A RECOMBINANT AVIAN ADENO-ASSOCIATED VIRUS AS A VECTOR FOR INFECTIOUS BURSAL DISEASE VACCINATION.

Un virus adeno-asociado aviar recombinante como un vector en la vacunación contra la enfermedad infecciosa de la bolsa.

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ABSTRACT

Infectious bursal disease is a worldwide distributed immunosuppressive disease of young chickens that need to be controlled by vaccination; it represents one of the main concerns for the poultry industry. The adeno-associated viruses are non-pathogenic viruses, capable of accommodating relatively long pieces of DNA, and of infecting a wide variety of cell types. A member of this family, the avian adeno-associated virus has been fully characterized and successfully used for gene delivery in chicken embryo tissues and cells. In this study, it was demonstrated by electron microscopy and immunocitochemistry the feasibility of generating recombinant avian adeno-associated virus (rAAAV) virions expressing the immunogenic viral protein 2 of infectious bursal disease virus (IBDV). Serological evidence of VP2 protein expression measured as IBDV specific antibody response after *in ovo* or intramuscular inoculation of the recombinant virus in specific pathogen free (SPF) chickens was observed. The use of rAAAV virions for gene delivery in poultry is a promising approach to poultry vaccination.

Key words: Avian adeno-associated virus, vaccination, immune response, infectious bursa disease.

RESUMEN

El virus de la enfermedad infecciosa de la bolsa es una enfermedad de distribución mundial que afecta a aves jóvenes que debe ser controlada mediante vacunación y constituye una de las preocupaciones principales de la industria avícola mundial. Los virus adeno-asociados aviares son virus no patogénicos capaces de dar cabida a porciones relativamente largas de ADN y de infectar una amplia variedad de tipos celulares. Un miembro de esta familia, el virus adeno-asociado aviar ha sido caracterizado por completo y utilizado como un vector para la entrega de genes en células y tejidos de embriones de pollo. En el presente estudio se demostró mediante inmunohistoquímica y microscopia electrónica la factibilidad de generar virus adeno-asociados recombinantes expresando la proteína viral 2 del virus de la enfermedad infecciosa de la bolsa. Luego de la inoculación *in ovo* o intramuscular de aves libres de patógenos específicos con el virus recombinante, se observó evidencia serológica de la expresión de la proteína VP2. La utilización de virus adeno-asociado aviar recombinantes para la entrega de genes es una opción interesante para la vacunación de aves domesticas.

Palabras clave: Virus adeno-asociado aviar recombinante, vacunacion, respuesta inmune, enfermedad infecciosa de la bolsa.

INTRODUCTION

Infectious bursal disease virus (IBDV) is a member of the *Birnaviridae* family, the genome of which consists of two segments (A and B) of double-stranded RNA [14]. Segment A encodes a polyprotein that can be cleaved by auto proteolysis to form mature viral proteins VP2, VP3 and VP4 [12]. The antigenic site responsible for the induction of neutralizing antibodies is highly conformation-dependent and located on the VP2 [4]. IBDV multiplies rapidly in developing B lymphocytes in the bursa of Fabricius, and causes infectious bursal disease (IBD) in chickens inducing clinical signs, immunosuppression, and can act as a predisposing factor for the development of several

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other diseases [1]. Control of IBD in young chickens have been primarily achieved by vaccination with live attenuated strains of IBDV early in life, as well as by transferring high levels of maternal antibody induced by the administration of live and killed IBD vaccines to the breeders [13].

Advances in the understanding of viral diseases pathogenesis and of the molecular mechanisms involved in the generation of protective immune responses, have opened new avenues for the prevention of these diseases. The use of recombinant viruses for gene delivery as a vaccination strategy in veterinary medicine has been previously documented [9]. Immunization of chickens against IBD using a vector that *in vivo* expresses VP2 has been previously reported using an avian herpesvirus vector [20], a fowlpox virus vector [17], a fowl adenovirus vector [10] and a Marek's disease virus vector [19].

In recent years, an extensive amount of work has been performed for the characterization and use of replication defective parvoviruses for the purpose of gene delivery [9]. These replication defective parvoviruses, the adeno-associated viruses (AAV), are non-pathogenic, capable of accommodating relatively long pieces of DNA, and capable of infecting a wide variety of cell types [18]. A member of this family, the avian adeno-associated virus (AAAV) has been fully characterized [3, 8] and successfully used for gene delivery in chicken embryo tissues and cells [7, 8]. The aim of this work was to generate recombinant AAAV virions (rAAAV) expressing the immunogenic VP2 peptides of the Edgar strain of IBDV and assess their ability to generate immunity in chickens.

MATERIALS AND METHODS

Amplification of the VP2 genes by reverse transcriptase-polymerase chain reaction (RT-PCR).

Amplification of the VP2 gene of the Edgar strain of IBDV (1734 bp) was performed using the forward VP2 (5'-AGCATCGATATGGACCGCGCCGTTA-3') and reverse VP2 (5'-AGCAGATCTTTACTAGCCAGACCTGGCTTCTCTA-3') primers. The *Cla*I and *Bgl*II restriction sites were included in the 5' end of the forward and reverse primers, respectively. Reverse transcription (RT) was performed with the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA. USA) while polymerase chain reactions (PCR) were carried out with the FailSafe PCR system and PCR 2X premix C (Epicentre, Madison, WI. USA.) following manufacturers' instructions. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml). The products of the proper size were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA. USA) using manufacturers' recommendations.

Cloning and sequencing of the VP2 genes.

The amplified VP2 genes were cloned into pCR 2.1 plasmids (Invitrogen, Carlsbad, CA) and transformed into TOP 10 *E. coli* cells following manufactures' recommendations. Positive colonies were selected and recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA. USA). The presence of the VP2 genes was verified by digestion of these plasmids with the *Cla*I and *Bgl*II restriction enzymes. Electrophortic analysis was performed as explained. In order to confirm the appropriated sequence, direct nucleotide sequencing of the VP2 gene was performed using the respective forward and reverse primers with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA. USA).

Generation of plasmid constructs to produce rAAAV particles coding for the VP2 genes.

DNA fragments of 1734 bp (VP2 gene) recovered from the pCR 2.1 plasmid after restriction enzyme digestion were cloned in a plasmid construct named p3.6 ITR-MCS containing the internal terminal repeats (ITR) of the AAAV [8]. Briefly, the p3.6 ITR-MCS plasmid was linearized with the *Cla*I and *Bgl*II restriction enzymes and dephosphorylated with calf intestinal alkaline phosphatase (CIP) (New England Biolabs, Inc., Beverly, MA. USA) at 37 C x 30 min. Equimolar concentrations of the VP2 genes were ligated using T4 DNA ligase and transfected using TOP 10 *E. coli* cells following manufactures' instructions. Positive transformed colonies were selected, the presence of the VP2 genes in the p3.6 ITR-MCS was verified after digestion with the *Cla*I and *Bgl*II restriction enzymes by electrophoresis analysis as previously mentioned.

Expression of the VP2 proteins.

Human embryo kidney cells expressing the E1A and E1B immediate early genes of human adenovirus type 5 (HEK 293) were obtained from ATCC (CRL-1573) and grown using Dulbecco's modified minimal essential medium (Gibco, Carlsbad, CA. USA) supplemented with 10% fetal bovine serum and antibiotics in 25 cm^2 cell culture flasks and subcultured in 35 mm^2 plates until 90% confluence. Ten micrograms of the p3.6 ITR-MCS plasmid containing the VP2 genes were transfected with the use of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA. USA), as recommended by the manufacturer. The expression of the VP2 proteins was detected 72 hr post-transfection by immunohistochemistry (IHC) using the Diaminobenzidine-Urea-Peroxidase complex (Sigma Chemicals Co., St Louis, MO. USA) following manufacturer's instructions. Commercial polyclonal antibodies against IBDV Edgar strain (SPAFAS Inc., Norwich, CT. USA) were used in the test. The presence of positive dark brown staining in the transfected HEK 293 cells was considered as positive protein expression.

Generation of rAAAV virions expressing the VP2 proteins.

Recombinant AAAV virions were generated by simultaneous transfection of HEK 293 cell monolayers, with three plasmid constructs: the p3.6 ITR-MCS containing the VP2, the pRC-CMV expressing the Rep and Cap coding regions of AAAV [8] and a commercial pHelper plasmid coding for the E2,

E4 and VR-RNA genes derived from the human adenovirus type 5 (Stratagene, La Jolla, CA. USA). The purification of the recombinant viral particles was accomplished 48 hr post-transfection using ultracentrifugation on cesium chloride cushion and density gradients [5]. The presence of the rAAAV virions coding for the VP2 gene (rAAAV-VP2) was confirmed by electron microscopy following standard procedures.

Systemic immune response against the rAAAV.

One hundred and twenty 18 day-old specific pathogen free (SPF) embryos (Sunrise Farms. Catskil. NY) were divided in 6 groups of 20 embryos and used to test the serological response to two different doses (10^8 or 10^9 virus molecules/ml) and two administration routes (*in ovo* or intramuscular inoculation of 0.1 ml) for the of the rAAAV-VP2 as described in TABLE I of the results section. Once hatched, birds were kept in BSL2 isolation units at the PDRC where appropriated husbandry was provided. Ten birds in each subgroup were wing bled at 14; 21; 28; 35 and 42 days of age and the serum was refrigerated until processed for the presence immunoglobulin G (IgG) against IBDV using a commercial ELISA test (IDEXX Laboratories Inc., Westbrook, ME. USA) following manufacturer's protocols.

Statistical analysis:

All statistical analysis was performed using the Sigma Stat 3.0 software (1- way ANOVA, multiple comparison tests). SNK test was performed at $P \leq 0.05$.

RESULTS AND DISCUSSION

The appropriate orientation of the VP2 gene with respect of the major late cytomegalovirus promoter was confirmed by immunohistochemistry as demonstrated by positive recognition of p3.6 ITR-MCS / VP2 transfected cells by IBDV specific antiserum. No staining was observed in non transfected HEK293 cells proving that the foreign gene induced the expression of the IBDV protein (FIG. 1). In the case of the VP2 protein, a correct structural conformation seems to be very critical due to the conformational dependence of the immunogenic epitopes formed by the close proximity of the hypervariable regions A and B [6].

Forty eight hours after transfection of HEK 293 monolayers with the p3.6 ITR-MCS containing the VP2, the pRC-CMV expressing the Rep and Cap coding regions of AAAV and a commercial pHelper plasmid coding for the E2, E4 and VR-RNA genes, assembly of rAAAV-VP2 virions was demonstrated by electron microscopy with the presence of viral particles exhibiting diameters from 17 to 22 nm (FIG. 2), which correspond to the normal size of the AAV assembled capsids [2, 5].

A humoral immune response measured as the geometric mean titer (GMT) of the antibodies against IBD was detected after the inoculation of the rAAAV-VP2 virions in SPF chickens, the results are shown in TABLE I. Statistically significant differences were observed 42 days of age between all the vaccinated groups and the mock inoculated controls, regardless of the dose or route of inoculation. Seroconversion against IBDV was observed as early as 14 days after inoculation of the rAAAV-VP2. The highest antibody titers (GMT of 3997) corresponded to the *in ovo* inoculated group using 10^9 genomic copies/ml at day 42. When applied by the intramuscular route the rAAAV-VP2 elicited a more consistent antibody response along the different time points tested. This is the first attempt to use a rAAAV for vaccination against poultry pathogens, previous work demonstrated that the recombinant virus was able to induce the production of a reporter gene in tissues derived from

TABLE I

ANTIBODY TITERS AGAINST IBDV AFTER VACCINATION WITH DIFFERENT DOSES AND ROUTES OF APPLICATION OF rAAAV-VP2./ TÍTULOS DE ANTICUERPOS CONTRA IBDV POSTERIOR A LA VACUNACIÓN CON DIFERENTES DOSIS DE rAAAV -VP2 Y DIFERENTES RUTAS DE APLICACIÓN.

Route	Inoculum	Dose (genomic copies/ml)	Sampling age (days)*				
			14**	21	28	35	42
In ovo	rAAAV-VP2	10^9	0 [b]	1095 [b]	0 [b]	0 [c]	3997 [a]
		10^8	0 [b]	0 [c]	0 [b]	0 [c]	1914 [b]
	control	-	0 [b]	0 [c]	0 [b]	0 [c]	0
Intramuscular	rAAAV-VP2	10^9	0 [b]	2177 [a]	0 [b]	581 [b]	1034 [c]
		10^8	304 2[a]	0 [c]	1469[a]	1331 [a]	2413 [b]
	control	-	0	0 [c]	0 [b]	0 [c]	0 [d]

*Geometric mean titer of ten birds per subgroup. ** Means with the same letter within column are not significantly different by the SNK test (P<0.05).

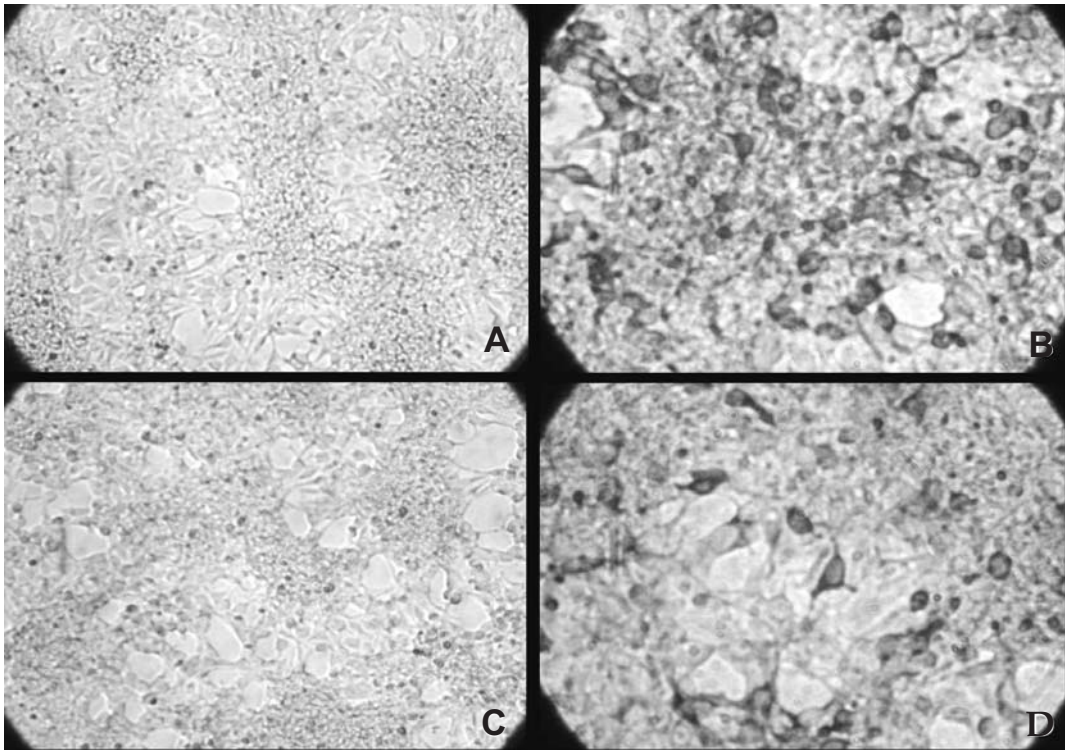

FIGURE 1. VP2 PROTEIN EXPRESSION 72 HR AFTER TRANSFECTION (B AND D). NO STAINING WAS OBSERVED IN THE NON-TRANSFECTED HEK 293 MONOLAYERS (A & C) / EXPRESIÓN DE LA PROTEINA VP2 72 HORAS DESPUÉS DE LA TRANSFECCIÓN (B y D). NO SE OBSERVÓ TINCIÓN EN LAS CÉLULAS EMBRIONARIAS DE RIÑÓN HUMANO NO TRANSFECTADAS.

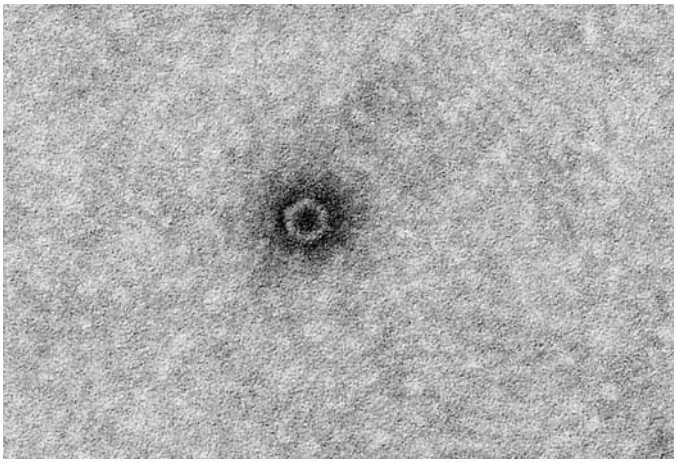

FIGURE 2. DETECTION OF RECOMBINANT AAAV VIRIONS BY ELECTRON MICROSCOPY. / DETECCIÓN DE PARTÍCULAS RECOMBINANTES DE AAAV MEDIANTE MICROSCOPÍA ELECTRÓNICA.

the rAAAV inoculated embryos [7], the presence of a measurable immune response confirms the ability of the rAAAV virions for gene delivery in poultry.

Even tough the presence of systemic humoral immune response measured as IgG titers against IBDV in the inoculated SPF birds was detected, the response was not uniform and relatively low in levels. The working mechanism of the gene delivery system used and the pathways for antigen presentation and recognition are not fully understood for chickens [9]. The variability in the results might be explained by a previously reported tendency of recombinant particles to agglutinate spontaneously leading to reduced yield and less efficient gene transfer [11, 15, 21]. Cell mediated immunity is known to be involved in protection against re-infection with IBDV [16]. In this pilot study, the effect of the rAAAV on the local level of protection was not evaluated. Further research including vaccine-challenge trials need to be conducted to assess the whether the rAAAV are able to elicit full protection against IBDV. The use of rAAAV virions for gene delivery in poultry represents a new and promising approach to poultry vaccination.

CONCLUSIONS

The feasibility of generating recombinant avian adeno-associated virions expressing the immunogenic viral protein 2 (VP2) of IBDV was demonstrated.

A detectable systemic immune response measured as antibodies against IBDV was elicited in SPF birds after inoculation with the recombinant virus.

Further research is required to determine the level of protection conferred by the rAAAV-VP2 virions against virulent IBDV and to compare the effects the replication defective recombinant product on the bursa of Fabricius with those of commercial vaccines known to induce bursal damage during viral replication.

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