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THE CULTIVATION OF ENDAMOEBA HISTOLYTICA AND THE IN-VITRO CHEMOTHERAPEUTIC TESTING OF AMEBACIDES[1]

By MAURICE E. CALLENDER

Endamoeba histolytica is the casual organism of the disease, amebiasis. Global distribution of high incidence makes this protozoan infection an enormous public health and economic problem. Cultural behavior and the ensuing search for effective chemotherapeutic agents has stimulated widespread interest in the parasitology and associated bacteriology of this organism.

A literature far too voluminous for presentation within the limitations necessary for the present paper has shown that it has not been possible to cultivate *E. histolytica* entirely free from microbial cells or their products. Apparently the concomitant microbial flora creates the physicochemical environment required by the amoeba for its continued well being. In 1949, Shaffer, Ryden, and Frye (21) published results of work which has been the closest approach to the goal of completely bacteria-free cultures. Their method, representing a few simplifications of an earlier one, used a medium containing a non-multiplying single species of an unidentified streptobacillus.

An adequate review of the literature dealing with the chemotherapy of *E. histolytica* is likewise beyond the limitations of the present paper. A few of the significant features may be briefly pointed out. Amebacidal properties of a number of antibioitcs have been studied. In the case of penicillin: Knoll and Howell (15) found no effect upon trophozoites. Streptomycin in concentrations of 10,000 u/ml was found to have no effect upon the cysts and some trophozoites were also unaffected. Belamuth and Wieboldt (3). Spingarn and Edelman (24) found that the addition of 1000-3000 u/ml of streptomycin prolonged the survival of cultures of *E. histolytica.*

[1] A portion of a thesis submitted in partial fulfillment of the requirements for the Master of Science degree in the Division of Graduate Instruction, Butler University. This paper has been greatly abbreviated for publication. The complete manuscript may be obtained on loan from the Butler University Library.

Using a combination of penicillin and streptomycin in equal unitage, Seneca, Henderson, and Harvey (19) published that 2000 u/ml kills amoeba in the first generation. However, Faust (6) found that a combination of 10,000 u of streptomycin and 5000 u of penicillin had no effect upon the amoeba. The present writer has found that a combination of 1000 u of penicillin and 2000 u of streptomycin per ml. can be used routinely in the isolation of trophozoites from their mixed bacterial flora.

Anderson et al. (1) found subtilin to be an effective amebacide in dilutions up to 1:400,000. Gould and Hansen (12) found that bacitracin, Polymixin B and streptomycin hydrochloride were lethal in "approximately 1/16 the minimal apparent amebicidal concentrations of the separate compounds when they were added together to the open or sealed cultures."

Falsenfeld et al. (7) claim, without publishing supporting data, that *E. histolytica* was inhibited in vitro by aureomycin, bacitracin and neomycin. Hewitt, Wallace, and White (13) have found aureomycin to be effective in dilutions as high as 1:100,000. Fuller and Faust (9) obtained good growth of *E. histolytica* in dilutions of aureomycin ranging from 1:300,000 to 1:1,000,000. Ishihara and Felsenfeld (14) found that neomycin inhibited growth in concentrations of 100 units/ml. Thompson et al. (26) found chloramphenicol to be effective as an amebacide in dilutions up to 1:3,496, but Smith et al. (23) found it effective only up to 1:1000 dilution and attributed the result to inhibition of associated bacterial flora. McCowen, Callender, and Lawlis (17) have found fumagillin, a new antibiotic, to be effective as an amebacide.

Emetine has given variable results when used as an amebacide, dilutions varying from 1:10,000 to 1:1,000,000 being effective (Fulton et al., 10; Dennis, Berberian, and Hansen, 5; Hewitt, Wallace and White, 13: Laidlaw, Dobell, and Bishop, 16; St. John, 25; Goodwin, Hoare and Sharp, 11). The last named workers also found cabarsone and chiniofon to be effective in dilutions of 1:10,000.

The present paper presents results from a study of several strains of *E. histolytica,* their cultivation on various media, and their exposure to antibiotics and known amebacides.

MATERIALS AND METHODS

CULTIVATION

This phase of the investigation was an attempt to establish strains of *Endamoeba histolytica* with various single species of bacteria and with various mixed bacterial flora. Three strains of *E. histolytica* have been used. These strains include the F-19, originally isolated by Dr. E. C. Faust, and NIH 200, obtained from Dr. C. W. Rees at the National Institute of Health. Nine species of bacteria were used in this study. All bacteria mentioned in this paper are named according to the 6th edition of Bergey's *Manual of Determinative Bacteriology.* Some of the bacteria were selected because they had been included in similar work by other investigators. Other species were selected because of their similarities to organisms in the first group; and still others were selected because of their widely differing characteristics from those of the first and second groups. Five media were utilized. These media included the Frye-Meleney (8) modification of the Boeck-Drbohlav (4) media, the Entamoeba medium of the Difco Laboratories, the Egg Infusion medium of Balamuth (2), the medium of Shaffer and Frye (20), and a medium consisting of nutrient agar slants overlayed with a serum saline mixture. Tables IV[2] through VIII[2] show the preparation of the various media.

The method used for experiments 1, 3, 4 and 5 was as follows: The media was inoculated with a 2 mm loop of bacterial organism obtained from a 48-hour culture grown on nutrient agar slants. The inoculated media was incubated for 24 hours at 37° C. After this period of incubation, sterile rice powder (Difco) was added and the media inoculated with 0 5 ml of a 72-hour culture of *E. histolytica* grown in the Shaffer-Frye (20) medium. The completely inoculated cultures were examined at 72 hours by taking a portion of the sediment containing rice powder and debris and examining under the microscope.

Experiment 2 differed in that the inoculation of *E. histolytica* was taken from the sixth culture of *E. histolytica-Bacillus cereus* from ex-

[2] Table numbers are those of the original thesis. Since their results are summarized in the published portion of the thesis, the tables are omitted from publication because of printing costs. They may be secured on loan from the Butler University Library.

periment 1. Experiment 6 was an attempt to establish the growth of *E. histolytica* upon a more simple medium, nutrient agar slants with an overlay of serum saline. Table X[2] lists the experiments with the various bacteria, amoebae and media used in each.

Chemotherapy

Five strains of *Endamoeba histolytica* have been used in the chemotherapy testing. These strains include the NRS strain obtained from Dr. J. G. Shaffer, Vanderbilt University; Luna, also obtained from Dr. Shaffer; NIH 103, obtained from Dr. Rees of the National Institute of Health; and the previously mentioned NIH 200 and F 19. The NRS and NI H103 have been used widely by many investigators seeking new amebacides. The NIH 200 is a strain highly pathogenic for laboratory animals and is being used as the infecting organism for *in-vivo* chemotherapy studies in rabbits, guinea pigs and rats. Strain F 19 has been utilized for the bulk of the work reported in this paper. The Luna strain is the most recently isolated of these five strains of amoebae. The isolation was accomplished by Dr. Shaffer at Vanderbilt University in February 1948. This was the first strain to be isolated from a human case of amoebiasis using the clear medium of Shaffer and Frye (20). Many parallel tests were run, using a compound against these several strains of amoebae. No appreciable difference was noticed in the behavior of the different strains. All of the strains employed were in regular association with mixed bacterial flora with the exception of strains grown and tested with the Shaffer-Frye (20) medium.

Test procedure I utilized a modified Boeck-Drbohlav medium, (tables IV[2] and IX[2]). The procedure of the test is as follows:

TEST PROCEDURE I

1. Add drug to overlay in tubes to make final desired dilution.
2. Add inoculum of 48-hour culture of *E. histolytica*.
3. Incubate 48 hours at 37° C.
4. Examine sediment for presence of amobae and subculture all negative tubes and faintly positive tubes.
5. Examine subcultures after 48 hours incubation at 37° C.
6. Record final results from the subcultures. (*Complete* absence of amobae in subculture tube is indicative of activity).

Test procedure II used the liquid Balmuth (2) Egg-yolk Infusion medium. See table VI[2] for preparation and composition of this medium. The procedure for this test is identical with procedure I.

Test procedure III also used the liquid Balamuth Egg-yolk Infusion medium. The details of this procedure follow.

TEST PROCEDURE III

1. Inoculate all tubes with 72-hour culture of *E. histolytica.*
2. Incubate 48 hours at 37° C.
3. Examine all tubes for presence of amoebae.
4. Add necessary amount of drug to tubes to give desired dilution.
5. Re-incubate 24 hours at 37° C.
6. Examine all tubes and make necessary sub-cultures.
7. Incubate subcultures for 48 hours at 37° C.
8. Examine subcultures and report.

Test procedure IV used the modified Shaffer-Frye medium described in table VIII[2]. The details are as follows:

TEST PROCEDURE IV

1. Place 2.0 ml of serum, saline, Penicillin G mixture in all tubes.
2. Place drug in first tube and make proper dilution to give desired drug dilution.
3. Add 2.0 ml of streptobacillus supernatant to each tube.
4. Add 1.0 ml of inoculum of *E. histolytica* to each tube.
5. Overlay tubes with sterile vaseline and incubate 48 hours at 37° C.
6. Examine each tube for presence of amoebae and make necessary subcultures.
7. After 48 hours incubation at 37° C. examine the subcultures.
8. Record final results.

Test procedure V also made use of the Shaffer-Frye medium.

TEST PROCEDURE V

1. Inoculate Shaffer-Frye media with *E. histolytica.*
2. Overlay with vaseline and incubate 48 hours at 37° C.
3. Examine each tube microscopically.
4. Add drug to each tube to give desired dilution.
5. Incubate 24 hours at 37° C.
6. Examine all tubes and make subcultures.
7. Examine subcultures after 48 hours at 37° C.

Test procedure I was used because it was the general method most often used by earlier workers. The medium used in this test has some serious faults. Prominent among these faults is the property of the egg slope to absorb certain chemicals. This can easily give erroneous results.

Test procedure II was the method most widely used in this paper. This method of testing utilizes a liquid medium which is low in protein and which also permits luxuriant growth of the amoebae.

Test procedure IV is the same method of testing as I and II, but utilizes an entirely different type of medium. This medium should give information as to the power of the amehacide to act directly upon the protozoan. This information is not clearly seen with the preceding test procedures.

Test procedures III and V, although using different media, are alike in that they test the power of the drug to destroy the organism as opposed to the power of inhibiting the growth of the organism.

In test procedures I, II and III, the inoculum of *E. histolytica* was in the range of 10,000-12,000 organism per ml of culture. This inoculum was obtained from 125 ml flasks containing the modified Boeck-Drbohlav media. A 72-hour culture of this was gently but well shaken and a representative sample counted in the manner described by Paulson and Morgenstern (18). Dilutions were then made to give the required number of organism per ml.

When utilizing the test procedures IV and V, smaller number of amoebae in the inoculum could be used. A number of amoebae amounting to 100 or 1,000 organisms per ml was used. This ten fold difference apparently led to no difference in results.

In all of the test procedures, many subcultures were made. All negative tubes were subcultured as were all faintly positive tubes. A number of heavily positive tubes were also subcultured for control purposes. Tubes designated as faintly positive contained less than 5 organisms per field when viewed through a microscope with 10 X objective and 10 X oculars. The effective dilution is that lowest dilution which is negative after the reading of the subcultures.

OBSERVATIONS AND RESULTS

Cultivation

Experiment 1: The inoculated cultures were examined at 72 hours by taking a portion of the sediment containing some rice powder and examining it under the microscope. This examination revealed motile organism only in the tube containing *Bacillus cereus*.

This tube was subcultured into fresh medium. Growth was maintained through a total of 17 subcultures at which time the cultures, still highly positive for *E. histolytica*, were discontinued.

Experiment 2: The growth of *E. histolytica* continued to be luxuriant with no apparent effects from the various bacterial mixtures. After 11 subcultures, with no demonstrable change in the numbers of *E. histolytica* present, the cultures were discontinued.

Experiment 3: Examination of 72-hour cultures failed to detect the presence of *E. histolytica* in any of the tubes.

Experiment 4: This was another repeat of experiment 1, and completely negative results were again obtained.

Experiment 5: No growth of *E. histolytica* was detected in any of the tubes inoculated.

Experiment 6: Both media used supported the growth of each of the amoebae-bacterial cultures through a series of 12 sub-cultures, at which time the various series were discontinued.

During this series of tests, it was noticed that if a drop of the positive cultures was placed on a glass slide, covered with a coverslip, ringed with vaseline or paraffin and reincubated at 37° C., the organism would survive and remain highly active and in large numbers for periods up to an additional 52 hours. This observation is of some use for classroom demonstration.

Chemotherapy

A total of 216 in-vitro chemotherapeutic tests are included in this report. Table XI[2] shows the complete tabulation of these tests. The results obtained using test procedure I correlated well with other investigators utilizing like methods. Emetine hydrochloride demonstrated activity at 1:20,000, Carbarsone oxide, 1:100,000 and Chloroquine, 1:500. Subtilin was the only antibiotic tested with this procedure, and no activity could be shown in a dilution of 1:500.

Test procedure II also correlated well with the observations of other investigators. This method of testing was applied to all of the drugs and antibiotics reported in this paper. Emetine hydrochloride demonstrated amebacidal activity in a dilution range of 1:100,000 to 1,500,000. Carbarsone oxide has amebicidal activity in the same

range as Emetine hydrochloride. Chiniofon was active in the range of 1:1,000 to 1:5,000 range. Carbarsone demonstrated no amebicidal activity in a dilution of 1,3,000. Chloroquine demonstrated amebicidal activity over a wide range, 1:500 to 1:5,000; but 84% of the tests performed showed activity at 1:1,000 or lower.

Among the antibiotics tested, Fumagillin was by far the most active substance tested. Dilutions as high as 1:262,144,000 completely inhibited the growth and reproduction of the amoebae. This antibiotic is especially interesting in that it possesses no anti-bacterial spectrum. Actidione demonstrated activity of a high order with a 1:10,000,000 dilution proving effective. Aureomycin was active at 1:64,000 dilution, with Terramycin showing activity at 1:32,000. Chloromycetin was active in a dilution of 1:4,000 while Bacitracin and Subtilin failed to demonstrate activity in a dilution of 1:500.

A modification of test procedure II by overlaying the medium with liquid vaseline was also used to test the activity of Fumagillin with identical results being obtained.

Test procedure III was used in the evaluation of Fumagillin. Here again, activity could be shown in a dilution of 1:131,072,000.

Test procedure IV was utilized to show the ability of the compounds to act upon the amoebae directly. The previous test procedure results are all difficult to evaluate because of the adsorption of the drug, interference of bacteria, and the action of the drug upon the bacteria. Emetine hydrochloride demonstrated activity in a range of 1:20,000 to 1:100,000. Carbarsone oxide continued to show activity in a range of 1:64,000 to 1:200,000. Chiniofon had activity at 1:2,000 while carbarsone was active at 1:8,000. Chloroquine failed to demonstrate activity at 1:500.

Fumagillin continued to demonstrate the highest amebicidal activity of any substance yet reported, with activity at 1:131,072,000. Actidione had activity at 1:20,000,000 to 1:50,000,000. Subtilin, which had failed to demonstrate activity in the earlier test procedures, now showed activity in the dilution of 1:20,000,000. The activity of Aureomycin decreased slightly to 1:32,000 while Terramycin dropped to 1:4,000.

Test procedure V was used only in the evaluation of Fumagillin. Activity was present in the dilutions of 1:131,072,000 to 1:262,144,000.

SUMMARY

Cultivation

A review of the cultivation of *E. histolytica* is given, and the possible role of bacteria in such cultivation is presented.

Attempts have been made to isolate trophozoites of *E. histolytica* with certain single species of bacteria. *Bacillus cereus* on one occasion supported the growth of the amoebae so that it could be cultivated with this single species. Other attempts to repeat this failed.

It has also been sbown that the addition of *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli* and *Micrococcus epidermidis,* either singly or in combination, to cultures of *Endamoeba histolytica* growing with *Bacillus cereus* failed to influence the growth of the amoebae.

Efforts to isolate the amoebae with pure cultures of *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Micrococcus epidermidis, Bacillus circulans* and *Aerobacter aerogenes* also failed.

The cultivation of *Endamoeba histolytica* with a mixed bacterial flora and with organism "t" has been accomplished with the use of nutrient agar slants overlaid with a serum-saline mixture and with rice powder added.

Chemotherapy

A review of the *in-vitro* chemotherapy of *Endamoeba histolytica* is given. Several amebacides of clinical value and many of the newer antibiotics have been tested in various methods using five strains of *Endamoeba histolytica,* and the results are given.. Of all the drugs and antibiotics tested, Fumagillin appears to be the most potent in every method. The antibiotic, Subtilin, presents a most interesting phenomena. This antibiotic, when tested with a mixed bacterial flora, demonstrates no amebicidal activity. When tested with a single species of bacteria, organism "t," activity is shown in a dilution of 1:400,000. Tests made with the Shaffer-Frye medium containing no actively multiplying bacteria, reveal activity in dilutions of

1:20,000,000. These last observations are in correlation with the direct microscopical examinations of Anderson, Villela, Hansen and Reed (1946). Table XII[2] compares the results reported in this paper with the results obtained by previous investigators.

CONCLUSIONS

1. *Endamoeba histolytica* can be isolated and grown with a pure culture of *Bacillus cereus*.

2. Addition of various other bacterial species failed to influence the growth of *E. histolytica* in association with *Bacillus cereus*.

3. It is possible to maintain cultures of *E. histolytica* growing both with a mixed bacterial flora and with a single species of bacteria upon a medium consisting of nutrient agar slants overlaid with a serum saline mixture with added rice starch.

4. Three methods of testing must be included for the complete evaluation of the compounds tested. One method should include a mixed bacterial flora to determine if the agent may be inactivated by such bacteria. Another method of testing should include no actively multiplying bacteria so that the direct effectiveness of the substance against the accompanying microbial organism may be ascertained. The third method of testing should be a method whereby the effectiveness of the substance to destroy as opposed to mere inhibition can be evaluated.

5. With the utilization of the fore-mentioned methods, one substance has consistently proved to be highly effective and should be thoroughly tested in cases of human amoebiasis. This substance is an antibiotic, Fumagillin.

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