1960

Serological studies of entamoeba histolytica.

Morello, Josephine A

Boston University

http://hdl.handle.net/2144/17825

Boston University
Thesis

SEROLOGICAL STUDIES OF ENTAMOEBA HISTOLYTICA

by

JOSEPHINE A. MORELLO

(B.S. Simmons College, 1957)

Submitted in partial fulfilment of the requirements for the degree of
Master of Arts
1960
Approved
by

E. E. Baker
First Reader
Professor of Microbiology

Matthew A. Denman
Second Reader
Assistant Professor of Microbiology
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Edgar E. Baker, Professor of Microbiology, Boston University School of Medicine for his patient guidance and invaluable advice throughout the course of this work.

I am also grateful to Dr. Matthew A. Derow, Assistant Professor of Microbiology, Boston University School of Medicine for his assistance with the fluorescent antibody technic and the preparation of this thesis.
This work was supported in part under contract # DA-49-007-MD-711 from the Research and Development Division, Office of the Surgeon General, Department of the Army.
# Table of Contents

- Introduction .................................................. 1
- Materials and Methods .................................. 18
- Experimental Results ..................................... 31
- Discussion .................................................... 37
- Summary ....................................................... 44
- Illustrations ............................................... 45
- Bibliography ............................................... 47
ILLUSTRATIONS

FIGURE 1. *E. histolytica* culture treated with fluorescein-conjugated anti-*E. histolytica* culture serum. 45.

FIGURE 2. *E. histolytica* trophozoite grown in culture with *B. symbiosus*. 46.
INTRODUCTION

I. Historical Review

In 1875, Lösch (26) published the first precise description of amebic dysentery. He found large numbers of active amebas, many with ingested red blood cells, in the stools of a patient suffering from relapsing dysentery in St. Petersburg, Russia. Post mortem examination of the patient revealed marked ulceration of the large intestine, and the presence of numerous motile amebas in the lesions. In an attempt to produce experimental infections, he administered dysenteric stools to 4 dogs by mouth and rectum. One of the dogs contracted dysentery, and active amebas were recovered from stools and intestinal lesions of the animal. Lösch did not, however, consider the amebas to be the primary cause of infection. He felt, rather, that they served only to delay or prevent the healing of an inflammation caused by some other agent. He included descriptions and drawings of the organism in his report, and suggested that it be named *Amoeba coli* because the colon appeared to be its primary site.

Koch, in 1883 (34), while conducting autopsies on 5 dysentery patients in Egypt, found that two of the infections had been complicated by liver abscesses. In stained sections from these patients, he observed "peculiar ameba-like structures" at the base of intestinal lesions and also, in one case, in the capillaries near a liver lesion. These
findings were indicative to him of a close relationship between the amebas and the disease.

Between 1886 and 1891, Kartulis (30, 31, 32) published several papers in which he concluded that amebas were the cause of "tropical dysentery." He found amebas identical to those described by Lüsch in the ulcers, and tissue beneath the ulcers of dysentery patients. Amebas were also present in the pus of liver abscesses which frequently accompanied the infections. In 1904 (33), he reported the presence of the same organism in abscesses of the brain following dysentery. He also recorded that he was able to infect cats by rectal injection of feces containing amebas, and thereby produce dysentery in them experimentally (32).

Hlava (23), in 1887, also decided that amebas were the cause of dysentery because he was able to infect 2 of 17 dogs and 4 of 6 cats by the rectal injection of human stools containing amebas. He was unsuccessful, however, in transmitting the disease to rabbits, chickens, or guinea pigs. His publication included a report of finding amebas in 60 cases of dysentery in Prague.

The subsequent designation of amebic dysentery as a specific disease characterized by definite pathological lesions produced by the amebas is attributed to Councilman and Lafleur (8). They made an intensive study of 15 cases of infection, and in their monograph of 1891, they introduced the terms "amebic dysentery" and "amebic abscesses of the liver." They renamed
the etiologic agent Amoeba dysenteriae.

Further proof of the role of the amebas in infection was provided by Kruse and Pasquale (35) in 1894. They reported the production of dysentery in cats by the rectal injection of liver abscess pus which contained amebas, but was bacteriologically sterile.

Schaudinn (58), in 1903, published an erroneous interpretation of the life cycle of the amebas, thereby causing confusion for many years. It was he, however, who coined the name Entamoeba histolytica for the organism "because of their capacity to destroy tissue."

II. Experimental Amebiasis in Animals

During the course of the work to be reported here, it was thought that one of the best methods for obtaining immune sera for serological studies would be to induce clinical or subclinical infections with E. histolytica in animals. As previously indicated, beginning with the discovery of the organism, numerous investigators have been active in the study of experimental amebiasis.

A. Types of Animal Employed

The kitten has been a popular animal for amebic studies because it is especially susceptible to the disease and does not harbor endamebas except when experimentally infected. Hlava (23), Kartulis (32), Quincke and Roos (52), and Kruse and Pasquale (35) were among the early workers who successfully produced lesions in kittens by the rectal inoculation of stools containing the amebas, or the oral administration of the cyst
form of the organisms.

Sellards and Baetjer (59), in 1914, reported that they were able to produce amebic infection in nearly 100 per cent of kittens by injecting stools containing the amebas directly into the cecum. All stages of the disease were produced including acute fatal infections, chronic infections with relapses and liver abscesses, and carriers. Not only was the disease propagated through 11 generations, but also the virulence of the infection was increased on repeated passages.

In 1925, Boeck and Drbohlav (4) were able to isolate and grow in cultures the organisms from 2 cases of amebic dysentery. They found both strains to be pathogenic for kittens even after prolonged cultivation. Injection of the organisms produced acute dysentery, colitis, and in two cases, liver abscess.

Meleney and Frye (42) developed a method for the injection of cultures of *E. histolytica* directly into the ileum, because only a small percentage of kittens became infected when given rectal injections. They felt that this allowed the amebas to enter the large intestine as they normally would in oral inoculations with cysts. The lesions produced in the kittens did not differ essentially from those produced by rectal infection, and the incidence of infection was more than doubled.

Although the characteristic lesions were usually super-
ficial, amebic infections in kittens were most often acute and fatal. The disease produced in this animal, therefore, bears no great similarity to the disease as present in man.

Despite the fact that Lösch (36) produced the first experimental amebic lesions in dogs, this animal was not used extensively for some time.

Faust (14, 15) conducted several studies with dogs. He was able to induce infection in animals with both human and canine strains of *E. histolytica* by the injection of trophozoites from infected dogs into the posterior levels of the ileum of uninfected dogs. Extensive microscopical studies were also made by him. Amebic lesions in dogs were usually confined to the large intestine with great numbers of amebas invading the underlying mucosa; lesions generally comparable to those found in man.

Swartzwelder (64, 65) attempted to infect dogs with both cysts and trophozoites of the organism. In 1937, he reported that 5 of 13 dogs who ingested trophozoites of *E. histolytica* in a cyst-free medium became infected. In 1939, he found that he could not produce infection of dogs by injection of cysts into the ileum and large intestine of dogs following laparotomy. Ingestion of cysts, however, was followed by excystation of the organisms in the ileum and large intestine with tissue invasion occurring in 24 hours.

Most observations of amebiasis in monkeys were made upon
animals who were naturally infected with the organisms (22,29).

Hegner, et al. (21), however, infected 7 monkeys by the rectal injection of trophozoites and 5 monkeys were infected when they received cysts per os. Infections produced with the former were more severe and were produced more rapidly than with the latter. The disease, as found in this animal, probably most resembles that in man with respect to location and pathology of the lesions and severity of infection.

After several unsuccessful, or partially successful attempts at guinea pig infection had been reported, Carrera and Faust (5) were highly successful in producing infection with a human strain of *E. histolytica*. Two hundred thousand to 7 million organisms from a culture were inoculated into the terminal ileum, and infection was produced in 34 out of 35 animals as determined by the presence of typical amebic lesions at autopsy.

Sadun, et al. (56), were able to establish the number of organisms necessary to produce infection in guinea pigs. They found that only animals that received inocula of from 1,000 to 5 million trophozoites intracecally became infected. Animals receiving smaller doses were not infected. The 50 per cent infective dose was 10,000 and the LD$_{50}$ was 63,000. All of the infected animals had typical amebic lesions in their ceca.

The use of the rabbit as an experimental animal for studies with *E. histolytica* was only slightly successful for
early investigators. In 1909, Huber (25) was able to produce infections in 4 out of 8 rabbits by feeding them human stools containing ameba cysts. Small, superficial lesions limited to the cecum developed in the infected animals.

Thomson (68), in 1926, was able to produce cecal ulcerations in one of 3 rabbits which were fed cysts obtained from a case of chronic human amebiasis. Amebas were observable microscopically in the lesions of the animals.

On the basis of the experiments which he reported in 1949, Tobie (69) concluded that the rabbit was probably a very satisfactory animal for immunologic studies of amebiasis. He was able to infect 6 of 33 rabbits by the intracesophageal inoculation of cultivated ameba cysts, and 10 of 11 animals were infected by the intracecal injection of trophozoites.

Active infection was diagnosed by the presence of flask-shaped lesions and "bottle" ulcers, and by the recovery of amebas from intestinal scrapings. The infections varied in intensity, ranging from those in which there were only a few superficial lesions to those in which the lesions were large, and death of the animal resulted.

Hunninen and Boone (26) were also successful in producing acute amebic infections in 88.4 per cent of rabbits inoculated with 5 strains of E. histolytica isolated from patients suffering from amebic dysentery. The infected rabbits developed crater-like lesions, intense diarrhea, and marked weight loss,
and large numbers of trophozoites containing red blood cells were isolated from them.

B. Factors Affecting Experimental Infection in Animals

The variety of results obtained by different investigators in their attempts to produce experimental amebiasis in animals has led to a study of some of the factors involved.

In general, diet has been found to affect the course of infection produced by intestinal protozoa. By the oral administration of unaltered canned salmon, Faust, et al. (16), were able to produce rapidly fulminating cases of amebiasis in dogs that had inactive or mildly chronic infections with *E. histolytica*. On the contrary, when the dogs were fed unchopped or finely chopped raw liver, there was a marked improvement in their condition and few intestinal lesions were present at autopsy.

Westphal (72) and Tobie (69) found that they were able to increase the number of experimental infections in rabbits if they were fed a diet consisting of a mixture of grains and whole wheat bread.

Sadun, et al. (57), were able to decrease the resistance of guinea pigs to infection by maintaining them on a diet that was inadequate in ascorbic acid. The animals were susceptible to infection with *E. histolytica* even with inocula as small as 1,000 amebas.

In 1957, Lynch (38) observed histologic alterations in the cecal mucosa of guinea pigs that were maintained on a
synthetic diet. In general, these alterations, which were absent in animals fed a commercial diet, consisted of a thinning of the mucosa and a dilatation of the glandular area with vacuoles (indicative of secretory retention). He concluded that the diet conditioned the cecal wall to permit invasion by the amebas and their associated bacteria. This then predisposed the animal to infection.

A second factor of importance in the production of experimental amebiasis is the relationship of amebas and bacteria. Luttermoser and Phillips (37), in 1952, found that 85 per cent of rabbits inoculated with amebas grown with a mixed bacterial flora became fatally infected with ulcerative amebiasis. However, 37 per cent of inoculated animals became infected when the inoculum contained amebas grown with one bacterial species, and only one of 24 animals became infected when treated with trophozoites grown in association with *Trypanosoma cruzi* in a bacteria-free medium. In 1954, Phillips and Bartgis (50) found that virulence could be restored to *E. histolytica* grown in association with *T. cruzi* by returning the amebas to cultures with selected bacteria, then reisolating with *T. cruzi*. These organisms then remained virulent again for from 3 to 8 weeks. In a continuation of their studies, Phillips, et al. (51), concluded that amebiasis is a disease of "synergistic etiology" which depends to a large degree upon bacterial associates. Although they could produce ulcerative amebiasis in the ceca of
conventional guinea pigs with *E. histolytica*-T. cruzi cultures, they were unable to do this in germ-free guinea pigs, even if the tissue adjacent to the site of inoculation was traumatized. According to these workers, the role of the bacteria is to condition the intestine of the host physically and/or chemically and provide a suitable environment for the amebas to become established and grow.

As more investigators used culture-grown material for the production of experimental amebiasis, many of them discovered that there was a marked decrease in the virulence of the organisms upon prolonged cultivation. In a study of 2 such strains of *E. histolytica*, Chang (6) found that the period of loss of infectivity came after the cessation of encystment in the cultures (3 years and 1 year, respectively). Infectivity was restored to both strains by inducing encystment in cultures. One of the strains, however, had considerably reduced pathogenicity which was restored only upon direct animal-to-animal passage.

Neal (49), in a study of various strains of *E. histolytica*, found that encystation in cultures did not alter the infectivity or invasiveness of 5 non-invasive strains of the organism. One highly virulent strain, however, while retaining its infectivity, lost its invasiveness (as determined by the size of lesion produced), even though it periodically underwent encystment. Neal, therefore, decided that the technique of
culturing does not affect the invasiveness of this organism.

Acute amebiasis was produced in 79 of 99 kittens which were exposed to a variety of environmental conditions by Sister M. Ann Josephine (62). The distribution and severity of the infections led her to conclude that nutritional status, the presence of intercurrent infection, and the response of the animals to stress factors were all involved in the production of overwhelming infections in otherwise only moderately susceptible animals.

The success of experimental infection of animals seems therefore to be dependent upon a complex of factors whose significance is still perplexing to many investigators in this field.

III. Serological Tests for Amebiasis

The development of an accurate serological test for amebiasis has been the subject of extensive research in the past. Most of the attention has been focussed on the use of the complement-fixation test. Izar (28), in 1914, reported that he was able to detect complement-fixing antibodies in the sera of 5 patients and 3 kittens infected with E. histolytica. His antigen was prepared from an aqueous extract of the feces of infected cats, and the pus from an amebic liver abscess. His discovery, however, remained almost unnoticed until 1927-1928 when Craig (9,10) reported his observations on the complement-fixing properties of an alcoholic extract of the
amebas, and its use in a complement-fixation test. Craig stated that he could correctly diagnose 92.3 per cent of cases of amebiasis (including carriers) which occurred among 225 people. He obtained a false-positive reaction in less than 1 per cent of his tests. The details of this complement-fixation technic, together with more impressive percentages confirming its accuracy were published by Craig (11) in 1929.

In addition to complement-fixing antibodies, Menendez (45) reported the presence of precipitin and amebolytic antibodies in the sera of rabbits that had been immunized intravenously with sedimented, washed cultures. He found that alcoholic extracts and suspensions of the organism in formalinized salt solutions were satisfactory as antigens in serological tests.

Further studies on the complement-fixation test were performed by Sherwood and Heathman (61). Their antigen, which they felt was more specific than Craig's, was prepared by adding small amounts of cholesterol to an alcoholic extract of dried amebas. They were able to obtain positive complement-fixation reactions with the sera of immunized rabbits, even after these sera had been absorbed with the bacterial flora accompanying the amebas. They attributed complement-fixation to the presence of antibodies which were specific for ameba lipo-protein.

Numerous other investigators continued studies with the
complement-fixation test. Tsuchiya (70), using an alcoholic extract of amebas was able to demonstrate specifically the presence of *E. histolytica* in 8 of 153 individuals. He obtained negative reactions when bacterial extracts were used as antigen, although he felt that some of the positive fixations obtained from clinically negative cases could be attributed to this factor.

In complement-fixation studies with experimental animals, Melaney and Frye (43) obtained positive complement-fixation reactions with infected dogs. Rabbits, that had been immunized intraabdominally with ameba antigens also had positive reactions, and retained them for at least 7 months. Negative results were obtained with monkeys who had natural intestinal infections, but no demonstrable lesions at autopsy. Positive reactions, however, were obtained in monkeys who were injected parenterally with extracts of *E. histolytica*.

Since only 42.5 per cent of patients with amebas in their stools gave a positive complement-fixation reaction, Melaney and Frye concluded that, in the negative cases, the parasite had not penetrated the tissue to an extent sufficient to produce antibodies in the serum.

Rees, *et al.* (53), prepared a complement-fixation antigen from cultures of amebas grown with a single organism (organism t). With their aqueous extracts, they correctly diagnosed 46 of 66 and 34 of 35 cases of amebiasis
and found no cross-reactions in patients with other parasitic infections.

When rabbits were immunized with amebas washed as free as possible from organism \( t \), complement-fixing antibodies developed in their sera. Only a minimum interference in their test by organism \( t \) was reported.

This type of antigen was found to be useful primarily in the diagnosis of extra-intestinal amebiasis. Husey and Brown (27) and McDearmon and Dunham (40) obtained a high percentage of positive reactions in patients with such infections, whereas the test was usually negative in patients with intestinal amebiasis.

This factor, as well as the non-specificity of existing antigens (27), the conflicting results obtained with the test in different laboratories (39), and the occurrence of numerous false positive reactions (55), have prevented the adoption of the complement fixation test for routine laboratory diagnosis of amebiasis. Investigators, therefore, have also sought other means of detecting antibodies in the sera of patients or experimental animals infected with \( E. \) histolytica.

An extract of scrapings from the ulcers of infected cats was prepared by Wagener (71) for use in precipitin tests. She was able to obtain positive reactions with the sera of cats that had been infected with the organism for one week
Spector (63), however, in 1932, found that the antigen which she prepared from the washed culture sediment of 7 different strains of *E. histolytica* was inadequate for use in skin tests or precipitin tests with human beings.

A technic for the immobilization of active *E. histolytica* trophozoites with immune sera was reported by Cole and Kent (7) in 1953. They found that the sera from rabbits that had been immunized with *E. histolytica*, as well as the sera from 5 of 13 individuals known to be infected with this organism, were able to cause the amebas to assume a rounded, contracted form. Maximum immobilization was observed at 20 to 30 minutes, after which the amebas gradually recovered their motility.

Goldman (18), in 1953, conjugated the globulin fraction of immune (anti-*E. histolytica*) rabbit serum with fluorescein isocyanate. When methanol-fixed amebas from cultures of *E. histolytica* were treated with the conjugate and examined under ultraviolet light, they were found to fluoresce brightly. Organisms from cultures of *Entamoeba coli* were also found to fluoresce with these sera, but not so intensely. Absorption of the sera with *E. coli* and substances present in the *E. histolytica* culture medium increased their specificity for *E. histolytica*, but decreased the intensity of the fluorescence.
Shaffer and Ansfield (60) observed that *E. histolytica* antisera inhibited the amebas from ingesting red blood cells. Antisera against 7 strains of the organism were found to have their greatest effect on the homologous strain, and varying abilities to inhibit phagocytic activity of heterologous strains. However, there was also some inhibition of phagocytosis with control sera which contained antibodies directed against the associated bacterial organism and the components of the culture medium.

The ability of immune sera to inhibit amebic growth in cultures was reported by Nakamura and Baker (48). When the sera from human cases of amebiasis, or from rabbits that had been immunized with "bacteria-free" *E. histolytica* were incorporated into the culture medium, growth of the amebas was inhibited by as much as 67.7 per cent.

A precipitin test, highly accurate in diagnosing severe cases of amebiasis has been reported by Moan (46). She mixed one drop of a water-clear antigen (essentially an aqueous extract of amebas) with a drop of clear serum on a slide, then examined the mixture microscopically for a specific precipitate. She obtained 85 to 100 per cent positive results in diagnosed cases of clinical amebiasis with virtually no false positive reactions.

None of these tests has gained widespread acceptance, and at the present time, definitive diagnosis of amebiasis
can be made only be extremely skillful laboratory personnel.

Studies of the serology of *E. histolytica* were therefore undertaken in an effort to develop a sensitive serological test which would simplify current laboratory diagnostic procedures.
MATERIALS AND METHODS

I. Strains of *Entamoeba histolytica*

Three strains of *E. histolytica*, NRS, 200, and DKB, were used during the course of this work. The NRS strain, with its associated bacterial flora, was obtained from Dr. C. T. Pan, Harvard School of Public Health, Boston, Mass. Strains 200 and DKB were acquired from Dr. R. E. Reeves, Louisiana State University Medical School, New Orleans, La. A culture of *Bacteroides symbiosus*, a gram-negative streptobacillus used as a cultural associate for the 200 and DKB strains was also provided by Dr. Reeves.

II. Culture Media

The NRS strain of *E. histolytica* was maintained on a modified Boeck-Drbohlav medium (4). This is a diphasic medium which consists of a coagulated egg slant overlaid with a Ringer's solution and serum mixture.

Egg slants were prepared by adding 150 ml of Ringer's solution to one dozen eggs and mixing for 5 sec in a Waring blender. The mixture was dispensed in 3-ml amounts in 125 X 15 mm tubes which were then plugged with cotton and sterilized in an Arnold steam sterilizer for 3 days (3 to 4 hours per day at 80 to 90 C). The slants were stored in the refrigerator until needed, at which time each was overlaid with 3 to 4 ml of a Ringer's solution and serum mixture. This overlay solution was prepared by adding 50 ml of serum
to 250 ml of sterile Ringer's solution. Horse serum, supplied by the Mass. Public Health Biologic Laboratories, Jamaica Plain, Mass., was originally used in all cultures, but because of its antigenic properties, it was replaced by normal rabbit serum when the material was to be used for immunologic purposes. Blood was obtained from normal albino rabbits by cardiac puncture and allowed to clot. The serum was then separated by centrifugation and sterilized through a Selas bacteriological filter before being used.

The Ringer's solution had the following formula:

- Sodium chloride: 6.50 g
- Calcium chloride: 0.12 g
- Potassium chloride: 0.14 g
- Monosodium phosphate: 0.01 g
- Sodium bicarbonate: 0.20 g
- Distilled water: 1000.00 ml

Stock cultures of the NRS strain were transferred three times a week. To each tube of culture medium was added a wire loopful (ca. 10 mg) of sterile Difco Bacto rice powder and 1/2 to 1 ml of a 48 to 72 hour culture of the amebas with their associated bacterial flora. The inoculum consisted primarily of the sediment found at the base of the egg slant because a majority of the amebas tended to accumulate there. All transfers were made with a capillary pipette.

The NRS strain was also carried for a few months on the diphasic charcoal medium of McQuay (41). This medium, which eliminated the antigenic substances present in egg and horse
serum, consisted of a charcoal agar slant overlaid with 3 ml of 0.5 per cent buffered saline at a pH of 7.4. The agar slants contained the following ingredients:

- Disodium phosphate: 3.0 g
- Monopotassium phosphate: 4.0 g
- Sodium citrate: 1.0 g
- Magnesium sulfate: 0.1 g
- Ferric ammonium citrate: 0.1 g
- Bacto-asparagine: 2.0 g
- Bacto-tryptone: 5.0 g
- Glycerin: 10.0 g
- Bacto-agar: 10.0 g
- Norite A: 1.0 g
- 1% cholesterol in acetone: 25.0 ml
- Distilled water: 1000.0 ml

The entire mixture was brought to a boil to dissolve the agar, then tubed in 3-ml amounts. The plugged tubes were autoclaved at 15 lb pressure for 15 min and slanted while still warm.

In order to prepare 1 liter of the buffered saline overlay, 5.0 g of NaCl was added to 190.0 ml of M/15 KH₂PO₄ and 810 ml of M/15 Na₂HPO₄.

Transfers into this medium were made in the same manner as into the modified Boeck-Drbohlav medium.

*E. histolytica*, strain 200, was grown in modified Shaffer-Frye (MS-F) medium (54). The medium consisted of

- Trypticase: 20.0 g
- Glucose: 10.0 g
- Sodium chloride: 2.5 g
- Dipotassium phosphate: 2.0 g
- Thiomalic acid: 1.5 g
- Distilled water: 1000.0 ml
- Sodium hydroxide to pH 7.0 ± 0.1
It was tubed in 10 to 12-ml amounts in 125 x 15 mm screw-cap tubes, autoclaved for 10 min at 15 lb pressure and stored in the refrigerator until needed. Prior to use, 0.1 ml of normal rabbit serum, 5000 units of Squibb penicillin G potassium, and 2 ml of an 18 to 24-hour culture of \textit{B. symbiosus} were added to each tube. The culture medium for \textit{B. symbiosus} was the same as that for the amebas with the addition of 0.2 per cent yeast extract.

New medium for the amebas was prepared once a week, and the bacterial medium was freshly prepared twice a week. Cultures of \textit{B. symbiosus} were transferred 7 days a week using a 2 to 5 per cent inoculum.

The ameba cultures were transferred 3 times a week or every other day depending upon the microscopical appearance of the organisms. 48 to 72-hour cultures of strain 200 were placed in ice-water for 2 minutes in order to detach the amebas which grew on the sides of the tubes. The contents of the tubes were then mixed several times with a pipette and 1 ml was transferred to each new tube of culture medium. The tubes were incubated at 37 C for from 48 to 72 hours in a slanted position which allowed the amebas to settle on the underside of the tubes. In this way, their growth could be observed microscopically through the glass of the tube without disturbing the culture.

The procedures used for the growth and maintenance of \textit{E. histolytica} strain DKB were identical with those used for
strain 200.

III. Production of Antisera

Attempts were made to prepare antisera from rabbits by inoculation of the amebas through a variety of routes. Albino rabbits weighing approximately 2 kg were used in all of the experiments.

A. Intracecal Inoculation

Two weeks prior to being inoculated intracecally, the rabbits were placed on a diet consisting of whole wheat bread and a mixture of cracked corn, oats and bran (69). They were maintained on this diet throughout the course of the experiment. The animals were anesthetized with 30 mg per kg of nembutal administered intravenously. A small incision (1 to 2 in) was made in the lower right quadrant of the abdomen and the muscles separated by tearing the tissue with the points of a small pair of scissors. Once the peritoneal cavity was reached, the cecum was located and live trophozoites of *E. histolytica* were injected into it with a 20-guage needle. After the inoculation, the incision was closed with 2 or 3 Michel wound clips. No special care was taken to maintain aseptic conditions during the operation.

Various culture combinations of the NRS strain were used as the antigen in this method. These included the injection into each rabbit of a total of 2 ml obtained from 1, 2, or 4 cultures grown in egg medium. The latter were
pooled in a 90 ml centrifuge tube and centrifuged at 1000 rpm for 10 min. At the end of this time, all except 5 ml of the supernatant fluid was drawn off through a 100 ml volumetric pipette. The sediment, which contained primarily amebas and starch granules, and the remaining supernatant fluid, were mixed until fairly homogeneous, and used as the inoculum.

Rabbits injected with the 200 strain received 1 ml of suspension from a pool of 6 cultures. The cultures were chilled in ice-water for 2 min, then centrifuged at 1000 rpm for 6 to 7 min. All except one ml of the supernatant fluid, which was used to resuspend the sediment, was withdrawn with a capillary pipette. The 1-ml suspension from 6 tubes was then pooled and used as antigen.

All of the cultures of both strains were 48 hours old. The animals were observed daily for signs of infection. Cultural and microscopical examinations of the feces of many of the animals were made, and after the death of each rabbit, its cecum was examined to determine whether amebic lesions were present.

B. Intraabdominal Inoculation

A series of rabbits was given intraabdominal injections of formalinized amebas for various time intervals. They did not receive a deficient diet.

Antigen made from the NRS strain was prepared by pooling 16 48-hour cultures, centrifuging them at 1000 rpm
for 10 min and withdrawing all except 5 ml of the supernatant fluid. After resuspension of the sediment, formalin was added to 0.4 per cent and allowed to act for from 5 to 6 hours.

Rabbits were inoculated intraabdominally with 2 ml of antigen either once a week for three weeks or 2 times a week for 3 weeks. They were tested for antibodies 1, 2, and 3 weeks after their final injection.

Antigen for intraabdominal injection was also prepared from cultures of the 200 and DKB strains of *E. histolytica*. The cultures were treated in the same manner as those used for intracecal inoculation. Formalin to 0.4 per cent was added after the cultures had been pooled, and it was allowed to act for 6 hours. Two or 3 times during the course of immunization, counts were made on the pooled cultures to determine the number of amebas present in the inoculum. The counts, which were made in a Levy counting chamber, averaged 250,000 amebas per ml.

A set of rabbits received 2 ml of either 200 or DKB antigen 3 times a week for 4 weeks. They were bled for antibodies 4 days after the 6th injection and 5 days after the final injection.

C. Intravenous Inoculation

Because the NRS culture medium contained so many foreign antigens, intravenous inoculations were made with
the 200 strain grown in MS-F medium only. Antigen was prepared as for intracecal injection and 1 ml of live trophozoites (3-400,000) was injected into the marginal ear vein of the rabbit. Each rabbit received 3 injections a week for 3 weeks.

Before inoculations with live cultures were begun, a normal rabbit received 2 ml of a 24-hour culture of *B. symbiosus* intravenously. This preliminary test established that the organism present in all cultures of strain 200 is not toxic when injected intravenously in a viable form.

Test for antibodies to the amebas were made 2, 3, and 4 weeks after the first injection.

All preliminary bleedings for serologic testing were made from the marginal ear vein. Final bleedings were made by cardiac puncture. After the serum was separated, merthiolate was added to a final concentration of 1/10,000.

IV. Absorbed Sera

The antibodies found in the sera of the immunized rabbits were not specific for the amebas because of the numerous antigens present in the cultures. In order to eliminate as many foreign antibodies as possible, 2 antisera were absorbed with NRS flora and whole, de-fatted egg. These sera were obtained from rabbits that had been immunized intraabdominally with strain NRS grown in egg medium.

NRS flora is a mixed bacterial culture consisting of the
enteric organisms that were present in the stool from which the NRS strain of \textit{E. histolytica} was originally isolated.

To prepare antigen for absorption, several tubes of egg medium were inoculated with NRS flora only. After 48 hours, the overlay solutions from these cultures were pooled, and formalin was added to a final concentration of 0.4 per cent. After it had stood overnight, the suspension was centrifuged at 2000 rpm until the supernatant was almost clear (ca. 30 min). The supernatant fluid was then decanted and the bacterial cells were washed twice with saline. One to 2 ml of the serum to be absorbed was added to the bacterial sediment. The mixture was stirred at intervals for 1 hour, then allowed to remain in the refrigerator overnight. The following day, the bacterial cells were removed from the serum by centrifugation at 2,000 rpm for 30 minutes.

The two sera, which had been absorbed in this manner with NRS flora, then underwent absorption with whole, de-fatted egg. Two beaten eggs which had been allowed to coagulate in the Arnold steam sterilizer were minced finely and placed in a desiccator for a few days. When dry, the granules were ground with a mortar and pestle, then washed several times in a 50 per cent alcohol--ether solution. The extraction process was continued overnight in the refrigerator. The egg particles were then filtered out and
allowed to dry in air. Before serum was added, the egg was moistened with saline to prevent excess adsorption of serum to the egg.

The remainder of the procedure was identical with that used with NRS flora.

Sera from 2 rabbits which had received intravenous injections of strain 200 were absorbed with *B. symbiosus*. Antigen was prepared from 24-hour cultures of this organism and the procedures employed with NRS flora were repeated until the sera had an agglutination titer of 0.

V. Serological Methods

A. The Agar-gel Precipitin Technic

The primary serologic method used was the agar-gel precipitin technic.

1. Preparation of Agar-gel Plates

One per cent of Special Agar Noble (Difco), an electrolyte-free agar, was made up in 0.85 per cent NaCl containing 0.6 per cent phenol. Ten ml of this were poured onto an agar-gel plate which consisted of a lucite ring 75 mm in diameter and 12 mm deep cemented with Weldwood Contact Cement (U.S. Plywood Corp.) onto a 3½ X 4 in Lantern Slide Cover Glass (Kodak). When the layer of agar had gelled, a mold with either 3 or 7 metal forms was placed on its surface. Another 10 ml of the agar solution were then poured onto the plate. After this second layer had become firm, the mold was removed leaving 3 or 7 wells
in the agar. Each of these could contain approximately 0.2 ml of antigen. As soon as the wells were filled, the plates were sealed by attaching another Lantern Slide Cover Glass to the top of the lucite ring with Grippit Paper Cement.

2. Preparation of Antigens

Ameba antigens for use in agar-gel plates were prepared from strains NRS and 200 in the same manner as the antigens used for intraabdominal inoculations. After formalinization, they were frozen rapidly in a dry ice--alcohol mixture, then thawed rapidly in warm water. This procedure was repeated 5 to 6 times until microscopic examination showed the absence of intact amebas (47).

Control solutions consisted of 0.85 per cent NaCl, sterile preparations of the various culture media, or formalinized suspensions of the bacterial associates.

The agar-gel plates were incubated at 30 C and observed daily. A reaction between antigen and antibody appeared as a white line of precipitate in the agar. Results were usually obtained within 4 or 5 days, and the plates were discarded at the end of two weeks.

B. The Fluorescent Antibody Technic

The fluorescent antibody technic was employed in the study of one set of sera only.

1. Preparation of Fluorescent Smears

One drop of the sediment of a chilled and centrifuged
culture of *E. histolytica* strain 200 was placed on each of several glass slides (1 X 3 inches), and smeared gently over a small area. When the smears had dried, they were fixed in absolute methyl alcohol for 1 min and treated with fluorescein-conjugated sera for 2 min. These sera were obtained from 2 rabbits which had received identical intravenous inoculations with strain 200. Studies with the agar-gel technic showed that both sera had similar antibody content, therefore they were pooled and the pool was divided into two parts. One part was absorbed 3 times with *B. symbiosus*; the other part was not absorbed, and therefore contained antibody to *B. symbiosus*.

Both the absorbed and unabsorbed sera were conjugated with fluorescein isothiocyanate by Dr. E. H. LaBrec, Walter Reed Army Institute of Research, Washington, D. C. After conjugation, the sera were dialyzed, and sterilized through an HA millipore filter. No preservative was added.

After treatment with conjugated sera, the ameba smears were rinsed in 0.85 per cent NaCl solution for 5 to 10 min, then rinsed briefly in distilled water, dried, and examined for fluorescence. The source of ultraviolet light was a Zeiss mercury arc lamp (HBO 200) that was used with BG12 and UG2 exciter filters, and a Wratten yellow barrier filter. The smears were examined with an American Optical bicentric darkfield condenser and an oil immersion lens stopped to N.A. 0.8. Glycerol immersion was used in place of oil to
eliminate scattered fluorescence.
EXPERIMENTAL RESULTS

I. Results with the Agar-gel Technic
A. Intracecal Inoculation

37 rabbits were inoculated intracellally with *E. histolytica*, strain NRS. Cultural examination of the feces of many of these was negative in all cases for both cysts and trophozoites of the ameba. Post mortem examination of the animals' ceca failed to reveal the presence of amebic abscesses, but in many of the rabbits which died within the first few days after inoculation, there was evidence of infection with some member of the NRS flora. No matter how carefully the intracecal injections were performed, some contamination of the peritoneal cavity usually occurred.

20 of the 37 rabbits survived at least 2 weeks and were bled and tested for antibodies. According to the results obtained with the agar-gel technic, 3 of these had a possible antibody against the amebas, 6 had antibodies against the associated bacterial flora and/or the components of the culture medium, and 11 had no detectable antibodies.

All 3 of the possible "positive" sera were from rabbits which had been injected with 2 ml from one ameba culture grown in the modified Boeck-Drbohlav medium with horse serum.

One of these sera reacted with ameba substance only when the organisms had been grown in the egg-containing, but not charcoal medium. At the same time, in addition to
the ameba line (i.e., line of precipitate on the agar-gel plate), a line representing antibody against the horse serum was also present. Sera obtained from the same rabbit 3, 4, and 5 weeks after inoculation gave reactions which showed that antibody against horse serum only was present.

Serum from a second rabbit was found to react with ameba antigens prepared from organisms grown in both egg and charcoal medium. No antibodies against NRS flora or horse serum were detected. This animal was re-inoculated intracecally with strain NRS grown in egg medium, then bled and tested 2 weeks later. At this time, several antibodies were present to components of the medium; no ameba antibody was discernible.

The third "positive" serum was obtained from a rabbit 3 weeks after it had been inoculated intracecally with strain NRS. Since this serum at first appeared to contain antibodies only against amebas, it was used as the control serum in subsequent experiments. Although it reacted variably with ameba antigens prepared in charcoal medium or egg medium containing rabbit serum, it always reacted with ameba antigens from egg--horse serum cultures, and not NRS flora or components of the culture medium. After several months, however, it became evident that other antibodies were present in this serum because it then began to react with antigens made from cultures which contained no amebas.
None of these 3 sera reacted with antigen containing organisms of strain 200 or DKB grown in MS-F medium with rabbit serum.

2 rabbits were inoculated intracecally with strain 200. They showed no physical signs of infection, nor were any detectable antibodies present in their sera 2 and 3 weeks after inoculation.

B. Intraabdominal Inoculation

Four rabbits which had shown no detectable antibodies after intracecal inoculation with the NRS strain, received 3 weekly injections of NRS strain intraabdominally. Only two of the rabbits survived the full course of the injections, but all 4 showed antibodies to the horse serum in the culture medium one week after their first injection.

One week after the final injection, numerous antibodies were present in the sera of the 2 surviving rabbits, however, none of these could be attributed to the amebas which were present in the antigen.

2 previously uninoculated rabbits were given 6 intraabdominal injections containing a total of 3,000,000 amebas. In order to eliminate the problems presented by horse serum, antigen was made from cultures in which rabbit serum was substituted for horse serum. The substitution was begun 15 transfers before the cultures
were used for antigen because even small amounts of horse serum were found to be highly antigenic.

One week after the final injection, the sera of these rabbits were found to contain several antibodies to egg components and NRS flora, but no detectable antibodies to the amebas. In an attempt to discover whether any antibodies to the amebas were actually present, two of these sera were absorbed with NRS flora and whole egg. Because of the complexity of the antigens involved, however, the absorptions were incomplete and no ameba antibody was revealed.

None of these sera reacted with antigens prepared from strains 200 and DKB.

3 rabbits were given a series of intraabdominal injections of strain 200 and 3 rabbits were treated in a similar manner with strain DKB. Each animal received a total of approximately 6,000,000 amebas.

All of the rabbits were bled 4 days after the 6th injection. At this time, one of the rabbits which had been inoculated with strain 200 was found to have antibodies against \textit{B. symbiosus}; two had no detectable antibodies. Two of the animals which had been inoculated with strain DKB also showed antibody against \textit{B. symbiosus}, but the third serum was negative for antibodies to both the amebas and bacteria.

Five days after the 12th injection, serological studies
on agar-gel plates showed that all of the animals' sera contained antibodies against the bacteria, but none to the amebas. Agglutination titers for the bacterial antibody ranged from 64 to 256.

C. Intravenous Inoculation

Four rabbits were given 12 intravenous injections of live trophozoites of *E. histolytica*, strain 200, over a period of 4 weeks. They received a total of approximately 3,300,000 amebas.

One week after the 6th and 12th injections, the animals were bled, and their sera tested for antibodies. All of these sera were found to contain antibody to *B. symbiosus* when tested on agar-gel plates, but no antibody against the amebas was detected.

In order to establish conclusively that no antibody directed against the amebas was present, 2 of the sera were absorbed with *B. symbiosus*. Their agglutination titers were reduced from 512 and 256 to 0.

The two sera were now found to react with neither ameba antigen nor antigen prepared from the bacteria. Samples of these sera, which were unabsorbed, reacted with both antigens when tested at the same time as the absorbed sera.

II. Results with the Fluorescent Antibody Technic

Methanol-fixed smears of *E. histolytica*, strain 200,
were treated with either of two fluorescein-conjugated sera and examined for fluorescence. One of these sera was absorbed 3 times with \textit{B. symbiosus}, and tested after each absorption; the other was a portion of the same serum which had not been absorbed.

An unstained smear prepared in the same manner as the stained smears was also examined. This showed a very slight autofluorescence of the amebas, but no fluorescence of the bacteria which were also present on the slides. When the smears had been treated with unabsorbed serum, both amebas and bacteria fluoresced brightly under ultraviolet light (Figure 1). The same was true of the organisms in smears that had been treated with serum absorbed only once with \textit{B. symbiosus}. However, a marked decrease in fluorescence of both amebas and bacteria was observed in smears prepared with serum that had been absorbed twice with the bacterial organism. After the third absorption, the amebas fluoresced no more than the unstained amebas, and no bacteria were visible.
DISCUSSION

Most of the problems encountered in serological investigations of *E. histolytica* are concerned with the lack of specific antigen preparations. To date, no-one has been able to maintain the amebas in a medium which is entirely free of other cellular material, and many additional antigenic substances are often present in the complex culture media. The presence of these foreign antigens makes it difficult to prepare antisera that are specific for *E. histolytica*. This was shown by the results obtained with sera from rabbits which had been immunized with ameba material administered intravenously or intra-abdominally.

It was thought that if clinical or sub-clinical amebic infections were induced in rabbits, antibodies directed against the amebas only would be produced. However, intracecal injection of *E. histolytica* trophozoites (strains 200 and NRS) was unsuccessful in producing amebiasis in rabbits. There was no physical evidence of infection, nor were ameba antibodies detectable in the sera of the animals.

The NRS strain of *E. histolytica* which was used throughout most of the experimental work, was originally isolated from a monkey. Toward the end of this research, it was discovered that this strain has always been relatively avirulent. The results reported here would seem
to support this fact.

Attempts to produce amebiasis in rabbits with strain 200, however, were unsuccessful also. This strain has been shown to be pathogenic for rabbits by Tobie (69). He was able to induce infections in 91 per cent of rabbits inoculated intracecally with trophozoites. The organisms used by him were obtained from cultures in which the amebas were growing in association with a mixed bacterial flora, whereas the organisms used in these experiments had a monobacterial associate only.

Although Luttermoser, et al. (37), were successful in establishing the 200 strain with NRS flora, attempts to achieve this during the course of this work were unsuccessful. Their experiments showed that the largest percentage of infections in rabbits could be produced with amebas which had been cultured with a mixed bacterial flora. Organisms growing with a single bacterial species produced infections in only 37 per cent of inoculated animals.

Because only a small number of rabbits (2) was inoculated with strain 200, it is conceivable that successful infections might have been produced if a larger group of animals had been employed in this part of the experiment.

Even if infections had been produced, however, it is evident that the sera would not necessarily have contained ameba antibodies only, especially when the NRS strain was used.
Contamination of the peritoneal cavity during the process of inoculation was responsible for the presence of many foreign antibodies. Also, various components of the culture medium may have been absorbed through the intestinal wall of the animals so that antibodies to them were then formed.

In 1932, Heathman (20), on the basis of her study of antigenic properties of amebas, concluded that both free-living and pathogenic amebas appeared to be good antigens. Most of the recent evidence, however, indicates that *E. histolytica* is not a strong antigen, a fact which serves to increase the difficulty of immunological investigation in this field. According to Meleney (44), unless there is actual invasion of the tissues by the organisms, antibodies are not detectable in the sera of patients, and recurrences of the disease are common. That some type of immunity to amebiasis can be produced, was shown by Swartzwelder and Muller (66) who were unable to infect 37 per cent of rats which had been immunized with aqueous extracts of amebas. In contrast, only 8 per cent of control animals, which had not been immunized, were not infected. Swartzwelder and Avant (67), despite repeated attempts, were unable to reinfect 24 of 29 dogs which had recovered from acute amebic dysentery. Perhaps the type of immunity present in this disease is one in which the antibodies predominate at the site of infection rather than in the circulation.

The NRS strain of *E. histolytica* maintained in the
modified Boeck-Drbholav medium is evidently a poor choice for use in serological investigations. The antigenic complexity of the culture medium and bacterial associates makes the absorption of non-ameba antibodies impracticable, and determination of the antibody content of antisera prepared with this strain is extremely difficult.

Use of strain 200 in MS-F medium for rabbit immunization allows almost all foreign antigens to be eliminated, especially when rabbit serum is substituted for horse serum. Precipitin tests in agar using antisera prepared with this strain indicated that no ameba antibodies were formed, even though a sizeable amount of ameba material was administered to the rabbits.

When ameba smears which had been treated with fluorescein-conjugated serum were examined under ultraviolet light, both amebas and bacteria were observed to fluoresce if the serum had not been absorbed with B. symbiosus (Figure 1). This would appear to indicate that antibodies directed against both the amebas and the bacteria were present in the sera. However, when smears treated with B. symbiosus-absorbed serum were examined in the same way, neither the amebas nor bacteria fluoresced. Therefore, antibodies against B. symbiosus only were present in the serum and were responsible for the fluorescence of the amebas. Examination of amebas with the phase microscope during the course of this work showed that the organisms contained large numbers of bacterial
cells (Figure 2). The fluorescein-tagged antibody apparently reacted with its specific antigen, in spite of the fact that it was now contained within an ameba cell.

Goldman (18, 19), in 1953 and 1954, described similar experiments with the fluorescent antibody technic, in which he was able to differentiate *E. histolytica* from *E. coli* and other intestinal amebas. He obtained antisera from rabbits by inoculating them subcutaneously with washed, intact *E. histolytica* and *E. coli* organisms which had been cultured with an associated bacterial flora. A certain amount of cross-reaction between the two sera was observed by him and absorption of *E. histolytica* serum with *E. coli* organisms resulted in a decrease of the fluorescence of *E. histolytica* as well as elimination of *E. coli* fluorescence. Absorption with non-ameba sediments was also found by him to reduce the titer of *E. histolytica* sera considerably due to the "non-specific" removal of protein from the conjugates. From these results, together with data obtained in the present study, it appears likely that Goldman was dealing with bacterial and not ameba antibody. The variations in staining intensity which he observed with different strains of *E. histolytica* and other amebas could have been due to differences in the bacterial flora rather than antigenic differences of the amebas.

Sister M. Ann Josephine (62) was able to detect complement-fixing antibodies in the sera of kittens that had been immunized intraabdominally with trophozoites of *E. histolytica*. 
However, no complement-fixing antibodies were demonstrable in the sera of kittens which had acute amebic infections. This experiment also seems to indicate that bacterial organisms were actually responsible for the antigen-antibody reaction.

With present cultural and serological technics, it is unlikely that anyone who has obtained E. histolytica antisera by immunization of animals can demonstrate conclusively that antibodies against the amebas exist in them. This is also true, to a limited extent, of serological tests conducted with human sera. None of the commercial or laboratory-prepared antigens contain only E. histolytica. It is not improbable that some positive reactions which are obtained with human sera actually represent cross-reactions of antibodies in the sera with foreign substances present in the test antigen. Many of the bacteria growing in association with the amebas in culture, and therefore present in antigen preparations, are common inhabitants of the intestinal tract. The invasion of an amebic lesion by any of these organisms would cause the production of antibodies whose reaction with test antigens could be mistakenly interpreted as representing the presence of ameba antibody. Nakamura obtained a strong positive serologic reaction with the serum of a patient with amebic dysentery when he tested it with antigen prepared from the NRS strain of E. histolytica. A short time later, this serum was again tested with NRS antigen, but no reaction was obtained (2). The only difference between the two antigen
preparations was that the associated bacterial flora was not the same. Apparently one of the patient's antibodies cross-reacted with a bacterial organism which was present in the first, but not the second batch of antigen.

After 45 years of investigation, no-one has been able to develop a specific, sensitive serological test for amebiasis. Unless the problem of growing the amebas in pure culture is solved, or investigators are able to deal in some other way with the wealth of foreign antigens present in the ameba cultures, success at developing this test will not be readily achieved.
SUMMARY

1. Attempts were made to produce anti-\textit{E. histolytica} sera in rabbits by means of the intracecal, intraabdominal, and intravenous inoculation of ameba-containing material.

2. Serological studies employing the agar-gel technic and the fluorescent antibody technic showed that antibodies were produced against bacteria growing in association with the amebas or components of the culture medium only. No ameba antibodies were detected.
Figure 1. *E. histolytica* culture treated with fluorescein-conjugated anti-*E. histolytica* culture serum.
FIGURE 2. *E. histolytica* trophozoite grown in culture with *B. symbiosus*. Phase microscope, dark contrast.


SEROLOGICAL STUDIES OF ENTAMOEBA HISTOLYTICA

Abstract of a Thesis

Submitted in partial fulfilment of the requirements
for the degree of Master of Arts

BOSTON UNIVERSITY GRADUATE SCHOOL

by

Josephine A. Morello
B.S., Simmons College, 1957

Department: Medical Sciences
Field of Specialization: Microbiology
Major Instructor: Professor Edgar E. Baker
The first recognition of *Entamoeba histolytica* in the stools of a patient suffering from dysentery was made by Lösch (Virchows Arch. f. path. Anat., 65:196, 1875). He, however, did not consider the amebas to be the primary cause of the infection, and it remained for later workers to prove that they were the etiologic agent of the disease.

Many investigators employed animals in their studies of amebiasis. Experimental infections were successfully induced in cats, dogs, guinea pigs, monkeys, and rabbits. The nature and severity of these infections were found to be partly dependent upon the animals' diet, the bacteria growing in association with the amebas, and the length of time the amebas had been in culture.

The development of an accurate serological test for amebiasis has been the subject of extensive research. Most investigators, especially Craig (Am. J. Trop. Med., 7:225, 1927; 8:29, 1928; 9:277, 1929), have focussed their attention on the complement-fixation test. However, the non-specificity of existing antigens, the conflicting results obtained with the test in different laboratories, and the occurrence of numerous false positive reactions have prevented the extensive use of this test. Skin tests, inhibition tests and precipitin tests for amebiasis have also been devised, but have not gained widespread acceptance.

In order to obtain anti-*E. histolytica* sera for use in this research, various methods of immunizing rabbits were
investigated. Attempts were made to induce infections in the animals by the intracecal inoculation of live trophozoites of strains NRS and 200. This method was unsuccessful in producing detectable clinical or subclinical infections in the animals. Their sera contained antibodies directed against the bacterial flora growing in association with the amebas, or components of the culture medium only.

As shown by the agar-gel technic, rabbits which had been immunized intraabdominally with formalinized organisms of strains 200 and DKB produced antibodies only against *B. symbiosus*, the streptobacillus present in all cultures of these amebas. Similar results were obtained with the sera of rabbits that had received a series of intravenous inoculations of live *E. histolytica* trophozoites of strains 200 and DKB. Studies with the fluorescent antibody technic confirmed the results obtained with the agar-gel technic.

These results indicate that none of the animals, regardless of the method of attempted immunization, produced ameba antibodies. Yet, antibodies directed against foreign antigens in the ameba cultures were produced in a majority of cases.

In the light of these findings, it seems likely that the results obtained by other investigators can also be attributed to antibodies to some foreign antigen, rather than to amebas. For example, Goldman (Am. J. Hyg. 58:319, 1953; 59:318, 1954) observed that the brightness of amebas treated with fluorescein-tagged sera decreased when they
were "non-specifically" absorbed with non-ameba sediments; Sister M. Ann Josephine (Am. J. Trop Med. Hyg., 7: 158, 1958) could detect complement-fixing antibodies in the sera of kittens that had been immunized intraabdominally with E. histolytica antigens, but not in those that had acute amebic infections; and Baker (personal communication) was unable to demonstrate the presence of previously existing "ameba" antibodies in a human serum after the bacterial flora in the test antigen had changed.

Since no commercial or laboratory-prepared antigens contain only E. histolytica, there is ample opportunity for cross-reactions of this nature to occur in serological tests. It therefore seems unlikely that great progress will be made in this field until bacteria-free cultures of E. histolytica are available.