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The development of piroplasmids in ticks has not yet been studied sufficiently. Data exist on the development of several species of Piroplasma (P. bigeminum, P. canis, P. caballi) and Babesiella bovis (=divergens). There are no special works on the development of Nuttallia in ticks. All we have is Tsaprun's short mention of the fact that N. equi reproduces in ticks in the same way as P. caballi (Tsaprun, 1957). Until now a dispute has existed as to whether there is a sexual process in the development of piroplasms in ticks or whether their developmental cycle goes on without this process and the parasites reproduce agamously. Some investigators (Christophers, 1907, Martsiovsky and Belitser, 1907, 1908, Dennis, 1932, Petrov, 1938, 1939, Tsaprun, 1952, 1954, 1957, and Riek, 1964) felt that there is a sexual process in the developmental cycle in the form of isogamous or anisogamous copulation. However, none gave convincing evidence of its presence. Other investigators (Koch, 1906, Crawley, 1915, Li, 1956; 1957) failed to find evidence of the presence of a sexual process but did not deny the possibility of its existence. A third group of investigators (Regendanz and Reichenow, 1933, Reichenow, 1935, Regendanz, 1936, Shortt, 1936, Polyansky and Kheysin, 1959, Kheysin and Muratov, 1959 and Muratov and Kheysin, 1959) denied the existence of the sexual process in piroplasmids, since they found no stage of development whatsoever which might be accepted as a sexual one. They felt that piroplasms reproduce only agamously.

It is clear that in order to resolve this question supplementary investigations on different species are necessary.

In 1963 we (Krylov and Zanina) described a new species, Nuttallia tadzhikistanica from the red-tailed gerbil (Meriones erythrourus). It was made clear that the vector of this Nuttallia was the tick Hyalomma anatolicum (Krylov 1963). The species of Nuttallia described was highly suitable for study, since its vertebrate host is easily raised in the laboratory, while the ticks can be cultivated all year round and are easily infected on the vertebrate host. This work gives some results of a study of the development of Nuttallia tadzhikistanica in the tick Hyalomma anatolicum.

MATERIALS AND METHODS

The gerbils used in the experiments were raised under laboratory conditions. The ticks were raised at 25-28 C. The tick larvae were fed on splenectomized gerbils with a large number of parasites in the peripheral blood. It was only in the presence of a heavy parasitic infection that we succeeded in infecting most of the tick larvae. However, not all of the ticks which drank the blood of heavily infected hosts had a multitude of parasites under the microscope. In some ticks the parasites could be found only with great difficulty, since they were present in such small numbers. Sometimes the Nuttallia were abundant in the 1st hours after the larvae dropped off of the gerbil but quickly disappeared, while in the other ticks a large number of parasites could be observed even 24 hours after they fell off the host.

The Nuttallia in the ticks were studied in sections and smears. For histologic examination the ticks were fixed with Zenker's fluid and formalin. The preparations were stained with eosin-azure II using Maximov's method and with

hematoxylin-eosin. Smears were prepared by crushing the entire tick, as well as from hemolymph and salivary glands. Staining was done by the Romanovsky-Giemsa method.

The tick larvae were examined in the semi-engorged state and then each hour for 6 hours after they fell off. Further examinations were made every 24 hours until the time of molting. In all, we studied 9 series of larvae, numbering about 1000, which had fed on different gerbils at different times of the year. Nymphs which had molted from infected larvae were examined when hungry and after feeding on Nuttallia-free gerbils for 1-5 days. These examinations were repeated 4 times. A total of 350 nymphs was examined. In addition, preparations were made from 100 infected nymphs.

As controls we prepared and studied preparations from ticks raised in the laboratory and fed on piroplasmid-free gerbils as well as on rabbits and white mice.

Parallel to our study of the structure of Nuttallia we studied the infective capability of various developmental stages of these parasites. For this a suspension prepared from 2-40 Nuttallia-infected, newly engorged larvae and larvae which had spent 24, 72, 144 and 216 hours since engorging was injected subcutaneously into 10 gerbils. With the same goal in mind nymphs washed in 0.85% NaCl solution and raised from larvae infected with Nuttallia were fed on rabbits for 1, 2, 3, 4, and 5 days, ground up and injected subcutaneously into 6 sterile, splenectomized and 6 sterile, intact gerbils. Each gerbil was given a subcutaneous injection of 4-22 washed nymphs. In all the experiments the gerbil blood smears were examined for 30-40 days.

The Stages of Development of Nuttallia tadzhikistana in Larvae

In preparations made from semi-engorged larvae which had not fallen off (on the 2nd day after the start of blood sucking) or from engorged larvae

one or 2 hours after they fell off we encountered, in addition to parasites contained in gerbil erythrocytes, many Nuttallia which had already emerged from the erythrocytes. This process is brought about by ameboid movement, which can be observed in studies of fresh tick intestinal contents under the phase contrast microscope.

In the erythrocytes we could see many rod-shaped, round, piriform and amoeboid Nuttallia. Often we encountered dividing forms in the shape of a Maltese cross. In the intestinal contents outside the erythrocytes we could see numerous mononuclear developmental stages of Nuttallia, very similar to the intra-erythrocytic forms. Such rod-shaped or amoeboid stages were 1.5-5.8 μ long and 1-3.6 μ wide (Fig. 1). Along with such mononuclear stages, which had probably left the erythrocytes not long before, we saw binuclear, trinuclear and tetranuclear Nuttallia (Figs. 2, 3). The tetranuclear stages were somewhat larger than the mononuclear ones. They were 2-6 μ long and 1.5-3.5 μ wide. We often saw almost rounded or irregular tetranuclear individuals, with a diameter of 4-5 μ. The binuclear stages (Fig. 3) were quite varied. They were given special attention, since they might be thought of as either stages of division or of copulation. Most often we saw the amoeboid forms with 2 nuclei; sometimes we observed stretched, cigar-shaped or oval individuals. It should be pointed out that the shape of the binuclear stages is quite varied, which attests to their considerable motility. The nuclei of the binuclear stages have identical dimensions and lie either right next to each another, or in the elongate forms, at opposite poles. The nuclei of the mononuclear and binuclear forms are identical in size. Among the binuclear individuals forms were some with an isthmus between the nuclei and features of body stretching. The body shape of the tetranuclear stages was very similar to that of the binuclear ones, but oval and irregular shapes were more common. The presence of trinuclear individuals of similar shape and size attested to the non-synchronization of nuclear division.

Such stages of nuclear division were found thruout the next 24 hours. It is interesting that for 24 hours after the larvae fell off the gerbil it was possible to find intact erythrocytes containing still undeveloped Nuttallia in the intestines. It is possible that they later degenerated.

The presence of mono- , bi-, tri- and tetranuclear Nuttallia in the larva's intestine 24 hours later attests to the fact that the parasite was multiplying at this time.

Again 24 hours later stages of multiple reproduction of Nuttallia (Fig. 4) were found in the hemolymph of larvae which had just fallen off. It was possible rather often to see various stages of developing Nuttallia. They were oval, rounded or irregular, and looked somewhat like small plasmodia. The number of nuclei varied from 6 to several dozen. Along with multinuclear parasites were groups of closely packed oval or piriform trophozoites united by a single membrane. This was the segmented, multinucleate plasmodium. We usually found groups of 16-32 trophozoites each. Sometimes several small trophozoites budded off the multinucleate plasmodium. The segmented plasmodia reached 24-28 μ in diameter. The trophozoites which were formed were 1.5-2.0 μ long and 1-1.5 μ wide. The multinucleate stages were found free in the hemolymph or in the hemocytes[1], where they occupied almost all of its cytoplasm (Fig. 4). Apparently such intracellular Nuttallia are a normal stage of development similar to that found in the hemolymph. Sometimes the hemocytes contained poorly stained multinucleate parasites. It is possible that these were Nuttallia phagocytized by hemocytes which later died without developing.

Within 48 hours after the larvae fell off the Nuttallia become fewer than before. The multinucleate stages were found comparatively rarely. It was likewise difficult to find the earlier developmental stages. The multinucleate stages continued to be found for up to 72 hours after the larvae fell from the

1 The uniform elements of tick hemolymph were described by Tsvileneva, 1961.

gerbil. It is probable that parasite reproduction does not cease for several days, but its intensity drops markedly. One can get an idea of this from the small number of dividing forms 72-96 hours after the larvae fall off.

The presence in the larvae of various fleeting stages of development is apparently associated with the fact that the parasites enter the intestine of the tick repeatedly during the blood-sucking, which lasts approximately 60-72 hours.

Not long before the molt (220-240 hours after the larvae fall off) it becomes very hard to find any Nuttallia in the larvae. At this time mononuclear oval or round individuals measuring 2.4-7.2 by 1.5-6 μ were found in the hemolymph. They were similar in form to the trophozoites formed after multiple division, but they were somewhat larger (Fig. 5; A-E). They are apparently formed by the growth of small individuals. We did not succeed in observing the developmental stages of these large trophozoites. During the larval molting period they were preserved in its body as if in a state of rest (Fig. 7; h). The very same developmental stages of Nuttallia were also seen in the hungry nymphs.

Development in the Nymphs

Rounded or oval mononuclear Nuttallia (measuring 2.4-7.2 by 1.2-6 μ--Fig. 5; A-E) were found comparatively rarely in hungry nymphs. All the parasites were found in the hemolymph, with none in the salivary glands.

Within 24 hours of the start of feeding, irregular-oval or rounded forms of Nuttallia (Fig. 5; F-K), measuring 2-8 by 1-7 μ began to be found more frequently in the hemolymph. There were transitional forms between the tiny and the large ones, indicating that the small ones were probably growing. It is probable that at this time the larger individuals were also dividing, since the number of parasites had increased in comparison with the number found in the hungry nymphs.

Within 48 hours of the start of feeding, developmental stages of Nuttallia were seen in the salivary glands. These were mononuclear, irregular-oval in form, measuring 2.4-3.6 by 1.2-2.4 μ (Fig. 6; B). Within 72 hours multinuclear plasmodia (Fig. 6; A, C, D) were being found in the salivary glands. The number of their nuclei reached several hundred. Within 96 hours multinuclear plasmodia were visible; they had broken down into a large number of piriform or rod-shaped trophozoites measuring 1.2-3 by 0.6-1.4 μ (Fig. 6; E). The nucleus of each trophozoite was usually close to the broad end.

The Possibility of Gerbils Being Invaded by the Developing Stages of Nuttallia from Larvae and Nymphs

The tick larvae were fed on heavily infected gerbils. The engorged larvae were then used 24, 72, 144 and 216 hours after dropping off to infect sterile gerbils. Each experiment used 20-40 larvae. They were washed with 0.85% NaCl solution and the suspension made from them was injected subcutaneously into the gerbils. A suspension of freshly dropped-off larvae was given to 5 gerbils. Of these, 2 became ill with nuttalliosis. Twenty-one gerbils were given a suspension of larvae which had dropped off 24-216 hours previously. None of them became infected. In the control larvae, which were examined microscopically, we observed the above-described stages of developing Nuttallia.

We must point out the interesting fact that attempts to infect gerbils with larvae taken 24 hours after they fell off were unsuccessful. At this period the larvae still contain many intact infected erythrocytes. Apparently they lose their ability to develop in a vertebrate host, and it is possible that they are not viable at all.

In order to explain on what day after the start of nymphal blood-sucking the nymphs are able to infect a vertebrate host, several series of experiments were set up. A suspension was prepared from nymphs which had fed 1-5 days on

rabbits which was then injected subcutaneously into gerbils. A total of 12 intact and splenectomized gerbils was used for infection. A suspension of 15-20 nymphs fed for 24, 48 and 72 hours was injected into 8 gerbils. None of them became infected. Suspensions of 5, 10 and 14 nymphs fed for 96 hours were given to 3 gerbils, respectively. All of them came down with Nuttalliosis with a characteristic parasitic reaction on the 20th to 25th day. Consequently, nymphs become infective only on the 4th day after starting to suck blood. Numerous small trophozoites are formed in the salivary glands at the 96-hour mark; these are the infective stage of Nuttallia.

EVALUATION

On the basis of an investigation of Nuttallia in the larvae and nymphs of Hyalomma anatolicum at various times after the end of blood sucking it is possible to imagine the development of the parasite in the following way (Fig. 7). The parasite's intra-erythrocytic stages get into the intestine of the tick during the blood-sucking process, rapidly leave the erythrocytes and continue their development within the cells in the intestinal lumen. This development occurs in the first hours after the start of blood sucking by division in 2 or into 4 individuals. No reproducing stages of the parasite could be observed in the epithelium of the intestine. In this sense Nuttallia differs from P. bigeminum and P. canis, which undergo mass division in the epithelial cells of the intestine (Regendanz and Reichenow, 1933; Muratov and Kheysin, 1959; Riek, 1964). The monocellular trophozoites formed as a result of division penetrate into the body cavity of the tick during the first days after the start of blood sucking, where they begin mass division. This can happen either in the hemolymph or in the hemocytes. During this division several dozen small, mononuclear, cigar-shaped or oval trophozoites are formed. Mass division can apparently be repeated

Approximately 72 hours after the larvae fall off the gerbil, the number of Nuttallia in the body cavity of the tick drops sharply in comparison with the earlier periods. Not long before molting one can find a few "resting" oval or amoeboid forms in the larvae. The same stages of development are found in hungry nymphs raised from infected larvae. Further development of Nuttallia in the nymphs begins only when the nymph begins to suck blood. The number of trophozoites in the body cavity of the tick increases, and 48 hours after the start of blood sucking Nuttallia are observed in the salivary glands. Here mass multiplication takes place, ending 96 hours later with the formation of a large number of small, piriform or rod-shaped trophozoites. During this time the nymph becomes capable of infecting gerbils. All the other stages of Nuttallia, in both nymphs and larvae are not infective for a vertebrate host.

Microscopic examination of larvae and nymphs raised in the laboratory and fed on rabbits has shown that they do not contain the parasites seen in larvae and nymphs which were deliberately infected with Nuttallia by feeding on invaded gerbils. There is therefore every reason to believe that the above forms are indeed Nuttallia tadzhikistanica.

Does a sexual process occur in the developmental cycle of Nuttallia? Using Haemosporidia as an analogy, the formation of gametes and their copulation ought to occur in the first hours after the parasites and the blood get into the intestine of the vector. Consequently, one might expect that in tick larvae which have just sucked blood some kind of stages would be observed which could be defined as gametes and zygotes. But this could not be done.

During the first 24 hours after the start of blood sucking, binuclear developmental stages which might be interpreted as either stages of division or as copulation stages of amoeboid isogametes were found in the intestine more than once. The following facts testify in favor of the first assumption. In the binuclear

stages one can often note an elongation or isthmus, both between the nuclei and on the surface of the body. In addition, tri- and tetranuclear stages of development of approximately the same size as the binuclear ones are encountered (Figs. 2, 3). Therefore it seems that the binuclear stages not only divide themselves but may also be transitional stages on the route to tetranuclear forms. The latter may break down into 4 trophozoites which again grow and become tetranuclear, passing thru a binuclear stage. The lengthy existence of such stages in the intestine is more of a proof that they are dividing forms than copulative forms. If we imagine that the binuclear forms are gametes combined by copulation, then the highly varied mutual position of the nuclei appears strange. In copulation the nuclei should lie close to one another, not at opposite ends of the body, as is often observed in the binuclear stages in the intestines of tick larvae. No clear picture of the mating of nuclei was observed. Not once did we find mononuclear stages which had combined, as could have happened during copulation. The nuclei of the mononuclear stages were the same size as those of the binuclear and tetranuclear ones. No stages with larger nuclei (such as one would expect in "zygotes") were seen. The dimensions of the mononuclear extra-erythrocytic stages were exactly the same as those of the erythrocytic stages one. Consequently, it would seem that the extra-erythrocytic mononuclear developmental stages were formed from erythrocytic ones and that the bi- and tetranuclear ones were formed from them by asexual reproduction. Mass division begins in the intestine of the larva in the first 24 hours after it fell off. If we assume that mass division of this sort is "sporogony," then it seems strange that this process repeats itself, which is not typical of "sporogony."

Thus, the stages of Nuttallia found in the tick larva cannot be interpreted as stages of sexual development. None of the investigators of other

piroplasmids has reported direct observation of a sexual process. The latest data from Riek (1964) on anisogamous copulation in Babesia bigemina in the tick Boophilus microplus are also debatable. Riek himself was not sure of the correctness of his interpretation of the piroplasmid stages he observed.

Some investigators (Petrov, 1938, 1939; Tsaprun, 1957; Riek, 1964) feel that one proof of the presence of a sexual process in piroplasmids is the fact that a large number of the parasites die in the body of the tick. In their opinion the agamonts die, while the gametocytes which got into the tick with the blood of the vertebrate host are preserved and develop further. Nuttallia also dies in Hyalomma anatolicum, since the number of parasites gradually diminishes. However, if death does occur it is not observed in the initial period of Nuttallia's development in the ticks. The sharp decrease in number of parasites usually occurs in the larvae no earlier than 48 hours after they have fallen off the gerbil. During this time repeated mass divisions occur in the hemolymph. In the first hours after the ticks fall off there is an increase in the number of parasites in the larvae because of their reproduction. At this time many bi- and tetranuclear stages appear. Apparently different stages die at different times in the tick, this process being determined by the development of protective mechanisms in the body of the tick. Actually, in some larvae Nuttallia dies in the very first hours after they get into the host with the blood, while in others this process is delayed for some time. If, as some investigators feel, the agamonts died, they could not increase in numbers with time, as they do in the case of Nuttallia in H. anatolicum.

Thus, having no convincing data on the presence of a sexual process, it seems possible that in Nuttallia development takes place as in Piroplasma, only by the asexual route. In Piroplasma, the parasite is transmitted transovarially, while this does not occur in Nuttallia. Hence there are substantial differences in their developmental cycles. Piroplasma is characterized by very

intensive multiplication of the parasite which increases with time in the engorged female. At this point a pin-shaped stage is formed, which enables the parasite to penetrate the eggs of the tick. In Nuttalia there is no intensive accumulation of parasites in the larvae, since even small numbers of parasites in the nymph will be able to form the necessary number of infective stages in the salivary glands. Nuttallia lacks the pin-shaped stage, which apparently limits its ability to penetrate the eggs.

In my conclusion, I consider it my pleasant duty to thank Prof. Ye.M. Kheysin for examining my preparations.

SUMMARY

The development of Nuttallia tadzhikistanica in the larvae and nymphs of Hyalomma anatolicum was followed. In the first hours after the larvae had begun blood sucking on infected specimens of Meriones erythrourus, Nuttallia which had got into the intestine of the tick, leave erythrocytes and multiply by division into two or four individuals. Two or 4 hrs. later, mono-nuclear trophozoites occur in the haemocoel of the tick. Here their multiple division occurs. Multinucleated developmental stages of Nuttallia occur in the haemocoel during 24 hrs. after the larvae become detached from the host. The multinucleated stages break down into 32 trophozoites. Sometimes several tens of them arise. Multiple division may evidently be repeated several times. The multinucleated stages sometimes localize in the cytoplasm of haemocytes.

In course of 24 hrs. after beginning of blood sucking by the larvea in the intestine of the tick, two- or four-nucleated Nuttallia usually occur; these are division stages. No developmental stages of Nuttallia which might be recognized as gametes or their copulation forms, were seen.

After 48 hrs. the number of Nuttallia individuals in the larvae considerably diminishes, and after 96 - 120 hrs. only the amoeboid forms of

Nuttallia which do not reproduce till the larvae molt are present. These are resting stages. They also occur in starving nymphs. The subsequent multiplication of Nuttallia in the nymph starts only then when the latter have begun blood sucking. The parasite then enters the salivary gland; here its growth takes place accompanied by multiple division of nuclei. On the 4th day, a great number of rod-shaped or pear-shaped trophozoites are formed in the salivary glands; these are invasive forms for the vertebrate host. The nymph cannot infect Meriones before the 4th day of blood sucking. An emulsion of larvae infected with Nuttallia, prepared 24 - 26 hours after the conclusion of blood sucking and injected into Meriones, failed to cause infection.

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