Institute for Avian Diseases, Veterinary Faculty, Ludwig Maximilian University, Munich, Veterinärstraße 3, 85764 Oberschleißheim, Germany

Development of an Experimental ELISA for the Detection of Antigens and Antibodies to the Virus of Pacheco Disease using Monoclonal Antibodies

NADIA ABDALA

Address of author: Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92717-4550, USA

With 5 figures

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Summary

Monoclonal antibodies (Mabs) against one of the strains of Pacheco Parrot's Disease (PPD) were produced and characterized. An enzyme-linked immunosorbent assay (ELISA) for the rapid detection of antigen and antibody to PPD was developed. The antigen ELISA could detect up to 117 ng/ml of purified virus material. To overcome the requirement for large amounts of purified viral antigen, the microtitre trays were initially coated with Mabs prepared against purified PPD virus (PPDV). A relatively crude viral preparation could then be used to coat the trays. Using one more step of incubation between the viral preparation and a test sera, the same ELISA protocol could be used for screening antibody against PPDV. The blocking ELISA with a detection range of 625 ng/ml of purified antibody was twice as sensitive as the serum neutralization test (SN), which detected 1.35 μg/ml.

Introduction

To date, a large variety of avian herpesviruses (HV) have been isolated from many avian species (BURTSCHER, 1965; TANTAWI et al., 1979; KALETA et al., 1980; DHARMA and SUDANA, 1982; MUELLER, 1990). Since about 8,000 species of birds have been described up to now (WOLTERS, 1982), it is expected that more different types of HV will be isolated in the future.

Pacheco parrots disease (PPD) is a highly contagious HV infection in parrots. PPD was diagnosed for the first time in Brazil (PACHECO and BIER, 1930) and has occurred since in sporadic outbreaks worldwide (WINTEROLL, 1977; RANDALL et al., 1979; GOMEZ-VILLAMANDOS, 1991). Natural migration, international trade and exhibitions contribute to the spread of HVs. To date, there are three HVs specific to Psittacine birds which can be differentiated by neutralization tests (KALETA, 1990). Apparently all of these viruses can infect any species of the psittacine birds (SIMPSON and HANLEY, 1977; EHRSAM et al., 1978; KRAUTWALD et al., 1988). Although latent infections of parrots with herpesviruses have not been demonstrated yet, it is probable that birds which have recovered from an acute disease may become latently infected,
as in the case of other diseases caused by herpesviruses. Stressful situations such as exposure to sudden cold weather, or moving birds to an unfamiliar location are predisposing factors for the appearance of symptoms (CHEVILLE, 1978). For this reason, disease outbreaks are commonly observed in recently imported birds (HITCHNER and HIRAI, 1978; MILLER et al., 1979; KALETA et al. 1980). Sudden, deadly and unrestrained outbreaks of PPD may occur after the introduction of a new presumably infected psittacine bird (MARTIN et al., 1979; MILLER et al., 1979; GOMEZ-VILLAMANDOS, 1991), but sometimes in the resident birds of a flock with no history of recent introduction of new birds (SIMPSON and HANLEY, 1977).

Although common clinical signs are anorexia, depression and yellowish diarrhoea, sometimes respiratory signs are also present (MUTLU et al., 1991; KORBEL, 1992). Systemic disease, and clinical manifestations such as gastrointestinal or neurological symptoms may be variously expressed according to the severity with which a system is affected. The acute form of the disease may cause sudden death without clinical or pathological signs of disease (EHRSAM et al., 1978; KRAUTWALD et al., 1988; GOMEZ-VILLAMANDOS, 1991). In many cases concurrent infections with various agents obscure the primary effects of HVs on the respective hosts.

Since the potential sources of HV disease in a susceptible population are inapparent carriers or recovered birds that shed the virus in their droppings (BURGESS and YUILL, 1981; VINDEVOGEL et al., 1982; SCHUH and YUILL, 1985), all newly introduced psittacine birds should be isolated and tested for the presence of herpesvirus. Also, the droppings of resident birds should be periodically screened for the presence of virus. Nevertheless, HV infections are characterized for intermittent phases of virus shedding and a virus investigation alone may give false negative results in those cases where infected animals are not shedding the virus (BURGESS and YUILL, 1981; SCHUH and YUILL, 1985). For this reason, it is also important to test birds' serum for the presence of antibodies.

Although the immunity to HVs is mainly cellular (ROBERTSON, 1977; PAYNE et al., 1978; YORK and FAHEY, 1990), the humoral immunity against HVs does not necessarily protect against an infection (RAGER-ZISMAN and ALLISON, 1976; DOCHERTY and ROMAINE, 1983; HERRING et al., 1989), and infected animals sometimes do not develop specific antibodies against the virus (VINDEVOGEL et al., 1980; BURGESS and YUILL, 1981), there is evidence that flock geometric mean titre correlates with protection against challenge. Thus serum antibody levels may be valuable flock-profiling indicators for checking immunity, as well as tools to check for exposure to virus in recent imported flocks of birds.

It is for this reason that both antigen and antibody detecting assays must be available in order to provide better means of prevention and control of HV diseases, thus improving health and livability of pet birds and aviary stocks, and protecting domesticated birds from additional infectious agents.

In this paper we report the preparation of monoclonal antibodies (Mabs) to one strain of the virus of the PPD and the further development of a diagnostic ELISA test for the identification of antigen and antibodies to the Pacheco disease of psittacines.

**Case report**

The specimens studied were clinical cases or tissues from dead birds submitted to the Institute for Avian Diseases for diagnosis. The virus strain of PPD used for the production of Mabs and the development of the ELISA test was isolated in 1988 from the liver and spleen of an African Congo Gray Parrot (*Psittacus erithacus* L., 1758) which had died following the disease outbreak in an imported flock of birds coming from Africa (MUTLU et al., 1991). The affected bird showed typical multifocal necrotic lesions in liver and spleen, and histological examination of the liver showed numerous eosinophilic intranuclear inclusions of Cowdry type A in hepatocytes. The bacteriological examinations showed only non-specific bacterial contents. The examination
of liver and spleen for infection with Chlamydia psittaci by the Landesuntersuchungsamt für das Gesundheitswesen Südbayern Oberschleißheim was negative. Mycoplasma isolation attempts were also negative and the allantoic fluid of infected embryonated eggs did not agglutinate chicken red blood cells. On the basis of the clinical observations and virus isolation attempts, the aetiologic agent of this disease outbreak was identified as the PPDV.

Materials and Methods

Organ samples
Liver and spleen of the gray parrot were homogenized, resuspended in phosphate buffer saline (PBS) and inoculated in chorio alantoid membranes (CAMs). After three passages, the infected CAMs were harvested, clarified and used to inoculate primary chicken embryo fibroblast culture (CEF).

Virus preparation
Tissue culture showing 90-100% cytopathic effects (cpe) were harvested, clarified and stored at -65°C. Infectivity titres (TCID50) were calculated after 7 days incubation at 37°C using Falcon 96-well microplates (Becton Dickinson).

Purification of virus
Viruses were pelleted through a 36% (w/v) sucrose cushion in TE buffer (10 mM Tris HCl, pH 7.4, 3 mM EDTA) in a SW 28 rotor (Beckman) at 100,000 x g for 1 h. The pellets were resuspended in TE buffer and layered onto 20-60% (w/w) continuous sucrose gradients. Gradients were centrifuged at 130,000 x g for 3 h and visible virion bands were collected pelleted at 130,000 x g for 1 h and stored at -70°C. For preparation of partially purified virus for performing the ELISA tests, clarified virus stocks were centrifuged through a 36% (w/v) sucrose solution at 100,000 x g for 1 h. This virus pellet was again clarified by low speed and the supernatant stored at -70°C. The protein contents of the virus samples were determined by the BCA assay (Pearce) according to the instructions of the manufacturer.

Electron microscope
Drops of virus samples were adsorbed to carbon-coated formvar grids, negatively stained with phosphoric tungstic acid and then examined by transmission electron microscope (Zeiss EM C/R).

Production of immune sera
One rabbit (Chinchilla hybrid, 2.5 kg) was immunized subcutaneously with 150 μg (total protein) of purified PPDV mixed with equal volumes of complete Freund's adjuvant (Sigma). Four booster injections at 3-week intervals using incomplete Freund's adjuvant (Sigma) followed.

Immunization of mice
A group of 6-8-week-old BALB/c mice were immunized with the purified strain of PPDV. The mice received two subcutaneous injections of virus (10 μg of total protein) 3 weeks apart; the first in Freund's complete adjuvant and the second in Freund's incomplete adjuvant. An intraperitoneal injection of virus was given 3 days before fusion.

Monoclonal antibodies to Pacheco parrots disease
Fusion was performed using a modification of the described method (KÖHLER and MILSTEIN, 1975). Spleens from mice immunized with PPDV were harvested, counted and mixed with P3-X63Ag8.653 myeloma cells at 2:1 or 5:1 ratio. The cell mixtures were fused with 50% polyethylene glycol 1500 (Boehringer Mannheim), resuspended in HAT media, and dispensed into 96-well Falcon tissue culture plates (Becton Dickinson). Beginning between days 10 and
14, the medium from the wells showing cell growth was tested for antibody activity using an ELISA with semi-purified virus as the capture antigen. Hybridomas secreting specific antibody were cloned by limiting dilution in 96-well plates, passed to tissue culture flasks and subcultivated until the amount of 800 ml/Mab could be obtained. The resulting antibody containing culture supernatants was harvested, clarified by centrifugation and affinity purified.

**Determination of the immunoglobulin subclasses of the antibodies**

The immunoglobulin isotype of the monoclonal antibodies was determined using culture supernatant in a commercial ELISA isotyping kit according to the manufacturer's instructions (Boehringer Mannheim).

**Purification of monoclonal antibodies**

IgG and IgM were isolated from tissue culture supernatant by affinity chromatography on Protein G-Sepharose and Concanavalin A-Sepharose columns (Pharmacia), respectively. After dialyzation against PBS, eluates were sterile filtrated (Millipore Filters 0.22 μm) and antibody samples were stored at −20°C. The protein contents of the samples were determined by BCA assay (Pearce) as recommended by the manufacturer.

**Detection of positive clones by ELISA**

Polystyrene microtitre plates (Nunc-Immunosorp, Nunc, Denmark) were coated with 1 μg/ml semi-purified virus in carbonate-bicarbonate buffer, pH 9.6 (100 μl/ml). Following overnight incubation at 4°C, the wells were blocked with 10 % FCS in PBS for 30 min at room temperature. After adding 0.1 ml volumes of the hybridoma supernatants to the wells for 1 h at 37°C, the plates were incubated with peroxidase conjugated anti-mouse immunoglobulins (Dakopatts) for 30 min at 37°C. ABTS substrate (Boehringer, Mannheim, Germany) was added and optical densities (OD) were measured at 405 nm with a Titertek Multiscan apparatus (Tecnomara, Germany). Extinctions higher than 0.2 (the double background) were considered positive. Between all steps, plates were washed five times with PBS containing 0.05 % Tween-20. To estimate non-specific reactions, samples were tested in the same way on plates coated with 1 μg/ml of mock-infected cells.

**Biotinylation of Mabs**

Antibodies were biotin conjugated by a method outlined previously (STAHLI et al., 1983). Immunoglobulin fractions isolated from culture supernatant were conjugated with Biotin-X-NHS (Sigma) dissolved in dimethyl formamide (Sigma). The antibodies were then extensively dialyzed against PBS and stored at −20°C.

**Competitive binding assays**

Unlabelled purified Mabs were adjusted to 0.1 mg/ml in ELISA diluent and 0.1 ml volumes added to ELISA plates which had been coated with antigen and blocked as described above. After incubation for 3 h at 37°C, the plates were washed and biotinylated Mabs were added for an additional hour at 37°C. Peroxidase-conjugated streptavidin (Amersham) served as detecting reagents. The plates were washed again, ABTS substrate added and absorbance values at 405 nm were read as described above. Each conjugated antibody was titrated beforehand to determine the amount of antibody needed to give an OD of 0.5–1.0 in the absence of a second unlabelled antibody.

Percent inhibition (%) due to the competing antibody was calculated from average corrected absorbance readings using the formula:

\[
\text{[ % inhibition } = \frac{[100 (A-B)]}{A}\]

where A = OD₄₅₅ without competing antibody and

B = OD₄₅₅ with competing antibody.

The binding curves were plotted. A decrease in binding to more than 80 % was considered...
Fig. 1. Competition antibody assay of PPDV specific Mabs, measured by ELISA. The boxes indicate groups of Mabs showing complete symmetrical inhibition as fully competitive, 40–80% was considered partial competition and less than 40% inhibition was considered as non-competitive.

**Avidity of Mabs**

In order to evaluate Mab-binding properties, Mabs were compared with respect to their activity in antibody binding assays. Relative binding activity was defined as the amount of antibody (µg/ml) required to yield an absorbance of 0.5 at 405 nm (20) and was classified as high (< 50 µg/ml), intermediate (= 50 µg/ml) and low (> 50 µg/ml).

**Virus neutralization**

The reaction of Mabs in the SN test will be described in details elsewhere (submitted). Briefly, dilutions of Mabs or hyperimmune serum were incubated with equal volumes of PPDV (100 TCID<sub>50</sub>) for 1 h at 37 °C. After 6 days of incubation, residual virus was assayed by plaque formation on CEF monolayers.

**Sandwich ELISA**

Purified Mabs were diluted in PBS containing 10 mM NaN<sub>3</sub> and used to coat 96-well Nunc microtitre plates. After incubation antibody solution was collected from the wells and reused to coat additional plates. After the blocking step, plates were incubated for 1 h with tissue culture supernatant of virus infected CEF cells. Plates were then incubated with detecting antibodies for 1 h. The following steps were the same as described for the indirect ELISA.
Fig. 2. Competition antibody assay of PPDV specific Mabs, measured by ELISA. The boxes indicate groups of Mabs showing complete symmetrical inhibition.

Results

Characterization of PPDV isolate

The PPD isolate induced complete cytolysis of CEF cultures in 4 days and the virus infectivity reached titres around $10^4$–$10^5$ TCID$_{50}$. Electron microscopy of the purified virus preparations indicated the presence of virus particles resembling herpesvirus in structure. The size of the virions ranged between 180 and 200 nm in diameter. The sucrose concentrations of collected virus bands were of 44.5%.

Production and selection of monoclonal antibodies

The procedures for immunization of the mice and production of hybridoma cultures secreting antibody reacting with PPD antigen in the indirect ELISA proved highly efficient. Both fusions that were carried out showed viable cells present in all wells of the 96-well Falcon plates, of which 50% secreted antibody which reacted with PPDV in the indirect ELISA. Sixteen Mabs that were positive in the ELISA test were chosen for further characterization. Eight of the selected Mabs were of the IgG1 isotype, five of IgG2a, two of IgG2b and one was IgM.

Competitive binding assays

All the PPDV Mabs retained their binding capacity after coupling to biotin and were used in reciprocal competition assays. Several Mabs exhibited complete, symmetrical inhibition, indicating that they recognized identical or overlapping sites.
Partial symmetrical competition between various Mabs was also observed (Fig. 3b, c). Here, differences in the degree of blocking efficiency between antibody pairs were seen despite having comparable avidities, suggesting that this could be due to the induction of conformational changes in the second epitope after the attachment of the first one. For example, Mab-7 competed fully with Mab-18 but Mab-18 only partially competed with Mab-7. In the same way, Mab-18 exhibited complete reciprocal binding with Mab-20 and Mab-24 but only partial binding with Mab-7 and Mab-15. On the basis of these results, the target of all Mabs presenting symmetrical competition were divided into nine groups considered to be overlapping or closely adjacent epitopes. Nevertheless, since even Mabs belonging to one same group displayed different serological behaviour (unpubl. data), all Mabs were considered to be specific for overlapping epitopes rather than specific for the same epitope. Figures 1 and 2 summarize the results of competition tests.
Virus neutralization

The rabbit specific antiserum showed a capacity of neutralizing the virus infectivity in concentrations up to 1.25 μg/ml. Mab-2, Mab-5F7 and Mab-8 showed neutralizing activities until concentrations of 9.7, 19.5 and 156 ng/ml, respectively.

Standardization of the sandwich ELISA

To determine the optimum concentration of reagents, checkerboard titrations with coating and detecting Mabs were carried out. Antibodies were tested in the concentration of 10, 5 and 2.5 μg/ml to find out the concentration which showed higher OD values and lower background reaction. The neutralizing Mab-2 and Mab-3 were chosen to coat the microtitre wells to capture biologically active PPDV.

A concentration of 5 μg/ml of Mab incubated in wells for 24 h in PBS bound the maximum amount of PPDV compared to other protocols without increasing the absorbance of negative control samples. The concentration of detecting antibody for maximum detection range was then determined. For this purpose, 5 μg/ml was taken as the concentration of catching Mab to coat assay plates, and constant amounts of virus infected CEF supernatants was incubated whereas detecting antibody was varied from 0.1 to 10 μg/ml. Non-specific reactions could be detected readily in this step by testing the sera against mock infected cells and heterologous HV antigens (infectious Laringo tracheitis virus (ILT) and Marek disease virus (MDV)). In all the following experiments, the concentration of detecting Mab was fixed at 5 μg/ml, and purified virus antigen was given in varied concentrations to determine the sensitivity of the test. None of the biotinylated Mabs showed satisfactory reaction compared to unlabelled antibodies, when used for detecting antigen. The incubation times were of 1 h and all steps were done at room temperature. Thirty minutes was chosen as a suitable time to read the OD of the enzyme-substrate reaction. The best Mabs used as catching antibodies were Mab-2 (IgG2a) and Mab-3 (IgM). As detecting antibodies, both Mab-3 and PPD-specific rabbit sera showed similar results. When Mab-3 was used as detecting antibody, plates were coated with Mab-2 and the reaction could be detected using conjugated anti-IgM specific immunoglobulins. When Mab-3 was used for coating plates, the PPD-specific rabbit sera was used as detecting antibody and species specific conjugated antibody was used to detect the reaction.

Specific amounts of bound PPDV were detected over a concentration range of 117–234 ng/ml. The use of different concentrations of Tween 20 (0.1 % and 1 % (v/v)) and blocking reagent (10 % FCS) in the diluting buffer did not affect the magnitude of the specific reactions. A typical standard curve for the assay is shown in Figure 4.

Using this same protocol and adding one incubation step between specific antigen and test serum, a blocking ELISA for testing the presence of antibodies in sera of healthy parrots was developed. Non-specific reactions were not found with SN-negative sera from older SPF chickens and negative rabbit sera in this test. Specific amounts of antibody up to 625 ng/ml in sera, corresponding to a dilution of 1 :32 768 of the rabbit specific hyperimmune sera, were detected using this method. Figure 5 shows the standard curve for this assay.

Discussion

Mabs raised against a strain of PPDV were produced and characterized. Based on the results of the characterization tests, all the 16 Mabs were considered to recognize different epitopes and were used in this work to study their efficiency in ELISA assays to solve PPD diagnostic problems.

A rapid and sensitive immunoassay for detecting PPD-antigen was developed. This sandwich ELISA utilizes neutralizing Mabs and has proved to be sensitive and specific for the detection of one of the strains of the PPDV. The neutralizing Mab-2 showed very high titres in the sandwich ELISA when used as catching antibody, and
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Fig. 4. Standard curve for PPDV sandwich ELISA. Each of the Mab coated wells was incubated with purified PPDV or control antigen (ILT virus). The data presented are for means of two independent experiments, each involving quadruplicate wells. Either Mab-2 as catching antibody with Mab-3 as detecting antibody, or Mab-3 as catching antibody with specific rabbit sera as detecting antibody presented identical curves.

Fig. 5. The blocking ELISA for detection of specific antibodies against PPDV consisted of the antigen-ELISA adding one incubation step between specific antigen and test serum. See Fig. 4 for details.

was therefore used for coating the plates. It recognizes an immunodominant epitope on the PPDV which easily induces an immune response in infected birds, as shown by the good immunization protocols of mice and rabbit. The use of PPDV-specific rabbit sera with anti-rabbit conjugate or of Mab-3 with anti-IgM conjugate as detectors enables the detection of antigen without using specific conjugates for the species of
bird tested, which is very important in light of the high number of different bird species, especially concerning the order of Psittaciformes. The antigen ELISA developed in this work can detect PPDV at levels as low as 117 ng/ml and did not show non-specific reactions with heterologous antigens or mock infected cells.

By preincubating test sera with PPD antigen before absorbing the virus material to the Mab coated plates (blocking ELISA), the presence and approximate concentration of anti-PPDV antibody in test sera could also be assessed. In addition, a relatively crude virus preparation could be used, since the trays were coated with specific Mabs in concentrations that did not show non-specific reactions with heterologous antigen, and for the detection of antigen specific antibodies again were taken. Specific antibodies to the PPDV resulted in high levels of inhibition of the Mabs reactivity with the PPD antigen, whereas antisera to heterologous viruses gave no inhibition even at low serum dilutions. Up to 625 ng/ml of specific antibody could be detected by this blocking ELISA. The antibody levels in the test sera may be titrated in the blocking ELISA by measuring the dilution of blocking serum which gives a particular level of inhibition. Variation may be controlled by use of a reference control positive serum, thus ensuring the reproducibility of the test.

In this system neither non-specific reactions of old chicken serum with the polystyrene ELISA plates, commonly described in avian ELISA tests (Slagh et al., 1978; Marquardt et al., 1981; Meulemans and Halen, 1981; York et al., 1982) nor of HV infected tissue preparations with normal immunoglobulins due to the binding of antibodies to the Fc receptors of infected cells (Evans et al., 1972; Michelson et al., 1989; Farrell and Shellam, 1990) were observed.

Although cross reactions between PPDV and other avian HV have not yet been described, both cross reactive proteins (Tantawi et al., 1979; Nakajima et al., 1990) and genetic homologies between different species of HV (Kishi et al., 1988; Griffin, 1989) have already been reported. For this reason, even though the developed ELISA assay exhibited no cross-reactivity with proteins of the tested avian HV, further studies will be necessary in order to determine the exact specificity of the assay developed in this study.

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References


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