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Cytokinesis Arrest and Nuclear Fission in Low Density Populations of Trichomonad Protozoan

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ABSTRACT—Cell growth of anaerobic protozoan *Tritrichomonas foetus* was analyzed. This protozoan usually proliferates in extremely high density, but protozoan parasites were dispersed uniformly in F-bouillon medium and cell division stopped temporarily. However, nuclear fission continued and giant polynucleated cells formed. Later, cell division resumed and cells returned to normal form. In conditioned medium, cytokinesis of the dispersed parasites did not stop. Results indicated that *T. foetus* cells secreted an extracellular factor that influenced cytokinesis.

Key words: Trichomonad, cytokinesis, polynucleated cell, cell density, cell cycle

INTRODUCTION

Trichomonads lack mitochondria, and branch very early in the eukaryotic tree (Viscogliosi *et al*., 1993; Gunderson *et al*., 1995; Riley and Krieger, 1995; Bui *et al*., 1996; Roger *et al*., 1996; Germot and Philippe, 1999; Keeling and Palmer, 2000). Evolution of the habitat of the trichomonads is uncertain, but they are parasitic in the digestive tracts or the genital organs of vertebrates, and have characteristics adapted to the anaerobic condition in parasitic sites of the host (Paget and Lloyd, 1990). In culture *in vitro* using liquid medium, *Tritrichomonas foetus* gather and proliferate at the bottom of the culture tube. Cell density was about 2.5×10^7 cells/ml, indicating anaerobiotic condition. To analyze growth of the protozoa, culture tubes were rotated slowly so that cells dispersed uniformly in the culture medium. In this condition, cytokinesis paused briefly and the protozoan transformed into unusually shaped polynucleated cells.

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MATERIALS AND METHODS

Protozoan

Tritrichomonas foetus (*Trichomonas foetus* Inui strain [Inoki *et al* ., 1961; Ito *et al*., 1975]) used in this experiment was provided by Dr. S. Inoki, Institute for Microbial Diseases, Osaka University. This protozoan has been maintained in liquid medium for 30 years or more in our laboratory.

Low cell density (LCD-) and High cell density (HCD-) culture of *T. foetus*

Experiments were in F-bouillon medium. 1 liter of medium contained 10g Extract Ehlrich, 5g yeast extract, 10g peptone, 10g glucose and 4g NaCl, and pH was adjusted to 7.0 with 10% NaOH. The medium was supplemented with 10% heat-inactivated bovine serum (Inoki and Hamada, 1953).

T. foetus cells were suspended at a concentration of about 1×10^4 cells/ml in F-bouillon. 4.8 ml of the cell suspension was poured into a sterilized 5 ml culture tube with a screw cap (assist tube; SARSTEDT). For low cell density culture (LCD-culture), tubes were rotated on the vertical axis at 0.5 rpm at 37°C to disperse cells by agitating the medium and air space. As a high cell density culture (HCD-culture) control, unrotated tubes containing the cell suspension were placed on a tube rack and the cells were cultivated statically at 37°C. In this stationary culture, cells gathered at a high density at the bottom of the culture tube. Cell densities were measured by ZBI Coulter counter at intervals of several hr.

Replacing air in culture tubes with nitrogen, carbon dioxide or oxygen gas

Culture tubes containing protozoan suspensions with loosely fitted caps were placed on a tube rack and then into a polyethylene bag. Some air in the polyethylene bag was removed with a pneumatic pump, and nitrogen, carbon dioxide or oxygen gas (Standard gas, GL Science, Japan) was injected into the bag. This operation was repeated 3–4 times, until more than 95% of air was replaced. Culture tubes in the polyethylene bag were incubated for 2 hr at 25°C in a constant temperature box. While culture tubes were still in the bag, their caps were fitted tightly. Culture tubes were then removed from the bag and placed in an incubator at 37°C.

Preparation of conditioned medium

Cell population increased to 5×10^5 to 1×10^6 cells/ml in the LCD-culture about 27 hr after the start of the rotated culture. Cells were removed from the medium by centrifugation at 3,000 rpm. Obtained supernatant was filtered by a sterilized 0.45 μm pore size filter to remove parasites. Conditioned medium was prepared by adding 10% fresh F-bouillon supplemented with inactivated bovine serum.

Observation of cultured *T. foetus* cells

Smear samples of parasites in LCD- and HCD-cultures were prepared using cells harvested by centrifugation at 900 rpm for 10 min at 5°C. Smeared cells were dyed by Diff-Quik staining (Xu *et al*., 1998) or Wright staining. The number of nuclei per cell was measured under a light microscope with about 500 cells per sample.

RESULTS AND DISCUSSION

When *T. foetus* cells were cultured statically in tightly capped culture tubes (HCD-culture), protozoa showed the massed state and cells proliferated exponentially (Fig. 1A). Doubling time of the parasite was almost 3 hr. In LCD-culture, growth of protozoa was not observed for about 30 hr from the start of culture (Fig. 1A). Oxygen suppresses

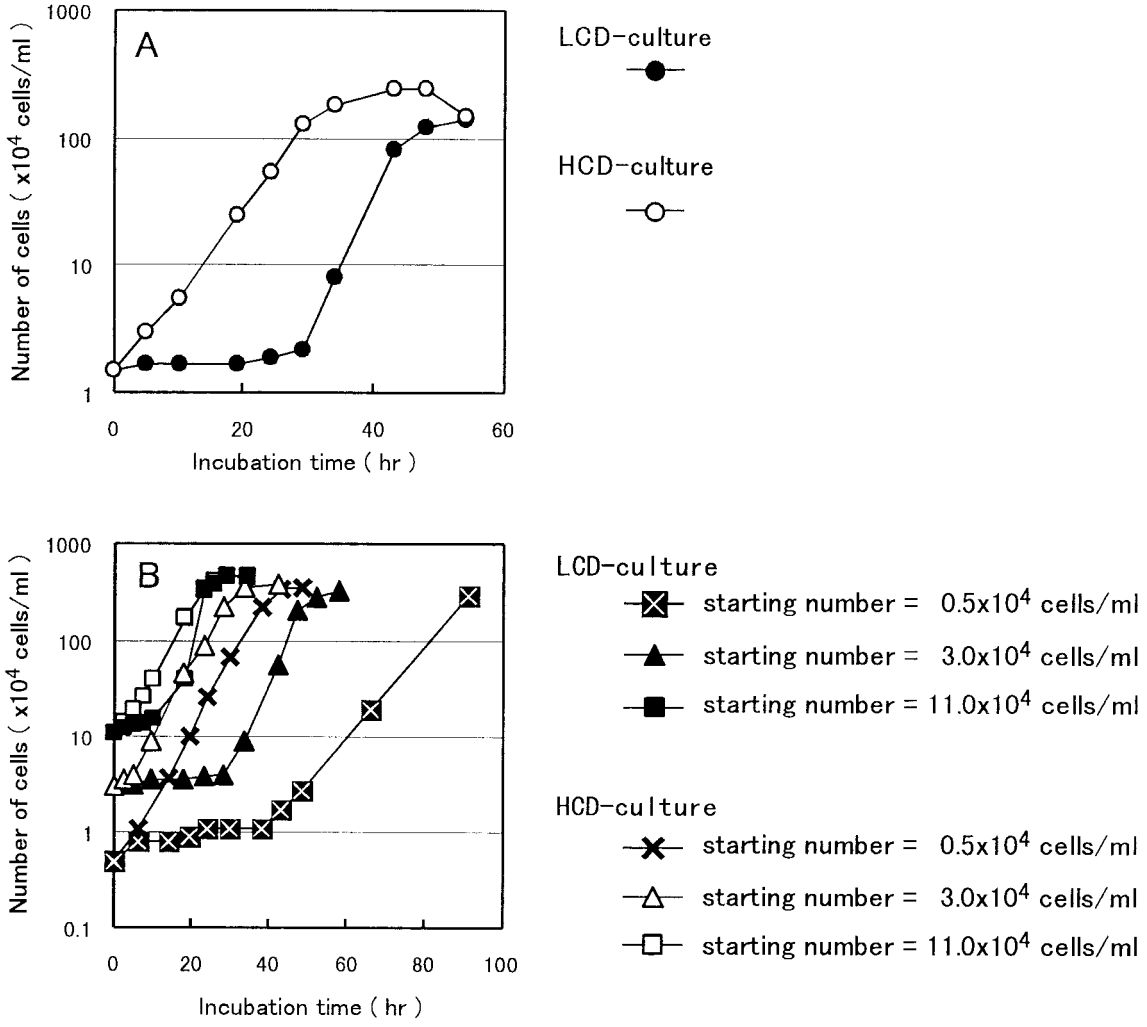

Fig. 1. Effects on the cell growth of *T. foetus* due to cell dispersion in the culture medium . (**A**) Cell proliferation pattern of the LCD-culture in rotated tubes and cell proliferation pattern of the HCD-culture in tubes which were not rotated. The cell density at the start of culture was 1.5×10^4 cells/ml. Culture medium was stirred well immediately before cell population was counted. (**B**) Effect of cell density on the cell growth of the trichomonad at the start of culture. Cell growth patterns were observed for 3 cases at the cell density of 0.5×10^4 cells/ml, 3.0×10^4 cells/ml and 11.0×10^4 cells/ml.

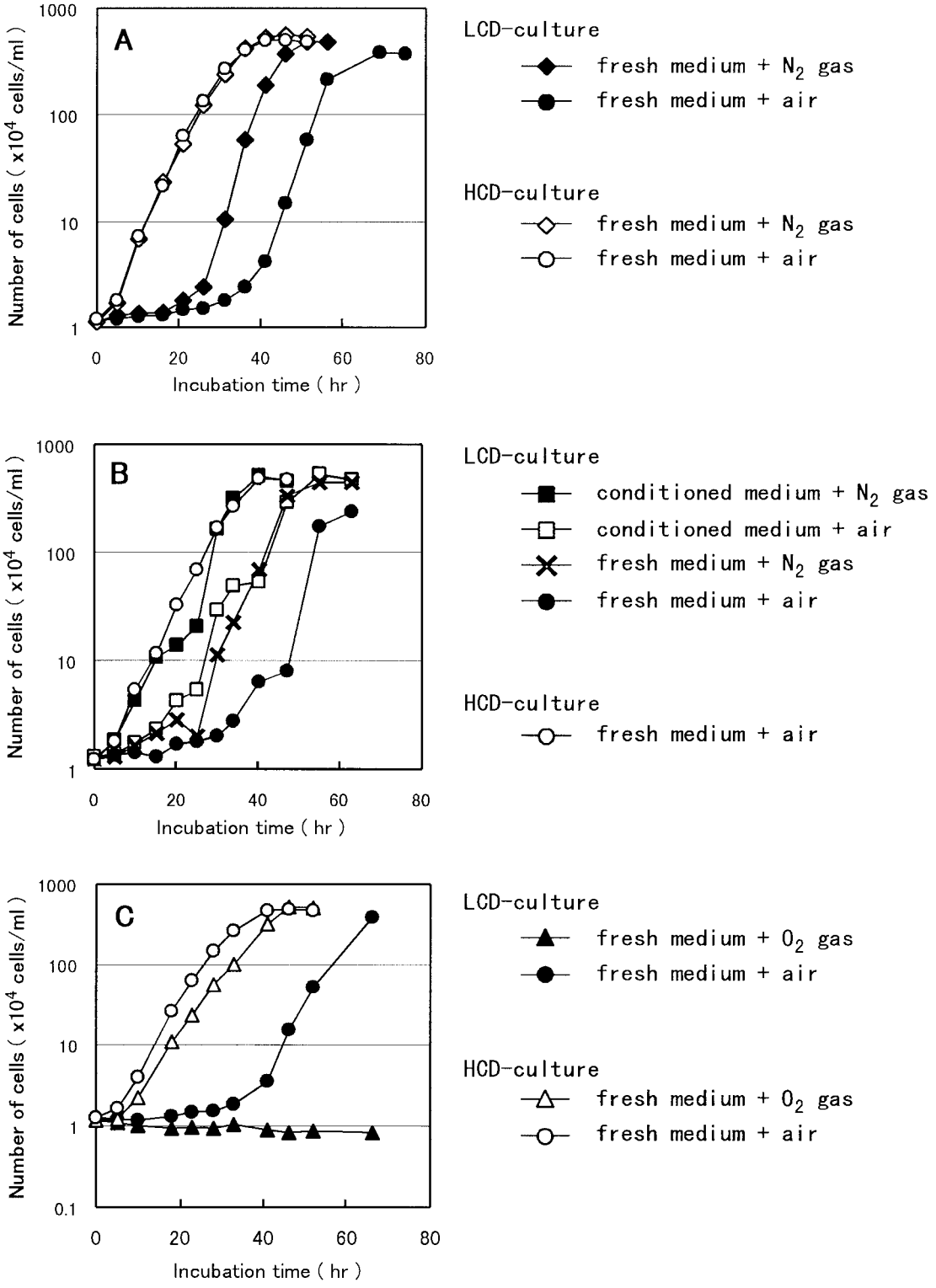

Fig. 2. The effect of oxygen on the cell growth of *T. foetus* and cell growth patterns of the protozoan using the conditioned medium in the LCD- and HCD-culture. (**A**) Cell growth patterns of the trichomonad in LCD-culture tubes in which air was replaced with nitrogen gas. (**B**) Cell growth patterns of the trichomonad using the conditioned medium. LCD-culture in the conditioned medium in which air was replaced with nitrogen gas, LCD-culture in the conditioned medium with air and LCD-culture in fresh F-bouillon medium in which air was replaced with nitrogen gas. (**C**) Cell growth patterns of the trichomonad in culture tubes in which air was replaced with oxygen gas.

growth of trichomonads (Paget and Lloyd, 1990; Mack and Müller, 1978; Ellis *et al*., 1994). LCD-cultures were carried out in an atmosphere in which air was replaced with nitrogen gas (Fig. 2A) or carbon dioxide (data not shown). When N_2 or CO_2 gas was bubbled into the culture medium, the period of arrested cell division of the protozoa was shortened in comparison with when the culture medium was bubbled with air. Nevertheless, the cell division of the protozoan was suppressed for about 20 hr in the LCD-cultures stirred with N_2 or CO_2. Since traces of air might have remained with the gas replacement method, a glass ball was inserted into the culture medium. Rotating the culture tube moved the glass ball and agitated the culture medium, producing a result similar to cases in which air was replaced with nitrogen gas (data not shown). Results indicated that temporary arrest of the protozoan cell division in the LCD-culture was not caused only by the presence of oxygen.

LCD-cultures were carried out with increased cell population at the start of culture and the period of cell division arrest was shortened in higher cell density cultures (Fig. 1B). Results indicated that protozoan cells proliferated cooperatively. Growth patterns of the protozoa in LCD-cultures using the conditioned medium are in Fig. 2B. After preparing conditioned medium, new cultures were started as soon as possible to prevent the aging effect. In LCD-culture tubes in which air was replaced with nitrogen gas, *T. foetus* cells proliferated immediately after starting the culture (Fig. 2B). Proliferation was similar to the protozoan growth in HCD-culture. This result indicated that *T. foetus* cells secreted a factor connected with protozoan cell proliferation (tentatively called "cytokinesis-regulating factor").

Unicellular eukaryotes seem to proliferate from single cells under suitable temperature and nutritional factors. There is extracellular secretion of the cell growth-promoting factor in a mutant of a ciliate (Takagi *et al*., 1989; Tanabe *et al*., 1990) like multicellular organisms of which cell growth is controlled by growth factors. The "cytokinesis-regulating factor" secreted by *T. foetus* seems similar to the substance promoting its own cell division found in the culture medium of a paramecium mutant. However, this factor seems different in the growth factor of paramecium. Under oxygen pressure of normal atmosphere, the cell proliferation of the protozoa could not be observed clearly in the culture using conditioned medium (Fig. 2B). Growth activity of the protozoan in HCD- and LCD-cultures was examined by replacing air in the culture tubes with oxygen gas. In the LCD-culture, there was no cell growth in the parasite during observation period (Fig. 2C). Viability of the parasites was very weak when observed by optical microscope. In the HCD-culture in which air was replaced with oxygen gas, cell proliferation of parasites started several hours later than in the control population exposed to air in tubes (Fig. 2C). After cell division had started, growth rate of the experimental population equalled that of the control population (Fig. 2C), indicating that oxygen affected the operation of "cytokinesis-regulating factor". Probably, protozoan cells gathered in high density in order to escape oxygen.

"Cytokinesis-regulating factor" may have a function in adjusting the microenvironment of the cell population. Octameric hemoglobin of a parasitic nematode *Ascaris lum-*

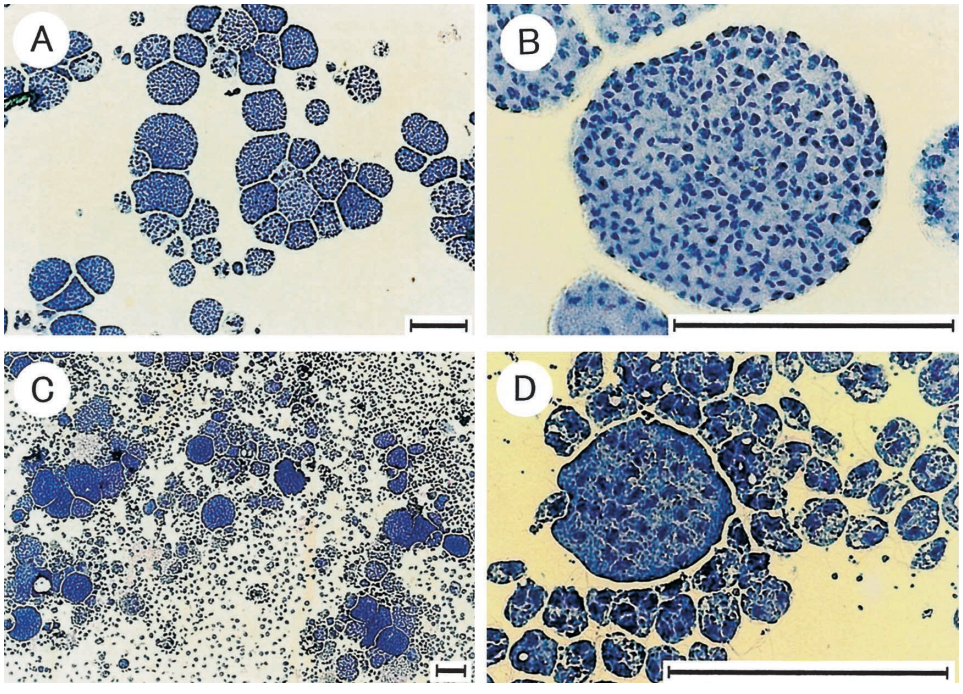

Fig. 3. Polynucleated trichomonal cells were in the LCD-culture. (Scale=100 μm) (**A**) and (**B**) Polynucleated protozoan cells immediately before the beginning of cell division in the LCD-culture, showing (**B**) photograph of a cell with more than 360 nuclei. (**C**) and (**D**) Once polynucleated protozoan cells after the beginning of cell division.

Table 1. Nuclei per cell and cell numbers of *T. foetus* in the LCD- and HCD-culture.

Low cell density culture

Number of nuclei per cell	Incubation time (hr)								
	0	5	10	19	24	29	34	43	54
1	365	206	77	2	10	5	195	243	130
2	126	184	206	19	13	12	172	184	204
3	8	37	42	3	8	11	47	42	53
4	3	30	110	107	14	14	20	25	93
5		1		3	3	6	8	1	5
6			8	15	7	8	8	3	8
7			5	22	19	13			
8		1	41	179	63	19	6	1	2
9			1		5	4	3		
10				2	8	15	3		1
11			1	5	10	13	1		
12				13		15	5		
13				6	16	25	1		
14				16	30	32	2		
15				16	47	24	1	1	
16				66	87	15	4		
17					14				
18				2	4	7	1		
19					3	8			
20				2	5	10		2	
21				3	5	18	3		
22				3	2	26			
23				4	10	8	1		2
24					4	10			
25						19			
26				4	18	22	4		
27					8	11	2		
28				1	29	14	1		
29					10	12	1		
30				2	17	8	1		
31				2	8	7			
32					11	13	2		
33					2	5			
34					1	2			
35									
36					3	16			
37					1				
38						4			
39					1	4			
40						8		1	
41									
42						4			
43						3			
44							1		
45					2	1			
46					1	4			
47					1	1			
48					1	1			
49						2			1
50					4	1			
51						2			
52					2	2	2		
53						1	2		
54						2	1		
55					2	2	1		
56							2		
57									
58						1			
59									
≥60					7	6	1		1

High cell density culture

Number of nuclei per cell	Incubation time (hr)								
	0	5	10	19	24	29	34	43	54
1	365	256	353	427	571	347	390	264	322
2	126	184	129	76	21	144	105	189	164
3	8	33	7	5		9	5	16	
4	3	21	6	3			3	25	6
5		4						2	
6								4	
7									
8		1	5					1	
16		1							

bricoides enzymatically consumes oxygen and keeps perienteric fluid hypoxic (Minning *et al*., 1999). It was unclear whether a single substance could adjust cell division condition and adjust microenvironment around the protozoan cells. To purify and identify the factor, we are trying to isolate and analyze it. Preliminary experiment using heat-treatment of this factor in the conditioned medium failed to induce cell growth of *T. foetus*.

Some cells formed polynucleated cells in the usual HCD-culture (Sakai *et al*., 1998). We examined the number of nuclei per cell using stained cell specimens from LCD-cultures. Strange giant polynucleated cells were visible under the light microscope (Fig. 3A, 3B). In the LCD-culture, cytokinesis-arrested cells continued nuclear replication, so the "cytokinesis-regulating factor" probably related to cytokinesis in the cell cycle. The number of nuclei per cell shown in Fig. 1A is in Table 1. Basically, nucleus numbers doubled and redoubled regularly, but the increase in nuclei per cell became less regular over time. Microscopic observation of *T. foetus* cells starting cell division showed normal size cells of the trichomonad with 1 or 2 nuclei around the giant polynucleated cells (Fig. 3C, 3D). This result suggested that small size protozoan cells were formed from the polynucleated cell. The mechanism of cell division of the polynucleated trichomonad is uncertain.

Our results confirmed that inhibiting cytoplasmic division induced the formation of giant multinucleated cells in the anaerobiotic *Trichomonas vaginalis* by a simple process for increasing oxygen tension (Wirtschafter, 1954). Buckner and Mikel (1983) observed "unusual parasites" that were large and multinucleated in a Papanicolaou smear from a young female. Our results confirmed speculation that large multinucleated cells were trichomonads that had undergone nuclear division without concomitant cytoplasmic division.

Like the pseudo-multicellular condition, the unique formation of polynucleated cells in this anaerobic parasite was probably related to the parasite evading oxygen. There is environmental adaptation because trichomonads are parasitic on the anaerobic site of the host. Future study could focus on the cell division cycle in parasitic protozoa.

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