Production and Characterization of Monoclonal Antibodies against Occluded Virion of Nuclear Polyhedrosis Virus from *Bombyx mori* (Lepidoptera: Bombycidae)

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To identify structural protein genes of the silkworm, *Bombyx mori* nuclear polyhedrosis virus (BmNPV), monoclonal antibodies (MAbs) were raised against the occluded virion (OV) and characterized. Out of 35 MAbs obtained, 10 belonged to immunoglobulin G (IgG) class. Two IgG class MAbs reacted with polyhedra. Three of the other 8 IgG class MAbs, designated 7D2, 8D5 and EHA, reacted with 2 to 9 polypeptides of BmNPV-OV. The periodate oxidation of OV suggested that 2 MAbs of 7C8 and CD6 are specific for carbohydrates. The electronmicroscopic observation indicated that 7D2 and 7C8 are reactive to antigenic determinants, present on the nucleocapsid and envelope, respectively. These results suggest that 7D2 can be a good tool as a probe to screen genes for nucleocapsid structural proteins from a BmNPV genomic library.

Key words: *Bombyx mori*, nuclear polyhedrosis virus, monoclonal antibodies

INTRODUCTION

Nuclear polyhedrosis virus (NPV) has two virion phenotypes, the occluded virus (OV) and budded virus (BV), in its life cycle (VAUGHN and DOUGHERTY, 1985). OV is occluded in polyhedral inclusion bodies, polyhedra, which are formed in a host cell nucleus. BV is released by budding, and enveloped with the host cell membrane. These two phenotypes are quite different from each other in morphology (SUMMERS and VOLKMAN, 1976), infectivity (KAWARABATA and ARATAKE, 1978) and envelope protein components (VOLKMAN, 1983).

Besides core proteins, the OV particle has more than 20 types of membrane proteins, which are considered to be coded by virus genomes (MATTHEWS, 1982). The nucleocapsid of NPV is a complex structure of viral DNA and basic core proteins (MAEDA, 1987).

NPV genomes are supercoiled and double stranded circular DNA and range from

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90 to 160 kilo bases in size (MATTHEWS, 1982). Virus genomes can code about 100 different proteins (MAEDA, 1987). Some protein genes were identified by cDNA cloning or Northern blot analysis. However, the genes, whose functions or locations are understood, are presently only a few. They are, for example, the genes for a polyhedrin, a DNA polymerase, a capsid protein, a basic DNA binding protein and a BV envelope glycoprotein from *Autographa californica* NPV (BLISSARD and ROHRMAN, 1990). In the case of *Bombyx mori* NPV (BmNPV), no protein genes have been identified except for polyhedrin (MAEDA et al., 1985).

To understand the genomic organization and functions of BmNPV, it is important to identify genes for different proteins. For such a purpose, monoclonal antibody (MAb) can be one of the powerful tools as a probe to select a specific gene in a genomic library.

As the first step to identify a structural protein gene of BmNPV, MAbs were raised against BmNPV-OV. In this report, we describe the production and characterization of these MAbs.

MATERIALS AND METHODS

Virus, host insect. Larvae of *B. mori* (J134×C135) were reared on an artificial diet (Nihonnosanko) at 25°C, and used as host insects. The plaque-purified BmNPV P4E (INOUE et al., 1990) was multiplied in 5th inster larvae. The hemolymph of diseased larvae was collected into polypropylene tubes at 4°C by cutting off the legs and then centrifuged at 3,500 rpm for 10 min. The pellet was washed with distilled water several times, filtered twice through a layer of nylon mesh to remove cell debris and subjected to Parcoll (Pharmacia) gradient centrifugation at 15,000 rpm for 30 min with Beckman 70Ti rotor. The band of polyhedra was collected, and washed several times with distilled water and kept at -80°C at a concentration of 1×10^9 polyhedra/ml. The OVs were purified from the polyhedra by dissolving in an alkaline solution (0.16 M Na_2CO_3 /0.05 M NaCl, pH 10.0) followed by centrifugation on a 20–60% sucrose density gradient as described previously (TANIAI and INOUE, 1988). Finally, OVs were suspended in phosphate buffered saline (PBS, pH 7.4) or CARLSON's solution (adjusted to pH 8.4 by KOH) (CARLSON, 1946) and kept at 4°C. Thus purified OV samples were used within a week.

Production of MAbs. MAbs against the OV were prepared by the usual cell fusion technique, as described previously (NISHIMORI et al., 1987). Briefly, 100 μl of OV suspension (300 μg of protein in 1 ml of PBS, measured according to LOWRY et al. (1951)) were intravenously injected 4 times into adult female BALB/c mice at weekly intervals. Two weeks after the last injection, the mice were boosted with the same amount of OV suspension. Three days later, the spleen cells were fused with mouse myeloma cells (P3-X63-Ag8-U1) in polyethyleneglycol 4000. The hybridoma cells were incubated at 37°C in 5% CO_2 and selected in HAT medium. Each hybridoma was cloned twice or 3 times by a limiting dilution using peritoneal macrophages as feeder cells. The immunoglobulin class and subclass of each MAb were determined by the indirect enzyme-linked immunosorbent assay (ELISA) using a sub-isotyping kit (American Qualex). Each hybridoma, which produces IgG class MAbs, was injected into peritoneal cavities of the BALB/c mice, pretreated with 2,6,10,14-tetramethylpentadecane. About 2 weeks later, the ascitic fluids were harvested and MAbs purified by an Affi-

Gel protein A MAPS II Kit (Bio-Rad).

ELISA. The microtiterplates for ELISA (Nunc-Immuno Plate I) were coated with the OVs at 4°C overnight and washed with 0.02% Tween 20 in PBS (PBST) 3 times. An aliquot of first antibody (50 μ l) was added and incubated for 60 min at room temperature. The plates were then washed with PBST 3 times and 100 μ l of substrate solution were added. After the plates were incubated at 25°C for 30 min, the absorbance of the sample in each well was measured by EIA-Reader (Bio-Rad). For the screening of MAbs, the culture fluid of hybridoma was used as the first antibody, and anti-mouse immunoglobulin goat serum conjugated with alkaline phosphatase (AP, Sigma) (NISHIMORI et al., 1987) as a second antibody. For the other experiments, purified IgGs of MAbs were used as the first antibody and horse radish peroxidase (HRP)-conjugated anti-mouse IgG (Bio-Rad) as a second antibody. As the substrate solution for AP, 1 mg/ml solution of 4-nitrophenylphosphate in 10% diethanolamine buffer (pH 9.8) was used and for HRP, 1 mM 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid in McIlvaine buffer (0.2 M Na₂HPO₄, 0.1 M citric acid, pH 4.0) and 0.3% H₂O₂ (v/v) were employed.

Western blot analysis. The OVs were suspended in a treatment buffer (2.5% sodium dodecyl sulfate (SDS), 8 M urea, 5% 2-mercaptoethanol, 0.005% bromophenol blue and 0.005% pyronin Y) and incubated at 4°C overnight. OV proteins were separated by electrophoresis in 15% SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) filters (Millipore). The filters were blocked for 2 h with 3% geratin and washed with PBST. The filter strips were incubated with 1 ml of MAbs (1 μ g/ml) overnight. After washing 3 times with PBST, the strips were incubated with 1:500 diluted solution of the second antibody (HRP-conjugated anti-mouse IgG goat IgG, Bio-Rad). MAbs on the filters were detected by staining with 0.05% 4-chloro-1-nophtol and 0.3% H₂O₂ (v/v).

Periodate oxidation. The method of periodate oxidation, described by WOODWARD et al. (1985), was modified slightly. Each well of the microtiterplates was coated with 50 μ l of OV suspension in PBS (3 μ g of protein/ml) by overnight incubation at 4°C, rinsed with 50 mM sodium acetate buffer (pH 4.5) and then exposed to different concentrations of periodate (0, 5, 10 and 15 mM) in sodium acetate buffer for 1 h at room temperature in darkness. After rinsing with sodium acetate buffer, the wells were washed 5 times with PBST. The microtiterplates were subjected to ELISA as described above.

Electronmicroscopic observation. An aliquot of virus suspension was dropped onto the carbon grid mesh. After 2 min, the solution was removed with by a piece of filter paper and a drop of PBS containing 2% bovine serum albumin (PBS-BSA) was laid on the mesh. Fifteen min later, the solution was again removed as above and an aliquot of the culture supernatant of hybridoma cells was dropped onto the carbon grid mesh and kept at 35°C for 20 min. The grid mesh was then washed 2 to 3 times with PBS-BSA. The gold-coloid labelled anti mouse IgG goat IgG solution, which was diluted with PBS containing 0.1% BSA, was added. The sample was kept for 10 min at room temperature. The grid mesh was washed several times with PBS-BSA and stained with 1% phosphotungstic acid for 5 min. This sample was observed by a transmission electronmicroscope (JEM 100C). As a control for the antibody, normal mouse IgG was used.

RESULTS

Production of MAbs

Out of 37 positive hybridoma cell lines, which were obtained from 5 fusion experiments, 35 stably proliferated cell lines were established. Of which, 10 cell lines belong to IgG class and the other to IgM class. Two MAbs of IgG class reacted to polyhedral

Table 1. Immunoglobulin classes and titers of MAbs

MAb	Class ^a	Titer ^b
2AC	IgG1	0.639
7C8	IgG2b	1.607
7D2	IgG2a	0.674
7GB	IgG1	0.517
8D5	IgG1	0.325
CD6	IgG2b	0.875
EHA	IgG2a	0.486
FE8	IgG2a	0.177

^a Immunoglobulin classes were determined by ELISA using a sub-isotyping kit (American Qualex).

^b Absorbance value at 414 nm of ELISA. Each well of microtiter plates was coated with 50 μ l of OV protein (3 μ g/ml) and then 50 μ l of MAb (40 ng/ml) was added. After the reaction, 500 fold diluted HRP-conjugated second antibody was added.

Fig. 1. Western blot analysis of OV proteins with MAbs. OV proteins were separated by 15% SDS-PAGE and transferred to PVDF filter as described in Materials and Methods. The filter was screened with each MAb.

protein (data not shown). So other 8 IgG MABs were assumed to be reactive to BmNPV-OV. Table 1 shows the classification and titers of the 8 MABs. The immunoglobulin subclasses were determined to be IgG 1 for the MABs of 2AC, 7GB and 8D5, IgG, 2a for 7D2, EHA and FE8 and IgG 2b for 7C8 and CD6.

Specificity of MABs

Figure 1 shows an immunoblot analysis of OV structural proteins by different MABs. Out of 8 IgG class MABs, 3 gave clear bands. Other MABs did not react with OV proteins under the conditions of the combination of SDS-PAGE and Western blot analysis. A MAB, 7D2 gave a strong band and a faint band. The apparent molecular weight of the strong band was 68 kDa. EHA reacted with 2 peptides of molecular weight 68 kDa and bigger than 68 kDa, but the intensity of the bands was very weak, compared with others. Another MAB, 8D5, reacted with at least 6 peptides with molecular weights of more than 54 kDa.

Effects of periodate oxidation of OVs on the reaction of MABs

All IgG class MABs were tested to investigate the effects of periodate oxidation of the OVs. Two MABs of 7C8 and CD6 exhibited a dramatic decrease of reaction with an increase of the periodate concentration (Fig. 2). On the other hand, other MABs were not affected so much even by the higher concentration of periodate.

Electronmicroscopic observation of MAB binding sites

Figure 3 shows the location of antigenic determinants of BmNPV-OV. One MAB, 7D2, bound to a surface of nucleocapsid, which had been released from the envelope (Fig. 3 left). On the other hand, 7C8 recognized the epitopes around the envelope of OV (Fig. 3 right).

Fig. 2. Effects of periodate oxidation of OV on the reaction of MABs. Different concentrations of periodate (0, 5, 10 and 15 mM) were tested. The reaction of MAB was measured by ELISA (For the details, see Materials and Methods).

Fig. 3. Electronmicroscopic observation for binding sites of MABs.

DISCUSSION

In the present study, we obtained 35 hybridoma cell lines that produce MABs. These MABs were subjected to SDS-PAGE or native-PAGE for the Western blot analysis. Seven MABs reacted with virion structural proteins, however, 13 MABs did not give any band (data, not shown). Fifteen MABs reacted with polyhedrin, indicating that a virus solution, which was used for immunization, was contaminated by polyhedral proteins.

To clarify the reasons why some of the MABs did not give any band in Western blot analysis, we characterized antigenic determinants of IgG class MABs reactive to the virion structural proteins. Treatments of virion by Triton X-100, urea and 2-mercaptoethanol did not have any effect on the reaction of 3 MABs, 7D2, 8D5 and EHA, which produced gave bands in SDS-PAGE by Western blot analysis. However, 2 MABs, 2AC and 7GB, were affected strongly by Triton X-100 treatment in native-PAGE. In addition, 3 MABs, 7C8, CD6 and FE8, which gave no band, were affected by Triton X-100 and SDS. Particularly, the reaction of 7C8 and CD6 as completely inhibited by urea and 2-mercaptoethanol (data, not shown). These results suggest that MABs, do not give any band by Western blot analysis after SDS-PAGE but may be reactive to carbohydrates, or recognize the three-dimensional structure of virions, which are easily removed or denatured by electrophoresis.

Previously, WOODWARD et al. (1985) reported a useful method to detect MABs, specific for carbohydrate epitopes using periodate oxidation. We applied the same method to our study. The results indicate that MABs of 7C8 and CD6 were reactive to carbohydrates because of their high sensitivity to a very low periodate concentration (5 mM). And other MABs were not affected significantly by periodate oxidation, suggesting no involvement of carbohydrates in their epitopes (Fig. 2).

Electronmicroscopic observation clearly demonstrated different binding sites of 2 MABs (Fig. 3). These results, combined with the data from Western blot analysis and periodate oxidation indicate that 7D2 reacts with 60 and 68 kDa structural proteins on the nucleocapsid.

Recently, MAbs were successfully used as probes to identify immunoreactive clones from gt11 expression genomic libraries of the multicapsid NPV from *Orgyia pseudotsugata* (QUANT-RUSSEL et al., 1987; BLISSARD and ROHRMANN, 1989). A MAb, 7D2, can be used for the isolation of 60 and 68 kDa nucleocapsid structural protein genes from a genomic library. Moreover, MAbs of 2AC, 7GB, 8D5 and FE8 should be also useful to clone other protein genes. Along this line, a molecular cloning of protein genes of BmNPV-OV is presently in progress in our laboratory.

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