferating fibroblasts was beginning to circumscribe the lesion. Polymorphonuclear leucocytes, increased in number, still retained their definite cell outlines and nuclear delineations. Among the fibroblasts were large numbers of clasmatoocytes with richly vacuolated cytoplasm, often enormous in size and occasionally having two nuclei. Trypan blue was found abundantly in these cells. Many clasmatoocytes had phagocyted bacteria, polymorphonuclear leucocytes and red blood corpuscles and showed only scattered dye granules. The blood

vessels were full, the endothelium swollen and proliferating and frequently endothelial lining cells jutted into the lumen of the vessel.

Rapidly advancing organization due to proliferating fibroblasts, together with marked activity of the clasmatocytes which were beginning to act as scavengers were the most marked changes at the end of 21-30 hours.

48-72 Hours after Bacterial Injection.

(a) *Controls.*—The control animal showed a marked degeneration of epidermis and corium. In the subcutis, the edema had decreased with no increase in cellular infiltration. The polymorphonuclears showed advanced signs of disintegration and bacteria, which previously were practically all intracellular, were now found in large numbers outside of the cells. Frequently large bacterial masses were seen, suggesting *in vivo* multiplication. Beginning signs of organization were seen in the lower part of the subcutis. In this zone, when the specimen had been stained with trypan blue, occasional cells took up the dye.

(b) *Broth-Protected.*—The broth animals showed a little greater thickening of the epidermis with broad deep papillæ. In the corium, there was a moderate cell increase, while in the subcutis there was a definitely walled off abscess. The zone of organization composed of fibroblasts and macrophages was thick. A large number of the cells in the peripheral portion of the exudate was enclosed in macrophages which were often engorged with phagocyted polymorphonuclears and debris. This was further shown by the fact that in the trypan blue specimens, few cells, except those at the extreme periphery, ingested the dye in large amounts, while it was present in many cells as scattered granules. The number of smaller mononuclear cells was decreased, while the number of large mononuclears was increased.

4-10 Days after Bacterial Injection.

(a) *Controls.*—In the control animals, the corium and epidermis had sloughed, leaving an ulcer with an organizing base just above the partially or wholly disintegrated muscle layer. In this zone, many cells now showed some avidity for trypan blue granules.

(b) *Broth-Prepared*.—In the broth-prepared animal, the abscess had definitely localized itself in the subcutis. Eventually, it either came to the surface and was evacuated or the clasmatoocytes took up the cellular exudate and ingested it, while the fibroblasts proliferated through the lesion and organized it. At the end of 10 days, the clasmatoocytes had largely digested their burden and the cytoplasm again showed marked vacuolization, so that the trypan blue was distributed in large amounts in the vacuoles.

To summarize, broth causes a marked stimulation of the cells of the subcutis, the clasmatoocytes and the connective tissue cells, which seemed to protect the animal against the overwhelming effects of the bacteria. The macrophages phagocyted bacteria, thus relieving the burden imposed upon the polymorphonuclears so that they in turn were not destroyed by the infection. In addition, the clasmatoocytes later phagocyted the polymorphonuclear leucocytes before they could disintegrate and free the bacteria. Concomitant with this was the rapid proliferation of fibroblasts walling off the lesion. The origin of the smaller mononuclear cells is difficult to determine. However, it was evident that they may develop into larger phagocytic cells and thus contribute largely to the number of these cells seen in the subacute stage of the lesion.

COMMENT.

Inasmuch as there is no question of specificity involved in these experiments, it remains to correlate the histological changes, *i.e.*, the increase of clasmatoocytes, fibroblasts and round cells, with the marked reduction in mortality and the definite alteration of the inflammatory reaction.

While it is generally recognized that the tissue macrophages play a part in the subacute stages of inflammation, it has not been emphasized until recently, that they can offer an effective barrier to bacterial infection.

Metchnikoff (11) and his pupils considered the large mononuclear leucocytes of the blood as the typical macrophages and thought that the large cells in the connective tissue were of lesser importance. In experimental streptococcus infection (12), they believed that the phagocytes of the connective tissue had nothing to do with the disposal of bacteria except in so far as they engulfed polymorphonuclear leucocytes which contained them.

In experimental inflammation of the subcutaneous tissue, both aseptic and bacterial, Maximow (13, 14) definitely separated the clasmatocytes from the fibroblasts by their marked difference in motility and phagocytic activity. Macrophages which were present in large numbers at an early stage in the inflammation, especially the aseptic lesion, he thought came from three sources, (1) through proliferation of fixed tissue macrophages, *i.e.*, clasmatocytes, (2) through the development from the polyblasts or small mononuclears present in the tissue and (3) by the development from lymphocytes and mononuclear cells coming in from the blood stream.

Tschaschin (15) confirmed these findings in experimental peritonitis. Many others (for full reference, see Gay (16)) have observed the early appearance of large phagocytic cells in peritoneal inflammation. Following the work of Evans and Scott (17) who established definite vital staining reactions for clasmatocytes as opposed to the connective tissue cell, it was determined that these phagocytic cells in the peritoneal exudate were of connective tissue origin (Cunningham (18)).

While Rous and Jones (19) and Smith, Willis and Lewis (20) showed that in tissue culture, clasmatocytes have marked phagocytic activity, it remained for Gay and Morrison (21) and Gay and Clark (22) to definitely link these cells with immunity. They found that substances such as infusion broth or diluted egg white, locally injected, produced a marked increase in the number of clasmatocytes in the pleural cavity of rabbits. Such an animal was thus protected against many times the fatal infective dose of streptococcus when injected intrapleurally. In the normal animal, the streptococci increased until the death of the animal (5-7 days), while in the broth-prepared animal, the cultures from the pleural cavity were sterile in from 3-4 hours. Substances such as aleuronat which produced largely an increase in polymorphonuclear leucocytes, gave no such protection. After bacterial injection, rabbits actively or passively immunized against streptococci showed a much earlier mobilization of clasmatocytes in the pleural cavity than control animals.

We cannot discuss the much disputed relationship between clasmatocytes, large mononuclears of the blood and endothelial cells. However, we believe that the large phagocytic cells seen after broth compresses are proliferated largely from the clasmatocytes of the connective tissue. Morphologically, they are similar, their staining reaction the same and their rapid development into large cells with richly vacuolated cytoplasm is different from the gradual change of the small mononuclear cells into phagocytic cells even under the added stimulus of bacteria. There is no doubt that these latter cells contribute to the number of macrophages later seen in the lesion. Furthermore, broth compresses cause a concomitant proliferation of the elongated connective tissue cells. This is evidence of the stimulation of the connective tissue cell as a whole.

The rapid increase in the number of fibroblasts plays an important part in the protective reaction. Their function would seem to complement the activity of the clasmatoocytes. The latter phagocyte the bacteria early, while the fibroblasts organizing around the focus hinder the spread of noxious material before it can be taken up by the phagocytic cells.

Polymorphonuclear leucocytes and small mononuclear cells certainly play a part later in the reaction. But these cell types, however, are too few in number directly after broth compressing and before bacterial injection for one to imagine that they are the effective agents in increasing the resistance.

After bacterial injection, the early disintegration of the polymorphonuclears in the control animal is of importance. This allows a proliferation of the organisms and a recrudescence of their activity before the clasmatoocytes are present in large enough number to assist by ingesting the degenerating leucocytes. We infer that the presence of a large number of clasmatoocytes previous to injection in the broth animal not only diminishes the virulence of the bacterial attack by the phagocytosis of the organisms, but by ingesting the leucocytes containing staphylococci, they prevent a recurrence of bacterial activity.

SUMMARY AND CONCLUSION.

1. Plain broth is just as effective as specific broth filtrate if used as a skin compress for the protection of guinea pigs against a subcutaneous injection of *Staphylococcus aureus*.
2. Plain broth compresses applied for 48 hours previous to bacterial injection sometimes prevent the death of the animal and practically always alter the inflammatory reactions.
3. This protection is not specific and is localized to the area "compressed."
4. The protection lasts at least 24 hours after removal of the compress.
5. Broth compresses applied to the abdominal wall of a guinea pig for 48 hours produced definite histological changes, especially in the subcutis, *i.e.*, edema, proliferation of clasmatoocytes, thickening of the epidermis together with a moderate exudation of polymorphonuclears and small mononuclear cells.

6. The histological response to the subcutaneous injection of staphylococci was different in the control and the broth-prepared animal.

7. In the broth-prepared animal, there was an increase in clasmatocytes and fibroblasts with a dense exudation of polymorphonuclears, which latter, in the main, did not degenerate. The clasmatocytes phagocyted bacteria early and later engulfed the polymorphonuclears, while the fibroblasts rapidly walled off the lesion. The result was a localized abscess which either came to the surface and ruptured or was absorbed and organized.

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EXPLANATION OF PLATES.

PLATE 29.

All sections described have been stained with hematoxylin and eosin.

FIG. 1. Section through normal abdominal wall. Low power.

FIG. 2. Section through abdominal wall after broth compresses had been applied for 48 hours. Low power.

FIG. 3. Section through abdominal wall of control animal (not "compressed") 48 hours after bacterial injection. Low power.

FIG. 4. Section through abdominal wall of broth "compressed" animal 48 hours after bacterial injection. Low power.

FIG. 5. Section through abdominal wall of control animal (not "compressed") 96 hours after bacterial injection. Low power.

FIG. 6. Section through abdominal wall of broth "compressed" animal 96 hours after bacterial injection. Low power.

FIG. 7. Section through abdominal wall of control animal (not "compressed") 6 days after bacterial injection, showing ulceration. Low power.

FIG. 8. Section through abdominal wall of broth "compressed" animal 6 days after bacterial injection. Low power.

PLATE 30.

FIG. 9. Section taken after broth compresses had been applied for 48 hours. Shows clasmotocytes, fibroblasts and mononuclear cells. Oil \times about 475.

FIG. 10. Control animal (not "compressed") 8 hours after bacterial injection. Shows degeneration of exudate cells. \times about 325.

FIG. 11. Broth "compressed" animal 8 hours after bacterial injection. Shows exudate cells well preserved and a proliferation of fibroblasts. \times about 325.

FIG. 12. Same as Fig. 10. Oil \times about 475.

PLATE 31.

FIG. 13. Same as Fig. 11. Shows clasmotocytes containing bacteria. Oil \times about 475.

FIG. 14. Animal (not "compressed") 21 hours after bacterial injection. Shows marked degeneration of exudate cells. \times about 325.

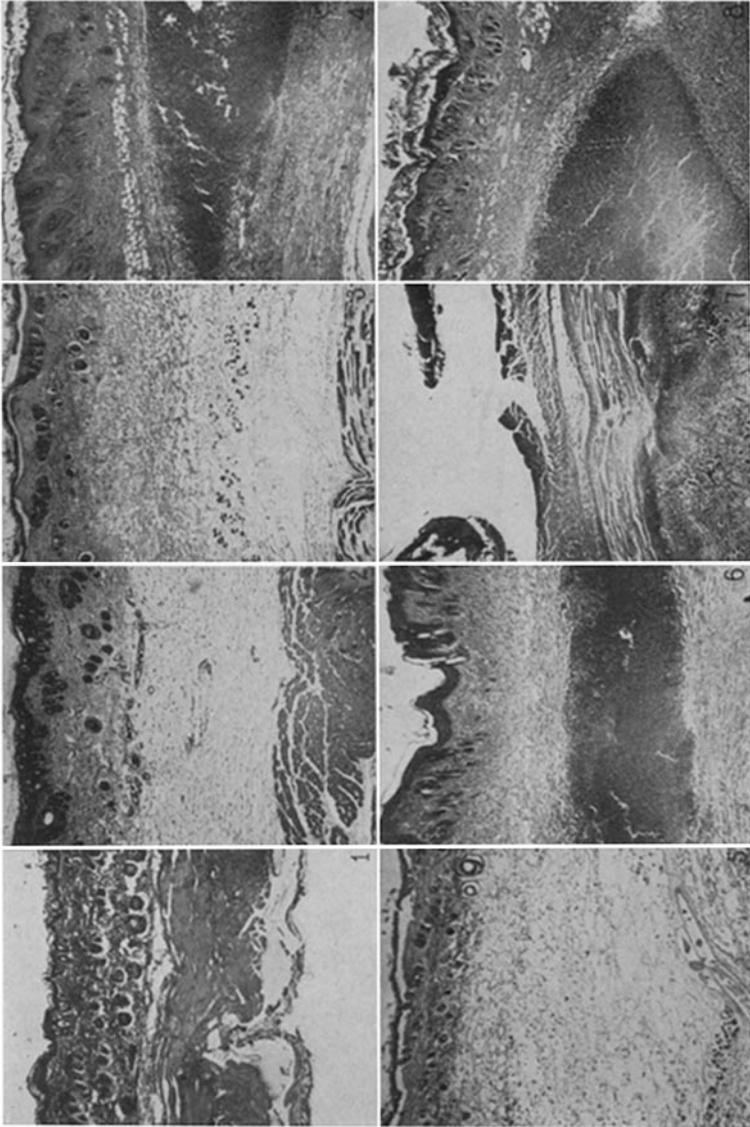
FIG. 15. Broth "compressed" animal 21 hours after bacterial injection. Shows many clasmotocytes, fibroblasts, small mononuclear cells. Cells of exudate well preserved. \times about 325.

FIG. 16. Same as Fig. 15. Oil \times about 475.

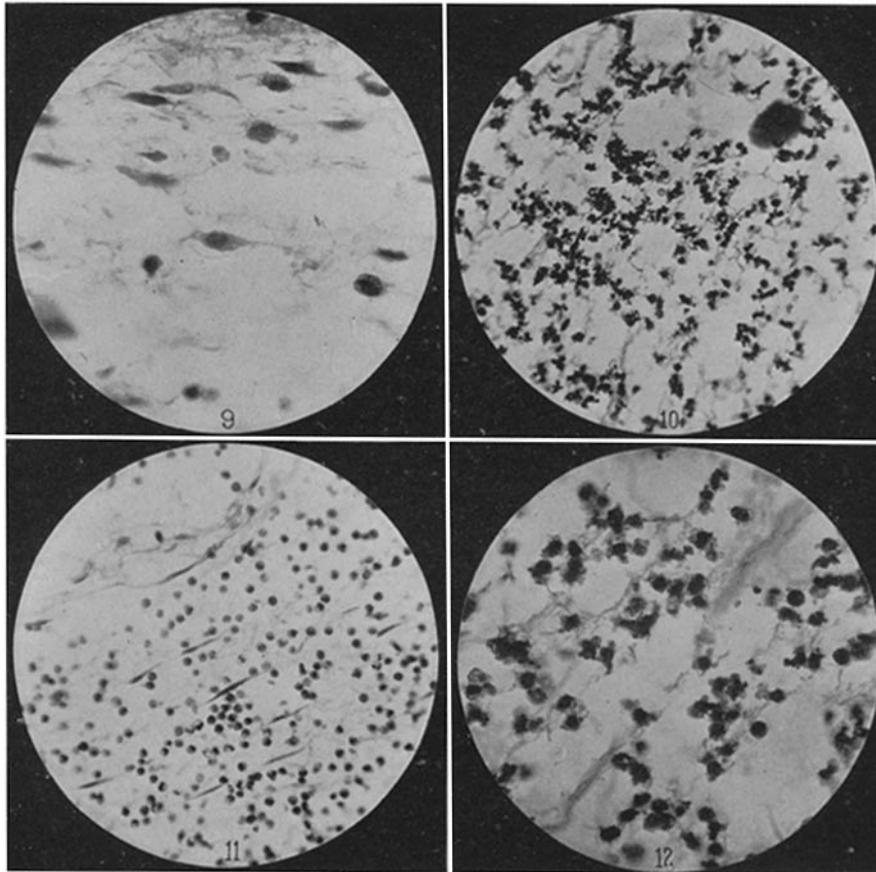
PLATE 32.

FIG. 17. Control animal (not "compressed") 72 hours after bacterial injection. Shows marked cellular degeneration and extracellular bacterial proliferation. Oil \times about 950.

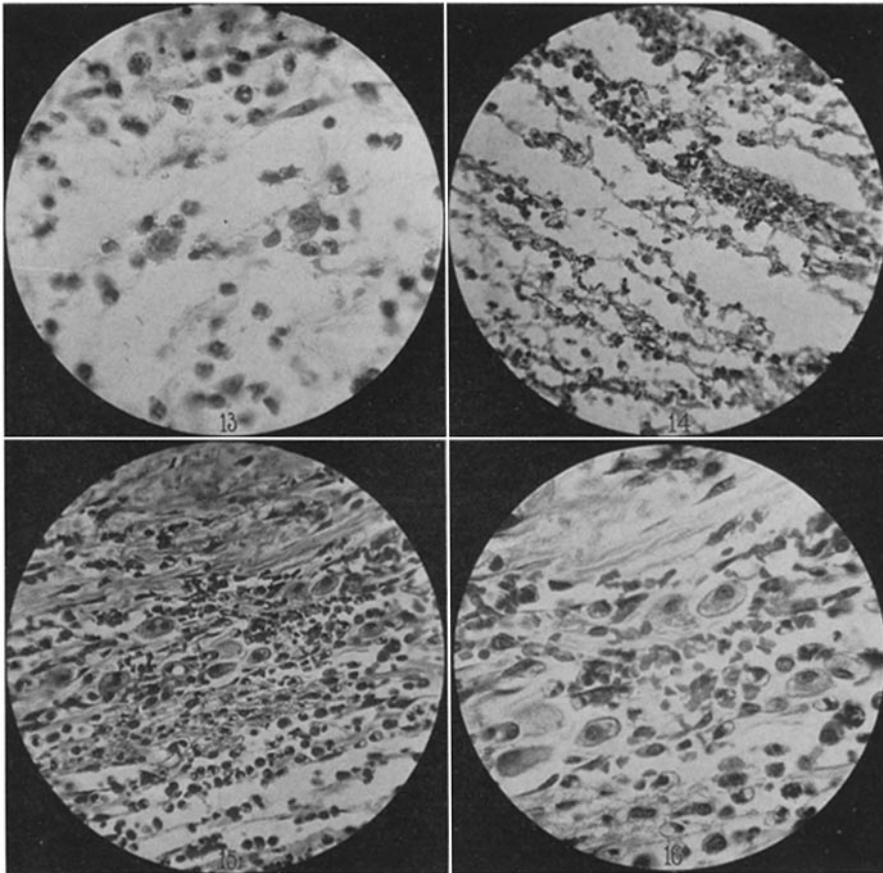
FIG. 18. Broth "compressed" animal 72 hours after bacterial injection. Shows phagocytosis of the cells of the exudate by macrophages. Oil \times about 950.



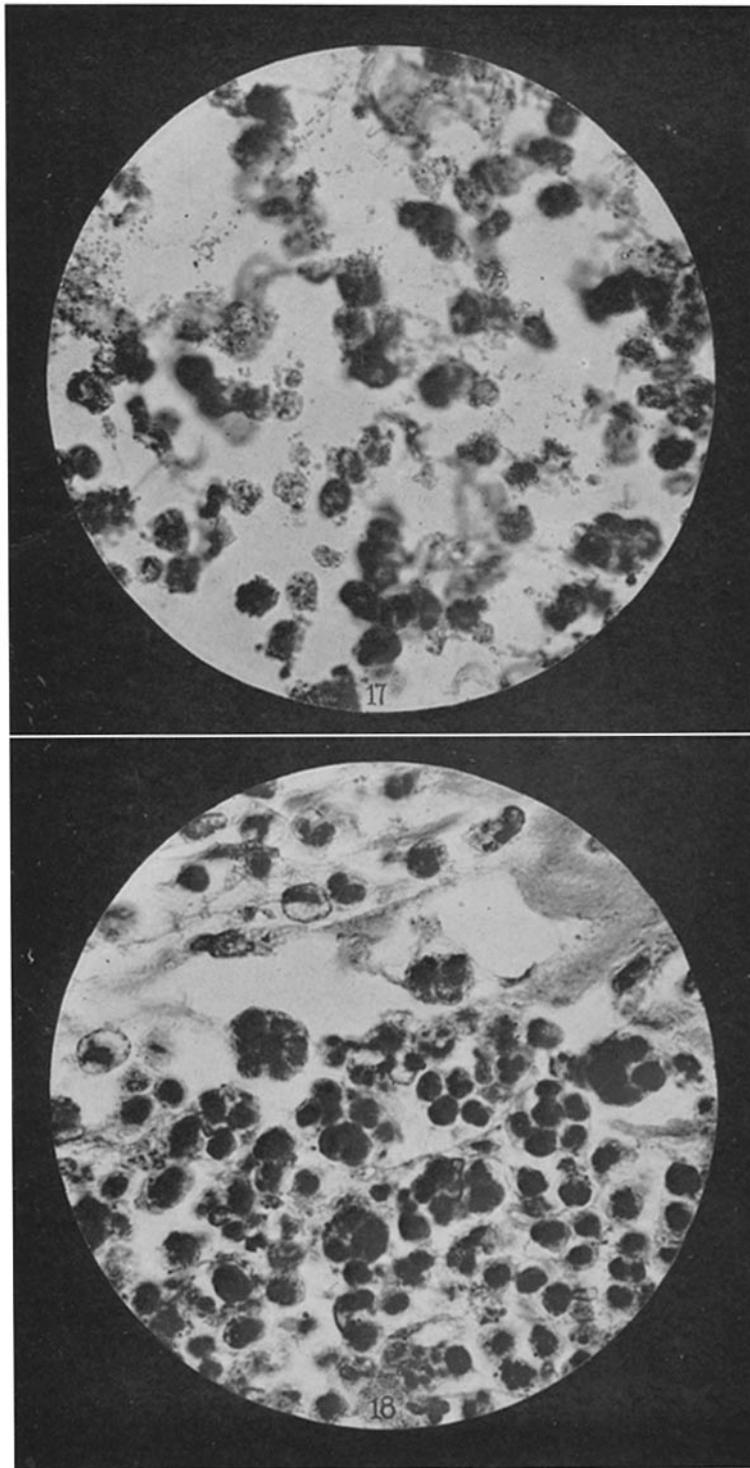
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