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Strains of Toxoplasma can be divided on the basis of their ability to kill white mice into virulent, weakly virulent and avirulent. It has been established that cultivation has no effect on the original degree of virulence of some strains of Toxoplasma which is stubbornly preserved, while in others it fluctuates.

The process by which a strain loses its avirulent properties is associated with the mass release of individual parasites from the cysts and their subsequent disseemination thru the entire body. However, the causes abetting such a process remain unclear. Knowledge of the reasons behind fluctuations in the virulence of Toxoplasma strains will help us to understand the polymorphism of the clinical symptoms and the pathologic picture in toxoplasmosis, as well as to develop more refined methods of diagnosis, treatment and prevention of this illness.

The use of such methods as electron and light microscopy, culturing the agent in tissue culture and chick embryos and immunochemical, biochemical and histochemical methods of studying it opens perspectives for determining the biological properties of Toxoplasma, in particular, the avirulence of some strains.

We studied avirulent Toxoplasma strains VFG_3, OCG, LEJ and ALG, isolated respectively from a red fox (Vulpes fulva), a rabbit, a hare and an arctic fox (Alopex lagopus). Our culture media consisted of primary trypsinized cells of chick fibroblast and human tissue, interwoven lines of HEp-2, He-La and pig embryo kidney (PES) cells, as well as developing chick embryos.

It was intended to trace the interrelationship of the avirulent strains and the cells and the change in virulence as related to the method of cultivation, as well as to study the antigenic and immunogenic properties of the avirulent strains.

Methodology. Test tubes containing a tissue culture were infected with cysts isolated from the brain of a white mouse (1, 2, 4, 6 cysts per test tube or jar) and a suspension of brain containing a large number of cysts; the chick embryos were treated only with brain suspension containing a large number of cysts.

The behavior of the strains was observed daily by studying fresh or Romanovsky stained preparations with a light microscope. For controls we used the bioprobe method on white mice. The observations in the experiments on tissue culture lasted 46 days and on chicken embryos 14 days.

In addition to this, a systematic observation of the behavior of the strains studied was conducted for about 2 years while the strains were constantly maintained on tissue cultures. Four avirulent strains were cultivated; for comparison so was virulent strain RH. When the culture medium was changed, which occurred every 2-4 days, control smears, stained by Romanovsky's method, were prepared from the centrifugate.

An obligatory condition of the cultivation was the parallel growing of no less than two lines of the same strain with alternating innoculation of the strains (to guarantee preservation of the strain in case of a contamination of one of the lines). The initial material for infection of the tissue cultures was a 10% suspension of white mouse and rabbit brain containing cysts, as well as culture skimmings containing infectious material (3-10 million Toxoplasma per ml.).

The strains were transferred from the initial material to the culture cells repeatedly: VFG_3 and LEJ 9 times each, OCG 5 times and ALG once. In all, 25 lines (reblendings of the initial material) were observed from 4 avirulent strains. From 20 to 36 passages were performed with most of the lines. The interval between passages on the primary trypsinized cells was 1-2 weeks, on reblended cells--2-4 weeks. Before each regular passage the parasites cultivated in the tissue culture were controlled on laboratory animals.

The antigenic and immunogenic properties of the avirulent strains were studied by the indirect fluorescent antibody method. Antigen smears were prepared from the Toxoplasma strains to be studied, which had been grown in cell culture. Three series of antigen smears were prepared from avirulent strains VFG_3, OCG and LEJ and one (control) from strain RH.

We studied sera from rabbits which had been experimentally infected with these same strains, as well as sera from spontaneously infected rabbits. Sera from the experimentally infected animals were studied to determine the progress of infection at intervals of two days for a month.

Results of observations. In the experiments on tissue cultures the avirulent strains grew and multiplied without showing any cytopathogenic effects on the culture cells. Smears taken from all tests, starting with the third day of cultivation, had proliferative forms of Toxoplasma both inside and outside the cells (Fig. 1, 2).

On the 10th to 12th days of cultivation we began encountering distinctly differentiated cysts (Fig. 3). In the preparations under examination we observed, in addition to the cysts and the proliferative individuals, a multitude of other formations of varying shapes. Thus, beginning with the third day of cultivation, we observed very tiny, pinpoint intracellular groups and clusters of freely distributed formations resembling vibrios. On the fourth day these and other formations had grown in size. On the fifth

day the intracellular pinpoint formations had emerged from the cells· they had become large, had overshadowed the nucleus and were appearing in paired groups (Fig. 4). The vibrionoid formations also got bigger. And on following days clusters were observed, consisting of tiny, vibrio-like individuals, and so were the larger, round forms, which were grouped in pairs. These clusters had no definite wall. Along with such formations we encountered small and large cysts, as well as free single toxoplasmas of the typical form with a well-differentiated nucleus and cytoplasm. All formations were found both in the cells and outside them.

It is important to point out that the cysts were of various shapes: the tiny, pinpoint clusters were light-colored, while the larger clusters were dark (Fig. 5). We also observed some from which Toxoplasma emerged thru apertures in the wall without any general breakdown of the cyst. We also saw "empty" cysts, without any Toxoplasma.

In the control biological probes on white mice using cultured material infected with various strains, various doses and harvested at different times during cultivation, we as a rule observed cysts (Fig. 6) which retained their original avirulent properties.

Avirulent Toxoplasma also develops well in chick embryos. The proliferative forms of Toxoplasma were encountered in almost all the infected embryos--in the blood, the chorioallantoic wall, the internal organs and the brain of the offspring. Cysts were observed in the blood of the embryo on the 11th day after infection. As a rule the parasites did not cause the offspring any harm. Experimental and control embryos developed identically. On the 13th day after infection the chicks hatched, but their development differed in no way from that of control chicks which had hatched at the same time. Cysts were found in the organs of practically all experimental chicks. Among them were individuals with tiny, budding daughter-cysts, as well as clusters of tiny cysts around one large one (Fig. 7).

In the control bioprobes on white mice using material from infected chick embryos cysts were observed. The avirulent strains passed thru the chicken embryos did not lose their original avirulence for white mice and other laboratory animals.

Thus, by cultivating avirulent strains we observed typical proliferative forms of the parasite and the cysts. In addition, we observed various forms which we are referring to provisionally as "intermediate."

In the control mice infected with intermediate forms, cysts were observed in the brain. We observed the emergence of Toxoplasma thru an aperture in the cyst without any general damage to the cyst; we also saw the development of cysts by budding of small daughter-cysts on the large mother-cyst (Fig. 8).

In the process of long-term cultivation of avirulent strains it was established that they live and slowly multiply on all the types of tissue which we experimented with without, as a rule, having any cytopathogenic effect on the culture cells. The parasites develop identically when they are transferred from one species of cell to another. Upon initial infection

of cultures with Toxoplasma cysts, the free proliferative forms of the parasites in the control smears are as a rule first observed on the third to seventh day. Development occurs--young individuals and intermediate forms (spheres and filaments, which we observed in a special experiment) are present. The cysts were formed in observable lines, at different times and independent of the strain being passed. The earliest observation of cysts was two weeks after transfer of the strain to the culture, and the latest was after eight weeks. A large number of proliferative forms of the parasites usually accumulated on the sixth to tenth week; by this time many cysts were encountered along with the proliferative forms.

When cultivating avirulent strains on blended lines of cells one can note that under favorable conditions of cultivation (good cellular plast, frequent change of support medium, optimal temperature) a more intensive accumulation of proliferative forms of Toxoplasma occurs. The number of parasites may reach 20 million per ml. They become cytopathogenic. Usually, under a great accumulation of parasites the cellular plast was destroyed somewhat faster (on the 20th to 21st day) than that in the control experiment (28-30th day), while virulent strain RH, when it had produced the same number of parasites, would break down a monostratum of cells in approximately 10 days.

However, under identical conditions of cultivation the most intense multiplication of Toxoplasma was observed in strains VFG_3 and OCG. When LEJ was cultivated it accumulated only 6 million parasites per ml., and its cytopathogenic activity was inapparent.

When a large number of parasites had built up in strains VFG_3, OCG and LEJ, a cellular suspension washed in physiologic solution was used to inoculate white mice. The dose for infecting a single animal was approximately 20 million parasites. In some cases the animals infected with strain OCG died on the 8th, 25th, 35th or 40th day. Toxoplasma was observed in small numbers in the peritoneal exudate of the mice that died on the 8th to 25th days. Those which died later (35th to 40th days) had large cysts in their brains. However, most of the animals infected with strain OCG, as well as all mice given strains VFG_3 and LEJ, remained outwardly healthy and were killed at 2-2.5 months. They had cysts in their brains.

Thus, our observations enable us to conclude that the degree of virulence of the avirulent strains under examination was not identical. Judging from the ability of a strain to have cytopathogenic effects, the degree of virulence appeared highest in strains OCG and VFG_3. Infection of laboratory animals showed that OCG was the most virulent strain.

One of the factors influencing the formation of conditions unfavorable to the reproduction of Toxoplasma is contamination of the strain by extraneous microflora (spore-forming bacilli, micrococci, etc.). When this happens the proliferative forms cannot stand the competition and quickly die. Toxoplasma undergoing lysis is observed in control smears. However, within several days the cysts appear. The strain is preserved in cyst form. If the strain can be successfully saved from the alien microflora by treatment of the contaminated strain with antibiotics (penicillin, streptomycin) and if optimal conditions can be reestablished, Toxoplasma again begins to multiply and continue its developmental cycle.

One interesting detail ought to be pointed out. Virulent strain RH reproduced slowly on a culture of swine kidney cells (PES). Infection of this culture of cells required a dose of parasites twice as large as was needed to infect any other culture. Sometimes strain RH formed cysts on PES. All this attests to PES cells' low sensitivity to the virulent strain. As we already pointed out, differences in the behavior of avirulent strains from one cell culture to another were not noted in our experiment.

We know that in the white rat virulent strain RH becomes avirulent and forms cysts. Passing through the body of the white rat, this strain becomes less virulent for white mice and rabbits. The literature contains references to the fact that this same strain can behave differently in the bodies of various species of animals. Thus, strains that are avirulent for many animals cause clinical illness in the suslik (Simitch et al., 1956; Galuzo and Krivkova, 1966, 1968). Savin, Simitch and Bordjochki (1963) observed how avirulent strains isolated from birds acted differently in different animals: susliks died, guinea pigs and white rabbits had chronic illnesses, while hamsters had both chronic and acute disease. As is apparent, the habitat and the infectious dose of the agent influence the formation of the degree of virulence of Toxoplasma.

A study of antigenic and immunogenic properties among avirulent strains of Toxoplasma has shown that the results of cross-reactions between antigen smears prepared from avirulent strains, and control antigen smears from virulent strain RH, with sera from animals infected with these same strains and spontaneously infected animals as well were identical. When rabbits were immunized with virulent and avirulent strains the boundary titers were identical, fluctuating from 1:40 to 1:64.

Whether rabbits are infected with virulent or avirulent strains of Toxoplasma, the same picture is observed in the dynamics of antibody formation. The antibodies formed in response to an infection by any strain invariably consist of 2 components: an early, thermolabile one and a later thermostabile one. Examination of the dynamics of the early and late antibodies, using antigens from avirulent strains, showed analogous results with antigens from virulent strain RH. In studying sera from rabbits infected with virulent and avirulent strains, we found that the early antibodies began to become recognizable on the second or third day after infection. On the 11th day they had reached a titer of 1:40; on the 14th to 16th day, 1:160. Later antibodies began to appear on the 8th to 11th day. Within two weeks the maximal titers were 1:40, and within 3 weeks 1:160.

Thus, the method of fluorescent antibodies enabled us to trace the dynamics of qualitatively different antitoxoplasmic antibodies. The application of this method for the study of the antigenic properties of virulent and avirulent strains of Toxoplasma has also enabled us to use antigens prepared to a definite standard, and likewise to standardize the experimental conditions, which is impossible in the complement fixation reaction.

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