Parasitol Res (1997) 83: 394–396

SHORT COMMUNICATION

Javier Viseras · Pedro García-Fernández
Francisco Javier Adroher

Isolation and establishment in in vitro culture of a *Theileria annulata* – infected cell line from Spain

Received: 21 September 1996 / Accepted: 1 October 1996

Abstract The isolation of *Theileria annulata*-infected lymphocytes using blood from an animal suffering from Mediterranean theileriosis as a source of parasites is described. The present work reports the first isolation and establishment in in vitro culture of a *T. annulata*-infected cell line from southwestern Europe, where Mediterranean theileriosis causes important economic losses, especially in southern Spain. The parasite was identified by staining of cells from culture with Giemsa, by immunofluorescent antibody techniques (IFAT), and by isoenzyme characterization. The possibility of using this *T. annulata*-infected lymphoblastoid cell line to obtain an antigen for diagnosis of Mediterranean theileriosis by IFAT and to develop a tissue-culture vaccine against this disease in our geographic area shows the significance of this isolation and culture.

Introduction

Theileria annulata is a protozoan parasite of cattle and other livestock that is transmitted by ticks of the genus *Hyalomma* and causes a disease named tropical or Mediterranean theileriosis. This disease affects cattle in a wide strip that covers southern Europe, northern Africa, and the Middle East and reaches the South of the former USSR, India, and China (Purnell 1978). In all the affected countries in which the disease has been studied in depth, research on the isolation and in vitro culture of the causative agent has been carried out (e.g., Pipano et al. 1989; Singh et al. 1993). In Spain, cases of Mediterranean theileriosis from the first third of this century have been described (Salvans-Bonet 1928), and the parasite has been identified in several areas of the country (García-Fernández et al. 1985, 1987). To date, no study has been carried out in Spain to isolate cell lines of the parasite or to characterize them, which would represent the first specific step in the fight against this disease. In the present paper we describe the isolation and establishment in in vitro culture of the first *T. annulata*-infected lymphoblastoid cell line from an enzootic area of Spain.

J. Viseras (✉) · P. García-Fernández
Departamento de Producción Animal,
C.I.D.A., Junta de Andalucía,
Camino de Purchil s/n,
E-18071 Granada, Spain,
Fax: +34–58–258510

F.J. Adroher
Departamento de Parasitología,
Facultad de Farmacia,
Campus Universitario de Cartuja,
Universidad de Granada,
E-18071 Granada, Spain,
Fax: +34–58–243862

Materials and methods

For the isolation of *Theileria annulata*-infected lymphocytes a 4-year-old cow from a farm in Algar (province of Cádiz, southern Spain) with an acute stage of the disease was used. The isolation was made from blood according to the method of Pipano et al. (1989).

Isolation method

Peripheral blood lymphocytes of cattle were collected by density-gradient centrifugation on Ficoll-Paque (Bøyum 1968) from Pharmacia. The lymphocyte layer was collected and washed in ethylenediaminetetraacetic acid (EDTA)-Na_2 (0.025%) in phosphate-buffered saline (PBS, pH 7.2). The cell pellet was resuspended in 5 ml of RPMI-1640 + 20% (v/v) heat-inactivated fetal bovine serum and was transferred to a 25-cm^2 culture flask and kept at 37°C in an atmosphere comprising 5% CO_2 in air. After 24 h the cells had attached to the inner surface of the culture flask, at which time the supernatant was discarded and fresh medium was added. The culture was monitored daily and the medium was renewed two to three times a week. The culture was considered to be established when a semiconfluent monolayer of cells was observed, and subculturing was done when necessary (Brown 1987; Pipano 1989).

Identification of the isolated parasites

For confirmation that the isolate was *T. annulata*, the culture of infected lymphocytes was stained with 10% Giemsa in PBS (pH 7.2) to identify the schizonts in the cells. An immuno-

fluorescent antibody test (IFAT) was also carried out to identify the schizonts using a previously tested, positive specific control serum. The same techniques were simultaneously carried out on a culture of cell line 529, an established *T. annulata*-infected lymphoblastoid cell line from Israel. In addition, the donor animal was checked and diagnosed for Mediterranean theileriosis after observation of typical clinical signs and laboratory confirmation.

Isoenzyme characterization

Melrose et al. (1980, 1984) reported that isoenzyme polymorphism studies applied to the characterization of *T. annulata* strains had shown that only one enzyme, glucose phosphate isomerase (GPI), was of taxonomic value. In this way, we compare the Spanish isolate (line 28E) with another from Israel (line 529). Noninfected bovine lymphocyte line BL-20 (Hall et al. 1990) served as a control for differentiation of the enzymes associated with the host cell versus the parasite cells. The cells were collected from cultures; after being washed, they were treated with 5% Triton X-100, and "pearls" were made by the introduction of 50-µl aliquots of the suspension into a glass containing liquid nitrogen (Maazoun et al. 1981; Martín-Sánchez 1992). Thereafter these pearls were kept in liquid nitrogen. Electrophoresis was carried out on starch gel prepared at 10% as described by Melrose et al. (1984) and Martín-Sánchez (1992), focusing on the following enzymes: malic enzyme (ME, E.C. 1.1.1.40), phosphoglucomutase (PGM, E.C. 2.7.5.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), and GPI (E.C. 5.3.1.9).

Results

Isolation of the parasites and establishment in culture

Using the blood of a dairy cow suffering from acute Mediterranean theileriosis and showing typical clinical signs of the disease (which led to death 3 days later), smears stained with Giemsa were prepared, revealing a high level of parasitemia (13% of the erythrocytes), but no schizont was observed by light microscopy (Fig. 1). Nevertheless, the infected lymphoblastoid cells were successfully isolated from the blood sample. A 4-day period of culture was necessary for observation of the cells attached to the inner surface of the flask. These cells grew until they formed a monolayer that could be subcultured as an established cell line named 28E (Fig. 2). The growing cells were stained with Giemsa and fluorescent antibody for identification of the *Theileria annulata* inside them (Fig. 3).

Isoenzyme characterization

On starch-gel electrophoresis the isolate from Spain (line 28E) showed a GPI isoenzyme pattern different from that of the isolate from Israel (line 529; Fig. 4). The other isoenzymes studied (ME, PGM, and MDH) showed identical isoenzyme patterns in a comparison of the 28E and 529 cell lines under our experimental conditions. The nonparasitized cell line (BL-20) was useful as a tool for control of the host-cell enzymes.

Discussion

In the present work we isolated and established an in vitro culture of the first *Theileria annulata*-infected

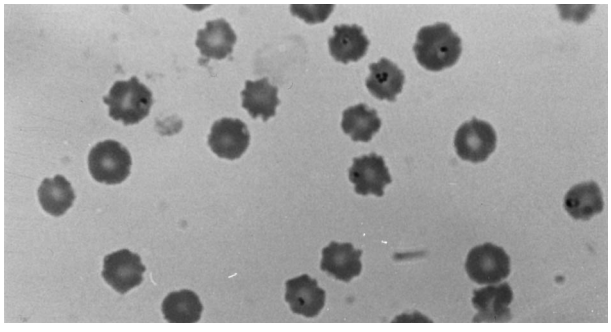

Fig. 1 Giemsa-stained thin-blood film obtained from the dairy cow infected with *Theileria annulata* from which the parasite was isolated. × 1200

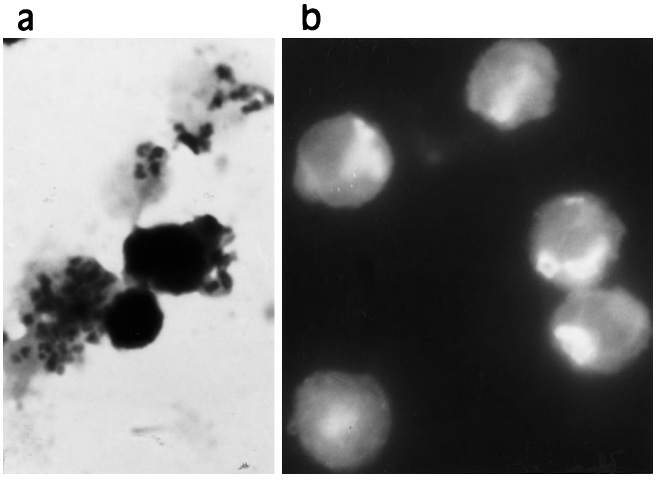

Fig. 3 a Giemsa-stained and **b** fluorescent smears of bovine lymphoblastoid cells containing *T. annulata* schizonts from established cultures. × 1200

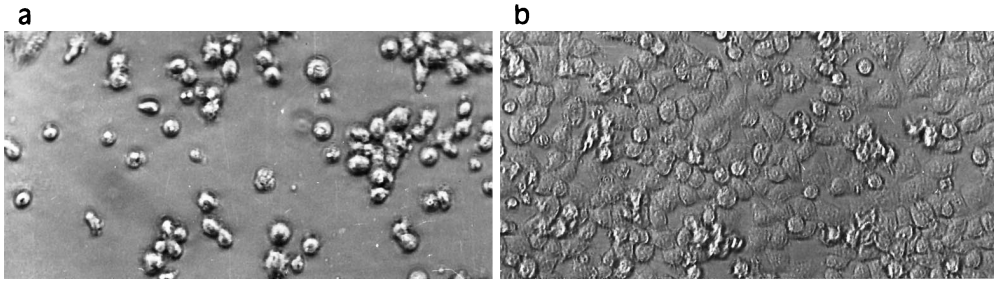

Fig. 2a, b *T. annulata*-infected lymphoblastoid cells established in culture. **a** At 10 days after isolation. **b** Confluent monolayer culture. × 260

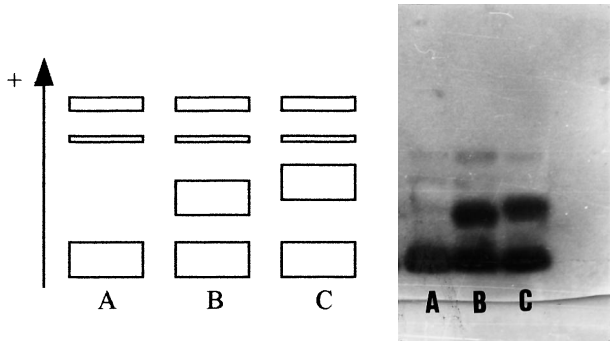

Fig. 4A–C Comparison of the GPI isoenzyme pattern detected in bovine lymphoblastoid cells. **A** Uninfected BL-20 cells. **B** *T. annulata*-infected cells, line 28E (Spain). **C** *T. annulata*-infected cells, line 529 (Israel)

lymphoblast cell line from southwestern Europe (Figs. 2, 3), where Mediterranean theileriosis has not yet been considered an important problem. However, numerous clinical cases are diagnosed every year by veterinarians in enzootic areas, at least in southern Spain, causing important economic losses.

According to Young (1981), the identification of *Theileria* species following morphological criteria is not precise enough, but immunology techniques such as the IFAT seem to be useful for the identification of *Theileria* species. To differentiate between strains of *T. annulata*, specific monoclonal antibodies or isoenzyme determinations have been used. In this sense, Melrose et al. (1980, 1984) have shown that GPI is the only enzyme that has taxonomic value for the differentiation of strains.

We carried out starch-gel electrophoresis of isoenzymes of ME, PGM, and MDH. The patterns of these isoenzymes were found to be the same between the two compared stocks (28E and 529), and although they have no taxonomic value, they serve to corroborate biochemically that our isolate is *T. annulata* as determined by comparison with cell line 529.

On the other hand, for confirmation of the GPI polymorphism (Melrose et al. 1980, 1984) between two stocks from different Mediterranean theileriosis-enzootic areas, starch-gel electrophoresis was used. As expected, because of the large geographic distance between the two isolates (the first being from Spain and the second, from Israel) the GPI band pattern of the 28E cell line was clearly different from that of cell line 529 (Fig. 4). Likewise, the identity of the patterns of the ME, PGM, and MDH isoenzymes as determined between cell lines 28E and 529 suggests a high level of conservation of these enzymes in *T. annulata*.

Finally, the isolated 28E cell line can be used to produce an antigen for the IFAT and also makes feasible the development of a tissue-culture vaccine against Mediterranean theileriosis for use at least in the South of Spain and, perhaps, in neighboring countries.

Acknowledgements We are very grateful to P.M. Arrones, D.V.M., for finding the clinical-case source of parasites. We thank Dr. E. Pipano, Kimron Veterinary Institute, Beit Dagan, Israel, and Dr. B. Shiels, Welcome Unit of Molecular Parasitology, Department of Veterinary Parasitology, Glasgow University, Scotland, for providing us with the cell lines 529 and BL-20, respectively, and Dr. J. Martín-Sánchez from our department for useful advice on starch-gel electrophoresis. We are also grateful to I. Martín for technical assistance and to L. Whiteside for revising the English text. This research was supported by project GAN90/0707 of CICYT, Spain. J.A. was recipient of a predoctoral fellowship from PAI, Consejería de Educación y Ciencia, Junta de Andalucía (Spain).

References

Bøyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 21 [Suppl 97]: 77–89

Brown CGD (1987) Theileriidae. In: Taylor AER, Baker JR (eds) In vitro methods for parasite cultivation. Academic Press, London, pp 230–253

García-Fernández P, Romero-Rodríguez J, Hueli LE (1985) Piroplasmosis bovinas en Andalucía. I. Estudio en reses procedentes de mataderos. Rev Iber Parasitol 45: 48–58

García-Fernández P, Romero-Rodríguez J, Hueli LE (1987) Piroplasmosis bovinas en Andalucía. II. Estudio en explotaciones agropecuarias. Rev Iber Parasitol 47: 7–13

Hall R, Coggins L, McKellar S, Shiels B, Tait A (1990) Characterisation of an extrachromosomal DNA element from *Theileria annulata*. Mol Biochem Parasitol 38: 253–260

Maazoun R, Lanotte G, Pasteur N, Rioux JA, Kennou MF, Pratlong F (1981) Écologie des leishmanioses dans le sud de la France. 16. Contribution à l'analyse chimiotaxonomique des parasites de la leishmaniose viscérale méditerranéenne. A propos de 55 souches isolées en Cévennes, Côte d'Azur, Corse et Tunisie. Ann Parasitol Hum Comp 56: 131–146

Martín-Sánchez J (1992) Identificación del agente etiológico de las leishmaniosis en sus diferentes hospedadores, canino, humano y flebotomo vector, en el sudeste de España: aplicación de la técnica de electroforesis de isoenzimas. Doctoral Thesis, Universidad de Granada

Melrose TR, Brown CGD, Sharma D (1980) Glucose phosphate isomerase isoenzyme patterns in bovine lymphoblastoid cell lines infected with *Theileria annulata* and *T. parva*, with an improved enzyme visualisation method using meldola blue. Res Vet Sci 29: 298–304

Melrose TR, Brown CGD, Morzaria SP, Ocama JGR, Irvin AD (1984) Glucose phosphate isomerase polymorphism in *Theileria annulata* and *Theileria parva*. Trop Anim Health Prod 16: 239–245

Pipano E (1989) Vaccination against *Theileria annulata* theileriosis. In: Wright IG (ed) Veterinary protozoan and hemoparasite vaccines. CRC, Boca Raton, Florida, pp 203–234

Pipano E, Shkap V, Frank M (1989) Comparison of three methods for initiating in vitro cultures of *Theileria annulata* schizonts. Rev Elev Med Vet Pays Trop 42: 529–533

Purnell RE (1978) *Theileria annulata* as a hazard to cattle in countries on the northern Mediterranean littoral. Vet Sci Commun 2: 3–10

Salvans-Bonet L (1928) Contribución al estudio de piroplasmosis bovinas en España. Rev Hig San Pec 18: 104–108

Singh DK, Varshney BC, Raghav PRS, Thakur M (1993) Response of crossbred calves to immunization with *Theileria annulata* schizont infected lymphoid cell cultures. Indian Vet J 70: 605–608

Young AS (1981) The epidemiology of theileriosis in East Africa. In: Irvin AD, Cunningham MP, Young AS (eds) Advances in the control of theileriosis. Martinus Nijhoff, The Hague, pp 38–55