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Short communication

Oral immunization using tuber extracts from transgenic potato plants expressing rabbit hemorrhagic disease virus capsid protein

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Abstract

Rabbit hemorrhagic disease, which is caused by a calicivirus, is a lethal infection of adult animals that is characterized by acute liver damage and disseminated intravascular coagulation. In this study, we report the production of the major structural protein VP60 of rabbit hemorrhagic disease virus in transgenic tubers of potato plants and its use as an oral immunogen in rabbits.

The rabbit hemorrhagic disease virus (RHDV) is the causative agent of a lethal contagious sickness characterized by an hemorrhagic syndrome and high mortalities in the wild rabbit population and affected farms (Liu et al., 1984). At the moment, there are several commercially available vaccines against rabbit hemorrhagic disease (RHD) on the European market (Peeters et al., 1992), elaborated from tissues collected from experimentally infected rabbits. Although they have proved to be effective tools for prevention of the disease, manufacturing this kind of killed vaccines gives rise to many problems resulting from the use of infectious virus and the risk of its dissemination from vaccine factories. Thus, it is important to develop alternative approaches for producing experimental vaccines. To this aim, previous works showed the expression of VP60, the major structural protein of RDHV using recombinant bacteria, yeast and baculovirus-infected insect cells (Boga et al., 1994, 1997; Laurent et al., 1994; Marín et al., 1995). However, these approaches are still expensive and their administration to wild rabbits cannot be conveniently done without stressing the animals which can be by itself the cause of high mortality rates (Argüello et al., 1988; Tesouro-Vallejo et al., 1990). Current research is dedicated to avoid such limitations by looking for alternative procedures to produce oral (edible) vaccines. Plant-based production of biologically active recombinant VP60 proteins has been reported and transgenic leaves and tubers have been successfully used for parenteral immunization of animals (Castañón et al., 1999, 2002). Moreover, other plant-derived proteins were also able to induce specific immune responses, even when administered orally (Haq et al., 1995; Mason et al., 1996; Arakawa et al., 1998; Tacket et al., 1998, 2000; Carrillo et al., 2000, Gómez et al., 2000). The possibility of using edible plants for oral immunization is an important issue considering that RHDV also infected wild rabbits (*Oryctolagus cuniculus*) (Villafuerte et al., 1994), which constitute an important prey for some protected carnivores such as *Linx pardinus*, and is the most important small game species in Spain.

Here we report the production of recombinant VP60 protein in transgenic tubers of potato plants

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and its use as an oral immunogen in rabbits. The plant expression vector pTUB2T7VP60 (Castañón et al., 2002) was used to transform potato plants (*Solanum tuberosum* cv. Desirée) as previously described (Castañón et al., 1999). Several clones that were able to root in presence of kanamycin were selected, and the presence *VP60* gene in the leaves of putative transgenic plants confirmed by PCR and northern analyses, according to Castañón et al. (1999). PCR and northern analysis showed the presence of the *VP60* gene and the expected size transcript (1.8 kbp) in all putative transgenic clones, whereas control plants, transformed with genes other than *VP60* or wild type potato plants, did not give detectable signals (data not shown). Fifteen independent transgenic potato clones were obtained by this methodology which were transferred to a sterile peat–perlite (1:1) mixture and grown under high humidity (95%) in a greenhouse. After about 2 weeks, the plants were transplanted to pots, and grown in a greenhouse under normal humidity and light conditions. From these, a clone named 1, transformed with pTub2T7VP60 was selected based upon its high level of VP60 expression in tubers. The relative amounts of the recombinant VP60 polypeptide in the tubers of this clone was investigated by enzyme-linked immunosorbent assay (ELISA) using an anti-VP60 hyperimmune serum obtained from a rabbit that survived the RDHV infection, according to Castañón et al. (2002). Proteins from potato tubers were obtained by homogenization with water in a blender. The resulting extract was centrifuged 3 min at $10{,}000 \times g$ and the supernatant used in ELISAs. Western blotting for detection of VP60 in the tuber extract sediments or supernatants were also done as previously described (Castañón et al., 2002). For this purpose the pellet and supernatant samples were mixed with electrophoresis sample buffer, boiled 3 min and subjected to SDS-PAGE using 10% gels. The size of the VP60 protein produced by transgenic potato tuber cells corresponded to that of native VP60, showing an apparent molecular mass of 60 kDa (not shown). Expression levels of recombinant VP60 protein in individual potato tubers of clone 1 ranged from 6 to 18 μg per g of fresh weight. The relative amounts of recombinant protein could be related to the different size and physiological development state of tubers. The maximum level of VP60 accumulation in the tubers of clone 1 was 3.5 μg of VP60 per mg of total soluble protein (PTS). This value was significantly higher than those found by Gómez et al. (2000) in transgenic tubers expressing S(N-gS) from transmissible gastroenteritis coronavirus, and was similar to the levels reported by Mason et al. (1996) for transgenic potato tubers expressing Norwalk virus capsid protein. An additional relevant observation was that the VP60 content in tubers stored and maintained in darkness at 4°C decreased from 18 to 6.25 μg per g of fresh tuber in a 2 months period. Thus to provide enough antigen for the whole immunization protocol and to avoid the variability inherent to the use of individual tubers, a freeze-dried homogenate made of all harvested tubers was made. Western blotting analysis showed that VP60 antigen content of the freeze-dried homogenate remained constant after several months of storage.

To investigate the oral immunogenicity of VP60 recombinant protein, 15 2-month old healthy rabbits were fed with extracts from potato tubers expressing VP60. It should be mentioned that the small number of rabbits used, that in other circumstances would not allow the application of a valid statistical analysis, can be considered adequate taking into account that under the experimental conditions used the mortality occurring after challenge in unprotected animals approached 100% (Boga et al., 1994, 1997; Marín et al., 1995). Antigen doses were made by reconstituting in water the appropriate amount of lyophilized material which was then orally administered to the rabbits by using a syringe. Animals were randomized to receive either 1.2 g of lyophilized transgenic tuber containing 100 μg of VP60 ($n = 5$); 6 g of lyophilized transgenic tuber containing 500 μg of VP60 ($n = 5$), or 6 g of lyophilized wild-type untransformed tubers ($n = 5$). All rabbits had nothing to eat or drink for 12 h before and after oral immunization. A second, third and fourth dose was administered to each animal on days 21, 42 and 63. The potato extracts were well tolerated. The animals were bled before the first immunization and 7 days after each inoculation. The endpoint titter of specific antibodies against RHDV VP60 was tested by ELISA according to Castañón et al. (1999), using recombinant VP60 as the antigen (Marín et al., 1995) and serial dilutions of sera from immunized and control animals. ELISA assays showed that only two animals fed with tuber extracts containing 500 μg of VP60 had measurable antibody titters after the third immunization, and two additional animals become seropositive after the following potato tuber ingestion (Table 1). The specificity of this anti-VP60 response was confirmed by western blotting. Interestingly enough, the antibody titters obtained in rabbits orally immunized with 100 μg of VP60 were

Table 1. ELISA titters of anti-VP60 antibodies in sera of control (1–5) and immunized (6–15) rabbits, after ingestion of lyophilized wild (A) or transgenic-type potato containing 100 μg (B) or 500 μg (C) of VP60

No. animal	ELISA titters (log_{10})					Survival
	Days after the first antigen ingestion					Hours after challenge
	0	27	49	70	79	
1A	0	0	0	0	[a]	45
2A	0	0	0	0	[a]	48
3A	0	0	0	0	[a]	50
4A	0	0	0	0	[a]	46
5A	0	0	0	0	[a]	44
6B	0	0	0	0	[a]	53
7B	0	0	0	0	[a]	49
8B	0	0	0	0	[a]	50
9B	0	0	0	0	[a]	45
10B	0	0	0	0	[a]	48
11C	0	0	0	1.30	[a]	66
12C	0	0	0	1.28	[a]	61
13C	0	0	2.10	2.10	3.00	[b]
14C	0	0	0	0	[a]	57
15C	0	0	1.10	1.40	[a]	96

[a] Dead animal.
[b] Survivor.

undetectable and in any case much lower than those reported by our group in previous experiments in which an equivalent amount of antigen was administered intramuscularly (Castañón et al., 1999, 2002).

To evaluate the protective efficacy of immunization with the orally-administered plant-derived VP60, all rabbits were challenged with 16,000 hemagglutination units of virulent RHDV 2 days after the last tuber extract ingestion. Only, the animal showing the highest level of anti-VP60 antibody titters survived. An ELISA analysis of the survivor serum indicated a further increase in specific antibody titters after the challenge (Table 1), supporting that this animal was only partially protected against virus infection. Similar results were previously reported by Plana-Duran et al. (1996) using VP60 particles produced in insect cells infected with recombinant baculovirus.

It should be also mentioned that although the three animals immunized with 500 μg of VP60 which had antibody titters from 1.28 to 1.4 log_{10} units did not survive the challenge, their mean dead times were in the range of 70 h after challenge, significantly latter than control or treated rabbits showing no measurable antibody titters which died within 48 h after RHDV administration. In spite of the low protection achieved in the immunization schedule summarized in this paper, these observations are relevant for future developments of oral vaccination protocols which should account for the high antigen doses required and the need of repeated antigen administration. Unfortunately, our data also indicated that the oral immununogenicity with recombinant VP60 could not be adequate using this approach thus requiring further studies to improve antigen concentration, stability and presentation to the gut immune system.

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