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Use of refrigeration as a practical means to preserve viability of in vitro-cultured IDE8 tick cells

Camila V. Bastos · Maria Mercês C. das Vasconcelos · Múcio Flávio B. Ribeiro · Lygia M. Friche Passos

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Abstract In vitro cultivation of the IDE8 cell line, derived from embryonic *Ixodes scapularis* ticks, constitutes an important system for the study of tick-borne pathogens, as these cells support growth of rickettsial species which are not normally transmitted by this tick. However, since cryopreservation of IDE8 cells is not always successful, there is a need to develop alternative ways to preserve these cells. In the present study, a suspension of IDE8 cells in culture medium was kept under refrigeration at 4°C for up to 60 days. Every 15 days, the suspension was mixed and aliquots were re-cultured in 2-ml tubes, under standardized conditions. In addition, three techniques for cryopreservation, using two different cryoprotectants (DMSO and glycerol), were evaluated. Medium changes were carried out every week and subculturing every 2 weeks. The development of cultures and their respective subcultures, after returning to standard culture temperature, was evaluated by percentage viability and by cellular morphology evaluated in Giemsa-stained cytocentrifuge smears. All cultures and subcultures appeared healthy, showing growth rates comparable to cultures that had not been kept under refrigeration. The results demonstrated that storage under refrigeration at 4°C is an efficient method for preservation of IDE8 cells for up to 60 days and that refrigeration may be preferable to cryopreservation for short-term preservation of IDE8 cells.

Keywords IDE8 cells · *Ixodes scapularis* · In vitro culture

C. V. Bastos · L. M. F. Passos (✉)
Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, CP 567, Belo Horizonte 30123-970 MG, Brazil
e-mail: lygia@dedalus.lcc.ufmg.br

M. M. C. das Vasconcelos · M. F. B. Ribeiro
Departamento de Parasitologia, ICB – UFMG, Belo Horizonte, MG, Brazil

Introduction

The production of antigens from pathogens using in vitro infected tick cell cultures is simpler, more economical and more ethical than the production of antigens through experimental infections in susceptible animals. Some tick cell culture systems allow the production of such antigens free from host or pathogen contaminants for use in diagnostic tests (Saliki et al. 1998; Rodgers et al. 1998) and for the development of vaccines (Blouin et al. 1998; Kocan et al. 2001; de la Fuente et al. 2002).

The IDE8 line was established from embryonic *Ixodes scapularis* ticks (Munderloh et al. 1994) and several pathogens have been established in IDE8 cells, such as *Anaplasma marginale* (Munderloh et al. 1996a), *Ehrlichia ruminantium* (Bell-Sakyi et al. 2000; Bell-Sakyi 2004), *Ehrlichia canis* (Ewing et al. 1995; Kocan et al. 1998), and *Ehrlichia equi* (Munderloh et al. 1996b) and *Ehrlichia phagocytophila* (Woldehiwet et al. 2002), reclassified by Dumler and collaborators (2001) as *Anaplasma phagocytophila*.

In general, cryopreservation in liquid nitrogen is a very advantageous method for preserving many cell lines for undefined lengths of time. However, as far as tick cell lines are concerned, cryopreservation has presented some practical limitations, as resuscitation is difficult and has not been always successful (L. Bell-Sakyi 2003 and E. Blouin 2004—personal communications). Therefore, there is a need to search for alternative ways to preserve these cell lines other than long-term cryopreservation in liquid nitrogen. In the present paper, a technique for short-term preservation of IDE8 cells at 4°C was evaluated in comparison to cryopreservation in liquid nitrogen.

Materials and methods

Culture conditions

The IDE8 cell line was kindly provided as a growing culture by Dr. Bell-Sakyi (CTVM, University of Edinburgh, UK), with permission given by Dr. U.G. Munderloh (University of Minnesota, USA). The cultivation was carried out at the Department of Preventive Veterinary Medicine, UFMG, Brazil, following standard procedures for maintenance (Munderloh et al. 1994), in flat-sided tubes (NUNC^{TM}) with 2 ml of supplemented L-15B culture medium. The tubes were kept in incubation at 30°C (±2°C). Medium changes were carried out weekly and subcultures every 15 days.

Storage of IDE8 under refrigeration

A suspension of IDE8 cells, with a concentration of 3.8×10^6 cells/ml was maintained under refrigeration (4°C) in a 15-ml Falcon® tube for up to 60 days. Every 15 days, the suspension was mixed and 1 ml-aliquots were re-cultured into flat-sided culture tube containing 1 ml of complete L-15B medium and were incubated at 30°C (±2°C), being three replicates for each condition. The day that the aliquots were brought to standard culture conditions was considered day 0. Medium changes were carried out weekly and subcultures were carried out on days 15, 30, 45 and 60 after recultivation.

Evaluation of cell viability and morphology

Cultures and subcultures were evaluated for viability and morphology. Determination of percentage of viable cells was done by the trypan blue exclusion method (Mary et al. 1994) and cell morphology was evaluated in cytocentrifuge smears made from 50 μl aliquots taken from each cell culture suspension. Smears were fixed twice with methanol (for 10 min), stained with an 8% Giemsa solution for 30 min and examined under oil immersion at 1,000× magnification.

Cryopreservation of IDE8 cells in liquid nitrogen

Three techniques, using two different cryoprotectants (DMSO and glycerol), were evaluated for cryopreservation. IDE8 cell cultures were resuspended and centrifuged at 250*g* for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in fresh medium. Half of this cell suspension was diluted with medium containing DMSO (final concentration of 6%) and the other half was diluted with medium containing glycerol (final concentration of 6%), resulting in a concentration of 3.1×10^6 cells/ml. Aliquots of each suspension were divided into three groups, each composed of a total of six cryotubes (three containing DMSO and three containing glycerol). The first group was frozen by rapid immersion directly into the liquid nitrogen tank (group 1). For the second group, the freezing procedure was carried out in three stages: the cryotubes were initially kept at 4°C for 30 min, followed by freezing at –20°C for 30 min and finally immersion in the liquid nitrogen tank (group 2). The third group was frozen through a gradual and slow technique of deep-freezing (temperature decrease rate of –1°C per minute), based on that described by Munderloh et al. (1994), with modifications, i.e., the final concentration of DMSO and glycerol was 6% and the temperature went down to –30°C (group 3). Eight days after cryopreservation, the contents of each tube were quickly thawed in a water bath at 37°C and centrifuged at 250*g* for 10 min at 4°C. The supernatants were discarded, to remove the excess cryoprotectant, and the cells were resuspended in 1.5 ml of medium specially prepared for resuscitation (adapted from Munderloh et al. 1994), supplemented with 20% fetal calf serum. Cell suspensions were individually placed into 24-well plates (Corning®). All cultures and their respective subcultures were evaluated for the percentage of viable cells and morphological aspects.

Statistical analysis

Comparisons between cell viability rates obtained for the different treatments were carried out using the Chi-square test, based on Sampaio (2002).

Results and discussion

Effect of refrigerated-storage on viability of IDE8 cells

The culture that had been kept for the shortest period of time under refrigeration (15 days) presented the best performance ($P < 0.05$) after 30 days in culture (mean viability of 97.96%). However, after maintenance for 45 days under standard conditions of cultivation, the cultures that had been kept under refrigeration for 15, 30, 45 and 60 days presented equivalent viability rates, with no significant differences

($P < 0.05$) (mean viability of 83.53%). This result demonstrates that the cultures recovered from any possible ''damage'' they might had suffered during storage at 4°C, as all of them achieved viability rates similar to those observed in cultures under refrigeration for only 15 days. The subcultures derived from cultures kept under refrigeration for 15 days also presented better performance (mean viability rate of 96.15%) when compared to the others on day 15 after subculture. However, after 30 days, all subcultures presented equivalent performances (mean viability rate of 89.39%) and no significant differences ($P < 0.05$) were seen between them. All cultures and subcultures appeared healthy, showing growth rates and morphological appearance comparable to cultures that had not been kept under refrigeration.

These results present a new approach to storing IDE8 cells for short periods of time. So far, the most commonly used method to preserve IDE8 cells has been cryopreservation in liquid nitrogen, which has several limitations (L. Bell-Sakyi 2003—personal communication). Researchers working with tick cell lines often affirm that cryopreservation in liquid nitrogen is not reliable and, in most laboratories, IDE8 cells have been cultured continuously in vitro in order to guarantee provision of viable cells whenever they are needed (L. Bell-Sakyi 2003 and E. Blouin 2004—personal communications). Therefore, these results demonstrate that IDE8 cells can be kept for up to 60 days under refrigeration without compromising their growth in vitro thereafter. This storage procedure can be very useful in laboratories that cannot afford to continuously culture this cell line or in those that, for any reason (vacations or any type of personnel restrictions) need to stop the cultivation of IDE8 cells for short periods of time. Although a short-term storage of IDE8 has been reported at 12°C (Munderloh et al. 1994), storage at 4°C would be more accessible to every laboratory.

Cryopreservation of IDE8 cells in liquid nitrogen

All cultures that had been cryopreserved by rapid freezing (group 1), as well as those cryopreserved by the 3-stage procedure (group 2) showed mean viability below 7% after resuscitation, with both DMSO and glycerol as cryoprotectant. On the other hand, cultures cryopreserved by the slow and gradual freezing procedure (group 3) showed significantly better results ($P < 0.05$) when compared to cultures cryopreserved by the other two techniques. In group 3, cultures cryopreserved with DMSO showed a mean viability rate of 74.96% after resuscitation, while this rate was only 23.04% for cultures cryopreserved with glycerol. Although glycerol is known to be a cell cryoprotectant, the viability rates obtained in the present study do not indicate that glycerol would be a good cryoprotectant for IDE8 cells.

It is interesting to observe that cellular viability rates obtained for IDE8 after refrigeration for 60 days were higher than those obtained for cells after cryopreservation in liquid nitrogen. Considering that cryopreservation in liquid nitrogen involves costs and requires skilled personnel, storage of IDE8 cells at 4°C appears to be an efficient way to preserve this cell line, with no cost, for short times (Fig. 1).

Conclusion

The results obtained in the present experiment allow the conclusion that the use of DMSO at a concentration of 6% with a slow and gradual freezing method is efficient

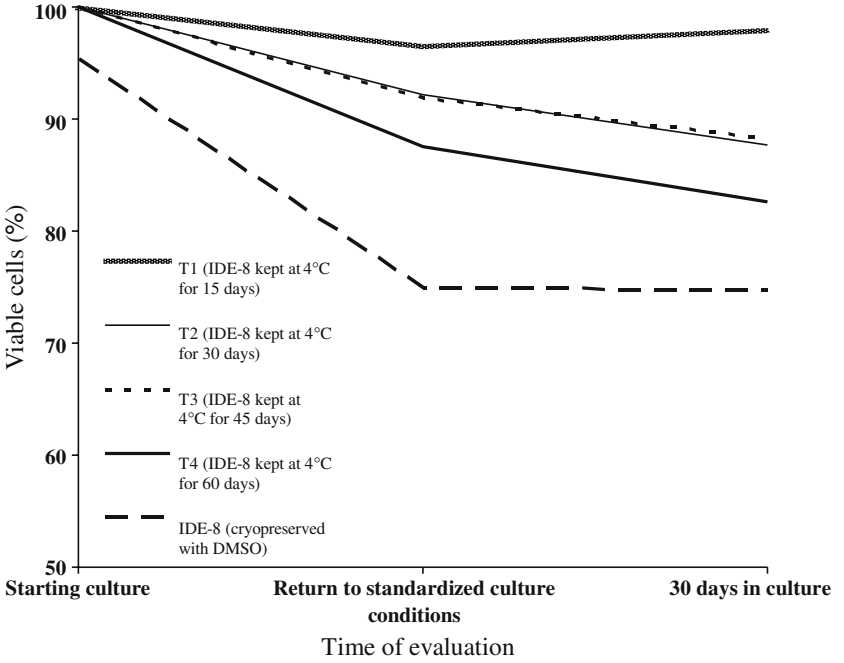

Fig. 1 Effect of refrigeration and cryopreservation on viability of in vitro cultured IDE8 cells

and can help to overcome the problems related to cryopreservation of IDE8 cells in liquid nitrogen. On the other hand, considering some limitations of cryopreservation in liquid nitrogen, particularly related to cost and manipulation, storage of IDE8 cells under refrigeration at 4°C constitutes an efficient and reliable method that may be preferable to deep-freezing for short-term storage of this cell line.

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