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Nanoparticle–Virus Complex Shows Enhanced Immunological Effect Against Baculovirus

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Insects protect themselves from majority of infections by a non-specific innate immune system (present in both vertebrates and invertebrates). *Bombyx mori* nuclear polyhedrosis virus (BmNPV), a baculovirus, causing the deadly grasserie disease is a scourge to silkworm industry and we report here the first success in combating this disease with the help of a nanosilica–virus complex. Hydrophobic aluminium silicate nanoparticles were mixed with live BmNPV *in vitro*. This mixture was injected into one day old 5th instar silkworm larvae (into the hemocoel at the third abdominal spiracle) before challenging the larvae with live BmNPV via a second injection. This led to substantial enhancement of longevity in the diseased silkworm larvae and $35 \pm 5.3\%$ larvae completed their life-cycle (i.e., formed normal pupae and enclosed as moth). On the other hand, 100% larvae infected with BmNPV alone died within 36 hours. The larvae treated with nanoparticles before infection had a longer lifespan but all of them eventually succumbed, not a single larva metamorphosed to adult stage. Results suggest two pathways of host protective response—one mediated by nanoparticle-alone and the second, more important, via non-specific innate immunological mechanism. AFM and confocal studies show that nanoparticles alter 3-D molecular structure of the virus envelope. Possibly this exhibits novel potent epitope(s) which stimulate(s) anti-viral machinery in infected silkworm larvae. SDS-PAGE results suggest that 39 KDa viral protein is the major target of the nanoparticles.

Keywords: BmNPV, Nuclear Polyhedrosis Virus, Nanoparticle, *Bombyx mori*, Grasserie Disease, Insect Immunity.

RESEARCH ARTICLE

1. INTRODUCTION

Bombyx mori nuclear polyhedrosis virus (BmNPV) is a circular double stranded DNA virus belonging to the family baculoviridae. The pathogenicity of baculovirus is confined to the invertebrates, especially in lepidopteran insects. In nature, infection occurs when occluded bodies (OBs) are ingested by silkworm larvae via oral route. OBs in the milieu of alkaline gut juice (pH 9.5–11.5) release polyhedra derived virion (PDV). PDVs enter the cells fusing directly with the plasma membrane of the microvilli.[1,2] Neutralization of BmNPV infection using anti-PDV antibodies has been demonstrated implying that the process of attachment and entry might be receptor mediated.[3] BmNPV causes primary infection in the midgut columnar epithelial cells followed by a systemic lethal spread in a number of silkworm organ tissues where progeny OBs are formed. OBs can withstand adverse environments and reestablish infection cycle when OBs from dead host contaminate silkworm feed.[3] Penetration of the peritrophic membrane by PDV (after mid gut infection) is known to be enhanced by a proteinaceous viral factor.[4] In cell culture, 24 hours post infection, a large number of budded virions (BVs) leave the host nuclei via budding of the nuclear membrane. BVs are responsible for the spread of infection in different tissues of silkworm.[5] BVs are released in the hemocoel and the generalized infection process ensues. At the later stages of infection, BV production terminates. In stead, PDVs are produced, occluded in the OBs and released from dead larvae to the environment. Until now, no specific antiviral antidote, therapeutic or prophylactic, exists. In recent years, depending on the tunability of physico-chemical properties (such as surface chemistry, size, solubility etc.), nanoparticles have been used in numerous biomedical applications from targeted drug/antibody delivery to imaging.[6,7] Here we show that

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Table I. Silkworm larvae survival percentage following treatment with AL60102–BmNPV complex.

Treatment lots	Challenged with live BmNPV	% of survival (Eclosion as moth)
50% Ethanol injected	–	100
50% Ethanol injected	+	0
Virus injected	+	0
50% Ethanol pre-incubated with virus and injected	+	0
AL60102 injected	+	0
Virus–AL60102 complex injected	+	35 ± 5.3

nanoparticle–virus complex can protect silkworm against deadly BmNPV disease.

2. EXPERIMENTAL DETAILS

2.1. Polyhedra Isolation

Prolegs of infected (36 hours post infection) nistari strain of silkworm larvae (*B. mori)* were punctured and hemolymph was collected in presence of phenyl-thio-urea (3 mM) to avoid melanization.[8] OBs were pelleted by centrifugation at 20000× g for 1 hour and washed three times with 5 volumes of distilled water until the supernatant was devoid of any turbidity or floating lipids. OBs were dissolved in alkaline dissolution buffer for 24 hrs at 8 °C for isolating polyhedra derived virions (PDV).[2] Pellets were re-suspended in 0.5% sodium dodecyl sulphate (1 ml per insect equivalent) by homogenization to disrupt the polyhedral aggregates. The polyhedral suspension was again pelleted at 20000× g for 1 hour and re-suspended in TE (0.5 ml/insect equivalent). For long-term storage, polyhedra was freeze-dried and stored at 4 °C. The OB concentration was determined by spectrophotometry at 550 nm. 1.0 $O.D._{550}$ is equal to 0.35 μg/ml. OB concentration where 1 μg equals to about 2×10^6 polyhedra or by counting OBs in a Neubauer counting chamber using a Zeiss Axiolab phase contrast microscope.

2.2. Preparation of Nanoparticle Solution

Aluminium silicate nanoparticles (AL60102) were gifts from Prof. Christian Ulrichs, Humboldt University, Berlin, Germany. The chemical formula of AL60102 is

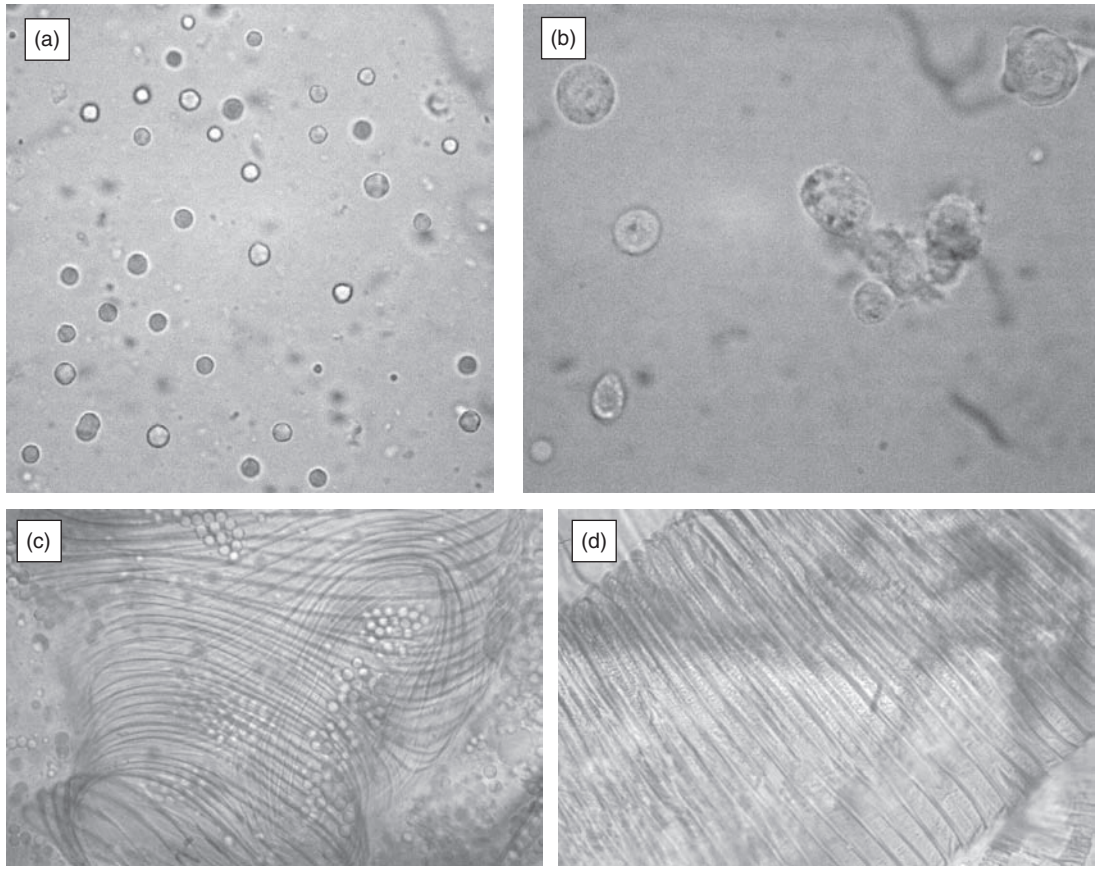

Fig. 1. Phase contrast photomicrograph of (a) BmNPV infected hemolymph containing large number of polyhedra bodies at 30 hours post infection (h.p.i.), (b) BmNPV–AL60102 complex treated infected hemolymph with very few polyhedra bodies and healthy hemocytes at 30 h.p.i. (c) BmNPV infected trachea at 30 hours post infection with large number of polyhedra particles and (d) BmNPV–AL60102 complex treated infected trachea at 30 h.p.i with very few polyhedra bodies.

$Al_2O_3(SiO_2)_{1.3–2·2.5}$, $3(H_2O)$ and this compound is amorphous in structure. It was produced by top–down approach from hydrothermal alteration products of feldspars. These biocompatible aluminium silicate nanoparticles are hydrophobic in nature and have average diameter of 6 nm. 10 mg of this dry AL60102 powder was dissolved in 1.5 ml ethanol so that the final concentration is 6.66 μg/μl (stock solution). In earlier experiments, it was found that the aforesaid concentration of AL60102 was physiologically compatible and can be used safely on silkworm larvae.

2.3. Infection of *Bombyx mori* Larvae

2.3.1. BmNPV Inoculation

One day old 5th instar *B. mori* larvae were infected by polyhedra according to Keddie et al.[5] with minor modifications in the protocol. Instead of oral route, the larvae were inoculated by injecting 5 μl polyhedra in the hemocoel at the third abdominal spiracle.[9] Polyhedra stock solution was diluted to a final concentration of 100 polyhedra/μl. Larvae were reared on fresh mulberry leaves for the entire experimental period.

2.3.2. AL60102-BmNPV Complex Inoculation

Polyhedra stock solution of BmNPV was mixed with ethanolic suspension of AL60102 (1:1) so that average concentration of polyhedra remains 100 polyhedra/μl and kept at room temperature for three hours with occasional shaking for polyhedra–AL60102 complex formation, if any.

2.4. Microscopy

Hemolymph and trachea of silkworm larvae were observed through the phase contrast microscope (Axiolab, Zeiss,

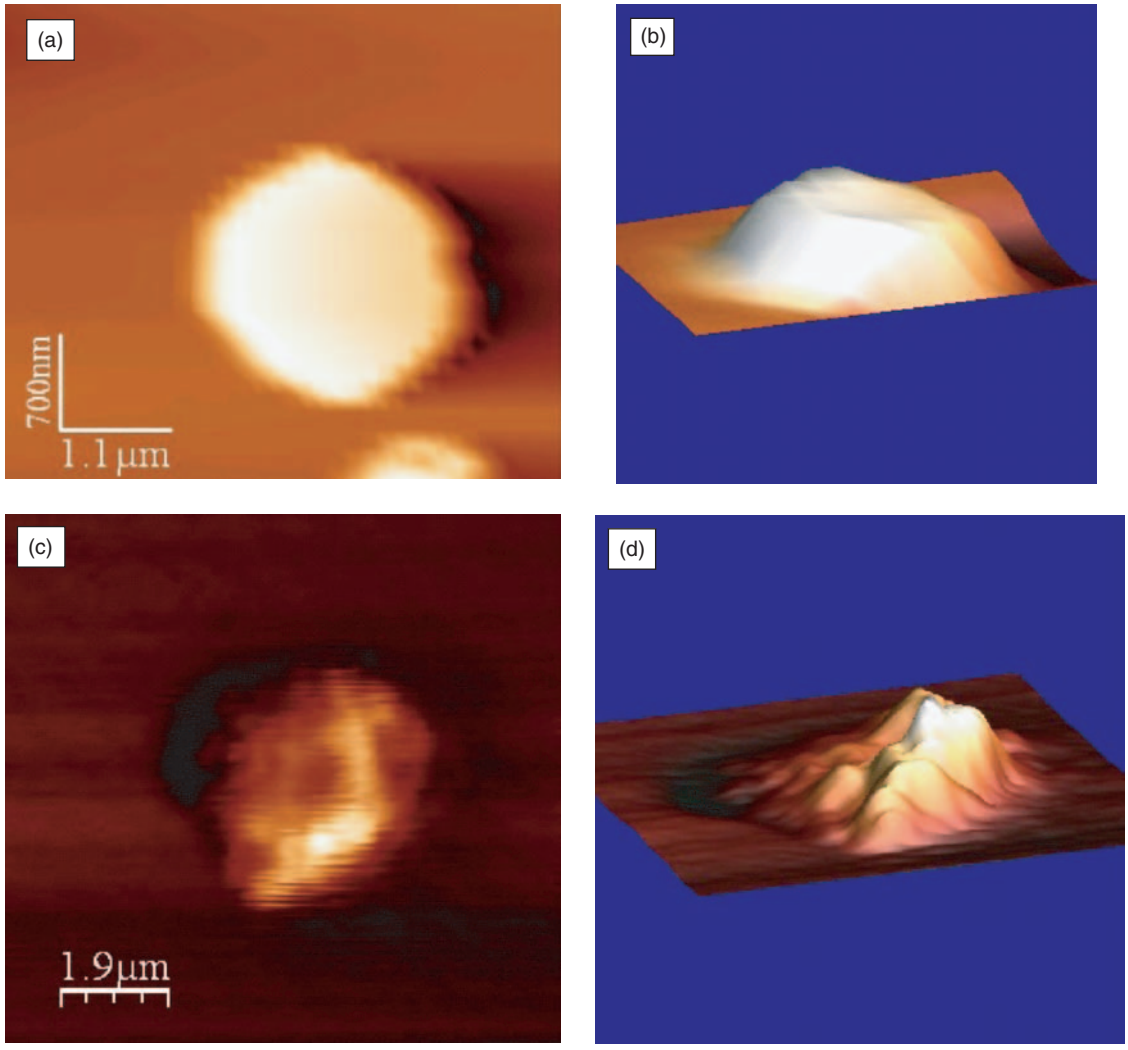

Fig. 2. Atomic force microscopic studies of (a) viral (polyhedra) particles and its (b) 3D image showing smooth hexagonal morphological structure. Confocal microscopic image of (c) AL60102 treated virus and its (d) 3D structure showing damaged morphology.

RESEARCH ARTICLE

Germany). Atomic force microscopic (AFM) and confocal microscopic (CM) studies were carried out using Nanonics Multiview 1000 microscope equipped with NWS software, with the samples drop-cast on clean glass slides. AFM studies were performed using Nanonics supersensor AFM probe glass tip (20 nm diameter), in tapping mode. Optical fiber of 100 nm diameter and the second harmonic, of 532 nm wavelength, of an Nd: YAG laser were used for confocal microscopy. The confocal microscopic data was collected in transmission (absorption) mode using the dual microscope. In both AFM and CM, the sample was scanned along x- and y-axes, while the z-movement was effected by a specially designed Z-Modulator.

2.5. AL60102 Polyhedra Binding Studies and SDS-PAGE

1 μg polyhedra (isolated as stated above) was diluted in 1.5 ml water and divided into two equal parts. To one aliquot, AL60102 (0.5 mg per 1.5 ml) was mixed and incubated at room temperature for three hours with occasional shaking. Equal volume of 2× protein extraction buffer (0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-β-mercaptoethanol and 0.004% bomophenol blue) was added to both the aliquots (with and without nanoparticles), boiled for 2–3 minutes, and centrifuged at 10000 g for 10 minutes. The supernatant were analyzed with 10% SDS-PAGE using standard molecular weight marker (M/S Bangalore Genei Pvt. Ltd., Bangalore, India).[10] The protein bands were stained with coommassie brilliant blue-R250 (Merck, Germany).

3. RESULTS AND DISCUSSION

400 silkworm larvae were divided into four equal lots- (a) 50% ethanol (control) injected, (b) polyhedra injected, (c) AL60102 injected, (d) polyhedra–AL60102 complex injected. Except in (a) lot, all the other three treatment lots were challenged three hours later with live BmNPV (Table I). Two other important internal controls were

(i) 50% ethanol injected larvae injected with live virus ($n = 125$) and

(ii) live virus incubated with 50% ethanol for three hours at room temperature and then challenged with virus ($n = 40$).

In both these cases, 100% larvae died within 24–30 hours and no cocoon formation took place. Table I shows 35 ± 5.3% larvae completed their lifecycle when treated with polyhedra–AL60102 complex.

In the hemolymph of BmNPV infected larvae, large numbers of polyhedra were found approximately 30 hours post infection (Fig. 1(a)) and 100% larvae died within 36 hours. In another experiment AL60102 was injected first into the healthy silkworm larvae and after three hours they were challenged with polyhedra. In this case, 100% larvae nevertheless died within 48 hours post infection (i.e., slight increase in larval lifespan). Significant reduction of polyhedra bodies in hemolymph were observed in the larvae treated with BmNPV polyhedra–AL60102 complex. 60 ± 3.9% larvae remained alive even after 72 hours and well formed hemocytes were observed with few polyhedra bodies present in the hemolymph (Fig. 1(b)). A similar result was obtained in the larval trachea associated with midgut basal membrane (Figs. 1(c and d)). This indicates substantial lowering of viral load. Decrease in viral load is inversely related to the survival rate of larvae treated with BmNPV–AL60102 complex.

Figures 2(a and b) show that pure polyhedra have a hexagonal shape with smooth surface (AFM studies) while the AL60102–BmNPV complex revealed damaged topological structure of BmNPV (Figs. 2(c and d); confocal microscopy studies). The enhancement of life span observed in larvae treated with AL60102–virus complex might be due to the damage of polyhedra 3-D structure. Binding of AL60102 to viral protein(s) could unravel potent novel epitope(s). This, in turn, might enhance innate immunological response in silkworm larvae.

In order to find out putative viral proteinaceous target of AL60102, *in vitro* binding studies followed by SDS-PAGE were undertaken. SDS-PAGE analyses show considerable reduction of a 35 kDa protein (lane 3; Fig. 3) when AL60102 was mixed with polyhedra bodies compared to pure polyhedra extracts (lane 2; Fig. 3). This indicates that AL60102 binds directly with 35 kDa viral protein affecting

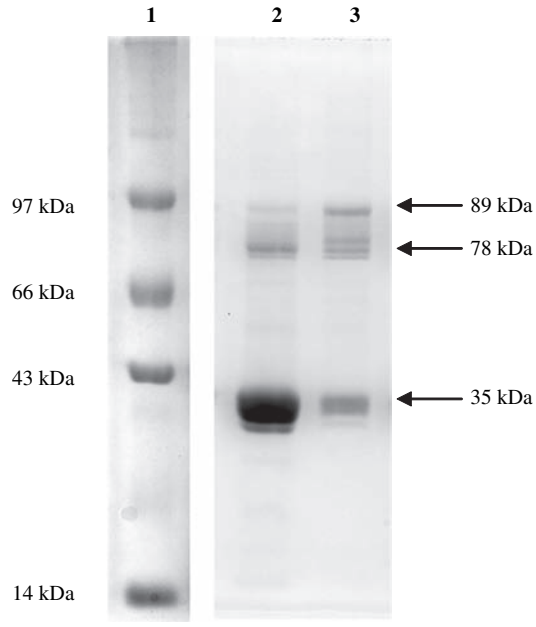

Fig. 3. Viral particle (BmNPV polyhedra) derived proteins with or without AL60102 treatment *in vitro* and analyzed with 10% SDS PAGE. Lane 1: Standard molecular weight marker. Lane-2: proteins extracted from pure BmNPV polyhedra. Lane 3: proteins extracted from BmNPV polyhedra incubated with AL60102.

RESEARCH ARTICLE

its stability. This process might expose novel host protective epitope(s) and triggers insect innate immune response. However proteins beyond 78 kDa show relatively minor changes compared to 35 kDa. Therefore enhanced lifespan of the silkworm larvae by AL60102–polyhedra complex injection could be correlated to 35 kDa viral protein.

4. CONCLUSIONS

For the first time the deadly grasserie disease caused by BmNPV, has been cured, albeit by only $35 \pm 5.3\%$. It is clear that AL60102 alone can not ensure this survival but a combination of AL60102 and polyhedral complex does so. AFM and confocal microscopic results show that AL60102 binds to the viral protein and alters the topological structure. This might exhibit novel potent epitope(s) which could stimulate the innate immune reaction. Viral protein analysis showed that the major target for AL60102 is a 35 kDa protein of BmNPV.

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RESEARCH ARTICLE