Morphology, Antigenicity, and Nucleic Acid Content of the *Bacteroides* sp. Used in the Culture of *Entamoeba histolytica*

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**ABSTRACT**

Albach, Richard A. (Lutheran General Hospital, Park Ridge, Ill.), James G. Shaffer, and Robert H. Watson. Morphology, antigenicity, and nucleic acid content of the *Bacteroides* sp. used in the culture of *Entamoeba histolytica*. J. Bacteriol. 90:1045-1053. 1965.—Certain changes in morphology, antigenicity, and nucleic acid content that occur in a culture of *Bacteroides* sp. in the presence of penicillin G in CLG medium are described. This "variant" is one of seven recovered in several laboratories, all of which are descendants of the original *Bacteroides* isolated by Shaffer and Frye. Penicillin-inhibited cells of this culture are currently being used in the routine propagation of *Entamoeba histolytica* in CLG medium. Evidence is presented for the loss of ability to react with antibody in these penicillin-inhibited bacteria in CLG medium, when studied by fluorescent-antibody techniques. The implications of the antigenic changes observed as they pertain to similar antigenic studies of the amoebas are discussed. A pronounced reduction in the ribonucleic acid (RNA) content of such penicillin-inhibited cells was also observed. The potential importance of the changes that occur in the RNA of these cells with respect to considerations of the growth requirements of the amoebas is also discussed.

During the course of extensive studies on the mixed bacterial flora of the NRS strain of *Entamoeba histolytica*, Shaffer and Frye (1948) isolated a gram-negative, anaerobic, nonsporulating rod which had a tendency to form short to moderately long chains in fluid thioglycolate medium. This organism was tentatively identified as belonging to the genus *Bacteroides* on the basis of certain morphological and cultural characteristics (Shaffer, 1952, 1953). Penicillin-inhibited cells of this organism were used to develop the Shaffer-Frye (S-F) and cysteine-lactalbumin-glucose (CLG) media for the propagation of *E. histolytica* (Shaffer, Ryden, and Frye, 1948; McDade and Shaffer, 1959).

As interest in the S-F medium increased, transplants of the original culture used to prepare this medium were sent to several laboratories. Over the years, for one reason or another, three separate transplants of the organism have been recovered from these laboratories.

Problems arose, for reasons which are not explainable, in the ability of the original organism maintained in our laboratory to support the propagation of *E. histolytica*. For this reason, three “variants” of the original isolate were produced by serial dilutions and heat treatment. Thus, a total of seven variants of the original *Bacteroides* sp. originally isolated by Shaffer and Frye have been recovered in our laboratory.

In a preliminary note (Albach, Shaffer, and McDade, 1965), it was shown that several of these seven variants of the *Bacteroides* sp. supported the propagation of amoebas in the above media, and several did not. These two “groups” of organisms were also shown to be different with respect to colonial morphology, growth characteristics in liquid media, carbohydrate fermentations, antigenic structure, and ability to produce round bodies (protoplasm) in the presence of penicillin. Those cultures which supported the growth of amoebas produced the largest number of round bodies in the presence of penicillin.

This paper deals with certain changes that occur in one of these variants in the presence of penicillin G in the CLG modification of the S-F medium. At present, penicillin-inhibited cells of this variant support growth of *E. histolytica* in this medium. Such cultures are currently being used in our laboratory for a series of antigenic and biochemical investigations of *E. histolytica*. It was therefore considered essential to determine whether any significant changes in antigenic and
biochemical makeup of these bacteria occur in the presence of penicillin.

The factors responsible for the production of each of several morphological bacterial types (spheroplasts, protoplasts, and swollen forms) observed in cultures of amoebas will be presented. Further experiments on fluorescent-antibody studies of these bacterial cell types will provide evidence for the lack of ability of penicillin-induced forms to react with antiserum produced in rabbits. These studies are of great importance in interpretations of similar studies done on the amoebas. Finally, the changes that occur in nucleic acid content of the bacteria in the presence of penicillin will be described. Such information may be of fundamental importance to the consideration of the dependence of the amoebas on such metabolic activities of the bacterial associate.

**Materials and Methods**

The studies to be reported here were done on the culture currently used for routine propagation of *E. histolytica* in CGM medium. It is possible that the observations to be described here are also applicable to the other variants which have been found to support the propagation of *E. histolytica*.

In routine cultures of this organism, hereafter referred to as *Bacteroides* sp., the typical rod is observed. In the presence of penicillin, three different morphological structures are produced: small spheroplasts, larger protoplasts, and swollen bipolar forms. The experiments outlined in this section were designed to reproduce those circumstances which were responsible for the formation of each of these aberrant forms. This was preliminary to obtaining sufficient numbers of each form for certain analyses of the antigenic and chemical differences in these structures. This information, as will be seen, permitted some important evaluations of certain changes that occurred in the bacterial associate in the medium used for the propagation of amoebas.

**Preparation of penicillin-inhibited cells.** Preliminary experiments on the *Bacteroides* sp. indicated that, by the appropriate manipulation of the conditions of the medium, populations of cells could be achieved in which each of the three cell types mentioned above (spheroplasts, protoplasts, and swollen cells) could be produced. The methods described below were those used to routinely produce large yields of each of these structures.

A stock solution of penicillin G (50,000 units per ml) was prepared in sterile saline (0.85%). The spheroplasts were produced as follows. Test tubes (20.3 by 2.5 cm) containing 40 ml of CGM medium were inoculated with 0.1 ml of a 48-hr culture of bacteria. After 24 hr of incubation at 37 C, 1.5 ml of the stock solution of penicillin (final concentration, 250 units per ml) were added to the culture. It should be noted that, in routine cultures used for propagation of amoebas, this is the same final concentration of penicillin used to prevent bacterial multiplication. The cells were then reincubated at 37 C. Samples were collected for microscopic examination at various time intervals (24, 48, and 72 hr) after the addition of penicillin. Such samples were washed three times with saline, affixed to slides, and stained with crystal violet.

Larger spherical structures (protoplasts) were produced as follows. Test tubes (20.3 by 2.5 cm) were prepared and inoculated with bacteria as described above. After 24 hr of incubation at 37 C, the cultures were centrifuged at 900 X g for 45 min and the supernatant fluid was collected. This procedure was repeated twice. The studies for preparation of routine culture tube containing 2.5 ml of a 24-hr “supernatant” culture of *Bacteroides* sp. collected by centrifugation at 900 X g for 45 min. To this was added 0.5 ml of a mixture of normal horse serum (0.1 ml) and 0.85% saline (0.7 ml); 0.5 ml of a 72-hr culture of amoebas was used to inoculate such tubes. The amount of penicillin carryover by the inoculum of amoebas resulted in a final concentration of approximately 35 units per ml. It was later found that the essential ingredient in the inoculum was the small quantity of penicillin in a somewhat degraded form. The cells, after incubation at 37 C, were harvested, washed, fixed, and stained, as described above, at various time intervals.

Slides of each of these structures were also prepared for fluorescent-antibody studies (see below). These specimens were air-dried on slides, fixed in 95% ethyl alcohol, air-dried, and placed in an incubator (37 C) for several hours to remove all excess ethyl alcohol (Eveland, personal communication).

**Antigenic analyses.** Certain preliminary observations of the three morphological variants induced by exposure to penicillin revealed the following. The swollen cells had a very rigid structure and were not osmotically fragile, and the smaller spherical structures (tentatively identified as spheroplasts) were not as osmotically fragile as were the larger spherical structures (tentatively identified as protoplasts). It was therefore considered likely that the swollen cells possessed cell-wall material. These structures resembled the swollen forms of *Bacteroides* described by Dienes and Weinberger (1951) which give rise to L forms. If these forms are equivalent to those described by these authors, they should possess
preformed bacteria within their structures. Hence, they should, perhaps, possess even more abundant cell-wall material than the "rodsform." The spheroplasts probably had some cell-wall material since they were not osmotically fragile, whereas the protoplasts probably had none.

When cells of the Bacteroides sp. were injected into rabbits, antibodies were produced, which, when examined by fluorescent-antibody (FA) techniques, were directed primarily against the cell-wall components (unpublished data). On the basis of this observation, it was decided to prepare labeled antibody against spheroplasts as well as whole bacteria. Such antibodies were used to stain each of these cell variants to test the hypothesis that each of the three cell variants differed with respect to the amount of cell-wall material each possessed.

The procedures used for preparation of labeled antibody are as follows. Antisera against cells of Bacteroides sp. grown for 24 hr in CLG medium and spheroplasts (see above) were prepared by inoculation of rabbits. The procedures used for rabbit immunization were essentially as described by Shaffer and Balsam (1954). Whole sera were labeled with fluorescein isothiocyanate (FITC; 1 part FITC-40 parts protein), and the globulin was precipitated three times with ammonium sulfate as described by Cherry, Goldman, and Carski (1960) and separated from unbound FITC on a Sephadex (G-25) column.

Slides of the three morphological variants of penicillin-inhibited Bacteroides sp., as well as whole bacteria grown for 24 hr in CLG medium, were stained with the normal and "immune" conjugates by the direct FA method. Stained specimens were evaluated for fluorescence by use of a Spencer microscope equipped with a Fluora-lume light source with an Osram HBO-200 mercury vapor lamp. A Schott BG-12 exciter filter with a Wratten no. 12 barrier filter was used.

Nucleic acid analyses. Previous observations (Zweig and Shaffer, 1961) suggested that the ribonucleic acid (RNA) content of the Bacteroides sp. was either diluted or decreased in value when protoplasts were produced. This hypothesis was tested by employing differential chemical extraction of the nucleic acids. Acetone powders of cells of the Bacteroides sp. grown for 24 hr in CLG medium and penicillin-inhibited bacteria (spheroplasts and protoplasts) were prepared according to the procedures of Umbreit, Burris, and Stauffer (1950). The acid-soluble fraction (ASF), RNA, and deoxyribonucleic acid (DNA) fractions were removed by differential perchloric acid extraction according to the procedures of Ogur and Rosen (1950), Woods (1957), and Albach (1963). The nucleic acids were then estimated by the absorbancy at 260 m\(\mu\) (Pearse, 1960). A recent discussion of the efficacy of these procedures has been given by Aldridge and Watson (1963). Lipids were removed prior to removal of RNA and DNA with three extractions of ethyl alcohol-ether (3:1) at 40 C.

**Results**

**Morphology of penicillin-inhibited cells.** The morphology of a typical 24-hr culture of Bacteroides sp. is shown in Fig. 1. The three cell variants produced in the presence of penicillin G are shown in Fig. 2, 3, and 4.

When penicillin (250 units per ml) was added directly to a 24-hr culture of this organism, complete dissolution of the cell walls did not occur. Small spherical structures, probably spheroplasts were produced (Fig. 2a to c). These structures could be seen within a few hours after the addition of penicillin and were produced by use of concentrations of penicillin ranging from 10 to 1,000 units per ml. Note also that cell walls persisted in these preparations even 48 hr after the addition of penicillin (Fig. 2a and b). Such structures were also present in abundance after 48 hr in control cultures to which no penicillin was added. The time required for producing these structures varied inversely with the concentration of penicillin. It is probable that they are equivalent to dying cells found in a normal 48- to 72-hr culture.

When penicillin (250 units per ml) was added to a 24-hr "supernatant" culture of Bacteroides sp., protoplasts were produced within several hours. These cells were larger in 48- and 72-hr

![Fig. 1. Typical 24-hr culture of Bacteroides sp. stained with crystal violet; \(X\) 970.](http://example.com/fig1.jpg)
Fig. 2. A 24-hr culture of Bacteroides sp. exposed to penicillin G (250 units per ml) 24 hr (a), 48 hr (b), and 72 hr (c) after the introduction of penicillin. Crystal violet; X 970. Note the presence of small round structures which are probably spheroplasts.

Fig. 3. A 24-hr supernatant culture of Bacteroides sp. (50 to 70 million cells per milliliter) exposed to penicillin G (250 units per ml) 24 hr (a), 48 hr (b), and 72 hr (c) after the introduction of penicillin. Crystal violet; X 970. Note the presence of round structures which increase in size over the 72 hr of incubation. These are thought to be protoplasts.
samples (Fig. 3a to c). These structures have previously been described and are ingested by *E. histolytica* in CLG medium (Shafer, Schuler, and Key, 1958). After harvesting and washing these structures, it was noted that the “cell pellet” contained two layers: an upper dark layer and a lower “whitish layer.” Slides of each of these layers were prepared and stained with crystal violet. The cells (protoplasts) of the upper layer were generally larger and did not stain as intensely as those of the lower layer. Those of the lower layer resembled spheroplasts as seen in Fig. 2.

The production of swollen cells (Fig. 4) was consistently obtained when a routine tube of *E. histolytica* was prepared in which the only penicillin available was that which was carried over by the 0.5 ml of inoculum of amoebas (see Materials and Methods). An intensive investigation of the factors involved in the apparent synchronous production of these structures and their nature is under way. These cell types were produced within 48 hr. Initial experiments indicate that the small carry-over penicillin in a degraded form yet to be resolved may be responsible for the production of these aberrant forms.

**Antigenic analysis.** The staining of cells of *Bacteroides sp.* with anti-*Bacteroides sp.* FITC-globulin was positive and yielded a titer greater than 1:160. Swollen cells produced after 72 hr exhibited an even greater fluorescence to the same antibody conjugate. Photographs taken of these cell types stained with a 1:10 dilution of anti-*Bacteroides sp.* FITC-globulin are shown in Fig. 5. Photographs of control specimens stained with normal FITC-globulin are also shown for comparison. The more intense fluorescence of the swollen cells (Fig. 5c) as compared to whole bacteria (Fig. 5a) is clearly evident. The areas of greatest staining intensity appear to be in the cell walls of whole bacteria and in the periphery of the central swellings of swollen cells (Fig. 5e).

Protoplasts, 24 hr after their formation, exhibited a minimal staining reaction with a 1:10 dilution of the same antibody conjugate used above, doubtful staining after 48 hr, and a complete loss of staining after 72 hr. Spheroplasts again showed only a minimal staining reaction with this antibody conjugate.

A 1:10 dilution of FITC-labeled antishpleftrightarrow{s}plast globulin only slightly stained the spheroplasts used for the production of this antibody. Apparently, spheroplasts are poor antigenic material when used as outlined in these experiments. This same labeled antibody, however, stained whole bacteria to a greater degree than it did spheroplasts, and swollen cells to an even greater degree. It should be noted that the intensity of staining of both whole bacteria and swollen cells with this antibody was much less than the staining observed when conjugate labeled with anti-*Bacteroides sp.* was used. Protoplasts yielded negative results with antishpleftrightarrow{s}plast antibody conjugate. Brenner et al. (1958) pointed out that the term protoplast should be applicable only when it is fairly well established that spherical forms of bacteria do not possess cell-wall material. Spherical structures of bacteria which are not shown to lack cell material have since been constantly referred to in the literature as spheroplasts. Thus, the terms spheroplast and protoplast as used in this paper seem appropriate.

**Nucleic acid analyses.** The RNA content of *Bacteroides sp.* harvested after 24 hr of growth was approximately 13% (acetone, dry weight), whereas RNA of spheroplasts was 6.4%.

Protoplasts increased in size between 24 and 72 hr after the addition of penicillin (see results, above). It was of interest to determine whether any differences could be observed in the nucleic
All photographs were taken on a fluorescence microscope. Exposure time was 30 sec, and 35-mm Tri-X film was used. (a to d) X 500; (e) X 970. (a) Bacteroides sp. grown for 24 hr in CLG medium and stained with a 1:10 dilution of anti-Bacteroides sp. conjugate; (b) same organism as in (a) stained with a 1:10 dilution of conjugate of normal rabbit serum; (c) swollen forms stained with a 1:10 dilution of anti-Bacteroides sp. conjugate; (d) same organism as in (c) stained with a 1:10 dilution of conjugate of normal rabbit serum; (e) same as (c), but at a higher magnification to show intensity of fluorescence in central swellings.

The acid content of such cells in these various "stages" of protoplast development. The results indicated that, as protoplast size increased, there was a concomitant decrease in the RNA on a dry weight basis. The values obtained for the RNA content of these cells were: 7.3% after 24 hr, 5.3% after 48 hr, and 4.4% after 72 hr.

The DNA content of Bacteroides sp. harvested after 24 hr of growth was estimated at 3% (acetone, dry weight). The values obtained for
spheroplasts and protoplasts (24, 48, and 72 hr) were approximately 4%.

**DISCUSSION**

The following evaluations of certain changes in morphology, antigenicity, and nucleic acid content that occur in the variant currently being used to support propagation of *E. histolytica* in CLG medium can be made. This organism will be referred to for purposes of this discussion as *Bacteroides* sp.

**Morphology of penicillin-inhibited Bacteroides sp.** The production of small spherical cells, almost completely devoid of cell-wall material (probably spheroplasts), and larger spherical protoplasts, which apparently possess no cell-wall material after 72 hr, are generally produced in the same culture. The percentage of each of these types in a given population is apparently dependent on the number of cells present at the time of addition of penicillin. If penicillin is added directly to a 24-hr culture of the *Bacteroides* sp., the small rounded structures (spheroplasts) are produced, whereas, if the 24-hr culture is harvested and penicillin is added to the supernatant fluid containing 50 to 70 million bacteria per milliliter, primarily protoplasts are produced. This latter procedure simulates those employed in our laboratory for the preparation of routine cultures of amoebas. Whether spheroplasts or protoplasts are produced in a given culture can probably best be explained on the basis of the availability of the penicillin to the cells. In the first instance, less penicillin per cell is available, and an incomplete inhibition of new cell-wall synthesis probably occurs in a population which has already reached its peak of growth. A complete investigation of the third cell type (swollen cells), probably precursors of L forms, produced in the presence of very small amounts of penicillin is still under way.

**Antigenic analysis.** The antigenic nature of the *Bacteroides* sp. when studied by FA techniques appears to be almost, if not completely, limited to the cell walls. Protoplasts (after 72 hr) do not stain with FITC-labeled anti-*Bacteroides* sp. globulin, whereas the smaller spheroplasts apparently do possess some of the antigenic character of the whole *Bacteroides* sp. This is significant when one considers the potential use of amoebas grown in CLG medium in the preparation of antibody in rabbits for experimental purposes with human sera.

The harvesting of large numbers of *E. histolytica* for the purpose of producing antigen for human serological tests, or the immunization of rabbits, involves differential centrifugation for removal of the bacteria or bacterial debris, or both. The presence of bacteria ingested by the amoebas, however, must be considered in any interpretation of such serological tests (Swart and Warren, 1962). This is especially true when the *Bacteroides* sp. is the associate, since antibody to such organisms may be present in human serum. Swart and Warren presented evidence indicating that antibody produced in rabbits from amoebas harvested from a culture containing *Bacteroides* is not directed against the bacteria. Also, Shaffer and Ansfield (1956) found that anti-*Bacteroides* sp. sera did not inhibit the phagocytic activity of amoebas grown in the presence of *Bacteroides* sp., whereas anti-amoeba sera did.

In CLG medium, *E. histolytica* ingests protoplasts (Shaffer et al., 1958). Since such structures (after 72 hr) do not stain with anti-*Bacteroides* sp. conjugate, it would seem likely that the presence of ingested protoplasts will not appreciably contribute to the antigenic character of *E. histolytica* when grown in CLG medium. Preliminary investigation by FA techniques of this problem employing amoebas which have ingested protoplasts or whole *Bacteroides* sp. show that, indeed, the contribution of bacterial antigen is negligible.

The intense fluorescence observed when swollen forms were stained with anti-*Bacteroides* sp. conjugate is of considerable interest. As mentioned previously, these forms resemble the structures observed by Dienes and Weinberger (1951) which give rise to L forms. The evidence presented here indicates that these swollen forms possess more antigenic material than do the cells (24-hr bacteria) used for antibody production. This appears to support the contention of Dienes and Weinberger (1951) that these forms possess preformed bacteria within their structure. Actually, upon close examination of these fluorescing swollen cells, it was simply observed that a great deal of cell-wall material was present in the periphery of the central swellings of such structures.

**Nucleic acid analyses.** Zweig and Shaffer (1961) presented evidence based on acridine orange staining techniques, that protoplasts of these gram-negative anaerobes when first formed might contain a considerable amount of RNA. As the structures enlarge, they apparently lose or dilute this substance. It was also noted by these authors that, at the time these structures lost their RNA, the medium lost much of its ability to support the propagation of *E. histolytica*. They also observed that the addition of RNA to the medium was stimulatory for propagation of the amoebas. Further, when amoebas ingested these structures in the initial stages
of protoplast development, they (the protoplasts) apparently rapidly lost their RNA. It may thus be inferred that the amoebas are possibly rapidly utilizing the RNA of such bacteria.

The data presented in this paper support the contention that protoplasts, formed in routine medium in which penicillin is added, do indeed lose much of the RNA originally present in the intact cell. This effect is noted 24 hr after the addition of penicillin to the cultures, and is even more pronounced 48 and 72 hr after the addition of penicillin. Thus, the RNA of the Bacteroides sp. in the presence of penicillin apparently degrades to a form which may be readily utilized by the amoebas upon ingestion of the protoplasts. This utilization of partial degradation products of the RNA of intact protoplasts (e.g., possibly labile nucleotides which might serve as coenzymes for biosynthesis) may well be one of the important contributions to the propagation of E. histolytica in S-F and CLG media.

This may be the reason that several of the seven variant cultures of Bacteroides fail to effectively support the propagation of E. histolytica in S-F and CLG media. These cultures do not effectively produce protoplasts in the presence of penicillin. Presumably, the RNA of these “non-supporters” remains relatively intact. It is possible that the RNA or RNA degradation products of such structures is not readily utilized by the amoebas upon their ingestion.

Such may also be the explanation of various reports that “...the incorporation of penicillin and streptomycin in cultures of E. histolytica being maintained in the laboratory in mixed bacterial flora or for primary isolation from stool material, enhances the propagation of amebae. It has been assumed that the function of the antibiotics in such cultures was to prevent excessive propagation of the accompanying bacteria. Although this inhibition of bacterial multiplication is probably of the greatest importance, it is possible that the effect of penicillin on certain of the bacterial cells may have some enhancing effect on the propagation of amebae” (Shaffer et al., 1958). This effect may be the partial degradation of the RNA of certain of the bacteria by the antibiotics. Upon ingestion of such structures by amoebas prior to the release of the breakdown products of RNA into the medium, potential growth factors (e.g., nucleotides) may now be readily available to the amoebas for biosynthetic reactions.

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