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Densonucleosis of bloodsucking mosquitoes

L. P. Buchatsky

T. G. Shevchenko State University, Kiev, USSR

ABSTRACT: Ultrastructure, development and histopathology of a new densonucleosis virus of bloodsucking mosquitoes are described. Mosquito densonucleosis virus is infective to all 4 larval instars, pupae and adults of both sexes. Incubation periods were significantly shorter in older fourth-instar larvae than in third-instar larvae. Infected larvae die during the fourth larval instar or as pupae. The limited field tests conducted on this virus have shown its efficiency for infecting larvae of natural mosquito populations.

INTRODUCTION

Densonucleosis of bloodsucking mosquitoes is a virus disease of larvae, pupas and imagines of mosquitoes characterized by compression of the affected cell nuclei, and leading either to death of insects or to disturbance of vital functions in surviving individuals.

The disease known as 'densonucleosis' was first revealed by French researchers from the laboratory of Professor Vago in *Galleria mellonella* caterpillars (Meynadier et al. 1964). At present, densonucleosis has been described for 10 species of insects belonging to different orders. In mosquitoes, densonucleosis was detected first at the Kiev State University in IV age larvae of an *Aedes aegypti* laboratory culture (Lebedeva et al. 1972, 1973).

Later, a number of reports appeared on isolation of the virus from larvae of mosquitoes of the genera *Aedes* and *Culex* which inhabit natural water bodies of Podmoskovie (near Moscow, USSR) (Yarneech et al. 1975), where the frequency of its occurrence was high.

Similar viruses were also isolated in cultures of *Aedes pseudoscutellaris* transplantable cells (Gorsiglia et al. 1980) and in *Simulium vittatum* larvae (Federici 1976). The assumption of Federici (1977) that Clark & Chapman (1969) had discovered in bloodsucking larvae of mosquitoes the densonucleosis virus rather than cytoplasmic polyhedrosis virus is, evidently, erroneous, since the virus was not isolated by the authors and the described symptoms of the disease of larvae and of the changes in nuclei are not typical of densonucleosis of bloodsucking mosquitoes.

RESULTS AND DISCUSSION

Disease symptoms in mosquito larvae

According to our observations, symptoms of the disease in larvae of bloodsucking mosquitoes, as a rule, arise in Stage IV. Affected larvae lose their mobility, respond only slightly to stimulation, hang near the water surface, and their body is shortened and deformed. In certain larvae the body is semitransparent while in others it exhibits a whitish colouration. Mosquito pupas affected by densonucleosis virus (MDV) also experience reduced mobility and sink to the bottom of the water. They twitch convulsively in response to stimulation.

When studying print smears of MDV-affected larvae, the hypertrophied nuclei typical of this disease are observed in cells of the fat body. Azureozine stains the nuclei violet, most of which have a darker circular zone over the periphery. Hypertrophied nuclei are 2 to 3 times the size of normal ones.

The diameter of some nuclei from the affected larvae reaches almost 24 μm, whereas in healthy larvae they are not seen due to small size and a great variety of fatty-protein-glycogenic granules (Lebedeva et al. 1972, Gonchar et al. 1974, Lebedeva et al. 1975).

In our laboratory we have elaborated a vital method for MDV diagnosis. The method permits diagnosis of the disease in mosquitoes without their destruction (Kononko 1976) and observations of affected individuals.

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Cytopathology

A distinct picture of the nucleus structure in affected mosquito larvae may be obtained on microscopical sections when staining them with ferrous hematoxylin according to Heidenhain or Karachi (Kuznetsova & Zelenko 1975, Tsarichkova et al. 1976, Kuznetsova & Buchatsky 1978). The immunofluorescent method developed by us for MDV diagnosis makes it possible to reveal the viral antigen in print smears and on paraffin sections at an early stage of infection (Kononko et al. 1984). MDV reproduces in almost all tissues and organs. In mosquito larvae, pathological disturbances are observed in cells of fat body, hypoderm, Malpighian vessels, imaginal disks, nervous disks, cerebral and abdominal ganglia, endocrine glands, muscles and tracheal matrix. It affects enocytes and all parts of the intestine as well. In larvae, cells of fat body, muscles and hind intestine are affected most intensively; in pupae, imaginal muscles, intestine and fat body; in imagines, muscles and fat body (Tsarichkova et al. 1976, Karpenko et al. 1977, Kuznetsova & Buchatsky 1978, Kononko 1979).

When studying the effect of MDV on the mosquito organism we have established (Kuznetsova & Buchatsky 1978) that the highest number of nuclei with typical disturbances is observed in the fat body of Stage IV larvae. In this case, simultaneously with cells having dense-dark-stained nuclei, there are cells with nuclei filled with light-refracting inclusions from 0.3 to 1.9 μm. The formation of nuclear inclusions of such a type is also observed during reproduction of other parvoviruses (Dawe et al. 1961, Rabson et al. 1964).

Electron microscopic studies have revealed a great variety of virus particles of spherical shape, 20 nm in diameter, in affected cell nuclei of the fat body in mosquito larvae (Buchatsky et al. 1979, Buchatsky & Raikova 1979). In early stages of virus reproduction, chromatin margination appeared in affected cells; in later stages, a great number of virus particles were observed in the cytoplasm where they formed paracrystalline inclusions. By the end of virus reproduction, such a great quantity of MDV virions had accumulated in affected cells that these replaced almost the whole cell material. Microtubes of 20 nm in diameter as well as different destroyed organellas with mitochondria included were met among accumulations of virions.

Influence on metabolism

The results of studies on gas exchange in MDV-infected mosquito larvae (Korzov et al. 1986) have shown that upon infecting with a low virus dose noticeable changes occur at developmental Stage II – the respiration level increases. The intensity of tissue respiration was the highest in larvae before their shedding for Stage III, and in Stage III, the intensity of oxygen uptake by tissue homogenates of infected larvae exceeded that of control larvae by 25%. In Stage IV larvae, the respiratory activity of affected larvae was sharply inhibited and reached only 68% of the controls. A certain rise of the respiration level was observed after shedding in pupas which had survived infection at the larval stage. This rise may be due to the fact that tracheal epithelium and fat body of pupas are less sensitive to the virus than in larvae (Kononko 1979), as well as to elimination of the most affected individuals at the larval stage. More intensive oxygen uptake is observed in certain pupas than in control individuals. Studies of heat exchange have established that intensity of heat production in infected larvae is lowered, maximum lowering being observed at the moment of larval death.

While studying MDV effects on mosquito larvae, we have shown that the virus disease is accompanied by considerable changes in metabolism of infected cells, and in particular of certain enzymes. It is established that MDV in the first stages or reproduction increases the activity of cellular ATP; 2 to 3 d later, its activity decreases. Seven days after infection, ATP activity approaches the normal level, decreasing essentially only before death of the larvae (Buchatsky et al. 1982).

Manifestation in ontogenesis

Mosquito larvae are poikilothermal; growth and development are closely related to various environmental factors, in particular temperature. Therefore, manifestation of densonucleosis in mosquitoes depends strongly on ambient temperature, as well as on the virus dose and the age of larvae at the moment of infection.

In mosquito larvae kept at 24 to 26°C, the incubation period of the disease lasts 6 d. Temperature reduction results in elongation of the incubation period, temperature rise shortens it (Lebedinets et al. 1978).

Larvae of all ages are sensitive to MDV (Lebedinets & Korz 1974, Tsarichkova et al. 1976, Kuznetsova & Buchatsky 1978). However, upon infection of younger larvae, maximal manifestation of densonucleosis is observed in pre-imaginal developmental stages, in particular at Stage IV. In mosquitoes infected with MDV in Stages III and IV, manifestation of the infection shifts towards the pupal and imaginal stages. Dose dependence is similar: the lower the virus dose, the later is densonucleosis manifested in subsequent phases of ontogenesis, with maximum values in imagines (Tsarichkova & Kononko 1982).

Death rate of males from densonucleosis is 3 times as

high as in females. Significant physiological changes are observed in surviving females: certain females do not suck blood, and both fertility and quantity of eggs laid are reduced. Several of these disturbances increase at gonotrophic Cycle II (Tsarichkova & Kononko 1982).

In infected individuals, oogenesis is sharply disturbed: phase lagging, asynchronicity in the development of follicles, as well as their partial or complete destruction (Karpenko et al. 1977). In this case ovaries together with muscles, fat body and intestine are also affected. This results in sticking of eggs, degeneration of mature eggs, and abnormal development.

Regardless of virus infection periods, non-differentiated tissues and ectoderm derivatives are the primary sites of virus reproduction (Tsarichkova & Kononko 1982).

Host range

Light and electron microscopy were used to study the sensitivity of various animals to the mosquito densonucleosis virus. Larvae of various insect species (Lebedeva et al. 1975, Lebedinets & Zelenko 1975, Buchatsky & Lebedinets 1977, Lebedinets et al. 1978) and of certain hydrobionts, dew worms, invertebrates, as well as cell cultures (Buchatsky & Lebedinets 1977, Sutugina et al. 1983, Buchatsky et al. 1986) were employed for experiments.

The studies conducted have shown that MDV affects only closely allied species of mosquito larvae of the genera *Aedes, Culex* and *Culiseta* when introducing the inoculative material into water containing mosquito larvae. Under intralymphal infection the virus reproduces in larvae of the genus *Anopheles* as well.

When studying species specificity of MDV, it was established that various mosquito species exhibit different degrees of sensitivity to this pathogen. Apart from the main host larvae of natural populations, *Aedes caspius caspius, A. vexans,* and *Culex pipiens pipiens* exhibit high sensitivities to the virus. Larval death rates were 59.3, 58.2 and 44.4 % respectively.

Larvae of *Culex pipiens pipiens* were less sensitive to MDV (16 % death rate). As in the main host, water temperature exerts an essential effect on disease development in larvae of other mosquito species. Temperature reduction not only increases the duration of one or another developmental stage of the insect but is also accompanied by an inhibition of virus reproduction. For instance, in *Culiseta annulata* larvae the disease lasts 14 d at 25 to 26 °C, but 18 to 19 d at 16 to 26 °C. In other mosquito larvae, e.g. of *Aedes geniculatus*, the disease proceeds slower and lasts 25 d after infection. In this case, the highest death rate is observed in pupas (Lebedinets et al. 1978). Evidently, this phenomenon is associated with the longer preimaginal developmental stage in larvae of this mosquito species (Daschkina 1968).

Larvae of flies, chironomids, butterfly caterpillars (5 species), mature bees, cockroaches, crustaceans (species of *Daphnia, Cyclops*), worms, fishes, fowls (chicken embryo) as well as albino mice, albino rats, and transplantable cultures of mouse cells and chicken fibroblasts proved to be non-sensitive to MDV (Buchatsky & Lebedinets 1977, Buchatsky et al. 1986).

Successive passages, except for primary infection, were also performed on mice-suckers (4 passages through the animal's brain). However, in this case also the virus did not reproduce – the animals developed normally, and females produced healthy progeny (Lebedinets et al. 1976). Other densoviruses also affect a narrow range of hosts. Thus, the densonucleosis virus of *Galleria mellonella* does not affect even closely allied species of insects (Giran 1966).

Experiments on possible toxic effects of MDV on warm-blooded animals and humans has shown its complete harmlessness.

Biological properties of MDV

When purifying virus, both full and hollow spherical virus particles of 20 nm diameter are revealed in preparations of MDV within the sucrose density gradient. In addition, there are particles involved in fragments of cell membranes (Buchatsky et al. 1985). One Stage IV mosquito larva contained 2 µg virus, i.e. 0.06 % of its body weight. This is much less than in other entomopathogenic viruses, for instance, of iridoviruses (Williams & Smith 1957). The sedimentation coefficient of the virus is 98 S (Svedberg units); its floating density in cesium chloride, 1.39 g cm^{-3}. The molecular weight (M) of the virus determined by the equation $S_{20,w} = 1.114 \times 10^{-3} M^{0.715}$ amounts to 4.6×10^6 Dalton. The virus has single-stranded DNA and 3 proteins with molecular weights of 46, 56 and 80 kDa. The protein with a molecular weight of 46 kDa is the basic one (Buchatsky 1982).

MDV has no hemagglutinating properties relative to erythrocytes of chickens, geese, mice, rats, rams, guinea pigs and man (Group I blood). The positive reaction was observed only with rat erythrocytes in dilutions of 1:2 and 1:4. However, a weak reaction was also observed in the controls. MDV is not related serologically to the *Galleria mellonella* densonucleosis virus, parvoviruses of rats and pigs, enteritis of minks and Aleutian disease of minks (Buchatsky & Goral 1982, Buchatsky et al. 1985). It is resistant to organic solvents (ester, chloroform), and after incubation of virus at 60 °C

some infectivity is still retained. Death rate of larvae after infection with pre-heated virus was 23 %. MDV is also highly resistant to different concentrations of hydrogen ions – it is not inactivated completely even after exposure for 24 h to acid (pH 1) or alkaline (pH 12) media (Buchatsky & Sinitsina 1984, Buchatsky et al. 1985).

Ecology of the virus

Variations in maintenance conditions and storage duration revealed limited effects on MDV infectious properties and thus demonstrated high resistance of the virus. MDV infectivity is preserved in water of 21 to 23 °C for 8 mo (Lebedinets & Buchatsky 1981). Such temperatures are common during summer in water bodies inhabited by mosquito larvae. Death rates of mosquitoes infected with the virus stored for 1.5 mo in water decreased by 33 %, however complete inactivation of the virus did not occur even after 8 mo.

MDV infectivity is preserved sufficiently safely in 50 % glycerol on phosphate buffer at 4 °C. Under these conditions, MDV infectivity was reduced considerably after 18 mo storage; 3 yr later, the virus activity was twice as low. When storing MDV in desiccated dead bodies of mosquito larvae at room temperature (21 to 23 °C) virus infectivity is already reduced 1 mo later; however, even in such a state, the virus maintained activity for 14 mo. We have observed that the incubation period of virus stored for a long time increases (Lebedinets & Buchatsky 1981).

As mentioned, MDV is resistant to organic solvent and different concentrations of hydrogen ions (Buchatsky et al. 1985). However, MDV is sensitive to UV irradiation, even 1 min exposure results in considerable inactivation, and ca 50 % of the virus is inactivated within the first 10 min (Buchatsky & Sinitsina 1984). Nevertheless irradiation over 12 h did not cause complete loss of infectivity. This was, evidently, due to the protective effect of cell fragments in the virus-containing liquid (supernatant after centrifugation of infected mosquito-larvae homogenates at 3000 × *g* for 15 min).

MDV is completely inactivated after 1 h exposure to 65 °C. The method of Stairs & Milligan (1979) was used to establish graphically the temperature threshold below which the virus is not inactivated. The threshold lies at 32 °C. Below this temperature MDV is not inactivated over 1 h, but above 32 °C gradual loss of infectivity occurs (Buchatsky & Sinitsina 1984). Evidently, in open water bodies where water temperature may exceed 32 °C the virus will be destroyed by heat. Above 28 °C, mosquito larvae develop quickly and have time to pupate.

Infection of mosquitoes with thermo-inactivated virus induces the production of an interferon-like substance (or substances) partially protecting mosquito larvae from MDV. The ability of cells of invertebrates to produce such a substance was shown in studies of arbovirus reproduction in cell culture of mosquitoes (Enzmann 1973).

Field tests have revealed that MDV is able to survive for a long time in natural water bodies – its activity was manifested 1 yr after introduction into the water body. The pre-imaginal death of mosquitoes in 1 yr-old biosamples was on average 14.5 %. Fat body affections typical of densonucleosis were detected in smears of dead larvae and pupas.

Experimental studies have documented transovarial MDV transmission (Buchatsky et al. 1986). Horizontal virus transfer within a host population may occur by cannibalism as well – healthy larvae readily feed on low-motility infected individuals. Females with non-apparent infection play an important role in the spread of the virus.

CONCLUSIONS

Application of chemical insecticides against bloodsucking mosquitoes should be limited on account of environmental pollution and development of resistant populations (WHO 1975, 1983). In spite of achievements in developing viral insecticides for leaf beetle control, preparations for inhibition of insects of medical and veterinary significance are presently not developed. One of the reasons for this is the low pathogenicity of baculoviruses isolated from mosquitoes and black flies.

In order to regulate the abundance of bloodsucking mosquitoes we have developed the MDV-based viral insecticide Viroden. Limited field tests of this preparation have shown its efficiency for larvae of natural mosquito populations. The death rate of individuals at pre-imaginal stages is on average 77.0 %; prior to the first gonotrophic cycle of females only 15.4 % of mosquitoes survived (Buchatsky et al. 1987a, b).

During storage at 4 °C, Viroden preserves 73.1 % of its initial activity. It is resistant to changes in concentrations of hydrogen ions within the range of 4 to 11, and endures 3 d incubation at 50 °C. Comprehensive tests have shown its safety for warm-blooded animals and humans, as well as for non-affected hydrobionts.

The study of the diseases of aquatic organisms opens up new vistas for the application of their agents. The detection of a virus, new for science, which affects mosquito larvae necessitates further profound studies on the virus itself, the development of methods for diagnosis under field conditions, and means for regulating the abundance of mosquitoes.

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