PSEUDO-COLONIES SIMULATING THOSE OF PLEURO-PNEUMONIA-LIKE MICROÖRGANISMS

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While attempting to cultivate filterable pleuropneumonia-like microörganisms from patients with rheumatic fever, we encountered pseudo-colonies, which resembled those previously described by Twort and Twort (1921) and Laidlaw (1925); and some additional information concerning these artifacts was obtained. Because of the current interest in pleuropneumonia-like microörganisms, it is felt that the confusion that might ensue from the appearance of these pseudo-colonies should be more widely appreciated.

METHODS

The media in which these pseudo-colonies were first seen consisted of 30 per cent horse serum agar. The serum was obtained from the New York Board of Health Laboratories, and was filtered under positive pressure of 20 lbs. through Seitz pads, grade EK. Freshly filtered serum was heated to 45°C. and mixed with freshly melted nutrient agar. About 20 ml. of the mixture were poured into Petri dishes having a piece of sterile filter paper under the cover. The dishes were sealed with parafilm, and incubated for 24 to 48 hours to insure sterility, then stored on a laboratory table. After being inoculated with suspected material, the Petri dishes were again sealed with parafilm and incubated at 37°C. Thus, during both the periods of storage and incubation, the filter paper prevented moisture from running over the surface of the medium; and the tight seal of parafilm insured a continuous moist atmosphere about the media. These cultural conditions are very favorable for growing several strains

of pleuropneumonia-like microörganisms; in fact, Dr. Albert Sabin (1938) has found them the most satisfactory for growing easily on solid medium those strains carried by many mice.

Cultures prepared in this way have an additional advantage: they may be repeatedly examined microscopically without being opened and exposed to air contaminants. The Petri dish is placed cover side down on an ordinary microscopic stage; and the filter paper in the cover is illuminated with a very fine beam of light obtained by almost completely closing the iris diaphragm under the condenser and sharply focusing this beam on the paper. By manipulating the mirror, and thus moving the beam of light, either flat or oblique illumination may be provided, and the contour of the colonies studied. Both the culture medium and the Petri dishes must be crystal clear. For preliminary examination, a low power, long focal distance planar objective has been found convenient, and for more detailed study the usual low power objective (10×) with a 10× ocular. A particular zone on the medium may be marked by sticking over this zone a piece of finely perforated paper. Scotch adhesive tape has proven a useful fastener for this paper. By numbering each bit of paper, the areas can be easily identified, and the underlying colonies can be repeatedly found and studied or used for subcultures.

Appearance of pseudo-colonies

On horse-serum agar a characteristic pseudo-colony begins as a highly refractile globule having a slightly yellowish tinge, the so-called "amber body"; around this there develops an irregular circular zone finely granular in texture and often with a radially arranged wrinkled appearance at the periphery (fig. 1). Oblique illumination gives the impression of a slight excavation of the medium, or the appearance of a very flat crater containing at its center the brilliant "amber body." This depression of the surface is especially noticeable when contrasted with the raised contour of a true pleuropneumonia-like colony (fig. 2). Under the cultural conditions described, these pseudo-colonies develop much more slowly than do those of most pleuropneumonia-like microörganisms; the "amber bodies" may not appear for 4 to 7

days after incubation at 37°C.; and the fully developed pseudo-colony may not attain its maximum size of from 50 to 150 μ for 10 to 15 days. Like true colonies, these present their characteristic morphology only when widely separated; when they are closely crowded, their macroscopic appearance on the surface of the medium is that of a film-like growth, and the microscopic picture that of numerous "amber bodies" with slight or no surrounding pseudo-colony formation (fig. 3). In old uninoculated plates, minute refractile bodies of various sizes form in the deeper parts of the medium; these sometimes resemble spores of molds, or appear like concave discs, and occasionally look simply like the globular "amber bodies." As noted by Laidlaw, similar microscopic globules develop in serum or serum broth that has been incubated several days, and when numerous, they form a fine sand-like sediment which may be seen macroscopically, or better with a hand lens, and which rises in the medium in the form of a fine whorl when the tube of liquid is shaken.

While the pseudo colonies have developed most vigorously on agar containing horse serum, they were also found on media made with rabbit, human and beef serum. The serum from each species gave pseudo-colonies with certain peculiarities. On rabbit serum agar the periphery of the pseudo-colony was more distinctly outlined and the surface more clear cut as though etched with a sharp tool; an "amber body" was often absent or small and excentrally placed (fig. 4). In the media surrounding the larger pseudo-colonies, the globules were usually less numerous and more uniform in size than those appearing in horse serum agar (fig. 4, fig. 5). On media made with ascitic fluid or human serum, the pseudo-colony appeared as a small concave disc (fig. 6), with no visible change in the surrounding medium; and in the depths of the agar relatively few refractile globules appeared. These pseudo-colonies were often distributed in streaks throughout the media and not uniformly as in solid media made with other sera. On beef serum agar there first appeared an "amber body" or concave disc, on one side of which there were subsequently formed curved or flat S-shaped lines radially ar-

ranged, so that the appearance was that of a cocks-comb (fig. 7). Subsequently, similar lines appeared about the entire pseudo-colony and gave it the appearance of a pin-wheel with a fairly large, highly refractile center. Relatively few round globules were seen in the deeper part of the agar.

While the forms described above were seen on uninoculated media that had been incubated at 37°C. for 10 to 14 days, or on plates that had stood at room temperature from 2 to 4 weeks, they appeared more readily on media inoculated with agar containing a few pseudo-colonies that had arisen spontaneously. For example, if a small block of agar containing one or two pseudo-colonies was cut out with a sterile spud and rubbed over the surface of a fresh plate, many pseudo-colonies developed along the line of inoculation, with characteristic morphology at the margins of the inoculated zone or where the pseudo-colonies were widely separated, and small, poorly defined pseudo-colonies in the middle of the inoculated areas. If this crowded zone were cut out and used as an inoculum, the subculture consisted almost entirely of the nontypical crowded forms. In this respect, as noted also by Laidlaw, the phenomenon of multiplication resembles that of microörganisms. After inoculating a serum agar plate with minced tissue or exudate, the pseudo-colonies usually developed more numerously along the inoculated streaks, a phenomenon which might easily mislead the observer into thinking that they had been derived from the inoculum. Moreover, simply disturbing the surface of the medium by rubbing with a spud, or by "inoculating" it with sterile salt solution or broth, sometimes similarly caused the pseudo-colonies to appear and "grow" most vigorously along the "inoculated" areas; hence it seems that the favoring factor may be mechanical rather than viable. When four blocks of horse serum agar, having on their surface approximately the same amount of "growth" were inoculated on the surface of fresh horse serum, rabbit serum, ascitic fluid, and beef serum agar, respectively, "growth" first appeared and was most vigorous on the horse serum agar subculture; it was somewhat slower in appearing and less vigorous on the rabbit serum agar; and only relatively few pseudo-colonies appeared on

the inoculated areas of the human ascitic fluid and beef serum agar. On each medium, moreover, morphology of the well-separated pseudo-colonies was typical for the respective medium. This phenomenon of formation of pseudo-colonies imitates the tendency for certain pleuropneumonia-like microorganisms to exhibit somewhat different but characteristic colonies on media containing serum from different animal species. "Cut-outs," placed in broth containing serum from these four different species of mammals, also showed the formation of refractile globules, most abundant in the horse serum and diminishing in intensity parallel to that on the respective solid media.

When agar was mixed with diminishing concentrations of horse serum from 30 to 0.1 per cent, all media containing over 5 per cent developed numerous pseudo-colonies, those containing between 3 and 1 per cent, less numerous; while those having only 0.5 to 0.1 per cent showed none. Media made with old or freshly obtained serum were equally favorable for the development of the pseudo-colonies, as were media made with citrated serum. On the other hand, the addition of 0.2 per cent sodium oxalate to the serum, completely inhibited the appearance of these pseudo-colonies.

The effect of applying certain measures that would influence the growth of filterable microörganisms was tested. Serum, which, unfiltered, yielded a good "growth" on serum agar plates, gave approximately as vigorous "growth" when filtered through Berkefeld V and W candles, Chamberland L3, L5 and L7 filters, and through Seitz EK pads of both American and German manufacture. Horse serum filtered directly through a Gradacol membrane of APS 100 m.mu. yielded a fair number of colonies when made into serum agar plates, but fewer than control plates made with unfiltered serum. Horse serum agar plates, made and sealed in the usual way, then heated to 60°C. for one hour, yielded many typical colonies; that heated to 65°C. for one hour still developed the characteristic pseudo-colonies to about the same degree as unheated controls. Plates made with serum heated even to 70°, although containing considerable coagulum, still gave some "growth," chiefly of highly refractile

bodies. Thirty per cent horse serum agar containing merthiolate in a concentration of 1:500, yielded pseudo-colonies like those on the control media, even though it was impossible to grow any bacteria on such chemically treated medium; and pseudo-colonies appeared on serum agar containing formalin in concentrations of 0.5, 1.0 and 5.0 per cent respectively; but in the 5.0 per cent concentration, the "growth" consisted chiefly of the finer globular bodies without the characteristic surrounding crater-like zone. The failure of these disinfecting measures to prevent the appearance of pseudo-colonies seemed to place the causation of the phenomenon outside the action of living microörganisms.

Laidlaw, by analyzing chemically the sediment consisting of highly refractile globules grown in serum, concluded that these globules consisted of calcium and magnesium soaps, and, by analogy, that the pseudo-colonies, he found on serum agar slants, were composed of the same material which, he thought, formed the spherocrystals that apparently comprise most of the pseudo-colonies. Because direct proof of the chemical constitution of these pseudo-colonies was lacking, we attempted to stain them in various ways. Contact films and sections of the serum agar pseudo-colonies stained with Giemsa revealed purplish globules of various sizes but with no uniform structures that could be definitely identified. Staining the colonies directly with Scharlach R or osmic acid did not give any differential diagnostic staining; but when the agar containing pseudo-colonies was first fixed a few minutes with 4 per cent formalin, then was stained with Scharlach R and counterstained with methylene blue, the pseudo-colonies took the typical red color of fatty substances against a blue background of agar. With this stain, pseudo-colonies on horse serum agar had a large homogenous central globule, which was probably the "amber body" seen in unstained preparations; this was surrounded by a disc-shaped structure composed of very fine red granules. Pseudo-colonies appearing on horse serum agar containing formalin, stained characteristically red immediately following treatment with Scharlach R. These staining reactions confirm Laidlaw's ideas concerning the fatty nature of the "growths."

DISCUSSION

The phenomenon here described is of interest from several angles. Unless recognized in their various forms, these pseudo-colonies, composed of material which takes a fat stain, may be confused with the growth of filterable microörganisms, for not only in their morphology do they mimic colonies of pleuropneumonia-like microörganisms, but by applying the cultural techniques commonly used to propagate these microörganisms in subcultures, the pseudo-colonies appear to multiply to a remarkable degree. Doubtless, in subculturing them by rubbing one or two over the surface of fresh medium, minute bits of these pseudo-colonies are distributed over the serum agar, and each bit forms a nidus specially favorable for the crystallization of new spherocrystals out of the medium. The procedure of adding small amounts of a given substance to a solution of that substance to favor and hasten its crystallization from solution is commonly employed by chemists. The peculiarity in the particular example of this general phenomenon here cited is that the resulting crystals so closely mimic the appearance of certain microörganisms, not only in their general morphology, but in the manner in which they present forms characteristic for the different sera out of which they may have crystallized. Here again chemical parallels may be cited, for the forms of crystals may be characteristically conditioned by the medium out of which they have precipitated. Finally, their spontaneous formation under suitable conditions or where the medium is physically disturbed, such as in areas inoculated with exudates or minced tissue, is comparable to the deposition of crystals from chemical solutions following the application of certain physical disturbances to that solution.

SUMMARY

Pseudo-colonies are described resembling somewhat those formed by filterable microörganisms, both in their morphology and in the manner in which they may be propagated on serum agar and in serum broth. Media made with sera from different

animal species form pseudo-colonies characteristic for that species. The pseudo-colonies are composed largely of spherocrystals that stain typically with fat stains. These observations extend and amplify those previously made by the Tworts and by Laidlaw.

REFERENCES

LAIDLAW, P. P. 1925 On structures which develop in certain culture media and resemble colonies of micro-organisms. Brit. J. Exptl. Path., **6**, 36–39.

SABIN, A. B. 1938 Identification of the filtrable transmissible neurolytic agent isolated from toxoplasma-infected tissue as a new pleuropneumonia-like microbe. Science, **88**, 575–576.

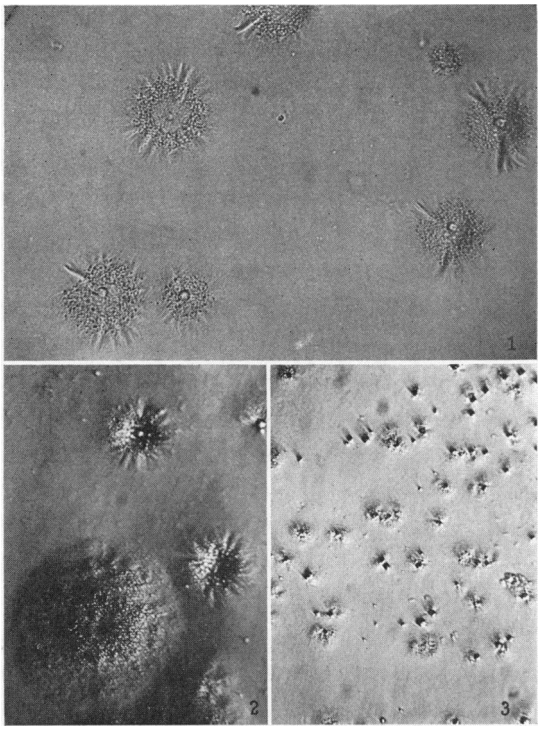
TWORT, F. W., AND TWORT, D. N. 1921 An investigation of influenza. J. Hyg., **20**, 85–98.

PLATE 1

FIG. 1. Thirty per cent horse serum agar incubated 14 days. Pure "growth" on 10th subculture. Flat illumination. Central "amber bodies" very marked. ×115.

FIG. 2. Thirty per cent horse serum agar. Inoculated with minced pneumonic mouse lung, incubated 7 days. Oblique illumination. Two smaller crater-like pseudo-colonies and one larger colony of pleuropneumonia-like microorganisms, Type A Sabin. ×115.

FIG. 3. Thirty per cent horse serum agar, heavily "inoculated," incubated 12 days. Crowded pseudo-colonies with numerous "amber bodies" and little peripheral "growth." ×115.



(T. M. Brown, H. F. Swift and R. F. Watson: Pseudo-colonies on Serum Agar)

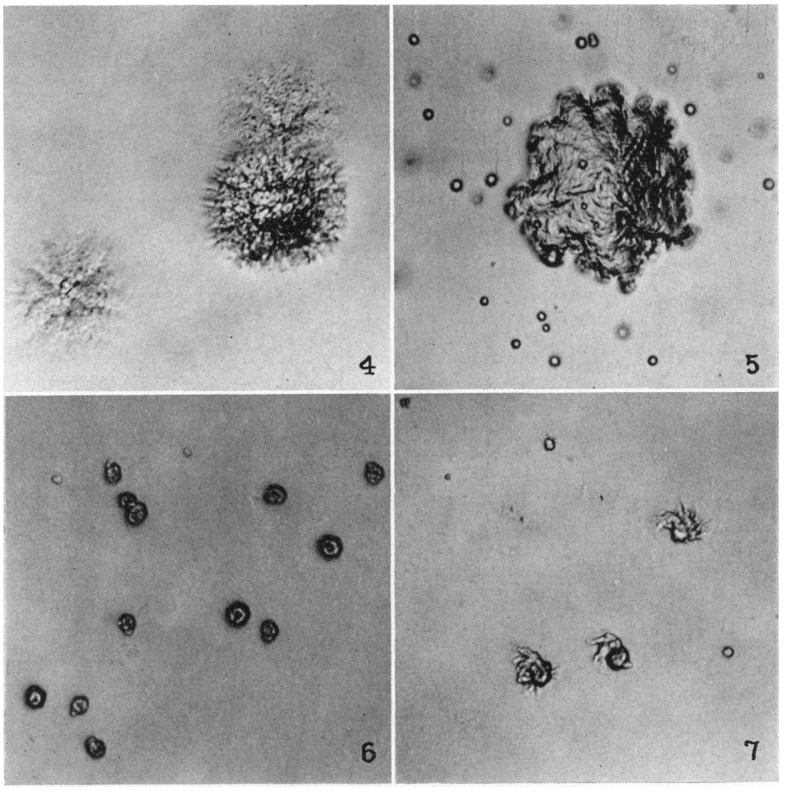
PLATE 2

FIG. 4. Pseudo-colonies in 30 per cent rabbit serum agar, incubated 2 weeks. ×115.

FIG. 5. Thirty per cent rabbit serum agar, incubated 6 weeks. Pseudo-colonies have marked chiseled appearance. ×115.

FIG. 6. Thirty per cent ascitic fluid agar, incubated 2 weeks. Pseudo-colonies in form of small discs with central depression. ×115.

FIG. 7. Thirty per cent beef serum agar, incubated 1 week. Small central concave disc with curved peripheral areas forming cocks-comb pseudo-colonies. ×115.



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