Review

Current Problems of Amebiasis in Japan and Recent Advances in Amebiasis Research

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SUMMARY: Amebiasis is epidemic in two major populations in Japan: male homosexuals and institutionalized people. Currently available diagnostic, chemotherapeutic, and prophylactic measures and their problems are discussed. Recent advances in basic research on amebiasis are also described with new findings of unique metabolisms and intracellular structures and organization. Discoveries and analyses of the unique features presented by this parasitic protist help in our elucidation of the pathogenic mechanisms of the parasite and may eventually lead to the development of new drugs and vaccines against amebiasis.

1. Introduction

The eukaryotic protozoan Entamoeba histolytica is a major cause of morbidity and mortality worldwide (1). There are an estimated 50 million cases of invasive amebiasis and 40,000-110,000 deaths annually (1). The most common clinical presentation of amebiasis is amebic dysentery and colitis; extraintestinal abscesses (i.e., hepatic, pulmonary, and cerebral) are, however, also common and often lethal. Infection of the human host occurs upon ingestion of water or food contaminated by the cyst form of the parasite. These cysts then excyst to form ameboid trophozoites that subsequently colonize the large bowel. Colonization of the human gut by E. histolytica most commonly results in an asymptomatic intestinal infection similar to that resulting from the closely related commensal E. dispar spp. (2). In approximately 5-10% of those infected with E. histolytica, disease and symptoms including bloody diarrhea, fever, tenesmus, and colitis develop. However, the rest of the infected individuals remain asymptomatic. Immunological, biological, and genetic mechanisms that contribute to whether or not a disease develops have not been elucidated.

The aim of this review is to summarize the current situation regarding amebiasis in Japan, and to highlight the remaining problems of diagnosis, treatment, and intervention. Recent advances in basic research to understand the pathogenesis and biochemical and biological peculiarities of this organism will also be discussed.

2. Epidemiology of amebiasis in Japan

Cases of amebiasis have often been found in two groups in Japan: male homosexuals (3-7) and in institutionalized people, including the residents of institutions for the mentally retarded (8-13). The high prevalence of amebiasis in male homosexuals is a unique characteristic of amebiasis in Japan. In the early 1980s, a high percentage of the male homosexuals in the United States (U.S.) and Europe were reported to be infected with E. histolytica. However, neither the presence of clinical symptoms nor positive serology correlated with the presence of the parasites in the stool. These cysts then excyst to form ameboid trophozoites that subsequently colonize the large bowel. Colonization of the human gut by E. histolytica most commonly results in an asymptomatic intestinal infection similar to that resulting from the closely related commensal E. dispar spp. (2). In approximately 5-10% of those infected with E. histolytica, disease and symptoms including bloody diarrhea, fever, tenesmus, and colitis develop. However, the rest of the infected individuals remain asymptomatic. Immunological, biological, and genetic mechanisms that contribute to whether or not a disease develops have not been elucidated.

The aim of this review is to summarize the current situation regarding amebiasis in Japan, and to highlight the remaining problems of diagnosis, treatment, and intervention. Recent advances in basic research to understand the pathogenesis and biochemical and biological peculiarities of this organism will also be discussed.
The prevalence of amebiasis in institutionalized people, e.g., the residents of institutes for the mentally retarded, has also been reported several times since late 1980s in Japan (8-13). A high prevalence of amebiasis in institutions was also reported in the U.S., and believed to be associated with typical abnormal behavior of the retarded (22). Besides the residents of institutes and daycare centers, a small percentage of health care workers are also found to be infected (e.g., 2%, [12]). There are also quite a few cases of imported invasive amebiasis, which presumably constitutes up to 20% of all cases in Japan (e.g., [7]). In contrast to the adult domestic cases, which are almost exclusively male, there is no reported bias on gender in the imported cases. However, it has been shown by field studies conducted in Vietnam that males have a higher risk of developing liver abscesses than females (Tannich et al., unpublished), suggesting that gender bias regarding a susceptibility to amebic liver abscess exists.

3. Current problems of amebiasis

1. Diagnosis

A majority of all diagnoses of amebic infection have been, and still are, based on light microscopic demonstration of hematophagous trophozoites in stool or liver aspirate. Physicians and lab technicians need to be careful not to overdiagnose E. histolytica by mistakenly identifying non-pathogenic amoebae, macrophages, or white blood cells. Unfortunately, microscopy does not differentiate between E. histolytica and E. dispar. This is not usually a problem in diagnosing invasive amebiasis, since a demonstration of hematophagous trophozoites in the specimen represents a definitive diagnosis. However, asymptomatic cyst passers of either the pathogenic E. histolytica or E. dispar need to be differentiated, since cyst passers of E. dispar do not require treatment. In contrast, asymptomatic E. histolytica cyst passers must be treated since they are a potential public health hazard, and long-term carriage could lead to later acute invasive disease.

A conventional method of differentiating the two ameba E. histolytica and E. dispar is cultivation and propagation of the isolated amoebae, followed by zymodeme profiling by isoenzyme electrophoresis. However, these procedures are time- and labor-consuming, and therefore are routinely performed in only a few laboratories in Japan, including the Department of Tropical Medicine and Parasitology, Keio University School of Medicine. However, antigen-capture enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies that distinguish between E. histolytica and E. dispar are commercially available (e.g., Techlab, Inc., Blacksburg, Va., USA). Alternatively, to detect parasite DNA in the specimen, a method to specifically amplify either E. histolytica or E. dispar genomic DNA by polymerase chain reaction (PCR) is available in some laboratories (e.g., Department of Infectious Diseases, Tokai University School of Medicine, and Keio University).

Infection with the pathogenic E. histolytica is often, but not necessarily always, associated with positive serologic tests, including gel diffusion precipitin test, hemagglutination test, immunofluorescence assay, and ELISA. These tests can be performed either at private laboratories, universities, or national institutes, where reagents are available. Some kits, including hemagglutination (E. histolytica HA, Japan Lyophilization Laboratory, Tokyo, discontinued) and indirect immunofluorescence assay (Ameba-spot IF, #72901, Biomerieux, Tokyo), are commercially available.

2. Treatment

As previously mentioned, E. dispar cyst passers require no treatment. This may pose the medical care provider with a question: whether or not asymptotic cyst passers need to be treated, because most physicians and clinical lab workers do not have immediate access to laboratories where isoenzyme characterization and PCR are routinely performed. However, consultation with these laboratories is strongly encouraged because asymptomatic E. histolytica cyst passers are a potential threat to public health, and there is also a possibility of future relapse. Freezing a stool specimen normally preserves the integrity of parasite DNA for at least a few months for future identification of the infecting parasites by a variety of PCR-based methods, and therefore is strongly recommended. After a diagnosis is made, the E. histolytica cyst passers should be treated with one of the lumen-acting amebacides described below.

The commonly used anti-ameba drugs metronidazole (Flagyl) and tinidazole (Fasigyn) have been proven to be effective against invasive amebiasis, including both intestinal and extraintestinal amebiasis. Extensive clinical experience and epidemiologic data showed that these compounds are not carcinogenic or mutagenic in humans (23). Chloroquine, emetine, and dehydroemetine have been successfully added to metronidazole treatment for the rare patient not responding to metronidazole or tinidazole alone (24,25). However, these compounds are well absorbed from the intestinal lumen, and, therefore, are generally not effective in the treatment of asymptomatic cyst passers unless therapy is prolonged for a minimum of 10 days. With shorter courses of metronidazole therapy, patients may suffer a relapse of invasive amebiasis months later (24,25). The lumen-acting drugs diloxanide furoate (Furamide), iodoquinol (Diodoquin), and paromomycin (Humatin) are often used to treat asymptomatic cyst passers and also to prevent relapses from persistent cysts after invasive amebiasis. Successful treatment of the cyst passers with metronidazole only or with metronidazole and diloxanide furoate on 5 consecutive days followed by 5 more days of metronidazole was also reported for several Japanese cases (11,13). Whether or not treatment is effective for each cyst passer could depend upon biological differences in the virulence of the infecting strain and/or genetic differences in the human immune background (see below).

The fact that not only institutionalized people, but also a fraction of health care workers are infected, suggests that transmission to healthy individuals actually occur. As this transmission could pose a serious threat to public health, those infected need to be identified and properly treated. To prevent transmission among homosexual males, people should be made aware of transmission by oral-anal sexual practices and of the relation between infection and sexual promiscuity.

3. Vaccine

E. histolytica exhibits a rather simple developmental transition in its life cycle, having only two known stages, trophozoite and cyst. It has been suggested that a great proportion of proteins are expressed in both developmental stages, providing logistic identification of target molecules expressed in both stages. There are no insect vectors or secondary hosts involved in the transmission of amebiasis. Furthermore, as E. histolytica is an extracellular parasite, an antibody is predicted to play a potential important role in vaccine-induced
immunity to amebic infection. All these suggest that we have a very good chance of developing a successful amebiasis vaccine. Several recombinant proteins have been tested for their efficacy in the gerbil model, including serine-rich *E. histolytica* protein (SREHP) (26), Gal/GalNAc-inhibitable lectin (27), and Peroxiredoxin (28). Animal studies using these recombinant proteins demonstrated the feasibility of developing a recombinant parenteral vaccine to prevent amebic liver abscess (29-38). Since *E. histolytica* first adheres to intestinal epithelial cells to cause disease, mucosal anti-amebic antibodies should play a critical role in defense. Stanley and colleagues, therefore, utilized an antigen delivery and presenting system that is capable of stimulating mucosal IgA antibody production. They first produced the dodecapeptide repeat of SREHP fused to the cholera toxin B subunit. When this antigen was orally administered, IgA anti-ameba antibodies were induced in the stool and in bile, and the numbers of antibody-secreting cells producing anti-SREHP antibodies were also significantly increased in mesenteric lymph nodes and the spleen (32). They subsequently explored the use of attenuated strains of *Salmonella* as carriers to deliver the SREHP or Gal/GalNAc lectin protein to induce mucosal and cell-mediated anti-amebic immune responses (33, 38, 39), showing that in the gerbil model, an oral vaccine combining the recombinant amebic proteins and the attenuated *Salmonella* strain can provide protection against amebic liver abscess. Successful utilization of the *Salmonella* and the *Vibrio* strains (35) for delivery of vaccine antigens offers the possibility of a combination amebiasis/typhoid fever and amebiasis/cholera vaccine.

Although all of these studies appear promising, there are still a number of important issues to be resolved. First, all of these studies were conducted to assess protection against amebic liver abscess with only one animal-model gerbil. Consequently, it is largely unknown whether or not the observed protection in the gerbil reflects the potential success of an ameba vaccine against human intestinal amebiasis. Second, the natural polymorphisms of these target molecules remains unknown. Therefore, it is not known whether immunization with recombinant proteins from one reference strain can protect gerbils against heterologous *E. histolytica* strains.

Third, currently used attenuated *S. typhi* strains still have, as shown in previous clinical trials in humans, side effects such as fever and diarrhea with vaccination. Thus, a truly innocuous host strain needs to be developed.

### 4. Recent developments in basic research on amebiasis

#### (1) Virulence factors

Quite a few virulence factors have been identified in the last 10-15 years. These factors include cysteine proteinases (40-44), Gal/GalNAc inhibitable lectin (45-49), amebapore (50-54), and phospholipase (55-57), and details on each factor should be consulted in the original references.

Antioxidative mechanisms of *E. histolytica* are also a part of the virulence function, as the parasite has to detoxify the host oxidative stress in order to survive in oxygen-rich tissues and organs, including the liver, lung, and brain. In contrast to other eukaryotic cells, *E. histolytica* lacks glutathione and glutathione-dependent enzymes (58). cysteine is the major thiol in *E. histolytica* and is presumed to play an important role in antioxidative defenses (58,59). cysteine has been shown to be essential in its attachment to the matrix (60), elongation, motility, and growth in vitro (61,62). As shown in Fig. 1, *E. histolytica* possesses a sulfur assimilatory cysteine biosynthetic pathway, where inorganic sulfur is fixed into an organic compound, cysteine, in the pathway that is absent in its mammalian hosts (63-66). Biochemical and reverse genetic studies of the major enzymes of this pathway showed that this pathway plays an important role in monitoring total cellular thiol concentrations and as a part of an antioxidative mechanism (65). Since this pathway is absent in mammalian hosts, it is a good target for developing a new chemotherapeutic. Peroxiredoxin (formally called as 29- or 30-kDa cysteine-rich protein [67-69]) is a ubiquitous antioxidant conserved from prokaryotes to higher eukaryotes, and has also been shown to be involved, in amoeba, in an antioxidative mechanism (70,71) together with NADPH:flavin oxidoreductase (70).

#### (2) Peculiar organellar structure

*Entamoeba* trophozoites appear to have a very primitive cytoplasmic structure (Fig. 2A). The major cytoplasmic components are the ingestive and digestive vacuoles, the residual bodies, the nucleus, and a number of smaller membranous vesicles. α-Glycogen is distributed throughout the cytoplasm. The rough endoplasmic reticulum (ER) is not present in the usual form, but is functionally replaced by shorter lengths of helically arranged ribosomes. These helices coalesce into bundles as larger crystalline arrays, forming the chromatoid bodies. The smooth ER is also poorly developed. Both typical Golgi apparatus and mitochondria are absent. The trophozoites also lack peroxisome and hydrogenosome. *E. histolytica* has long been thought to be an ancient eukaryote due to a lack of mitochondrion, a major source of ATP in most eukaryotic cells. However, a recent demonstration of two *E. histolytica* genes encoding proteins that in other eukaryotes are localized in the mitochondrion, i.e., pyridine nucleotide transhydrogenase and the chaperonin cpn60, unequivocally proved that *E.
Enzymes from E. histolytica showed a close phylogenetic association with ORF of the enterobacteria and T. vaginalis, which targeted its ORF to hydrogenosome (76). Moreover, a gene encoding iron hydrogenase, demonstrated in hydrogenosome of T. vaginalis (77), is present in Entamoeba, and its recombinant protein is enzymatically active in vitro.

Vesicular transport in E. histolytica is believed to share a common basic machinery with that in other eukaryotes (Fig. 2B). Common proteins involved in vesicular transport and shared by both E. histolytica and other eukaryotes include small GTP-binding proteins (Rab [78–80] and Arf [81]), ER chaperons BiP (81,82) and putative disulfide isomerase, signal recognition particle (Srp54) (83) and ER membrane protein Sec61, which is involved in transport from the cytosol to the ER lumen, Golgi protein Erd2 (84), and lysosomal vacular ATPase (85). More than ten Rab genes have been identified and characterized from E. histolytica (78–80). All of these Rab genes obtained by sequence homology or from EST databases show high identities to Rabs from other species. Furthermore, Rab5 from E. histolytica, for example, does not reverse the growth defect of a yeast rab5-mutant, and is involved in phagocytosis, whereas, in yeast and mammalian cells, Rab5 is located in endosomes and is involved in vesicular transport from the plasma membrane to early endosome and in the homotypic fusion of early endosomes during endocytosis (78). Aley et al. showed that E. histolytica trophozoites possess a unique non-acidified rapidly exchanging endocytic compartment (86). These data suggest that Entamoeba has developed unique machinery in protein trafficking, especially in the endocytic and phagocytic pathways, that significantly differs from that of other eukaryotes, although E. histolytica mostly shares identical proteins for vesicular transport from ER to Golgi.

(3) Prokaryote-like energy metabolisms

E. histolytica is a unique eukaryote with respect to energy metabolism (87,88). As a mitochondria-lacking, and anaerobic or microaerophilic eukaryote, E. histolytica largely depends on glycolysis and fermentation for its energy production. E. histolytica lacks several typical eukaryotic glycolytic enzymes. Pentose phosphate shunt enzymes are completely missing. Another unusual feature of glycolysis is the utilization of pyrophosphate instead of ATP in several enzyme reactions: phosphofructokinase (89), phosphoenolpyruvate carboxytransphosphorylase (90), pyruvate phosphate dikinase (91), and acetate kinase. E. histolytica also possesses fermentation enzymes that resemble those of anaerobic bacteria. These enzymes include POR, ferredoxin, and alcohol dehydrogenase. Phylogenetic analyses strongly suggest that these amebic genes encoding fermentation enzymes are laterally transferred from bacteria that amebae have phagocytosed (76). This so-called “net hypothesis” is supported better than “tree hypothesis”, which proposes that these amebic genes were present in a common eukaryotic ancestor that lived under anaerobic conditions, but were lost from other eukaryotes because fermentation enzymes are not necessary for growth in the presence of oxygen (76).
5. Remaining problems in amebiasis research

Why does disease occur only in 5-10% of those infected? This has been a long-standing question since the first description of amebiasis in 1875 by Lesh (Losch) ([92], for history of amebiasis also see [93,94]), and we still do not have a proper answer to it. There are three possible explanations. 1. Commensal theory. E. histolytica is usually viewed as an innocuous gut commensal. However, with a yet-unconfirmed stimulus (e.g., nature of gut flora, dietary implications), it turns to a tissue-invading form that lost its capacity for cyst production. Thus far there is no direct evidence supporting this hypothesis of a possible conversion from a non-virulent to a virulent type, such as an identification of differentially expressed pathogenic factors. 2. Host immune theory. It depends on the genetic background of host immune systems with regard to whether amebiasis develops in the infected individual. It was reported that individuals with a certain type of human leucocyte antigen (HLA) in Mexican mestizo (DR3) are more susceptible to amebic liver abscess (95). Although no such association was found between HLA types and amebic colitis (96), the contribution of the host genetic background on the susceptibility to infectious diseases is widely known (e.g., [97,98]). In E. histolytica infections, macrophages play a crucial role in host defense involving both oxidative (e.g., O₂⁻ and H₂O₂) and non-oxidative (e.g., tumor necrosis factor [TNFα] and nitric oxide [NO]) mechanisms (99-103). Therefore, it is reasonable to investigate whether polymorphisms of the genes involved in TNFα and NO production are associated with susceptibility to amebiasis. 3. Spectrum of virulence among the E. histolytica isolates. Not all amoeba strains are equally virulent: i.e., there is a spectrum for the strength of virulence in the E. histolytica population. It is known, based on zymodeme characterization, that there are at least three E. histolytica subtypes (104). Recent studies on the diversity of the E. histolytica field isolates revealed that genetic polymorphisms of several genes, including SREHP and chitinase, exist in E. histolytica (105). Clark and his colleagues have also initiated an investigation of clonal diversity within E. histolytica spp. using novel polymorphic alleles (Clark, unpublished).

There are more than a dozen unanswered questions that are not discussed in this review. These questions were also summarized by Graham Clark in the opening lecture at the XIVth Seminar on Amebiasis, Mexico City, 2000. Important basic questions regarding amoeba biology, molecular biology, and biochemistry include: Are there any other stages, including extra-intestinal propagation of amoebae? As E. histolytica genome database is rapidly growing, enormous information regarding unique and, therefore, applicable aspects of metabolisms, biological pathways, and gene expression should be readily available. Such information from genomics, together with conventional research results, should help in further exploring possible targets in the development of new chemotherapeutic and prophylactic measures. Considering the rapid occurrence of multidrug resistant strains of protozoan and prokaryotic pathogens, e.g., Plasmodium and Mycobacteria, drug-resistant strains of E. histolytica will probably emerge in the future. Therefore, we need to continue to look for parasite-specific cellular events not only to elucidate pathogenic mechanisms of the parasite, but also to eventually develop measures to intervene in transmission of the disease.

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