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THE EFFECT OF VIRULENCE ON CULTIVATION OF *THEILERIA ANNULATA* STRAINS IN LYMPHOID CELLS WHICH HAVE BEEN CULTURED IN SUSPENSION (*)

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INTRODUCTION

Interest in production of an effective vaccine against **Theileriasis** due to **Th. annulata** has steadily increased for the past two decades. Early investigators in this field, Sergent **et al.** (1945), introduced the method of vaccinating the susceptible cattle by using 5 to 10 ml. blood from a donor animal infected with a mild strain. This vaccine used to be derived from different animals at different passage levels and, naturally, was of varying virulences. Moreover, it could be used in the field only for 3 to 4 days after preparation, so that its quality could not be properly evaluated prior to issue. The experiments conducted at the Razi Institute by Rafyi **et al.** (1965) show the inconveniences of the use of vaccines prepared from infective blood and emphasize development of the tissue culture technique for propagation of **Th. annulata** schizonts. Significant contribution concerning cultivation of **Theileria** schizonts in tissue culture was made by Tsur & Adler (1962), who described cultivation of **Th. annulata** schizonts in monolayer culture.

Theileriasis in Iran due to **Th. annulata** has ever been a major problem to those importing exotic cattle either for improving the local breeds or for dairy purposes. Production of a vaccine, as a control measure for the disease, has been under consideration at the Razi Institute for nearly six years, and during this period many strains of **Th. annulata** were tried from different points of view. During the experiments which were conducted to cultivate the **Th. annulata** schizonts in lymphoid cells it was noticed that the virulence of the strains play an important part in their cultivation. This paper deals with the differences in cultivation of **Th. annulata** strains with variant virulences and presents a technique for suspension culture of the lymphoid cells. The results obtained from vaccination of the cattle by a tissue culture vaccine will be discussed elsewhere. The term "infected lymphoid cells" in this paper means the lymphoid cells containing **Th. annulata** schizonts.

Materials and Method

Strains: Three strains of **Th. annulata** were used throughout the experiments on cultivation of **Th. annulata** schizonts in tissue culture and were identified as follows:

Strain No. **3**, a very virulent strain which has been kept at this Laboratory by serial blood passages for six years.

Strain No. **11**, a very mild strain, kept at this Laboratory for nearly four years.

Strain No. **15**, a virulent strain but its virulence is less than that of the strain No. **3**.

Thechnique of suspension culture of the infected lymphoid cells-An infected calf's lymph-node which contained good many number of **Th. annulata** schizonts was punctured by a needle and the obtained pulpa was immediately transferred into a conical centrifuge tube containing Eagle medium. After washing with the medium and centrifuging, the pulpa was cut into small particles, dispersed in the medium and distributed into small culture bottles. The infected lymphoid cells and the fibroblast cells grew in association; infected lymphoid cells showed "emperiopolesis" in the mixed culture. Medium was changed every fourth day until there was sufficient number of the infected lymphoid cells available in the supernatant media of the cultures. Supernatant media of the cultures were pooled, centrifuged and then the sedimented cells were resuspended in fresh Eagle medium. The final number of the cells in the medium was adjusted to 100,000/ml. and the suspension was distributed into Roux flasks, each receiving 80 to 100 ml.. The infected lymphoid cells started to grow in 2 to 4 days time, and suspension culture, without shaking or use of any mechanical means, was established after a week or ten days. Subcultures could be made by simply transferring an amount of the cell suspension, containing enough number of cells to establish a culture, into another Roux flask and fresh medium was supplied.

Culture medium — Medium used for supension culture was Eagle with 10% calf serum.

EXPERIMENTS and RESULTS

A—Cultivation of the infected lymphoid cells in suspension:

Main aim in our works was to cultivate the infected lymphoid cells alone, without the association of other cells. At first, in order to study the effect of soft surfaces, experiments were carried out to grow the infected lymphoid cells on agar gel surface and the experiments proved to be successful as far as the multiplication of the lymphoid cells was concerned; most of the cells remained attached to the gel surface and could not be detached even by trypsine or trypsine-versen. Subcultures could be made only by using free floating infected lymphoid cells in the supernatant media. Although cultivation of the infected lymphoid cells on agar gel surface did not give satisfactory results but it showed that the role of fibroblast cells for the growth of the infected lymphoid cells was not a physiological and indispensable one. Then cultivation of the infected lymphoid cells alone

without the presence of fibroblast-like cells, on the glass surface, was tried in small culture bottles but the results were not very much encouraging. At last a strain of the infected lymphoid cells which had been grown in association of the fibroblast-like cells for nearly four months was tried, according to that described in the "technique", in large flasks and this time the cultivation of the infected lymphoid cells was accomplished. The same strain as well as other strains of the infected lymphoid cells which had been grown in association of the fibroblast cells for different period of time were tried by using the same technique but the results were always the same. The only important point in suspension culture of infected lymphoid cells in large flasks was to give them enough time to get adapted the this method of cultivation and then they would grow in abundance.

B—Differences in cultivation of different strains of Th. annulata with variant virulences:

All the trials for cultivation of the very mild strain (St. No. 11) proved to be successful. lymphoid cells containing the schizonts of this strain could be easily grown in cell culture. Although its cultivation was quite easy but this strain could not keep its pathogenicity for cattle, when passaged in lymphoid cells, for a long time and completely lost its virulence after being passaged in lymphoid cells for about three months. Cultivation of the strain with a medium virulence (St. No. 15) was difficult at first but feasible. This strain could maintain its virulence for cattle, although decreasing in the length of time, for a longer period and after being maintained in the lymphoid cells for ten months could produce a **Theileriasis** in cattle with 3 to 4 days fever, with persence of schizonts in the liver and the lymph-node. Several trials to cultivate the very virulent strain (St. No. 3) were proved failure. The experiments which were set up to cultivate this strain in the lymphoild cells were as follows:

In order to evaluate the effect of the nutrient medium, different media such as **YLH-YLE-Eagle** were tried but the results were always negative. Percentages of calf serum, ranging from 10 to 40%, were used with each of the medium without success. Lymphoid cells of different organs of different calves were tried without any result. Results are summarized in table No. 1

DISCUSSION

Cultivation of **Th. annulata** schizonts in tissue culture was introduced for the first time by Tsur (1945) who used bovine tissues in explant culture technique. Tsur & Adler (1962) later on applied the monolayer culture technique for the bovine lymphoid cells containing **Th. annulata** schizonts. They described the presence of two types of cells, fibroblast cells and lymphoid cells, in the cultures and found that **Theileria** schizonts grew only in lymphoid cells. Mode of multiplication of **Th. annulata** schizonts in the lymphoid cells was much more elucidated by Hulliger (1964). Hulliger (1965) described that the infected lymphoid cells can be grown only in association of other cells and fibroblast cells exclusively can support their growth. We have observed at this Laboratory that the infected lymphoid cells perform "emperiopolesis", a phenomenon described by Laochim (1965), and as the fibroblast cells are the only cells to permit such phenomenon to take place that is the reason why the infected lymphoid cells grow only in

association of the fibroblast cells. In this paper it has been shown that the infected lymphoid cells can be grown in suspension culture, without stirring or any mechanical means, independent from any associate cells in Roux flasks, provided they are adapted to this method of culture in the length of time. It is stipulated that in the first passages a selection takes place and only those which can grow in suspension culture survive. Application of this technique has facilitated the production of the anti-theileriasis vaccine on a large scale.

Cultivation of different species of **Theileria** was first tried by Tsur (1957). Hulliger (1965) described the cultivation of three species of **Theileria** and found that there is difficulties in establishing culture from **Th. parva** schizonts but it is quite easy with that of **Th. annulata.** None of these investigators has mentioned differences in cultivation of strains of **Th. annulata** or any other species. We have found that there are differences in the response of different strains of **Th. annulata** to the **in vitro** cultivation and the virulence is a factor which interferes the most; cultivation of the very virulent strains is very difficult, if possible. In case of strain No. **3** it is thought that the schizonts of this strain are either very toxic to the lymphoid cells or they need the animal's body condition for their growth. It is most possible that the same phenomenon prevails in the cultivation of **Th. parva** and if a less virulent strain is tried the result might be more successful.

TABLE No. 1

Results of cultivation of different strains of **Th. annulata.**

Strains	Number of trials	Tissues	Number of calves	Media	Range of serum %	Result
No. **3**	4	lymph-node	4	YLH-YLE-Eagle	10—40	—
"	5	spleen	5	" " "	" "	—
"	4	liver	4	" " "	" "	—
"	2	blood	2	" " "	" "	—
No. 11	3	spleen	3	"	"	3
"	4	lymph-node	4	"	"	4
No. 15	4	spleen	4	"	"	4

SUMMARY

Differences in cultivation of **Th. annulata** strains with variant virulences is described and it is concluded that the cultivation of the very virulent strains in lymphoid cells is very difficult, if possible. A technique for cultivation of the lymphoid cells containing. **Th annulata** schizonts in suspension, without stirring

or any mechanical means, is presented. Suspension culture of such cells could be better obtained in large flasks such as Roux flasks. Simplicity of this technique has greatly facilitated the cultivation of **Th. annulata** on a larger scale, for vaccine production.

RESUME

On a expliqué la difference des souches de Th. annulata, par une diversité de leur virulence, **in vitro,** en culture. On a précisé que la culture des souches trés virulentes, si c'est possible, ce sera certainement beaucoup plus difficile. Pour culture des cellules lymphoïdes infectées aux schizontes de Th. annulata on a proposé une technique beaucoup plus simple qui aboutit à des resultats nets. C'est à dire on prépare une suspension des cellules infectées sans les agiter et sans profiter des moyens mecaniques. En outre la culture des cellules lymphoïdes en suspension dans les boites de Roux sera beaucoup plus facile. De toute façon la simplicité de cette technique a rendu facile la culture des schizontes de Th. annulata afin de produire du vaccin en grande quantité.

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