

Pathogenicity of *Entamoeba* Species Depends on Cell Line Conversion, Genome Reprogramming and Epigenetic Gene Regulation

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Abstract

The protist life cycle is certainly much more than a simple sequence of trophic and non-trophic stages (cysts), and trophic cells (trophozoites) do not divide categorically into two identical daughter cells. The pathogenic amoebae *Entamoeba histolytica* and *Entamoeba invadens* exhibit complex life cycles including stem cells and cell lines following various biological tasks such as virulence and encystment. Intrinsic and extrinsic molecular mechanisms controlled by environmental cues, develop in both species a PST stem cell lineage that consists of primary, secondary and tertiary self-renewing cell lines. Oxygen gradients controlled by the host intestine and bacteria initiate the stem cell lineage machinery and are responsible for cell line conversions. *Entamoeba dispar* has a similar PST stem cell lineage despite being less pathogenic. These three amoebic species begin their life cycle with a primary multipotent stem cell line (p-SRL) that starts from metacystic amoebulae. The p-SRL line converts to progenitor cell lines depending on the environmental oxygen content. Progenitor cell lines are of reduced potency. The secondary s-SRL line produce mitotic arrested MAS cells (cyst precursor cells) committed for terminal differentiation; they continue development entering the endopolyploid cell cycle, a developmental cycle opponent to mitosis and form cysts. The tertiary t-SRL line does not form cysts. It produces mitotic quiescent MAT cells that enter a state of G0 and mature to invasive cells of variable genotypic virulence. MAT cells reentering mitotic cycle form new t-SRL variants. In hypoxic conditions the t-SRL line changes to symmetric cell fate.

Keywords: *Entamoeba histolytica*; *Entamoeba invadens*; *Entamoeba dispar*; Ecosystems; Stem cell lineages; Cell lines; Differentiation; Invasiveness; Virulence

Abbreviations

OCB: Oxygen Consuming Bacteria; ATD: Autonomous Terminal Differentiation; ITD: Induced Terminal Differentiation; p-SRL: Primary Stem Cell Line; s-SRL: Secondary Progenitor Cell Lines; t-SRL: Tertiary Progenitor Cell Lines; t-SRL(P): Tertiary Stem Cell Line Generated by Primary Cells (P/T conversion); t-SRL(S): Tertiary Stem Cell Line Generated by Secondary Cells (S/T conversion); t-SRL(ISH): Tertiary Stem Cell Line Generated by ISH Cells (ISH/T conversion); SRP: Self Renewing Primary Cells; SRS: Self Renewing Secondary Cells; SRT: Self Renewing Tertiary Cells; RSC: Reserve Stem Cells; MAP: Primary Mitotic Arrested Cells; MAT: Tertiary Mitotic Arrested Cells; MAS: Secondary Mitotic Quiescent Cells; ISH: Identical Strong Hypoxic Cells; ILH: Identical Low Hypoxic Cells; A/P, P/S, P/T: Cell Line Conversions; ALA: Amoebic Liver Abscess; A: Amoebulae; P: Primary Cells; S: Secondary Cells; T: Tertiary cells (PST lineage)

Ecosystems and Stem Cells Niches

The life cycle of pathogenic amoebae begins in the ileo-jejunal region of the human duodendum, where ingested cysts hatch. Amoebic cells passing the ileo-cecal valve (ileocolic orifice) colonize the colon, in particular the cecum and the sigmoidal region. *Entamoeba invadens* living in reptiles is in a biological sense the close relative of *Entamoeba histolytica*. It has played a key role in deciphering the amoebic life cycle and stem cell lineages of pathogenic and less pathogenic amoebae. In this author's opinion the closely related stem cell lineages of *E. invadens*, *E. histolytica* and *E. dispar* are inherited from the common eukaryotic ancestor and therefore closely related [1]. They are each subjected to the oxygen gradients of the intestine. The key role of oxygen gradients could be clarified for the first time in cultures sediments with oxygen consuming bacteria (OCB cultures) [1]. OCB grown amoebae regulated by oxygen gradients reveal much more about the stem cell biology

of *Entamoeba* species than axenic grown amoebae do. Symbiosis with oxygen consuming bacteria modulates all three species with respect to phenotypic and genotypic changes and exacerbation of pathogenicity [2-5].

Natural oxygen gradients

The intestinal lumen is largely devoid of oxygen [6]. pO₂ values measured by electrodes are as little as 0.5 mmHg [7,8]. EPR oxymetry recorded a linear oxygen gradient from the stomach to the distal sigmoid colon [9]. The measured pO₂ levels decreased from 58 mmHg in the stomach to 3 mmHg near the distal sigmoid colon [6]. Other researchers found distal lumen values of less than 0.5 mmHg. On the other hand there is evidence that oxygen diffusion from the intestinal tissue into the intestinal lumen also forms a radial gradient. More oxygen at the mucosal surface increases the abundance of oxygen-tolerant organisms' adherent to the mucosal surface (mucosally-associated oxygen tolerant microorganisms). Gut microbiota residing adherent to the mucosal interface consumed the oxygen diffused from the tissue [6]. On this way, communities of aerobic and facultative anaerobic bacteria open the way for colonization of the intestine by anaerobes.

The studies above demonstrate, firstly, that radial segregation of gut microbiota depends on the radial oxygen gradient and secondly that the distribution of the tissue associated mucus serves as a nutrient

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source for gut microorganisms [10]. According to Nagalingam et al. [11] the aerotolerant organisms normally residing at low levels in the intestinal mucus “could serve as a keystone community that expand during intestinal inflammation facilitated by the flux of oxidative metabolites and oxygen into highly hydrated (diarrheic) feces”. On the other side, the inflammatory response to the oxidative agents may affect gene expression and function of intestinal microbiota [12-15].

The small intestine

The small intestine has the most oxygenic lumen. The mucous gel at the surface of the intestine wall is one-layered and discontinuous. The intestine wall layers are: mucosa with the epithelium, lamina propria and muscularis mucosa; submucosa, muscularis propria, subserosa and serosa. Mucosa contains numerous microvilli with central blood vessels (arteriole with capillary network) and is highly oxygenated, as is the capillary abundant serosa. When villi are cut across they appear as islands protruding in the lumen¹.

The one-layer mucous gel overlying the epithelium consists of mucin (glycoproteins) which are secreted by goblet cells. It is a discontinuous layer that allows easy penetration of nutrients. The mucous layer has a dynamic nature. Its function and chemical composition is controlled by a broad range of luminal stimuli and mucus-resistant bacteria [16]. Glycoproteins adjacent to the epithelium form a compact inner sheet that is nearly sterile [17]. The luminal side of the mucous layer is more hypoxic and the inner side more oxygenic [17,18]. The radial oxygen gradient inside the mucous gel gives rise to distinct microbial niches thus partitioning gut microbiota.

Resource contents (nutrients, mucin and oxygen) decide which microorganisms colonize the mucus and which reside in the lumen. Usually, the small intestine contains a sparse microflora and small bacterial populations of $\sim 10^4$ cells/ml lumen content [19]. Microvilli and crypts are colonized by adherent species include the segmented filamentous bacteria, *Lactobacillaceae*, *Helicobacter sp.* and the facultative anaerobe *Entamoeba coli*. Colonization by colonic anaerobes is abnormal. Stable lumen colonization is suppressed by gastric acid, bile and peristaltics [20]. Mucolytic bacteria with a sufficient repertoire of genome-encoded glycosidic enzymes inhabit some of the more oxygenic niches.

The cecum

The cecum is the first part of the large intestine. In contrast to the one-layered mucus of the small intestine, the mucous sheet of the large intestine (including cecum) is two-layered and more hypoxic. It provides a space for mixing bacteria with the partially digested food from the small intestine. Most resident bacteria are aerobic, namely facultative anaerobes as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus* and *Proteus*. They reach high densities of approximately 10^7 - 10^8 cells/ml [19]. Pioneer infantile bacteria can modulate expression of genes in host epithelial cells preventing colonization by other bacteria.

The large anoxic intestine

The mucous sheet of the large intestine (absorbing water) is also two-layered and more hypoxic. It contains a complex and dynamic microbial ecosystem with high densities of living bacteria and achieves the maximally attainable concentration of up to 10^{11} - 10^{12} bacteria/g of luminal content [19,20]. 60% of the faecal mass consists of bacteria. The most common bacteria (99%) are anaerobes such as *Bacteroides*,

anaerobic streptococci and clostridia and less facultative anaerobes such as *E. coli*. Non pathogenic bacteria occupy the lumen and adhere to mucosa. Pathogenic bacteria such as *Shigella*, *Salmonella* and *Campylobacter* [20] can penetrate the mucous surface. However, bacterial penetration is a rather uncommon event.

Similarities with OCB culture sediments

The ileocecal region has many similarities with OCB culture sediments [1]. Different populations of aerobic and facultative anaerobic bacteria colonize the surface and the depth of the mucous gel and support amoebic development. At the surface and inside of the mucous layer amoebae found sufficient nutrients and stimuli to switch from one cell form to another. The radial oxygen gradients of the ileocecal region offers the best conditions and appropriate oxygenic/hypoxic niches needed for amoebic stem cell conversion, genotyping and virulence development

Amoebiasis

Non-invasive stages

GarlandScience² describes *E. histolytica* as a pathogen “that exhibits a wide spectrum of virulence, ranging from an avirulent commensal to a highly invasive and destructive pathogen. In a historical sense, some of the virulence differences observed in the past, are explained by the existence of a morphologically identical, but avirulent species *E. dispar*” However, 85-90% of the true *E. histolytica* infections remain without visible clinical manifestations also, while some others are accompanied by diarrhea or light symptoms such as abdominal pain or cramps [21]. After a few months, the infection may self-resolve or persist as a chronic non-invasive disease [22] that can last for years mimicking inflammatory bowel diseases [23].

Mucosal erosion and penetration

Experimental studies on animals show that mucus depletion and mucosal inflammation occurred as a response to the adsorption of amoebic toxins and preceded mucosal erosion and amoebic entry in the intestinal crypts [24,25]. Crypts are also the normal habitat of intestinal stem cells³. Examination of pathologic events in an animal model of amoebic colitis showed that goblet cell mucin stores are depleted before amoebic invasion occurs. The authors speculated that unknown amoebic secretions may be responsible for mucin depletion [26] and consider invasion as a result of epithelium destruction.

Invasive disease

Alternatively to the chronic non-invasive form, amoebiasis may exacerbate to an invasive disease. Amoebae of the mucous layer migrate deeper into the intestine wall killing epithelial cells. They give rise to a small area of necrosis (small mucous ulcers) associated with amoebic colitis and dysentery, respectively, with stools containing blood and mucus⁴.

Subsequently these small ulcers expand to large submucosal flask-shaped ulcers⁵ beneath the intestinal epithelium, becoming necrotizing cecal and rectal ulcers⁶. Amoebae ingesting host cells and erythrocytes are rarely present in the highly necrotic areas but rather are present

² http://www.garlandscience.com/res/pdf/9780815365006_ch03.pdf

³ <http://jpcck.zju.edu.cn/jcyxjp/files/ge/05/MT/0511.pdf>

⁴ http://www.garlandscience.com/res/pdf/9780815365006_ch03.pdf

⁵ <http://www.propath.com/companies/press-clippings/26-newsletters/166-entamoeba-histolytica-winter-2002>

⁶ <http://www.meb.uni-bonn.de/dtc/primsurg/docbook/html/x11281.html1>

¹ <http://www.pathologyoutlines.com/topic/smallbowelnormalhistology.html>

at the boundary to the healthy tissue. Ulcers and necrosis continue to expand laterally and downwards in the ascending and transverse colon [27]. Deepening to adjacent tissues they produce fulminant colitis and bowel necrosis leading to perforations and peritonitis with high mortality rates [23]; In some extreme cases severe inflammations of the mucosa (from rectum to sigmoid colon) were observed in biopsies, together with amoebae. Occasionally, the intestine wall forms an inflammatory thickening mass around the ulcers (amoebic granuloma, ameboma) sometimes confused with intestinal tumors [22,27,28].

Sigmorectal colonization

Cysts and amoebae carried down by faeces and peristaltic colonize the sigmorectal region. In 1996 William Sodeman write: "*The slow transit of the intestinal contents in these two locations (cecum or sigmoid colon) seems an important factor in invasion of the mucosa, both because it affords the ameba greater mucosal contact time and because it permits changes in the intestinal milieu that may facilitate invasion. The initial superficial ulcer may penetrate into the submucosa and muscularis, to become the characteristic flask-shaped chronic ulcer*" [29].

Intrasigmoidal and intrarectal infections are delayed and occur gradually. Already in 1928 James reported that at the first examination the rectum and lower sigmoid colon only about 50% of infections were positive for *E. histolytica* but in later examinations this increased to 70%-90% [30].

Transfer from cecum to liver and other extra-intestinal symptoms

The liver is the most commonly affected organ and amoebic abscesses (ALA) are the most common form of extra-intestinal amoebiasis; amoebae from the large intestine are transported directly to the liver via the mesenteric blood vessels and the hepatic portal vein⁷. The portal vein drains most of the blood from the cecum and ascending colon infections in the liver [22]. Pulmonary, cardiac or brain involvement are rare [23].

Cell Differentiation in Historical Wisdom

There is a wide spread dogma that single celled organisms such as *Entamoeba* divide by binary fission and the resulting daughter cells are identical cells (symmetric cell fate). Another belief is that the unique differentiated state is the cyst and that cysts differentiate in turn to trophozoites. Consequently, the life cycle would be a sequence of trophic and non-trophic stages (trophozoite and cyst) controlled by favorable and non-favorable environmental conditions [31]. In contrast, current state of the knowledge shows that pathogenic *Entamoeba* species have a complex multicellular life cycle consisting of a multitude of differentiated cell types [1]. The author of this review considers encystment as a process of terminal differentiation and ex-cystment as a process of de-differentiation. Products of de-differentiation are the undifferentiated totipotent amoebulae.

Initially, only two basic phenotypes were considered: the magna and minuta forms. Later one spoke of non-invasive (commensal) amoebae producing cysts and invasive tissular amoebae that do not form cysts. Molecular studies reveal the presence of stage specific lectines, antigenic structures and isoenzymes pools specific for xenic and axenic grown amoebae. More recently, genomic encoded genotypes were added. Lately, *Entamoeba*'s life cycle was enhanced by the discovery of stem cell lines and the developmental endopolyploid cell cycle opponent

⁷ http://www.garlandscience.com/res/pdf/9780815365006_ch03.pdf

to mitosis [1]. All these findings move *Entamoeba*'s life cycle and its intrinsic control mechanisms in an unexpectedly complex light.

Excystment

Ingested cysts of *E. histolytica* hatch in the oxygen rich mucosa of the small intestine around the protruding microvilli⁸, however, authors do not completely agree if excystment takes place as the cysts enter duodenum⁹ or later as they arrive in the ileum¹⁰. Several authors mention the cecum as the site of excystment¹¹. Excysting cysts release the metacyst (innercyst cell) that gives rise to eight amoebulae by binary fission. Amoebic cells adhere to bacteria bound to the mucus sheet¹² ingesting the bacteria and debris [32]. Amoebae released and multiplying in the duodenum assume the "typical morphology" observed in cecum and colon. What does this mean? How many amoebic lineages really occur in the small intestine?

Magna and minuta phenotypes

The terms "forma minuta" and "forma magna" were introduced by Gnezdilow [33]: minuta-as the smaller commensal cell type from the ascending colon subsisting on bacteria; magna-as the large invasive tissue form producing intestinal and extra-intestinal diseases. According to Gnezdilow, the proliferating minuta is the former cell type that means the "normal" cell type producing cysts, while magna is the latest trophozoite that does not represent an indispensable stage in the life-history of this species. According to Gnezdilow, *Entamoeba* proliferates and encysts as commensals quite satisfactorily preserving their biological specificity. Thus, the biological meaning of minuta/magna conversion remains unclear and factors favouring conversion from non-invasive and invasive amoebae we're not understood. Gnezdilow presumed that intestinal flora played a key role in managing *Entamoeba*'s life cycle.

Asymmetric cell division vs. binary fission

More recently, studies affirmed: *The "adult" trophic form inhabits the anterior part of large intestine of host; it multiplies by repeated binary fission in the intestinal wall. Some of the daughter Entamoebae grow into normal adults while others stop growing. These are distinctly smaller than the normal trophozoites and are called minuta form*^{13*}. This quote illustrates how confused and unusable the old terms magna and minuta are. The authors confirm the existence of asymmetric division in *E. histolytica* leading to mitotic arrested daughter cells but do not understand it: they confuse mitotic arrested cells (MAS cells) [1] cells with minuta cells.

Such conflicting findings, incomplete results and misinterpretation prolonged Hoare's dilemma of 1952 [34]: "*Though countless observations have been made on the host-parasite relations in infections with Entamoeba there is still considerable controversy about important points in the life-history and pathogenicity of this parasite*".

Cyclic encystment and cysts dissemination

Previous researchers believed that amoebae transported by feces and peristalsis encysted in the luminal content of the anoxic colon that absorbed water [27]. In contrast, other researchers thought cysts were

⁸ <http://www.pathologyoutlines.com/topic/smallbowelnormalhistology.html>

⁹ http://www.atlas-protzoa.com/Entamoeba_histolytica.php

¹⁰ <https://www.msu.edu/course/zol/316/ehisgut.htm>

¹¹ <http://thedoctorz.com/entamoeba-histolytica>

¹² <http://www.propath.com/companies/press-clippings/26-newsletters/166-entamoeba-histolytica-winter-2002>

¹³ www.biologydiscussion.com/2735

produced in oxygenic sites of the intestinal wall, adjacent to mucosa/submucosa and that mucin layers trigger encystment¹⁴. Cysts and some of the trophozoites were excreted with feces. Excluding dysentery stools - vegetative amoebae in feces are not accompanied by cysts; usually, only 50% of the cases are positive. In contrast, dysenteric stools contain more often a mixture of cyst forming cells together with hematophagous cells [30,35]. Depending on the stage of disease cysts predominate in formed stools of the non-invasive infection while vegetative amoebae are found in diarrheic stools [35]. Loose dysenteric feces facilitate transport of hematophagous amoebae¹⁵. In the invasive disease encystment goes back². Cysts are never found in tissue lesions [22].

In the 1960s there was a tendency to consider encystation as “cyclic and possibly intrinsically biologic in nature, rather than generated by environmental factors” [36]. Unfortunately, this valuable idea of Lin was not further pursued. More and more researchers induced encystment by manipulating environmental conditions¹⁶ especially nutrients resources. This experimental method of induced encystment allowed the study of molecular mechanisms and gene expression in cyst differentiation and many researchers lost interest in studying the natural encystment process occurring in xenic cultures and intestine. Today we know that encystment is a cyclic process following mitotic cell cycle. MAS cells differentiated by the asymmetric cell cycle are committed for encystment [1,37].

Repress SCFAs cysts production *in vivo*?

Some reports tried to explain encystment breakdown as the inhibitory action of SCFAs (short-chain fatty acids) observed *in vitro* [38,39]. In our opinion this statement does not apply *in vivo*. SCFAs are the major end product of bacterial metabolism in the colon and depend on the types and numbers of fecal bacterial populations [40]. SCFA are derived from the bacterial break-down of complex carbohydrates, especially in the proximal large intestine (high concentrations of SCFA) and would be differently absorbed in the colon. SCFA concentration varied quantitatively in the colon from higher concentrations in the proximal region (cecum, ascending and transverse colon) to lower concentrations in the distal region (descending and sigmoidal colon).

In other words, luminal SCFA pools are abundant in the small intestine, cecum and proximal colon [41-43] but cannot explain satisfactorily encystment breakdown *in vivo*.

The Developmental Endopolyploid Cycle

Recent findings in *E. invadens* [44,45] re-evaluate encystment and excystment as parts of an endopolyploid cell cycle of differentiation and de-differentiation opposing the mitotic cycle. The key biological role of the endopolyploid cycle is totipotency recovery (conversion from cell of restricted potency/unipotent cells to totipotent amoebulae). Only mitotically arrested cells exiting definitively mitotic cell cycle can start the developmental endopolyploid cycle.

The endopolyploid cycle can finish in one and the same host. Newly formed autonomous terminal differentiated cysts (ATD cysts) may excyst as long as they are in the colon (autoreinfection or self-infection). This is a highly interesting but little noticed mechanism that gives rise to new lineages and more viable cell lines not yet immunologically suppressed by the host.

Molecular Aspects of Pathogenicity

On their long life cycle way from amoebulae to highly virulent cell types all pathogenic *Entamoeba* species undergo different cell line programs and cell line conversions. Before discussing genomic pathogenicity in the next sections we review what is known concerning amoebic pathogenicity.

Lectins and zymodemes in populations of *E. histolytica* and *E. dispar*

In the 1970s different lectin agglutination patterns were observed in amoebae isolated from asymptomatic and symptomatic patients [46]. One believed initially that there were stage-specific differences. Then it turned out that the different agglutination patterns represented different species [47].

Agglutination arises from lipophosphoproteoglycans which occur on the surface of *E. histolytica*, but not on the surface of *E. dispar* [48,49].

At the same time isoenzymes (zymodemes) [50-52] and monoclonal antibody differences were observed between pathogenic and non-pathogenic cell types of *E. histolytica* [50-54] however, the authors considered that non-pathogenic forms should be identified as *E. dispar*. Some years later, several authors observed isozyme interconversions between “non-pathogenic” and “pathogenic” zymodemes, were occurring during axenisation attempts [55-59]. They described the interconversions as protein modifications and changes in gene expression. Other studies could not confirm zymodemes conversion. They concluded that the apparent conversion of the isoenzyme pattern is actually due to cross contamination.

In reality, *E. dispar* cannot be made to grow axenically under the standard conditions of *E. histolytica* [60,61]. It was discovered over time that axenic media are not well suited to study the biology of *Entamoeba* [45]. One could better elucidate the isozyme question by using controlled monoxenic culture methods such as OCB culture sediments. The cross contamination hypothesis [60,61] is difficult to reconcile with the pure innoculum experiments of Mirelman et al. [56] carried out in the axenic TYI-S-33 culture.

E. dispar strains are non-pathogenic for humans but are potentially pathogenic for animals [62] although, they are less virulent than *E. histolytica*. We have known since 1989 that *E. histolytica* secretes up to 1000 times more cysteine proteases than *E. dispar* [63] and has a three times higher amebaporesis activity [64]. *E. dispar* secretes toxic products in lower amounts, with lower activity than *E. histolytica* [2]. It produces in hamsters only focal inflammatory infiltrate lacking necrosis and granuloma [65].

However, in 2000 researchers using xenic grown *E. dispar* strains isolated in Brazil observed a high degree of pathogenicity in infected hamsters, similar with the results obtained in the past with the nonpathogenic strains (NP strains) of *E. histolytica* [66,67] considered more recently to be an *E. dispar* strain. Hamsters infected by this strain show hepatic and intestinal lesions similar to those caused by *E. histolytica*. According to Costa et al. [5] the capacity of xenic grown *E. dispar* to produce tissue damages in experimental infected animals indicates a considerable unknown pathogenic potential of the monoxenic grown *E. dispar* amoebae. The explanation of the acquisition of pathogenicity is that adequate environmental cues of monoxenic cultures modulate cell type conversions and virulence (genotypic virulence) a conclusion also reached by others [3-5].

¹⁴ <http://www.pathologyoutlines.com/topic/smallbowelnormalhistology.html>

¹⁵ <http://www.parasitesinhumans.org/entamoeba-histolytica-amoebiasis.html>

¹⁶ http://www.jstor.org/stable/3278046?seq=1#page_scan_tab_contents

Mechanisms destroying host's cells and tissues

Destruction of cells and tissue of the host organism is the goal of each invasive *Entamoeba* cell. It occurs by digestive enzymes and trophocytosis. Trophocytosis is the process of ingesting pieces of living human cells [68].

The initial contact and adhesion to host's cells surface carbohydrates occurs by specific amoebic galactose-inhibitable lectins [69] and other proteins [70]. Pore forming peptides (so called amebapores) insert them into the target cell membrane. Substrate degradation occurs by proteases that destroy host connective tissue. Such proteases act *in vitro* on a variety of host substrates and lead *in vivo* to host tissue breakdown. Crude extracts of *E. histolytica* had specific collagenase activity [71]. Incubation with collagen promotes not only collagenase activity but also increases the secretion of other proteases [72]. Collagen seems to be able to induce the activation of several amoebic genes related to amebapore 5 and cysteine protease 5 and extracellular cysteine proteases are the virulence factors cleaving collagen [70]. The collagenolytic activity of *E. histolytica* is a virulence factor [73-75]: the more virulent strains have the highest collagenolytic activity [70].

Cell lines and Stem Cell Lineages

It was the discovery of the amoebic stem cell lineage (protolignage) in *E. invadens* that puts the life cycle of all pathogenic *Entamoeba* species and their capacities for multiple cell differentiation in a new light [1,37,45,76]. Based on evolutionary considerations, it can be said that stemness and stem cell line conversions assured *Entamoebae* success as pathogen protists [77]. This applies to *E. histolytica* and *E. dispar* also.

Cell lines in cultures

E. invadens starts in appropriate OCB culture sediments as a primary p-SRL line generated by the totipotent amoebulae and A/P conversion [1,37,45]. The multipotent primary stem cell line (p-SRL line) proliferates asymmetrically forming new cycling cells (D1 daughter cells) and mitotic quiescent MAP cells (D2 daughter cells) working as reserve RSC stem cells. The primary p-SRL is a short living stem cell line soon converting to secondary s-SRL or tertiary t-SRL lines. When passaging in subcultures, RSC cells replenish the mother p-SRL line, generating then more specialized s-SRL and t-SRL lines. Cell line conversion depends on the oxygen content of the OCB culture sediment (pO₂ level). In more oxygenic sediments the p-SRL line converts into a secondary s-SRL by P/S conversion (conversion of primary to secondary cells); in more hypoxic sediments it converts into a tertiary t-SRL line by P/T conversion.

The s-SRL line persists in cultures as long as the environment remains oxygenic [1]; it proliferates by fast cycling (5-6 hrs) and gives rise to MAS precursor cells for ATD encystment. Increasing hypoxia stops growth of the s-SRL line; it converts into a long living facultative hypoxic t-SRL line that produces abundant MAT cells (mitotic arrested tertiary cells). MAT cells are precursors of the invasive amoebic cells. They mature in OCB cultures to large cells/hematophagous cells (unpublished observation by the author).

The *E. invadens* lineage has more t-SRL variants namely t-SRL (P) generated by primary cells, t-SRL (S) generated by secondary cells and t-SRL (ISH) generated by ISH cells (identical strong hypoxic cells). By each passage MAT cells replenish the t-SRL line giving rise to new sub-lines or even to unknown subsequent stem cell lines. In OCB cultures the t-SRL line manifests as a unipotent cell line however, *in vivo* it could be omnipotent.

t-SRL lines survive and proliferate in a broad range of pO₂ values from more oxygenic to more hypoxic. Similarly with the s-SRL line it proliferates in oxygenic conditions by fast cycling (5-6 hrs). In multilined OCB cultures, the t-SRL line is usually dominant. Strong hypoxic conditions, such as those occurring in the anoxic colon, stop t-SRL line proliferation and its asymmetric cell fate. Anoxia induces tertiary cells into identical daughter cells (ISH stem cells) that are arrested in the G2 or G2/M phase. In less hypoxic conditions these ISH cells convert back to an asymmetrically proliferating t-SRL line.

Concluding, the oxygenic s-SRL line is a precursor cell line for autonomous ATD cyst production by intrinsic mechanisms of terminal differentiation, while the facultative hypoxic t-SRL line is a precursor of invasiveness, lacking capacity of ATD encystment. *In vitro* it may be induced to produce ITD cysts (induced terminally differentiated cysts) from the early G1 phase.

Cell lines *in vivo*

The transitory p-SRL line: As described, ingested cysts of *E. histolytica* hatch in the oxygen rich mucosa of the small intestine surrounding the protruding microvilli¹⁷ and amoebic proliferation begins directly at the place of excystment independent from whether it is the duodenum, ileum or cecum^{18,19,20}. Thus, it can be concluded that environmental conditions met by amoebulae at the lumen/mucus interface around the microvilli favor A/P conversion and the development of the primary cell line p-SRL. Whether mitotically quiescent MAP cells find RSC niches in the small intestine wall and persist therein is unknown.

Oxygen preferences: Both oxygenic and facultative hypoxic cell lines (s-SRL and t-SRL) for proliferation prefer oxygenic environments. The "aerophilic" cell cycle lasts in optimal oxygenic conditions approximately 5-6 hr [1]. This is the reason why both s-SRL and t-SRL colonizes *in vivo* the oxygenic regions of the intestine. In contrast, hypoxic regions slow down the cell cycle progression of the t-SRL line and stop s-SRL line proliferation. It is unknown if the oxygenic P/S conversion takes place in the more oxygenic regions of the small intestine and the hypoxic P/T conversion in the more hypoxic lumen of the cecum. According to the life cycle description of Sampurna Roy (2016)²¹ both s-SRL and t-SRL lines proliferate in the cecum. Abundant nutrients (bacteria and mucus particles) and luminal pO₂ values allow self-renewing SR lines to colonize the mucous gel, ingesting mucus-adherent bacteria from the outside and mucolytic bacteria from mucus sheet inside.

The oxygenic s-SRL line produces cysts via MAS cells. In author's opinion [1,37,45] the s-SRL line *in vivo* proliferates in the more oxygenic surface layers of the cecum around microvilli. Increasing hypoxia stops s-SRL proliferation converting it to a tertiary invasive t-SRL line. Mitotically arrested MAS cells which are confronted by hypoxia interrupt development to ATD cysts and wait for re-oxygenation [78]. They may be taken by feces together with MAT cells and ATD cysts along the ascending/ transverse colon and may encyst later if they encounter appropriate oxygenic niches [76]. Encystment breakdown [35] occurs in each case as the s-SRL line reaches the too hypoxic colon and converts into a t-SRL line. The high ileocecal SCFA level [38,39,41-43] has no major influence on ATD cyst breakdown *in vivo*.

¹⁷ <http://www.pathologyoutlines.com/topic/smallbowelnormalhistology.html>

¹⁸ <http://www.atlas-protozoa.com/Entamoebahistolytica.php>

¹⁹ <https://www.msu.edu/course/zol/316/ehisgut.htm>

²⁰ <http://thedoctorz.com/entamoeba-histolytica>

²¹ <http://www.histopathology-india.net/amco.htm>

In this author's opinion, in the non-invasive phase, the ileocecal region is colonized by the oxygenic s-SRL stem cell line that produces ATD cysts. This could explain why the greatest number of cysts is released by asymptomatic individuals [79]. In asymptomatic patients with high cyst excretion rates the ileocecal region offers more constant oxygenic niches, assuring long term proliferation and survival of the s-SRL line. Most infections with s-SRL lines remain without visible clinical manifestations; while others are accompanied by diarrhea or light abdominal pain or cramps [21].

The facultative hypoxic t-SRL line proliferates and survives in oxygenic and hypoxic surroundings [1]. MAT cells that form the dominant cell fraction of the vegetative amoebic population prefer the deeper layers of the intestinal wall. Young MAT cells ingesting bacteria and debris increase their size and cell membrane extent. Mature MAT cells become capable of hematophagy (author's unpublished observation concerning MAT cells development in OCB cultures) and tissue invasion. Invasive cells are known to produce proteolytic enzymes [68-74] and are resistant to complement-mediated lysis [77]. Following suppression of the protective mucus layers invasive tertiary cells attach to the epithelial cells of the colonic epithelium disrupting the extracellular matrix [80]. MAT cells enter submucosa, produce flask-shaped ulcers and disseminate in the more oxygenated regions of submucosa and serosa, near to the oxygen carrying capillaries²².

During invasive proliferation t-SRL lines are dominant [1] and the s-SRL line ceases to exist. ATD cyst production gradually decreases and stops while symptoms worsen.

Are t-SRL variants genetically different?

There is no information about whether t-SRL line variants and sub-lines are identical. At this time one cannot say whether the t-SRL(P), t-SRL(S) and t-SRL(ISH) variants generated by primary, secondary or complete self-renewing ISH cell fractions are genomically identical or are different regarding genomic virulence and tissue invasiveness. This applies to t-SRL sub lines (variants) started by reactivated mitotic quiescent MAT cells under different conditions and time points. *In vitro* MAT cells mature in time and increase in cell size and membrane extent [1] becoming greater as >20 µm and potentially hematophagous²³.

Invasive cell lines and variants in liver invasion

In this author's opinion, MAT cells of increased cell size or more invasive t-SRL variants penetrate the intestinal wall and superficial ulcers (initial ulcers) deep into adjacent tissues. It must be assumed, some of the invading tertiary cells (cycling SRT cells or MAT cells) are more pathogenic than others. Alternatively, it is also conceivable that extrinsic cues such as luminal stimuli, necrotic tissue products and host defense factors, and specific microbiota induce the t-SRL to additional cell conversion to further descendants, genome reprogramming and increased pathogenicity. Meanwhile, modern *E. histolytica* researchers accept the idea that distinct virulent and non-virulent cell types in unique amoebic infections are responsible for the different disease stages (non-invasive, invasive and extra-intestinal disease). The pathogenicity of *E. histolytica* is considered now to be controlled by inherent genomic features²⁴.

Accordingly, MAT cells that invade small blood vessels disseminate through the blood stream passing to the liver. Whether the mother t-SRL line follows the invasion route is unknown, however,

it is widely assumed so. Amoebic liver invasion occurs by the portal system. Invasive amoebae provoke the enzymatic focal necrosis of liver hepatocytes and multiple micro-abscesses that coalesce to form a single lesion [81].

Amoebic liver abscesses were studied in detail in animal models [82-86]. Human and animal abscesses have a similar evolution. A few hours after inoculation, animal liver models develop a large number of inflammatory foci formed by the recruitment of neutrophils and, later on, by macrophages that surround each amoebic cell [82]. Most of the invading amoebae are killed by the host immune cells and the majority of inflammatory foci are free of amoebae. In author's opinion surviving amoebae proliferate and divide forming new invasive x-SRL lines by unknown T/X conversions and single inflammatory foci that gives rise to abscesses and necrotic areas. New inflammatory foci can result from the metastatic spreading of amoebae throughout the liver tissue.

We believe that both intestinal MAT cells and cycling self-renewing SRT cells participate to ALA abscesses. Many of the invading MAT cells will be successfully destroyed by the immune cell system of the host. Single amoebic cells could be observed only at the edge of the lesion and in the pus or within the abscess cavity itself [87]. Resistant amoebae are cycling SRT cells capable of proliferation. Whether they belong to the original intestinal mother t-SRL line or to a new x-SRL line formed in the liver is unknown.

Encystment *in vivo*

Until recently it was not understood that the so called "spontaneous encystment" observed in xenic and polyxenic cultures (clinical isolates), is in fact the same process of ATD cyst formation taking place in the oxygenic regions of the cecal mucous layer. Studying the stem cell lineage of *E. invadens* in OCB cultures [1,37,45] it was found that MAS cells are the precursors of oxygenic ATD cysts: they withdraw from the mitotic cell cycle, enter the endopolyploid developmental cell cycle and, in nutrient rich cultures, form cysts by intrinsic mechanisms of terminal differentiation (cyclic encystment). ATD cyst formation takes place in OCB cultures during the early more oxygenic growth phase [37,45] or by aeration in the pre-stationary/stationary growth phase [1].

Cycling tertiary cells (SRT) and MAT cells produced by the t-SRL line do not encyst in nutrient-rich media. However, early G1 cells arising from hypo-osmotic treated SRT, MAT and ISH cells are capable of forming ITD cysts when induced by appropriate extrinsic stimuli such as nutrient free hypo-osmotic media [1,37,45].

Why the double strategy of ATD and ITD cyst formation? Do both encystment processes occur *in vivo* and what are the benefits of this dual cyst forming system for the pathogenic *Entamoeba* species? These questions will be discussed in the following sections.

Re-infection by ITD cysts?

As described in preceding sections re-infection by self-produced ATD cysts is probably a frequent event during infections with *Entamoeba* pathogens and occurs during the movement of cysts along the proximal and distal colon. In OCB cultures ATD cysts excystment occurs frequently by passaging [1].

In contrast, ITD cyst production and re-infection by ITD cysts is so far unknown. Thus, the first question to answer is: do MAT cells form ITD cysts *in vivo*? Searching for answers, we found a lesser known work of Jones [88]. This author reports an induced two-step infection with

²² <http://www.histopathology-india.net/amco.htm>

²³ http://www.atlas-protzoa.com/Entamoeba_histolytica.php

²⁴ http://www.garlandscience.com/res/pdf/9780815365006_ch03.pdf

E. histolytica in rats. A primary infection was established in kittens by rectal injection of culture material. It was followed by the inoculation of kittens' dysenteric stools into the rat cecum. Examination post-mortem, showed cecal ulcerations occurring during the first 24 hrs. Infection reached a peak in 3-6 days and gradually disappeared during a period of 7-28 days. Infection was not readily detected by direct microscopic examination of feces. However, cysts were occasionally detected during the 14-28 day post infection period in tissue samples²⁵.

It must be assumed that the dysenteric stools of initially infected kittens [88] contain invasive amoebae (MAT or SRT) not able to produce ATD cysts; in the first 14 days after inoculation no cysts were produced. In light of these findings the question is: how does cyst production arise between days 14 and 28, and which type of cysts (ATD or ITD cysts) were these late occurring cysts?

We think, some of the amoebic cells from the kitten's dysenteric stool (MAT cells, cycling SRT cells) were induced by extrinsic stimuli occurring at the time of transfer and intracecal injection (aeration, transport media / washing buffers) to form sporadic ITD cysts, as observed sometimes in *E. invadens* [1,78].

However, the dominant fraction of amoebic inoculum remains vegetative and invasive and gives rise within 24 hrs to cecal ulcerations. We believe that some of the sporadically formed ITD cysts excyst intracecally, forming over 14 days a second amoebic lineage (re-infection). The corresponding new s-SRL line migrate into the more oxygenic mucous layers giving rise to the ATD cysts, detected post mortem in cecal tissue (days: 14-28). If this supposition is correct, we do not have evidence that the invasive cells (MAT or SRT cells) form ITD cysts during the normal infection but rather evidence that the transfer of invasive amoebae from kittens' dysenteric stools (MAT or SRT cells) into the rat cecum induces some of these cells to form ITD cysts. Subsequently, some of these cysts hatched out and give rise via p-SRL to a new s-SRL line and MAS cells. These MAS cells form the ATD cysts observed between day 14 and day 28. The initial infection with the invasive cells from the kitten's dysenteric stool disappeared gradually during day 3 and 7 and the days afterwards.

Genotypes and Pathogenicity

In a recent paper concerning the regulation of virulence in *E. histolytica* [89] Marie and Petri Jr. summarized what was currently known and noticed that virulence is not predictable and depends on multiple factors, many of them still unknown. We believe that many of these unknown factors are strongly related to the intrinsic mechanisms of cell differentiation and lineage development (stem cells, stem cell lines, genotypes). Extrinsic factors such as microbiota, step oxygen gradients, host resources and host immunity combine to induce stem cell conversions inside amoebic lineages.

A "cross talk" process between pathogens and liver?

According to the classical ALA concept, the ability of *E. histolytica* to destroy host tissues and survive in the liver is determined/ accomplished by a strong adaptative response, which requires the specific regulation of a number of amoebic proteins [90]. The authors compared RNA expression between vegetative cells from liver abscesses and those grown under restrictive axenic culture conditions and identified a number of specific genes up- or down regulated during pathogenicity. These results are not surprising: axenic cultured cells are far from the natural environmental conditions and its complete repertoire of environmental cues [44,45].

²⁵ <http://www.cabdirect.org/abstracts/19472900544.html;jsessionid=DFDDA334FD8A9C448A9D6AE4D718ECF0>

Supporters of the adaptative theory cannot explain why some pathogens resist and some succumb in the same microenvironment during liver infection [82] and believe that local and individual adaptation lead finally to subpopulation specialization. They consider that some of the amoebic cells residing in the colon penetrate the portal system and resist the stress presented by the new environment (fever, high pO₂), while other amoebae are failing and die.

Genomic reorganisation in the host?

In recent years increasing numbers of authors assumed that the acquisition of invasive behaviour is accompanied by changes in gene expression leading to expression of genes that promote invasion [91]. This applies to the different invasive outcomes, which depend on the intestinal cell type (diarrhoea and colitis cell type) and the extra-intestinal cell types producing amoebic liver abscesses (ALA) [92]. It is suggested that the outcome of infection may be determined by a parasite's genotype. Genotypes from the paired intestine/abscess samples seem to be genetically different. This leads to the question of whether there is a reorganization or recombination of DNA during the life cycle²⁶.

Already Ali et al. [93,94] had revealed that intestinal and extra-intestinal amoebae are genetically distinct and considered liver invasion as caused by a subpopulation of varying organ tropisms. The authors meant DNA reorganisation would take place "prior to or during metastasis from intestine to liver". The authors analysed intestinal and extra-intestinal subpopulations by PCR and found a serine rich protein gene (SREHP) [94-96] that encodes an immunodominant surface antigen; intestinal amoebae and ALA amoebae were different from each other at this locus [94]. Authors suggested alternatively: (i) the original intestinal infection may have contained multiple genotypes of which only one migrates to the liver (as a bottleneck in parasite genotypes) or (ii) DNA reorganisation takes place when amoebae migrate from the intestine to liver [94].

Other reports also favor the genetic bottleneck hypothesis [97] and suppose that specific differences in microbiomes translate to differences in the outcome of *E. histolytica* infections [98,99]. They conclude that "parasite genetics contribute significantly to virulence, and in particular, not all strains are capable of causing liver abscesses". On the other side two different ALA genotypes were found in two simultaneous and independent liver abscesses from the same patient [100].

Stem cells, epigenetic silencing and reprogramming

We believe that pathogenesis, invasiveness and "genetic bottleneck" in *Entamoeba* are controlled by cell line conversion mechanisms [1] and are basically epigenetic. Recently we reviewed literature on the field of genome reprogramming by "epigenetic patterns" that occur during development and differentiation as a response to intrinsic and extrinsic stimuli [45]. During its developmental progression *Entamoeba* reduced its potency from a totipotent state (amoebulae) into a multipotent state (the p-SRL stem cell line) and then to more restricted unipotent states (s-SRL, t-SRL line variants and followers). Reik stated that "development is, by definition epigenetic" [101]. This means, during early stages of development, genes that are required later in development are transiently held in a repressed state by histone modifications which are highly flexible and easily reversed when expression of these genes is needed. In other words, genes that are required later in development are repressed by histone markers, which confer short term and flexible epigenetic silencing [101]. The lineage

²⁶ http://www.medscape.com/viewarticle/737810_6

differentiates into several cell types that develop a narrower functional potential.

We believe that the proliferating t-SRL lines from the intestine and its tissue specific variants, including buried MAT progeny activate or silence genes for invasiveness and enzymatic tissue necrosis in an epigenetic fashion. Epigenetic changes increase gene expression activating silenced genes. These epigenetic changes occur via cell line conversion in responses to luminal and tissue specific stimuli. Some of them are more oxygenic, others are more hypoxic. Environmental cues play a key role in activating genes promoting virulence and increased invasive activity.

Conclusions

Researchers had to recognize that the development and differentiation biology of *Entamoeba* pathogens was little understood. In the past, there was no lack of interesting and promising clues but they were not recognized (asymmetric daughter cell fate, intrinsic mechanisms of differentiation, multicellularity). Equivalents in related biological fields such as in mammalian and human stem cell biology were not yet discovered. Only in the last few years were the observations made that revealed that amoebic stemness, cell lines, genotyping and pathogenicity are closely linked together.

There are many questions concerning cell type pathogenicity that remain to be answered: (i) Are hematophagous amoebae in fact MAT cells performing a phase of maturation or mitotic arrested products of a subsequent x-SRL line? (ii) Are ALA genotypes and high virulent cells products of further cell line conversions? (iii) Are the high proteolytic and collagenolytic activity specific characteristics of mitotically arrested cell pools? (iv) What are the phenotypic molecular differences between primary, secondary and tertiary cell types?

The next generation of researchers will have opportunities to advance this fascinating field of research and develop new OCB culture techniques which can resolve the questions posed above

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