

BODY OF REPORT

SEATO Medic Study No. 120 Amebic Dysentery, Experimental Studies on Pathogenesis

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S. E. Asia

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Principal Investigator: David Weinman, MD

Assistant Investigator: Mrs. Pirom Phisphumvidhi

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Objective: To study and identify factors which determine the pathogenicity of amebic infection.

Description:

1. Cultures of Entamoeba histolytica, either bacteria-free, or grown with a mono-bacterial associate which can be inhibited by antibiotics, have been available for experiment.
2. By using these cultures, experimental pathogenicity will be investigated.
3. With the above results as a base-line, studies then will center on the pathogenicity of pure or antibioticly inhibited cultures to which single types of micro-organism are added. It will be necessary to devote initial efforts to finding test systems suitable for the various microbial agents to be studied.
4. The problem of amebic liver abscess will be particularly studied. These

are usually reported as sterile by bacteriological tests. If amebae alone are non-pathogenic, then the pathogenesis of liver abscess is obscure. It is possible that a hepatic lesion caused by an additional microbial agent allow the amebae to become established initially. Weeks to months later, when a liver abscess has formed, the additional agent may no longer be demonstrable. This is particularly true if the additional agent is a virus for which short term recovery is the rule. The problem will be investigated by processing human liver aspirate for the recovery of persisting virus and experimentally by investigating the effect of virus infection on localization and survival in the liver.

5. In vitro studies of the relationship of *Entamoeba histolytica* to other microorganisms will complete the investigation.

Progress. Background. Approximately 10 months ago the laboratory became functional, experimental work began and has been carried on by the two investigators listed, the principal investigator being able to devote approximately half-time to the project.

The working-out of appropriate methods was the first problem, and this has received major attention during the report period. As a major point to be investigated was the influence of animal viruses on amebic pathogenicity, a test system had to be provided which would permit survival of viruses and amebae. This system was limited to one providing living mammalian cells. A further requisite was that the system should reduce the possibility of introducing additional infectious agents to a minimum. This excluded all customary procedures involving intestinal inoculation. Procedures investigated have been the inoculation of embryonated eggs, of hamsters inoculated intra-abdominally and into the liver, and micr intra-cerebrally.

Experimental:

1. Entamoeba histolytica preparations employed.

Nearly all experimental inoculations were performed with strain 301 of Entamoeba histolytica, and all references to amebae or to E. histolytica are to this strain except as stated. Strain 301 was obtained from Dr. Louis Diamond of the National Institute of Health as a bacteria-free culture, and a certain number of inoculations were performed with these bacteria-free cultures. Extreme morphological peculiarities were noted in the cultures on arrival, and it proved impossible to maintain this strain in a bacteria-free state in medium provided by the donor or made locally. The reasons for this are unknown. After experimentation it was found that rich cultures capable of unlimited transfer could be obtained on whole egg medium to which Clostridium perfringens was added. This medium is sensitive, and an inoculation of 4 to 10 amebae has given cultures in each of 17 trials according to data from this laboratory provided by Mr. Somkiet Vongtangswad. Clostridium perfringens is sensitive to penicillin, and a concentration of 10 units per ml prevents growth in the Reinforced Clostridial Medium recommended for anaerobes. Inoculations of amebae have been made employing

bacteria-free cultures of amebae, penicillin-inhibited cultures containing Clostridium perfringens, and, in two trials, directly from liver aspirate containing amebae into eggs and hamsters. These results were then compared with inoculations in which amebae and bacteria and/or virus were combined.

2. Test Systems Employed and Results.

a. Chick embryo

The chick embryo being susceptible to herpes infection, the first virus to be tried, has been investigated extensively. Experiments using 10-day-old embryos inoculated on the chorio-allantoic membrane and incubated at 37° have been performed, using pure cultures of amebae, penicillin-inhibited cultures, cultures containing viable Clostridium perfringens, and eggs given herpesvirus either simultaneously with the amebae or several days in advance. One preparation of liver abscess aspirate was also inoculated directly on the C-A membrane. The numbers of amebae inoculated varied from 10^3 to 2×10^5 , that is, each inoculum was capable of growing out under good cultural conditions. Eggs were opened one to four days after inoculation. No normal amebae were ever seen or recovered in culture from the inoculated eggs under any circumstances. As will be shown below, strain 301 does cause lesions in animals. It is apparent, therefore, that the CAM method is not a satisfactory test system with the experimental procedures employed. Limited trials of yolk sac inoculations also have not yielded viable amebae.

b. Hamsters

Observations on hamsters are on a small scale as yet, require additional experiments to be conclusive, but have already yielded information of interest.

The inoculation of less than 50,000 amebae has given no lesion and no recovery of amebae in 20 animals. This includes 6 inoculated with bacteria-free preparations: 4 with 10^3 amebae of strain 301 directly in the liver and 2 with liver aspirate containing an estimated 10^3 amebae inoculated intra-abdominally. Fourteen other negatives followed intra-hepatic inoculation with mixed 301 and Clostridium perfringens. As noted, 10 amebae suffice to initiate a culture.

Nine animals have thus far been inoculated with larger doses. Intra-abdominal injection of 4 to 5×10^5 amebae with viable Cl. perfringens produced no lesions in 3 animals, an extra-hepatic abscess containing amebae and bacteria in the fourth; whereas the same dose of the same preparation given to 5 animals which had received herpesvirus 24 hours before (either by intra-hepatic or intra-peritoneal injection) produced liver abscess containing amebae in three, and extra-hepatic ameba-positive abscess in one and no lesion in the last animal. Repetition of the factors involved is under way.

c. Mice

No effect was detected in young adult mice inoculated intra-cerebrally with 2700 to 4500 bacteria-free amebae, and no amebae could be seen in or cultured from the brain when the animals were sacrificed. Likewise with a mixed

inoculum containing 10^3 to 10^4 amebae and Clostridium perfringens, no amebae could be detected either by direct examination or by culture at elective autopsy. The effect of herpes as a potentiating pathogenic agent will be studied, but thus far we have no evidence that brain inoculations in mice provide a satisfactory test system.

3. Studies on Aspirate from Amebic Liver Abscess.

Specimens from twenty-eight different cases of amebic liver abscess have been made available for study by the Royal Thai Army Hospital, the School of Tropical Medicine and other cooperating institutions.

Seventeen of these specimens have been processed in collaboration with the Department of Virology. Isolation methods used were tissue cultures of monkey kidney and Hep 2 lines and the inoculation of suckling mice. No viruses have been isolated. This indicates that no viruses isolable by these methods persisted in the liver abscess, but furnishes no information on the possible role of viruses at the time of initial seeding of the liver with amebae. A bacterium isolated from one set of infant mice was identified through the courtesy of the Department of Bacteriology as belonging to the Salmonella C1 group. The organism is presumably of murine origin as it did not agglutinate with the patient's serum at dilutions of 1:10 or above.

Eleven additional aspirate samples are under way, for virus isolation. For specimens in which E. histolytica was observed, three new strains of amebae have been established in culture. These strains are now available for comparative studies and have served as thesis material in a Master of Science dissertation from this laboratory.

Summary and Conclusions:

1. The etiology of amebic disease is obscure. In an attempt to clarify some factors involved, Entamoeba histolytica is being tested for pathogenicity. Attention has been devoted to: (1) the selection of a satisfactory test system and (2) comparing in such a system the effects of bacteria-free amebic inocula with the effects of amebae associated with a single species of bacterium (Clostridium perfringens) and a single virus (herpesvirus).

2. A satisfactory test system would allow growth of both amebae and viruses, and it should be also a procedure which will minimize the risk of introducing additional infectious agents. The systems used have been (1) the embryonated chicken egg, by inoculation into the chorio-allantoic membrane and into the yolk sac; (2) mice, by intra-cerebral injection; and (3) hamsters by intra-hepatic and intra-abdominal inoculation.

3. Bacteria-free preparations of amebae have provoked no lesions, and have not been recovered after inoculation of 10^3 to 10^4 organisms in chick embryos, hamsters or mice, whereas, under good cultural conditions, 10 amebae can al-

ways be expected to initiate a culture.

4. Amebae associated with Clostridium perfringens and inoculated into hamsters produced no amebic lesions, and E. histolytica was not recovered with an amebic inoculum of less than 5×10^4 . Inoculation of larger numbers of amebae in mixed cultures has been done on only a limited scale, but approximately half the hamsters have developed abscesses, liver or other, from which amebae were recovered. Analysis of the factors involved is under way.

5. The chick embryo has not yet proved satisfactory. Regardless of dose of microbial associates (Cl. perfringens and herpesvirus), recovery of amebae has not been obtained following a large number of inoculations on the chorio-allantoic membrane and a smaller number in the yolk sac.

6. Mice have been used on only a limited scale and with small doses. Neither bacteria-free preparations nor combined inoculations Cl. perfringens produced brain abscess containing E. histolytica when the amebic inoculum was in the 10^3 range.

7. Aspirate from seventeen cases of liver abscess has been processed for virus, using infant mice and monkey kidney and Hep 2 cell lines. No virus was recovered, indicating virus detectable by these methods did not persist in the liver abscess material. Eleven additional liver aspirates are under study.

ADDENDUM: Educational Commitment.

As referred to in the previous Annual Report of 1 April 1963, one thesis student, Mr. Somkiet Vongtanswad, from the Faculty of Graduate Medical Education was welcomed into the laboratory for experimental work on E. histolytica. The thesis completed, the M.Sc. degree was awarded this month. Reference to this work is included in the body of the report.