SOME OBSERVATIONS ON THE EFFECTS OF ENVIRONMENTAL CHANGES ON THE BIOLOGY OF ENDAMOEBA HISTOLYTICA

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Review of Literature and Purpose: The *Endamoeba histolytica* Losch[1] 1875, is the specific etiological agent in amebiasis in man and certain lower animals. There is, however, considerable ignorance and confusion in regard to the reason why one person shows the severe symptom-complex of amebic dysentery while many another individual harboring a similar organism is relatively free from such symptoms and, to a large extent, free from the anatomical changes associated with those symptoms.

Poindexter[2], in 1932, found in Puerto Rico, as many other observers have found in various tropical regions, a high incidence of *Endamoeba histolytica* infection; 12.4 per cent of the 564 stools examined were positive upon a single stool examination. Repeated examinations, as shown by Faust[3], would undoubtedly have increased the percentage. Only one of the positive cases had active dysenteric symptoms and showed the active trophozoites in the stool. This high incidence of infection in the tropics, frequently reported, is primarily due to poor sanitation. The explanation of the difference between the number of cases with history of dysentery and those carriers without any definite dysentery symptom is not known. Among the suggested theories may be mentioned native tolerance or accumulative group resistance, climate, dietary habit of the people or the presence in the environment of some other associated organism with varying degrees of antagonism.

It has been observed that Puerto Ricans who migrate to New York City frequently develop symptoms of dysentery even though they do not give a history of dysentery before leaving Puerto Rico. It has been observed, also, that persons who have lived continuously in temperate climates,

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upon migrating to the tropics and upon equal exposure, develop symptoms of amebic dysentery in proportion to the carriers far above the natives.

Poindexter [2] was interested in the diet of the Puerto Rican of the lower income group and found that it consists chiefly of rice, beans, and other food products of high carbohydrate values. Since individuals from the temperate climate where meats are an accustomed article of diet may take his dietary habit with him to the tropics, while a Puerto Rican coming to New York may change to a more meaty diet, we thought that by approximating these two extremes of diet in animal experiments some light might be thrown on the problem of difference between carrier and acute case incidence, and the above mentioned change which results from migration.

The earlier work of Kartulis [4] 1887; Kruse and Pasquale [5] 1894; Quincke and Ross [6] 1893, the more recent observation of Dale and Dobell [10], of Faust [11] 1930, and others, have established the kitten and the puppy as good animals for experimental work with *E. histolytica.* Kittens are better for acute observation, while the puppies are used for subacute or more chronic studies.

The observations of Baetjer and Sellards [12] in 1914, and of Chatton [13] in 1918, in which granulomatous lesions resembling a neoplasm were observed in the region of the cecum in guinea pigs infected with *E. histolytica,* give hope of being of definite value as a possible study of the type of local tissue reaction to *E. histolytica* infection. It has not been adequately confirmed. Guinea pigs still are not used to any extent for experimental research with *E. histolytica.* Several of the other laboratory animals have been used for work with *E. histolytica.* Hegner [14] recently reported observations in which monkeys served as *E. histolytica* carriers even without tissue invasion by the organisms. If the *E. histolytica* can live and multiply in the lumen of the intestinal tract indefinitely without invading the tissue, a non-symptomatic carrier state is possible. The question then arises as to what enhances its invasive power or lowers the portal of entry to invasion. We hope to throw some light on this question from our experiments here.

Kittens and puppies were used in our experiments.

Material, Methods and Results: During the last 4 years we have used in these experiments 49 kittens with weights varying at the time of their infection from 320 grams to that of 1,180 grams and varying in ages from 4 weeks to 8 months. All of these kittens were infected per rectum using the technique described by Faust[15] for infecting puppies. The kittens were used in 4 groups of 12, except in one group in which there were 13 kittens. The experiments were carried out during the months of December, January and February, at which time the material was used as teaching material for the class in parasitology. In these groups the animals were placed in separate cages and alternate kittens of comparable weights were placed alternately on (*a*) a high carbohydrate diet that consisted chiefly of bread, rice, beans and water, and (*b*) a high protein diet consisting chiefly of milk, fish, and liver. Each animal under ether anaesthesia was then given per rectum from 5 to 8 cc. of a 24 hour growth of *E. histolytica* culture grown on the solid Boeck-Drbohlav coagulated Locke's egg-base and a mixture of 1 part of rabbit, horse or human sera or of hydrocele or ascitic fluid in 6 parts of Locke's solution as the liquid part of the media. In the cultures from kittens showing a high blastocystis or coccidial infection we occasionally used St. John's[22] media.

A cotton tampon to which a string was attached was used to plug the rectum after the inoculation to prevent the animal from expelling the fluid upon coming from under the ether. After from 12 to 14 hours the kittens were again examined. If they had not at this time expelled the cotton plug, it was removed by pulling the string.

The "takes" in the medium size kitten of from 450 to 800 grams were good. In the older kitten, the "takes" were poor and in some cases after waiting from 7 to 10 days without any symptoms of dysentery or trophozoites in the feces, repeated inoculations were given, in some cases to a third time before we got a "take". In the younger kittens between the weights of 320 and 450 grams, especially those taken from their mothers and put on the high carbohydrate diet free from milk, the results were poor. Eye infection was common. Pneumonia following the ether was frequent and in some cases they refused to eat and died

from emaciation. The kittens in this young group, 320 to 450 grams in weight, that were permitted to continue nursing and were given high protein diet of minced liver and fish in milk were in better condition so far as the eye infection was concerned. They were not emaciated and seemed to show a greater resistance than those of the intermediate weight group. Only 13 of the kittens were in the very young group; 6 were on the high carbohydrate diet and 7 were on the high protein diet. Because of the reasons given here, these 13 will not come in for much consideration in our conclusions. The emphasis will be laid on the other 36. It was observed that in the 18 on the carbohydrate diet it was harder to obtain "takes." They had a longer average incubation period: 5 to 7 days, against 3 to 5 days for those on high protein diet. The pathological lesions in the cecum and rectum were less pronounced in the carbohydrate group, the dysentery when once established was less fulminating and the duration of the disease in those that terminated fatally after the prepatent period was prolonged. One kitten after being injected 3 successive times and developing diarrhea and later dysentery upon two occasions, during which time active trophozoites were demonstrated in the feces, is still alive, 7 months later. All symptoms of dysentery have disappeared. No amebae are now demonstrable in her stool. We have shifted this cat to a high protein diet to see if she will show a return of the symptoms. Up to this time she has shown no relapses.

We have used 15 puppies in this experiment, and the same system of feeding alternate puppies on high carbohydrate diet and high protein diet. When infected with *E. histolytica,* those on the protein diet showed more symptoms of dysentery and more pronounced lesions. The disease is normally more chronic in puppies than in kittens, but we have not been able to produce cysts in either kittens or puppies that may be compared with the cysts observed in chronic conditions of amebiasis in man. There would appear in the stools of these dogs organisms that morphologically resemble cysts of *E. histolytica,* but there were certain biological characteristics generally associated with *E. histolytica* cysts that these morphological cysts from the

dogs did not have. Other dogs fed on emulsions of cysts from dogs did not become infected. These apparent *E. histolytica* cysts when washed and suspended in isotonic saline solution were killed by a temperature of 47°C. in 5 minutes, while the thermal death point for *E. histolytica* cysts similarly treated from human carriers is 68°C. in 5 minutes. These cysts from the dogs were killed by chlorine solution in the proportion of one part of free chlorine per million gallons of water in 12 minutes. This is in the same proportion as is used in swimming pools. The cysts from human carriers were not killed when similarly exposed for 30 minutes. In each instance we used as a test of viability the ability of the organism to excyst and grow when washed in saline and inoculated into suitable culture media.

After observing that these morphological cysts from these experimental animals did not possess the same criteria as were possessed by cysts from human carriers, we decided to check the cysts which several observers have reported as occurring in culture. From the time of the discovery of a practical culture medium by Boeck [16] and Drbohlav in 1924, various observers have from time to time reported encystment of *E. histolytica* in culture by alteration of the cultural environment. Poindexter [17], in 1932, reported encystment in culture in which the hydrogen-ion concentration was changed by sealing the tube with paraffin which not only increased the acidity and the carbon-dioxide tension, but produced partial anaerobiasis. In view of the observation with the morphological cysts obtained from the puppies cited above, these morphological cysts from culture were subjected to a similar test and found that they too behaved, not like the cysts found in human carriers in regard to resistance, but like those from the puppies. We attempted also to ship from Washington, D. C., some of the cysts produced in culture to Dr. A. Packchanian of New York City. He reports that they were not viable upon arrival in New York City. We know that cysts from human carriers may be viable after 2 months or more of shipment if kept moist. Realizing the value of being able to consistently and at will cause encystment in vitro, and the value of being able to ship these cysts to distant laboratories while they remain viable, we welcome the observation of Stone [18].

Stone has used a medium consisting of sodium chloride, 8 grams; calcium chloride, .20 gram; potassium chloride, .20 gram; magnesium chloride, .01 gram; sodium phosphate, (secondary, (Na_2HPO_4) 2 grams; sodium bicarbonate, .40 gram; potassium phosphate, monobasic (KH_2PO_4), .30 gram, and distilled water, 1,000 cc. With this media he reports consistent encystment at will. We have not tested morphological cysts produced by this method according to the above viability criteria. The buffer action of the KH_2PO_4, and Na_2HPO_4 in Stone's media is made use of by Gladys M. Craig[19], to keep the pH more constant. She found that between pH 6.8 and pH 8.3 the rate of growth and encystment of the *E. histolytica* increased toward the more alkaline end; in 24 and 48 hour cultures, the pH 7.4 to 7.6 were the most favorable for encystment.

For the last two years, instead of using human, rabbit or horse sera as we have done in our previous experiments, we used hydrocele and ascitic fluid. A large quantity of this was readily available from certain services of Freedmen's Hospital. It proved to be entirely satisfactory as a substitute, producing good growth and large endamoebae 35 to 40 microns in diameter. We also tried to use fluid from pleural effusion obtained chiefly from cases of tuberculous pleurisy, but we found it was not very satisfactory. The *E. histolytica* multiplied very slowly; they were unusually small in size, averaging 21 microns in diameter, and tended to die out after several transfers. Kittens infected with the culture from the hydrocele or ascitic fluid mixture showed the same incubation period as did kittens infected from human, rabbit, or horse sera. The kittens infected with the culture in pleural effusion fluid mixture showed fewer "takes" from single injections and longer incubation periods. Of course, the number of organisms were less here per c.c. of emulsion.

Since the kittens of weaning age and above, and the puppies on high carbohydrate diet showed more resistence to infection with *E. histolytica* than those on a high protein diet, we decided to make some comparative observations on the *E. histolytica* in vitro with change in concentration of available carbohydrates.

For a long time workers have used rice starch, or wheat flour as an accessory growth factor in the cultivation of *E. histolytica* even though it is not essential to their cultivation. We used powdered rice starch in our culture and were able to count as many as 32 particles of starch in an amoeba in a 24 hour culture. The amoebae also showed more bacteria in their endoplasm than is generally seen in more active non-degenerating *E. histolytica* in culture. The starch granules showed various stages of digestion. The amoebae from these cultures though in most instances numerous, were very sluggish. They would remain in one place from 4 to 6 minutes, often rounded up like a fat globule and only occasionally putting forth a pseudopod here and there and then withdrawing it. This lack of desire to progress in a definite direction differs from the slug-like progression of organisms of the same strain cultured in starch-free media, which even though smaller in number and size, would have progressed ½ the diameter of the high power field of the microscope.

By increasing the volume from the cultures of starch-free media, we were able to inject an equal number of trophozoites into each kitten per rectum. The starch-filled, sluggish amoebae did not give as high a percentage of "takes" as did the more active amoebae from the starch-free medium. If, however, the rectum were sealed for from 12 to 24 hours to prevent expulsion, the percentage of "takes" was about the same.

It has been observed that the trophozoites obtained from liver abscesses, which in most cases are filled with glycogen particles, are very sluggish and do not infect the kittens as readily as do the more active trophozoites which are not so filled, such as those from acute cases of dysentery.

Discussion: From the standpoint of the organisms themselves, and in regard to penetration of the mucosa, we believe that the mechanical action of the active pseudopodia is equal in importance to the cytolytic action of the organism described by Craig [20]. If the pseudopodia action is slowed for any reason, even in the presence of moderate necrotic changes from the cystolytic substances, the invasive power of *E. histolytica* is decreased. This decrease in invasive power may be counteracted by associated bacterial toxins, chemical irritants, the devitalizing effect on the mucosa of hot water passed

into the colon or abnormal intestinal stasis which will permit even a very sluggish endamoeba to penetrate the mucosa. The latter may account for the greater number of ulcers in the region of the cecum, ascending colon, and rectum where stasis is more pronounced.

We believe that the apparent tolerance on the part of the Puerto Rican is due in some degree to his high carbohydrate diet which gives the *E. histolytica* a rich supply of carbohydrates. This produces a lethargic state which is not conducive to active penetration of the mucosa. The few animal experiments and the in vitro observation tend to support that theory. This lack of tissue penetration and the multiplication of *E. histolytica* in the lumen of the intestine where plenty of carbohydrates are present may account for "nonsymptomatic healthy carriers." Craig [21] does not feel that there is multiplication without tissue invasion. The type of body defense in regard to *E. histolytica* infection is not known. The lymphocyte infiltration and connective tissue proliferation around the ulcers in the colon are the chief responses. They are not pronounced. It is not unusual to find endamoebae, when unaccompanied by bacteria, lying between the muscularis mucosa fibers or below them in a small area of cytolysis, without any pronounced cellular infiltration.

Endamoeba histolytica infection is usually without dramatic onset. The fulminating types are generally associated with some member of the Shigella genus or a hemolytic streptococcus. The hemolytic streptococcus produces an acid form of toxin that will inhibit the growth of *Escherichia coli.* This bacterial antagonism may serve to give *E. histolytica* a chance to proliferate without the normal protective flora of the colon. A greater knowledge of the effect of climate alone and of the variation in intestinal flora in different climates will be of value in the solution of this problem.

Summary and Conclusion: *Endamoeba histolytica* trophozoites that were heavily filled with starch granules from cultures containing rice starch were more sluggish than were those cultures in starch-free media. These sluggish endamoebae showed less invasive power when injected into kittens.

Kittens weighing from 450 to 1,180 grams and fed on a high carbohydrate diet consisting chiefly of wheat bread,

rice, beans, and water, showed more resistance to infection with *E. histolytica* than did the kittens of the same weight, fed on a high protein diet consisting chiefly of minced fish and liver in milk.

Puppies placed on similar diet showed corresponding results to the kittens; the puppies in each case being naturally more resistant. Morphological cysts obtained from the puppies when subjected to biological criteria were shown to differ from the cysts obtained from carriers in human beings. They were less resistant to chlorine, had a lower thermal death point, did not infect kittens or puppies when given by mouth to these animals, and could not be cultured after shipment from Washington, D. C., to New York. The morphological cysts produced in culture by alteration of the media corresponded in biology to those obtained from the puppies just mentioned. Hydrocele and ascitic fluid were used as satisfactory substitutes for the human, rabbit, and horse sera in the culture of *E. histolytica.* Fluid exudate from pleural cavity in cases of tuberculous pleurisy with effusion was not a good substitute. Kittens under 450 grams of weight were not considered satisfactory for *Endamoeba histolytica* experiments where special diets are used.

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