E. Pipano [1]
V. Shkap [1]

Observations on the *in vitro* multiplication of bovine lymphoid cells infected with *Theileria annulata* schizonts

PIPANO (E.), SHKAP (V.). Observations sur la multiplication *in vitro* de cellules lymphoïdes bovines infectées par des schizontes de *Theileria annulata*. *Revue Élev. Méd. vét. Pays trop.*, 1990, **43** (4) : 485-488

La multiplication des cellules bovines de lignée lymphoïde infectées par les schizontes de *Theileria annulata* a été étudiée. Dans les cellules à division par mitose, le schizonte occupait une position centrale pendant les dernières phases de la division, partagée en deux cellules nouvellement formées. Des cellules binucléées avec des schizontes situés entre les noyaux ont également été observées. On ne peut tirer aucune conclusion définitive quant au fait de savoir si ces cellules appartiennent à des phases situées dans la division sans mitose, ou si elles sont le résultat de la fusion de cellules infectées. On a noté de larges variations dans le nombre des noyaux des schizontes par cellule infectée, avec une moyenne de 12,2 noyaux par schizonte. La grande majorité des cellules en contenait 4 à 16. *Mots clés* : *Theileria annulata* - Schizonte - Cellule lymphoïde bovine - Infection *in vitro*.

INTRODUCTION

Theileria annulata schizonts were first cultivated in vitro in plasma-clot cultures of liver and spleen explants from animals with theileriosis (7). Later, mass cultures were made in monolayers of trypsin-dispersed cells originating from internal organs of acutely infected cattle (8) and in cultures grown in suspension (2). Multiplication of the schizonts in culture was studied in Giemsa-stained preparations of sedimented suspensions from a mixed culture system containing infected bovine lymphoid cells and baby hamster kidney cells (3).

The present study reports on monolayer cultures of infected bovine lymphoid cells fixed *in situ* and stained with acridine orange and examined by fluorescence microscopy. Phenomena related to the division of schizont-infected cells are described.

1. Division of Parasitology, The Kimron Veterinary Institute, Beit-Dagan, POB 12, 50250, Israel.

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MATERIALS AND METHODS

Cultures

Theileria annulata schizont cultures were initiated from liver biopsy material obtained from an infected calf using previously described techniques (6). Initial cultures were grown as monolayers in 75 ml plastic culture flasks (Falcon Plastics, Los Angeles, 3012). Serial subculture was carried out by transferring cells dispersed by exposure to 0.025 % ethylene diamine tetra-acetic acid into new culture flasks. The present observations were made on the 160 to 170th passages of an established line of schizont-infected cells (8).

To study the multiplication of the infected cells, dispersed cells were transferred to Leighton tubes (Weaton, 3505-F22) bearing cover slips and incubated for 24 or 48 h at 37 °C. For counting schizont nuclei in infected cells the cultures were incubated up to 5 days.

Acridine orange staining

Coverslips removed from Leighton tubes were rinsed in phosphate-buffered saline and fixed in Carnoy's fixative (glacial acetic acid-absolute ethanol, 1v : 3v) for 3 min. The coverslips were rinsed twice with the staining buffer (1MKH PO_4 ; 0.5M Na_2 $HPOß^4$ $2H_2O$ pH 6.5) diluted 1 : 25 before use, and immersed for 4 min in 0.01 % acridine orange made up in staining buffer. They were then rinsed twice in staining buffer and mounted cell-side down on a small drop of buffer on microscope slides. Observations were made with a fluorescence microscope (UV excitation) using an oil immersion objective.

Immunofluorescence

This technique was used to detect specific *Theileria* antigen. Immunoglobulins obtained by ammonium sulfate precipitation of serum from cattle recovered from *T. annulata* infection were labelled with fluorescein isothiocyanate as described by GOLDMAN (1). Coverslips from Leighton tubes were rinsed with PBS and dried at room temperature. The cells were fixed in anhydrous acetone for 10 min. Fluorescein-labelled antiserum diluted 1 : 20 in PBS was

layered over the cells on the coverslip and allowed to react for 30 min at room temperature. After rinsing with PBS the coverslips were mounted with buffered glycerine and examined with the fluorescence microscope.

RESULTS

Monolayer cultures stained by the direct fluorescent antibody technique showed that almost all cells in the culture contained schizonts (photo 1). In the acridine orange preparations both mitotic and what appeared to be amitotic division of schizont-infected cells were observed. In the early stage of the mitotic process (prophase) the multinucleated schizonts were usually located beside the agglomeration of chromosomes (photo 2). At the beginning of metaphase the schizonts were situated between the separated and aggregated chromosomes (photo 3), and in some cases in the center of the cells (photo 4). After completion of the mitotic division each newly formed cell contained a portion of the schizont (photo 5). A considerable number of cells showed lobed nuclei (photo 6) or 2 complete nuclei with schizonts situated between the lobes or the nuclei (photo 7). In other cells two nuclei could be seen at opposite ends of a stretched out cytoplasmic band or strand with schizont material scattered in the cytoplasm connecting the nuclei (photos 8-10). A few cells were seen with parasite-free cytoplasmic projections extending towards, but not touching other cells (photo 11), or within schizont-free cytoplasmic bridges in contact with other cells (photo 12). Mono- and binucleated cells containing large schizonts with numerous nuclei were also seen in the cultures, but only rarely (photo 13). Disintegrated nuclei and free schizonts attached to the slides also occurred in 3-4 day cultures (photo 14). The centrifugate of the supernatant medium harvested from 3-4 day-old cultures revealed a few free schizonts. Figure 1 shows the percentage distribution of the number of schizont nuclei per cell in 1600 acridine orange stained cells. About 75 % of the cells contained 4 to 16 schizont nuclei with an average of 12.2 nuclei per cell. The average number of schizont nuclei per cell in cultures counted every 6 or 12 h over a 24-to 114 h period varied from 10.8 to 13.4 with a trend towards more nuclei in older cultures.

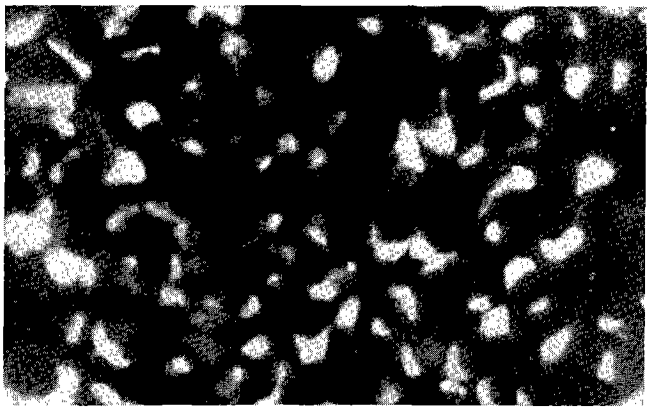

Photo 1 : T. annulata*-infected lymphoblastoid cells stained by the direct fluorescent antibody technique (x 400).*

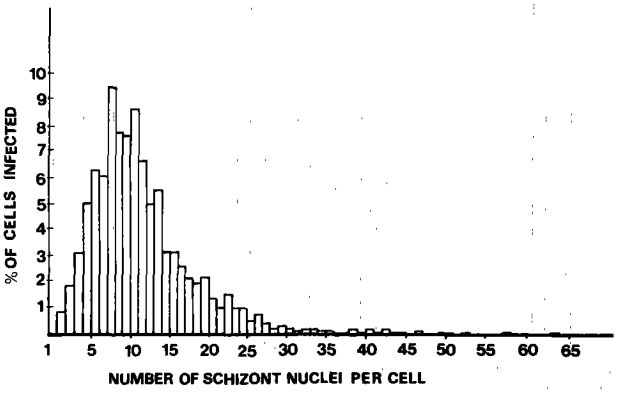

Fig. 1 : Percentage distribution of T. annulata schizont *nuclei in 1600 cultured bovine lymphoid cells.*

DISCUSSION

The multiplication of *T. annulata*-infected cells described in the present work was similar to that described previously by others (3, 4). The probable sequence of events in cultured cells appeared to be as follows : at the beginning of the process, when chromosome formation occurred, the schizont was located in the cytoplasm along the chromosome agglomeration. In the following stages the schizont moved to the center of the cell and occupied this position while the cell was dividing. As a result, each daughter cell received a part of the original schizont. In a previous study (5) schizonts were found in later stages of cell division on the margin of the cells also, laterally to the chromosome agglomerations. This is in contrast with the present finding which showed that the schizonts were always located in the central cytoplasmic area during the above stages of cell divisions. This difference might be due to the fact that in the present study the cells were fixed and stained *in situ* while in previous studies, cells were collected in suspension and then spread and fixed onto slides (5). As a result, the latter technique might not reflect the natural position of the living schizont in the dividing cells.

A considerable number of cells appeared to be in an amitotic binary fission. At no stage of this sequence did the structure of the nucleus show changes characteristic of a mitotic process. This phenomenon was not stressed in previous investigations on *T. annulata*-infected cells in culture. On

the other hand, the cytoplasmatic projections of some cells towards others and the binucleated infected cells described here might be stages of a fusion rather than a division process. Fusion of *T. parva* infected cells was demonstrated by autoradiography of isotope-labelled cultures (5). However, in the absence of unequivocal evidence in the case of *T. annulata* cultures, the apparent amitotic division of schizont-infected cells must remain speculative.

The number of nuclei in individual schizonts was from 4 to 16 but the average number per schizont was 12.2. This figure is similar to observations of other investigators (4). For most of the infected cells there is a balance between the division process of the host cell and the growth and multiplication of the schizonts. In a few cells very large schizonts were present and it may be assumed that such cells were no longer capable of dividing. The presence of residues of disintegrated cells and free schizonts adhering to culture vessels or suspended in the medium indicate that some cells were destroyed in these cultures. Nevertheless, the fact that cultures can be propagated for years without adding fresh cells (6) shows that only a limited number of cells are affected in such a way.

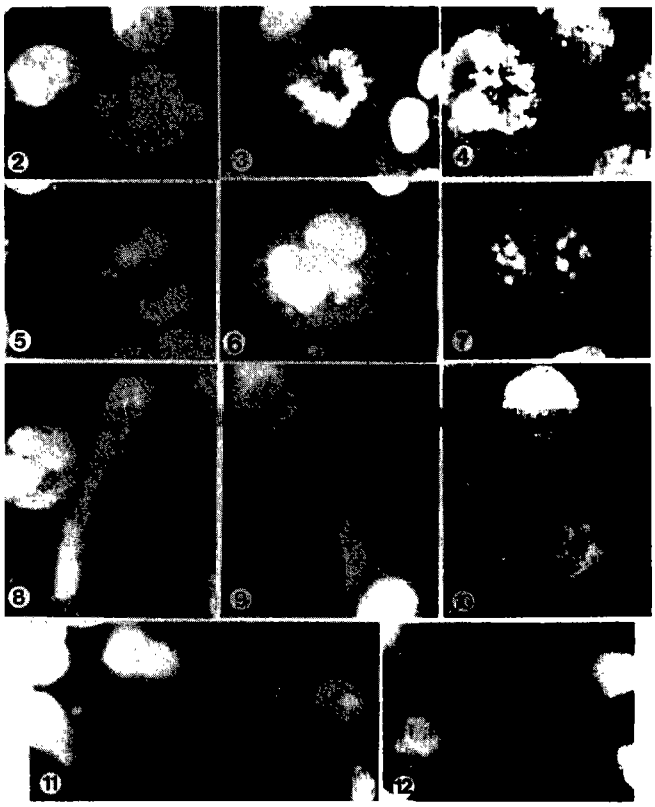

Photo 2 : Prophase, condensation of the chromosomes with the schizont along-side. (Photographs 2-14 show division of T. annulata*-infected cells stained with acridine orange only. The cells were photographed at 1000 x magnification.)*

Photo 3 : Metaphase, schizont located marginally between the two clusters of chromosomes.

Photo 4 : Anaphase, schizont located centrally between the separating chromosomes.

Photo 5 : Telophase, each daughter-cell contains schizont material.

Photo 6 : A bilobed nucleus with a schizont located in the hilus between the lobes.

Photo 7 : Binucleated cell with the schizont between the nuclei.

Photos 8-11 : Schizonts in cytoplasmic bridges connecting host cells.

Photo 12 : Schizont-free cytoplasmic bridge between two host cells.

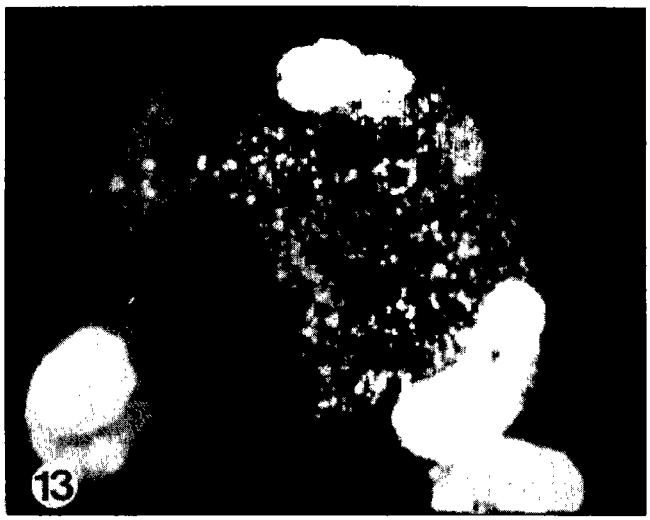

Photo 13 : Giant binucleated lymphoid cell with multinucleated schizont.

Photo 14 : Extracellular schizont apparently released from disintegrated host cell.

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PIPANO (E.), SHKAP (V.). Observations on the *in vitro* multiplication of bovine lymphoid cells infected with *Theileria annulata* schizonts. *Revue Élev. Méd. vét. Pays trop.*, 1990, **43** (4) : 485-488

The multiplication of a bovine lymphoid cell line infected with *Theileria annulata* schizonts was studied. In cells dividing mitotically, the schizont occupied a central position during the late stages of the division and was shared by the two newly formed cells. Binucleated cells with schizonts located between the nuclei were also observed. No definitive conclusions can be drawn as to whether these cells are stages in amitotic division or result from fusion of infected cells. Wide variations in the number of schizont nuclei per infected cell occurred with an average of 12.2 nuclei per schizont. The great majority of the cells contained 4 to 16 schizont nuclei. *Key words* : *Theileria annulata* - Schizont - Bovine lymphoid cell - Infection *in vitro*.

PIPANO (E.), SHKAP (V.). Observaciones *in vitro* de la multiplicación de células linfoides bovinas infectadas por esquizontes de *Theileria annulata*. *Revue Élev. Méd. vét. Pays trop.*, 1990, **43** (4) : 485-488

Se estudió la multiplicación de células bovinas provenientes de líneas linfoides infectadas por esquizontes de *Theileria annulata*. En las células en las cuales existe una división mitótica, el esquizonte ocupó una posición central durante las últimas fases de la división, repartiéndose en las dos nuevas células formadas. También se observaron células binucleares con esquizontes entre los núcleos. No es posible concluir en cuanto a lo que sucede en células con división no mitótica o bien en cuanto al resultado de la fusion de células infectadas. Se notó una gran variación en cuanto al número de núcleos de esquizontes por célula infectada, con un promedio de 12,2 núcleos por esquizonte. Una gran mayoría de las células contenía entre 4 y 16. *Palabras claves* : Esquizonte - Célula linfoide bovina - *Theileria annulata* - Infección *in vitro*.

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