Production of African Cassava Mosaic Virus (ACMV) Specific Polyclonal Antibody BY Oral Immunization of Mice

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Abstract: *Serological techniques are commonly used in the detection and characterization of plant viruses. These methods employ the use of antisera produced by highly purified preparations in intramuscular, intradermal and intraocular. In this study oral route was explored using crude extracts. Two groups (control and experimental) of Swiss albino mice consisting of two replicates were immunized via the oral route with crude extracts from uninfected cassava plants (Manihot esculenta) and cassava plants systematically infected with African Cassava Mosaic Virus (ACMV). Uninfected and infected leaves were grinded separately in saline solution (0.15M) at 1:2 (w/v) with laboratory mortar and pestle and then filtered with double layered cheese cloth of 75μm to obtain extracts. Clarified extracts were orally administered to the mice in daily doses of 200μl per mice for 21 days and booster doses were also given at day 28 and 35 respectively. Antiserum were obtained from the mice for 6 consecutive weeks after the commencement of immunization and were analyzed using antigen coated plate (ACP) and triple antibody sandwich (TAS) indirect enzyme- linked immunosorbent assay (ELISA). Group A antisera gave negative reactions (OD values < 1.5) while group B antisera reacted positively (OD values ≥ 1.5) in the two methods used. The polyclonal antisera obtained were very specific to ACMV in ACP and TAS ELISA. This appears to be the first antisera specific to ACMV obtained by oral immunization of mice. Oral immunization is considered less stressful for animals, the method is a fast, simple and cheap way for producing antisera to plant virus compared to the traditional methods of using purified preparations for immunization. We have used this procedure in the production of antisera yet there is room for improvement in immunization strategies to enhance antibody production. Immunization dosage can also be tried and manipulated in bigger animals like rabbits and chicken. This research work leaves room for further exploration of similar procedure in bigger experimental animals like rabbits and chicken for greater antiserum production.*
Keywords: *ACMV, Antibodies, Immunogens, Mice, Oral immunization*

I. Introduction

Detection and characterisation of plant viruses are key aspects in combating pathogens. Several techniques are employed in these procedures. In routine methods purified preparation of viruses are used for intramuscular, intradermal and intraocular immunizations for antiserum production. A major factor limiting this procedure is the need for a highly purified preparations. In this study, the problems were bypassed using the raw fluid through the oral route. Ingestion of antigen stimulates the synthesis and release of specific secretory immunoglobulin A (sIgA) antibodies into saliva, tears, colostrum, milk, gastrointestinal, respiratory and cervical mucosal fluids [1, 2, 3, 4 & 5]. A common mucosal immune system (CMIS) has been postulated to consist of the inductive sites, gut- associated lymphoreticular tissue, that is Peyer's patches (PP), and bronchus associated lymphoreticular tissue and the effector sites, laminae propriae of the gastrointestinal, upper respiratory, genitourinary tracts and the salivary, lacrimal and mammary glands.

The preferred routes of administration are subcutaneous and intramuscular. However, oral immunization routes are generally considered less stressful for animals, and there have also been a welcome development in oral immunization strategies for routine production of antibodies. These routes include oral immunization by voluntary intake of the antigen or through the gavage, as well as oral - nasal administration through the exposure of the animal to antigen containing aerosol of the antigen and adjuvants [6]. Oral immunization is an employed route for inducing a mucosal immunoglobulin A (IgA) response following antigen uptake at Peyer's patches of the small intestine.

The mucosal immune system comprises of several anatomically remote and functional distinct compartments, it is firmly established that the oral ingestion or intranasal administration of antigens will induce humoral and cellular responses not only at the site of antigen exposure but also in other mucosal compartments [7& 8]. This is due to the dissemination of antigen- sensitized precursor B and T lymphocytes from the inductive (e.g. intestinal Peyer's patches) to the effector sites. Other studies have also shown that oral immunization stimulates the immune system and the intermittent oral exposures to immunogens have been found to result in the immergence of significantly high serum titers of specific antibodies [9]. According to [10], small amounts of protein orally, administered escape the enzymatic digestion in the intestine and are absorbed as

intact antigens. To the best of our knowledge, no work has been done on the production of African cassava mosaic virus specific polyclonal antibody by oral immunization in mice. Therefore, this study aims to produce polyclonal antibody specific to African cassava mosaic virus in mice and also determine the ELISA protocol that will be more sensitive to detect the antibodies produced.

1.1 African cassava mosaic virus (ACMV)

African cassava mosaic virus (ACMV) of the genus Bergomovirus is a plant pathogen virus that is part of the group of cassava mosaic geminiviruses (CMGs) in Manihot esculenta (cassava), a highly valuable African food crop. This plant virus causes severe mosaic to cassava. Plants infected with ACMV are not killed but show mosaic leaves appearance or loss of chlorophyll [11]. CMGs are plant viruses with a restricted dicotyledonous host range, comprising of twinned (germinate) particles, and possess a unique capsid structure of approximately 20- 30nm in size, containing a bipartite, single stranded, circular DNA genome [12]. Their circular single stranded DNA (ssDNA) is packaged into two incomplete TI icosahedra, which are formed by multiple of a single coat protein (CP) [13].

II.   Materials And Methods

2.1 Animals

Twenty, Eight week- old Swiss Albino female mice were purchased from the Veterinary Physiology Department of University of Ibadan, Oyo State, Nigeria. The animals were acclimatized for a week before the commencement of immunization.

2.2 Plants

Healthy leaves and leaves systematically infected with ACMV from cassava were obtained from International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria.

2.3 Preparation of Crude Extracts

Crude extracts of both healthy and infected leaves were prepared by grinding in 0.15M saline solution at a ratio 1:2 (w/v) that is, 1g of leaf to 2ml of saline solution [14]. These extracts were filtered through double layered cheesecloth, and stored at -20°C in 1ml aliquots.

2.4 Experimental Animals and Oral Immunization

Two groups of mice A (control) and B (experimental) were used for the experiment. Each group has two replicate which is made up of five mice. The mice were starved for four hours before the start of oral immunization. Group A mice were given by gavage 200µl crude leave extracts from uninfected cassava leaves daily. Group B were fed by gavage with 200µl crude leave extracts from ACMV infected cassava leaves.

2.5 Blood Collection

The mice were bled from the tail to obtain antiserum at days 7, 14, 21 after the commencement of the daily oral immunization and at days 28, 35 and 42, after booster immunization. The antiserum was centrifuged at 1500rpm and the supernatant was collected in eppendorf tubes for storage and analysis [15].

2.6 Enzyme- linked immunosorbent assay (ELISA)

The antisera were tested by two indirect ELISA, ACP and TAS indirect enzyme- linked immunosorbent assay (ELISA). Results were analysed by dividing the optical density (OD) by values from wells loaded with antigen (D) by values of healthy controls (H) (Diseased/Healthy). Results were interpreted as follows: < 1.5 is negative; 1.5- 1.9 is mildly positive; 2.0 -2 .9 is positive; > 3.0 is strongly positive.

2.6.1 ELISA protocols

In TAS- ELISA, the plates were coated with polyclonal antiserum at 100µl per well at 1:500 to 1:256000 dilution in coating buffer and were incubated at 37°C for 3 hours. The plates were washed thrice with PBS- Tween. 100µl per well of antigen prepared in grinding buffer was added to each plate at 100µl per well and was incubated overnight at 4°C. Plates were washed and blocked with 200µl per well of 5% w/v solution of non- fat skimmed milk powder in PBS- Tween. Plates were incubated at ambient temperature for 1 hour and were emptied. Monoclonal antibodies at 1:500 dilution in PBS- Tween was added and plates (100µl per well) were incubated at 37°C for 3 hours. Each plate was washed and 100µl per well of ram anti- mouse alkaline phosphate conjugate at 1:1000 dilution was added. Plates were incubated at 37°C for 3 hours and washed. P- Nitrophenyl phosphate substrate in substrate buffer was added at 150µl per well. The plates were incubated at ambient temperature and were read after 1 hour and overnight using ELISA Bio track II reader at 405nm absorbance.

In ACP- ELISA, 100μl of antigen grinded in coating buffer was added to the plates, the plates were incubated at 4°C overnight. The plates were washed thrice with PBS- Tween and blocked with 200μl per well of 5% w/v solution of non- fat skimmed milk powder in PBS- Tween. Plates were re-incubated at 37°C for 1 hour and were tapped dry. 100μl of polyclonal antibody diluted in conjugated buffer was added to each well and incubated at 37°C for 3 hours. The plates were washed and treated with ram anti- mouse alkaline phosphate conjugate diluted in conjugate buffer (100μl per well) and incubated for 3 hours and washed. P- Nitrophenyl phosphate substrate in substrate buffer was added at 150μl per well. The plates were incubated at ambient temperature and were read after 1 hour and overnight using ELISA Bio track II reader at 405nm absorbance.

III.   Results

Fig. 1 shows the highest absorbance values of antiserum dilutions in two indirect ELISA at weeks 2, 4 and 6 after the commencement of immunization. The antisera obtained from the control group were negative to ACMV with the highest OD values < 1.5 at week 2, 4 and 6 in both ACP and TAS ELISA. In ACP ELISA, the antisera obtained from the experimental animals were mildly positive to ACMV with the highest OD values $\geq$ 1.5 at week 2 and 4, and were strongly positive to ACMV with the highest OD value > 3.0 at week 6 while In TAS ELISA, the antisera obtained from the experimental animals were negative (< 1.5), mildly positive ($\geq$ 1.5) and positive ($\geq$ 2.0) to ACMV at week 2, 4 and 6 respectively. The result shows that ACP ELISA was more sensitive in detecting the virus than TAS ELISA. In addition, the antibody titres increased relatively with increase in weeks after immunization for both methods.

The result of the antibody titres produced in orally immunized mice at series of antiserum dilutions against African Cassava Mosaic Virus (ACMV) in ACP ELISA is shown in fig. 2. The peak antibody titres were recorded at 1:2000 antiserum dilutions for week 2 and 1:1000 antiserum dilutions for week 4 and 6. The working dilutions are as follows: - 1:4000, 1:8000 and 1:64000 for week 2, 4 and 6 respectively.
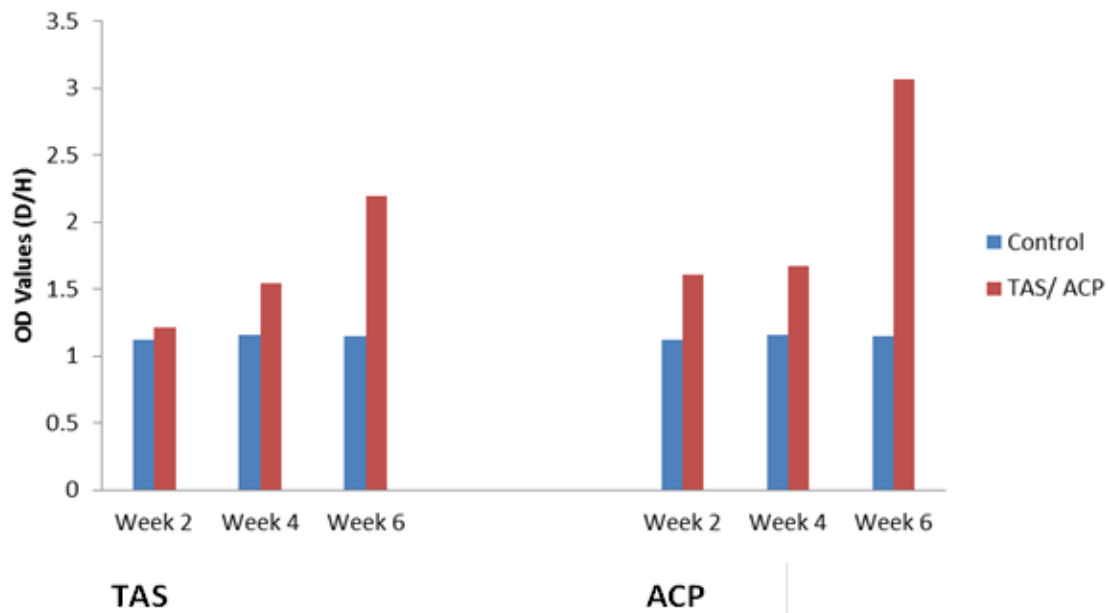
Figure 1: Highest absorbance values of antiserum dilutions in two indirect ELISA at 2, 4 and 6 weeks after immunization.
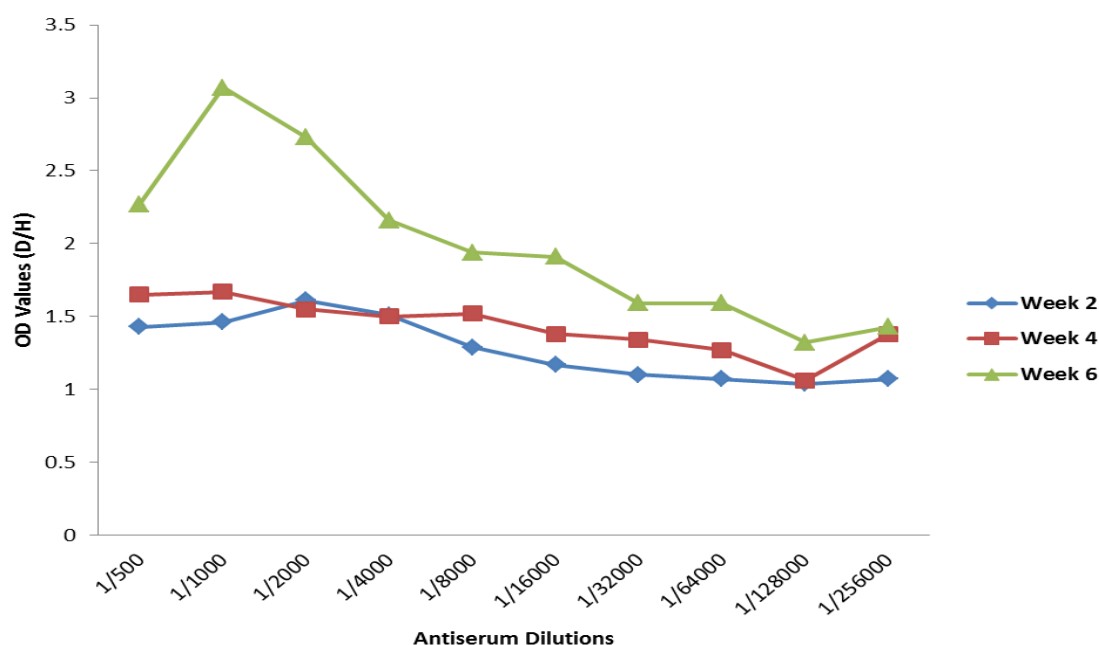
Figure 2: Antibody titres produced in mice by oral immunization at series of antiserum dilutions against African Cassava Mosaic Virus (ACMV) in ACP ELISA.

IV. Discussion

The study showed a strong systemic immune response in mice immunized orally with crude leaf extracts of ACMV- infected cassava plants in the absence of extract purification and immunoadjuvants. The specific antibodies synthesized against the ACMV coat protein were detected by ACP- and TAS- ELISA. The result showed that the crude ACMV infected leaf extracts given orally to experimental animals did not compromise their immunogenic capability amidst the digestive enzymes in the gastrointestinal tract by eliciting specific immune response against ACMV. This corresponds to reports made by [10] that small amount of protein orally administered escape the enzymatic digestion in the small intestine and are absorbed as intact antigens. [16], also suggested that plant virus are 'bio encapsulated' within the plant cell walls and cell membranes and have therefore increased resistance to digestion.

Since it is unlikely that murine epithelial cells possess receptors for ACMV, presumably the size and particulate nature of the virus particles allow them to be efficiently taken up and retained by M- cells in gut and nasal- associated lymphoid tissues which present them to the lymphoid cells in the underlying tissues. [15], also reported that internalization of the virus through the gastrointestinal tract might be based on the structural properties of the antigen molecule. The strong immunogenic property of ACMV can be ascribed to the icosahedral structure of ACMV which could be important in facilitating mucosal uptake and immune recognition.

The relative increase in OD values with increase in weeks after immunization resulted from re-exposure of experimental animals to the same antigen (ACMV). This support the report made by [9], that intermittent oral exposures to immunogens could not favour oral tolerance phenomenon and may result in significant high serum titres of specific antibodies. The high sensitivity results from ACP- ELISA were due to the adsorption of healthy plant antibodies produced with healthy cassava leaf extract. This ensured that antibodies specific to cassava plants proteins were manually eliminated to prevent cross reactivity.

V. Conclusion

In conclusion, the result of this study with oral administration of crude ACMV infected demonstrated an induction of a durable mucosal and systemic immune response without the presence of adjuvant and without purification of virus. Therefore, ACMV is a good immunogen and can be used for oral immunization to produce antibodies.

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