Experimental Parasitology 126 (2010) 14–21

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Balamuthia mandrillaris: The multiple nuclei of *Balamuthia* amebas; their location, activity, and site of development

Thelma H. Dunnebacke *

Viral and Rickettsial Disease Laboratory, California Department of Public Health, 850 Marina Bay Parkway, Richmond, CA 94804-6403, USA

ARTICLE INFO

Article history:
Received 22 September 2009
Received in revised form 18 October 2009
Accepted 21 October 2009
Available online 24 October 2009

Keywords:
Nuclei
Nucleoli
Fusing nuclei
Polyploid nucleus

ABSTRACT

Multiple nuclei were first noted in the pseudopodia of *Balamuthia mandrillaris* amebas feeding on mammalian cells. Phase microscope observations of live amebas in vitro reveal that while many amebas have a single nucleus, others have multiple nuclear-like structures, now confirmed as nuclei with hematoxylin and Feulgen stains. In the live cultures, two nuclei located near the tip of an extended pseudopodium were seen to fuse resulting in one larger morphologic unit. Such merging of nuclei has not been previously reported. Other nuclei were located at positions that subsequently became the site for the outgrowth of an additional pseudopod branch. A newly discovered large structure, a polyploid nucleus, was located in the mid-part of the ameba. Nucleoli of uniform size were seen to develop from the central mass of chromatin and each became surrounded by a vesicular component as they moved into the protoplasm as morphologically complete nuclei.

1. Introduction

The *Balamuthia mandrillaris* amebas are free-living opportunistic pathogens found in soil and dust (Schuster et al., 2003; Dunnebacke et al., 2004; Niyyati et al., 2009). First recognized and isolated from a mandrill baboon, they now are recognized as the causative agent for usually fatal granulomatous encephalitis in humans and other animals (Visvesvara et al., 1990, 1993). The symptoms are insidious and the disease may not be recognized in the early stages of the infection (Schuster et al., 2009). The diagnosis, often postmortem, is based on the microscopic examination of fixed, stained, sectioned pathological material used for the visual identification of the amebas and cysts in the tissue (Visvesvara and Schuster, 2008). Additional diagnostic measures include immunologic detection of amebic antibodies in the serum by direct immunologic staining (Schuster et al., 2006), by enzyme-linked immunology (Schuster et al., 2008), and by the detection of the amebic specific 16S rRNA gene by polymerase chain reaction (Yagi et al., 2005).

The nuclei of the *B. mandrillaris* amebas have been described as vesicular with a single, large, dense, centrically located nucleolus (Martinez et al., 1993). The nuclei, however, may have more than one nucleolus (Martinez and Visvesvara, 2001). The amebas have been described as uninuclear, and binuclear. During their mitotic division, the nucleolus appears to split in two. The nuclear membrane remains intact at first, followed later by the breakdown of both the nucleolus and nuclear membrane (Visvesvara et al., 1993).

Multiple nuclear-like bodies in *B. mandrillaris* were first noted during observations of the amebas while feeding in culture with monkey kidney cells (Dunnebacke, 2007, Fig. 2); they were 2–4 μm in diameter, with a large, dense central body; they were present near the tips of extended pseudopodia; their numbers per ameba varied. Their movement along with the protoplasmic granules in the pseudopodia was fluid and their outward or inward direction followed the extension, or the retraction of their pseudopodium. Although they were basically round, they were pliable as shown when they were squeezed around a sharp angle while moving from one pseudopod branch to another. Since the available information was that the *B. mandrillaris* basically had a single nucleus, the question arose about the identity of these additional "nuclear-like" structures in the pseudopodia. Their central area was about the same size, shape and density of the nucleoli in the monkey kidney cells posing the possibility that they could be nuclear fragments from the monkey kidney cells that had been ingested by the amebas. This reasoning seemed remote because: (1) there was a slight photographic difference in the densities of the nucleoli of the cells and that in ameba-structures; (2) the ameba preferentially feed on the cytoplasmic portions of the cells; (3) and the *B. mandrillaris* apparently did not feed by phagocytosis of cellular material (Dunnebacke, 2007). Even so, it was our interest to question the origin and nature of these multiple nuclear-like structures in the amebas.

The observations reported have been made from cultures of *B.* mandrillaris that have been propagated in axenic medium, free of tissue cells. The isolates examined include those obtained from

* Address: Viral and Rickettsial Disease Laboratory, California Department of Public Health, 850 Marina Bay Parkway, Richmond, CA 94804-6403, USA. Fax: +1 510 307 8605.
E-mail address: dunnebacke@gmail.com

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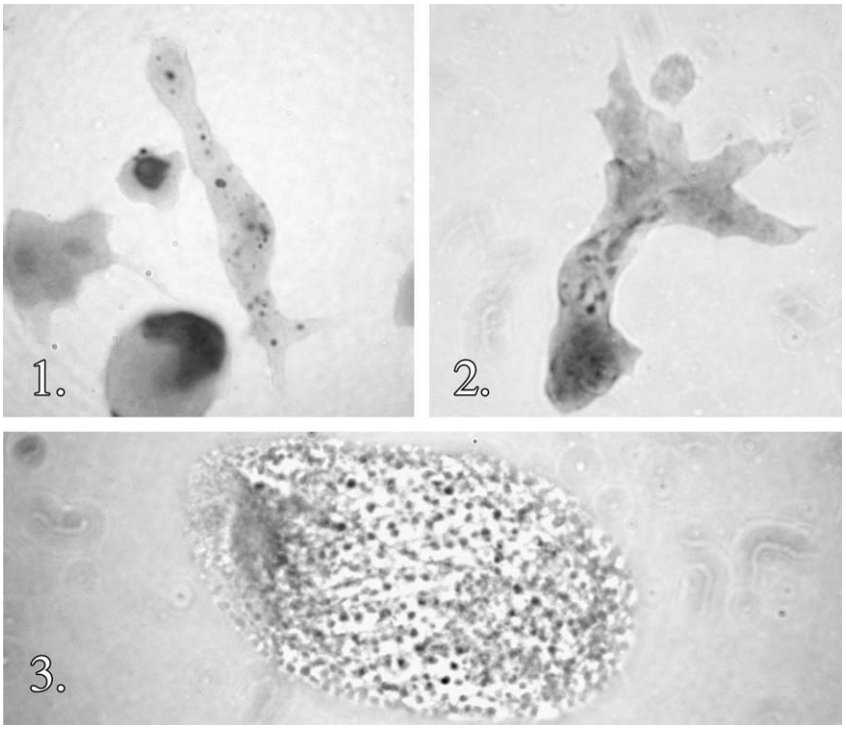
doi:10.1016/j.exppara.2009.10.012



Figs. 1–3. Feulgen stained amebas (Fig. 1) from a V188 culture show the presence of a thinly spread ameba with two Feulgen negative nuclei, two rounded amebas each with a single nuclear Feulgen positive unit; adjacent to the smaller nucleus are two minute units. The elongated fourth cell contains multiple nuclear units that vary in size. The Feulgen stained ameba (Fig. 2) from a V426 culture contains a large unit plus scattered smaller units throughout the elongated cell. An ameba (Fig. 3) from V188 stained with iron hematoxylin displays numerous small nuclei arranged in lines plus a large nuclear component at one end of the ameba. Photographed at 1200×.

two humans, a baboon and a sample of soil. Amebas with multiple nuclei are present in cultures fixed and exposed to nuclear specific stains. In live cultures, the findings show: (1) the presence of multiple nuclei in a portion of an ameba population; (2) the ability of the nuclei to fuse; (3) an association between the site of a nucleus in a pseudopodium and the subsequent development of a new branch of the pseudopodium at that location; and (4) the discovery of a polyploid structure in the amebas, likely a macro-nucleus, that is a source of nuclear formation.

2. Materials

Three of the *B.* mandrillaris amebas observed in these studies were originally from the laboratory of Dr. G.S. Visvesvara at Centers for Disease Control and Prevention, Atlanta, GA. The cultures, V039, V188 and V426 had been shared with Dr. Fred Schuster of this laboratory, and subsequently with the author. The culture V039 originated from a baboon in California (Visvesvara et al., 1990), culture V188 from a man in Georgia (Gordon et al., 1992).

The culture V426 was from a child in California (Bakardjiev et al., 2002) and ameba culture RP5 was isolated from the soil of a plant (Schuster et al., 2003). Stocks for each of the cultures were maintained in axenic medium BM-3 (Schuster and Visvesvara, 1996) at 37 °C in closed flasks.

3. Methods

Preparations of live cultures were observed by phase contrast microscopy in cover slip-microscope slide chambers sealed with paraffin (Dunnebacke, 2007). The active amebas were able to survive in these chambers at room temperature for 2–4 days. The live cultures were observed with a Nikon microscope with 40× and 100× objectives and photographed with a digital camera, Canon Power Shot G3 (4× optical zoom) or a Canon Power Shot S5 IS (12× optical zoom) camera attached to a Martin Microscope Adaptor with a 3× lens. Most photographs of the live cultures were taken at a magnification 1200× with a few at 3600×. Manipulations of the photographs involved only cropping and adjustment of brightness and contrast.

Cultures of the amebas attached to sterile cover slips that had been placed in Petri plates were washed in two changes of PBS and fixed in 5% formalin in PBS for 10 min. After rinsing with PBS and tap water they were air dried for storage. Fixed cultures were stained with either iron hematoxylin or Feulgen following the procedures in Kirby (1950), mounted and photographed at magnifications of 1200×.

4. Results

The *B. mandrillaris* amebas observed live were in axenic medium. Within a culture, the ameba population was composed of two basic body types; the majority were generally rounded in shape and thinly spread on the surface. Many of these amebas had a single nucleus. Among them were dividing amebas that moved apart until the strand of protoplasm that connected them broke. Other amebas within the same culture were highly active and displayed multiple, elongated pseudopodia that were frequently adjusted as the ameba moved in a crab-like fashion (Schuster et al., 2003) on the surface or floated in the medium. Also present in the stock cultures were clusters of amebas whose active pseudopodia extended beyond the edge; individual amebas could emerge from such aggregates (Dunnebacke, 2007).

Amebas with both of the body types were present in each of the *B. mandrillaris* isolates. The "rounded" spread amebas were predominant in the cultures V188 and RP5 while the active amebas with extended pseudopods were more numerous in the cultures V039 and