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Toxoplasma gondii in vitro cultivation: economic and efficient mass production

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Abstract

Based on a serum-free and CO_2-independent culture of *Toxoplasma gondii* in vitro, a simple single arrangement mass production of trophozoites of the highly virulent BK strain of this parasite was developed. Starting simultaneously, 10^7 host cells (HEp-2) and 10^7 Toxoplasma trophozoites were cultured in one cell-factory® for 5 days, removing unattached cells, cell remnants and extracellular parasites by flushing daily. Each unit yielded about 0.5×10^9 parasites, which were almost free of any contamination with host cells and cell debris. As extensive manipulation of the cultures were avoided, a simple and reliable method for a mass production of Toxoplasma trophozoites has been developed, distinguishing itself by a low medium consumption and a low work-expenditure.

Keywords: Cultivation in vitro; HEp-2 cells; Mass production; *Toxoplasma gondii*

1. Introduction

Despite a continuous propagation of Toxoplasma strains in vitro, frequently a second demand is required to satisfy the need for antigens used in serological tests, i.e. large numbers of pure, extracellular, and sometimes intact parasites have to be provided. The goal of a mass production technique is usually a rapid breeding of intact trophozoites, mostly tachyzoits, with small amounts of host cell remnants. Therefore, the system is adjusted to achieve a complete destruction of all host cells, thereby accepting a considerable portion of non-infective, because immobilised parasites. Optimizing a mass production technique means balancing factors like the multiplication rate of the host cells and of the parasites, and their generation periods under given culture conditions. We based our technique on a serum-free and CO_2-independent culture technique, which has proven to be advantageous with respect to financial and organisational reasons [1].

2. Materials and methods

Mass-production of Toxoplasma trophozoites was performed under a considerable number of different culture conditions. Conditions leading to parasite suspensions which were considerably contaminated (>2.0% w/w) with host cells or host cell remnants were excluded from the study. We compared the cumulated multiplication rates (number of cells after harvesting, washing, and counting/number of cells

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actually bottled) of parasite strain Vienna-BK (for strain characteristics see [2]) applying different tanks and different nutrition media. As hosts, HEp-2 cells (CCl 23, American Type Culture Collections, Rockville, MD, USA) were used exclusively. All experiments were performed at least three times. Seven other Toxoplasma strains were also tested for mass production [3].

All experiments started at the same time and with the same stocks of parasites and host cells. Different types of culture tanks were compared: First, culture flasks (Costar, Cambridge, MA, USA), eight 150 cm^2 flasks were handled as one unit, representing 1200 cm^2 culture area. Each flask contained 30 ml nutrition medium during tissue culture.

Secondly, 22 Petri dishes (each 55 cm^2; Sterilin, Stone, GB) were considered as one unit (=1210 cm^2), and each dish required 10 ml medium.

And thirdly, a Cell-Factory® (Nunc Inc., Roskilde, DK) with a culture area of 1260 cm^2 and a nutrition medium need of 130 ml.

Different types of blended nutrition medium were compared: e.g. mixtures of one part of PC-1 medium (Hycor, Irvine, CA, USA) and one or three parts of the CO_2-independent medium (CO) (Gibco, Gaithersburgh, MD, USA) respectively. Moreover, mixtures of SFH medium (Sigma, St. Louis, MO, USA), QBSF medium (Sigma), SF-900 medium (Gibco) and CO were tested, too. All these mixtures were enriched with 1% L-glutamin, (Life Technologies, Paisley, GB), 1% serum supplement (Hycor) and 1% antibiotics/antimycotics.

A total of 10^7 HEp-2 cells, which were obtained from a separate tissue culture, were mixed with 10^7 Toxoplasma tachyzoites obtained from the permanent propagation (Part I) in the appropriate volume of medium. This suspension was evenly distributed on the growing surface and the tanks were incubated for 1 day at 37°C without any fumigation or agitation. Then, a medium exchange was done, removing all non-attached host cells and all non-invasive parasites. From this point of time onwards medium was replaced daily for 5 days, thereby harvesting the extracellular tachyzoites. Cell density and the contamination rate was determined in a Bürker-Türk counting chamber; means and S.D. were calculated with SPSS 6.0.1 (University of Vienna, A).

3. Results and discussion

We based our *Toxoplasma gondii* cultivation in vitro on a previously developed serum-free and CO_2-independent tissue culture technique [1]. Such techniques offer several advantages like lower investment costs, lower running costs, less organisational efforts, and no need for searching for batches of Toxoplasma antibody-free fetal calf serum [3].

Using the technique of 'outburning' tissue cultures large numbers of trophozoites can be produced, almost without any contamination with host cells and cell debris (<1.5% w/w). Best purity results and best output were obtained with highly virulent parasite strains only, thus, strain BK-Vienna — and RH strains [3] — were chosen for further experiments. This technique is especially suitable for a production of parasite cells used for antigen preparation in serology, it is less suitable for the breeding of parasites used for physiological studies. Almost all host cells have been parasitised and destroyed after 5 days of incubation, thus the cultures must be terminated at that time. But, some parasites may have outlasted extracellularly for many hours, thereby loosing some of their physiological characteristics.

Checking the parasites microscopically we could not detect any differences in exterior, appearance, and vitality, and checking them in an in vitro culture system there were no differences in the penetration behavior. But, some physiological changes due to different cultivation conditions have been found previously, especially an antigen shift between in vivo and in vitro bred parasites has been observed [4], and a minor variation of the enzymatic equipment has been demonstrated [5]. Due to changes in the public judgement of values, the rank of in vitro cultivation techniques is increasing, thus any comparative studies including in vivo systems are impracticable.

The main factors influencing Toxoplasma breeding in vitro are the suitability of the tank and of the nutrition medium, the number and the depletion characteristic of the host cell type, and the multiplication rate of the parasite strain. Best results in breeding Toxoplasma strain BK in vitro were achieved by using a Cell-Factory® and a 1:1 mixture of PC-1 medium and CO_2-independent medium

Table 1
Mean cumulated multiplication rate (CMR±standard deviation; CMR, number of cells after harvesting, washing, and counting/number of cells actually bottled; cumulated) of the parasite after x hours post infection (h p.i.) using different mixes of nutrition media and different culture tanks

Tank	Media mixture used	M (l)	HT (min)	CMR (48 h p.i.)	CMR (72 h p.i.)	CMR (96 h p.i.)	CMR (120 h p.i.)
CFY	PC-1/CO (1:1)	0.65	40	19.5 (±4.2)	56.8 (±4.2)	69.2 (±4.2)	71.7 (±4.2)
F	PC-1/CO (1:1)	1.2	120	20.1 (±2.8)	51.3 (±3.3)	54.1 (±1.6)	54.7 (±0.4)
F	PC-1/CO (1:3)	1.2	120	11.2 (±2.9)	28.7 (±3.9)	30.3 (±1.8)	30.3 (±2.2)
F	SFH/CO (1:1)	1.2	120	12.9 (±3.1)	32.8 (±3.0)	34.6 (±3.2)	34.7 (±1.9)
F	QBSF/CO (1:1)	1.2	120	10.3 (±2.4)	26.1 (±4.2)	29.3 (±1.7)	29.3 (±2.1)
F	SF-900/CO (1:1)	1.2	120	12.1 (±3.1)	30.8 (±4.8)	32.9 (±2.7)	34.1 (±0.4)
PD	PC-1/CO (1:1)	1.1	132	5.6 (±1.6)	11.0 (±1.5)	13.2 (±1.3)	14.5 (±2.5)

CFY, cell-factory® (Nunc Inc., Roskilde, DK).
F, culture flasks (Costar, Cambridge, USA).
PD, petri dishes (Sterilin, Stone, OB).
Media mixtures: see Materials and methods (Section 2).
HT, mean handling time required in min per unit.
M, consumption of the media mix in l after 120 h/unit.

(Table 1). We were able to breed 5.6×10^8 Toxoplasma-trophozoites within 72 h, investing 10^7 host cells, 10^7 parasite cells, and 0.65 l nutrition media mixture. Comparing references of similar techniques, other authors yielded between 1 and 3.5×10^8 parasites, but in most cases the cultures were incubated longer (4–8 days) [6–10]. Chang and Gabrielson [11] reached a yield of even 3.9×10^9 trophozoites after 4 days breeding, but these results have never been confirmed.

Handling a Cell-Factory® is extremely simple, as medium exchange can be performed using one way syringes (or, semiautomatic, a peristaltic pump). Moreover, this technique distinguishes itself by a minimal medium consumption and by a less chance of any contamination. A disadvantage of the Cell-Factory® may be the compulsion of a complete destruction of the culture in case of a contamination. Besides, we tried to reuse cell factories for a second breeding cycle after different refinement steps, but in vain — host cells never depleted.

The Toxoplasma tissue culture systems described, the long-term propagation and the mass-production, are both based on a common technique, a serumfree and CO_2-independent cultivation of the parasite. Depending on the requirements they may be performed separately, but more efficiently both techniques are combined to one course of procedure. First, both systems need a separate, but equal breeding system for host cells; secondly, all the technical equipment and the handling are the same; and thirdly, surplus tachyzoites bred in the long-term propagation system can be used for starting the mass production. Thus, by combining a technique of feeding parasites (Part I) and a technique of 'outburning', the main goals of parasite cultivation in vitro, a long-term continuous preservation of the strain without resorting to laboratory mammals and a distinct, cheap production of large numbers of parasite cells may be achieved.

References

[1] Harmer, Ch., Hassl, A. and Aspöck, H. (1994) Vergleich verschiedener Nährmedien zur Zucht von *Toxoplasma gondii* in der Gewebekultur. Mit. Österr. Ges. Tropenmed. Parasitol. 16, 123–126.
[2] Hassl, A., Manafi, M., Sommer, R., Kundi, M. and Aspöck, H. (1994) Characterization and check of identity of different strains of *Toxoplasma gondii* by enzymatic profiling. J. Protozool. Res. 4, 48–55.
[3] Harmer, Ch. (1995) Versuche zur Optimierung der Zucht von *Toxoplasma gondii* in vitro. Thesis, University of Vienna, 113 pp.
[4] Hassl, A. and Aspöck, H. (1992) In vitro propagation of *Toxoplasma gondii* in PC-1 serum-free medium. Folia Parasitol. 39, 285–286.

[5] Harmer, Ch., Manafi, M., Hassl, A. and Aspöck, H. (1993) Vergleichende Untersuchungen über Enzymmuster unterschiedlich kultivierter Toxoplasmen. Mitt. Österr. Ges. Tropenmed. Parasitol. 15, 113–118.

[6] Chamberland, S. and Current, W.T. (1991) Use of mouse macrophage cell lines for in vitro propagation of *Toxoplasma gondii* RH tachyzoites. Exp. Biol. Med. 197, 150–157.

[7] Couatamaranche, A., Andre, P., Le Minous, D., Martin, L., Robert, R. and Deunff, J. (1991) In vitro culture and cloning of *Toxoplasma gondii* in a newly established cell line derived from TO 180. Int. J. Parasitol. 21, 129–132.

[8] Hughes, H.P.A., Hudson, L. and Fleck, D.G. (1986) In vitro culture of *Toxoplasma gondii* in primary and established cell lines. Int. J. Parasitol. 16, 317–322.

[9] Srinivasa, H., Mithal, S. and Balaya, S. (1982) Maintanance of *Toxoplasma gondii* in Vero cell line. Indian J. Med. Res. 75, 521–528.

[10] Suresh, K., Mak, J.W. and Yong, H.S. (1991) In vitro culture of *Toxoplasma gondii*. Southeast Asian J. Tropic. Med. Public Health 22, 449–451.

[11] Chang, G.N. and Gabrielson, D.A. (1984) *Toxoplasma gondii*: growth in ovine fetal kidney cell culture. Exp. Parasitol. 57, 81–85.