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Short Communication

A DNA vaccine encoding foot-and-mouth disease virus B and T-cell epitopes targeted to class II swine leukocyte antigens protects pigs against viral challenge

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ABSTRACT

Development of efficient and safer vaccines against foot-and-mouth disease virus (FMDV) is a must. Previous results obtained in our laboratory have demonstrated that DNA vaccines encoding B and T cell epitopes from type C FMDV, efficiently controlled virus replication in mice, while they did not protect against FMDV challenge in pigs, one of the FMDV natural hosts. The main finding of this work is the ability to improve the protection afforded in swine using a new DNA-vaccine prototype (pCMV-APCH1BTT), encoding FMDV B and T-cell epitopes fused to the single-chain variable fragment of the 1F12 mouse monoclonal antibody that recognizes Class-II Swine Leukocyte antigens. Half of the DNA-immunized pigs were fully protected upon viral challenge, while the remaining animals were partially protected, showing a delayed, shorter and milder disease than control pigs. Full protection in a given vaccinated-pig correlated with the induction of specific IFNγ-secreting T-cells, detectable prior to FMDV-challenge, together with a rapid development of neutralizing antibodies after viral challenge, pointing towards the relevance that both arms of the immune response can play in protection. Our results open new avenues for developing future FMDV subunit vaccines.

Foot-and-mouth disease (FMD) is one of the most devastating and contagious animal diseases that affects cloven-hoofed animals. Although useful to control and eradicate the disease from different regions around the world, traditional inactivated FMDV vaccines induce short-term protective responses, thus making re-vaccination of animals necessary to maintain protection. This is a serious limitation for implementation in many areas of the world. Moreover, classical FMDV vaccines involve the large-scale production, chemical inactivation and purification of infectious virus, which is risky and expensive. In addition, a cold chain must be maintained until vaccine distribution. Therefore, development of novel vaccines avoiding the problems associated with current vaccines, based on inactivated viruses, is important for FMD control (Laddomada, 2003; Uttenthal et al., 2010). Thus, different approaches have been explored, such as subunit vaccines, recombinant viruses or DNA vaccines, expressing FMD virus (FMDV) antigenic determinants (Beard et al., 1999; Cedillo-Barrón et al., 2001; Cubillos et al., 2008; Li et al., 2008; Niborski et al., 2006; Pacheco et al., 2005; Rodriguez and Grubman, 2009; Sanz-Parra et al., 1999; Sobrino et al., 1999; Wang et al., 2002; Wong et al., 2002). Previous results obtained in our laboratory had demonstrated that a DNA vaccine encoding B and T cell epitopes from the type C FMDV (C-S8c1), conferred protection in mice (Borrego et al., 2006), although results were less consistent when plasmids were used to immunize pigs (Ganges et al., 2011).

Besides the benefits concerning biosafety, easy production and storage, a remarkable advantage of DNA vaccines is the feasibility in obtaining complex vaccine formulations incorporating several protective antigens and/or marker genes to generate DIVA-vaccines. In addition, DNA vaccines offer the possibility of incorporating different strategies for optimization and/or modulation of the immune responses induced, such as those aiming to drive antigens to the sites where the immune responses take place, for instance by using single chain variable fragments (scFv) that recognize surface markers on antigen presenting cells (APCs) (Demangel et al., 2005; Nchinda et al., 2008). In this study we aimed at improving the immune response induced and the protection afforded against FMDV by fusing the FMDV B and T cell epitopes to the scFv of the 1F12 mouse monoclonal antibody (named as APCH1), that specifically recognizes Class II Swine Leukocyte Antigens (SLA II) and also the MHC II from other animal species (Bullido et al., 1997). Mouse and rabbit immunization with subunit

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vaccines previously demonstrated the adjuvant potential of APCH1 (Gil et al., 2011) and, more recently, these results have been extended to DNA vaccines encoding African swine fever virus (ASFV) antigens, both in mice and pigs (Argilaguet et al., 2011). Here we show that a DNA vaccine encoding FMDV B and T-cell epitopes fused to APCH1 (pCMV-APCH1BTT), improves the protection afforded to pigs, opening new avenues for developing future FMDV subunit vaccines.

The C-S8c1 FMDV sequences encoded in the DNA vaccine corresponded to three viral epitopes fused in tandem: the B cell epitope spanning residues 133–156 of the VP1 protein, and two T cell epitopes: one from the VP4 protein (residues 20–34) and the second being a highly conserved peptide from the 3A protein (residues 11–40) (Blanco et al., 2000, 2001; Cubillos et al., 2008). The ORF encoding these BTT epitopes was obtained from plasmid pCMV-spBTT (Ganges et al., 2011) after digestion with the restriction enzymes *BamH*I and *Bgl*II, and was subsequently subcloned into the unique *Bgl*II site of pCMV-APCH1 (Argilaguet et al., 2011), to obtain the plasmid pCMV-APCH1BTT, encoding BTT fused in tandem to the carboxy-terminus of APCH1 under the control of the CMV promoter.

Once the correct *in vitro* expression of the antigen-encoded plasmids by immunofluorescence was confirmed (data not shown), their immunogenicity was tested. Four Swiss outbread mice [Hsd:ICR (CD-1); Harlan] received 3 intramuscular injections of 100 μg of the endotoxin-free plasmid at 15 day-intervals (optimal protocol for mouse DNA immunization); 1 month after the last dose, mice were sacrificed and their splenocytes used to test the specific cellular responses by IFNγ-intracellular staining. For comparative analysis, a group of four mice was immunized with pCMV-BTT (encoding the FMDV epitopes alone) and finally, four extra mice were immunized with pCMV-APCH1 as a negative control for the assay. Spleen cells were *in vitro* cultured for 6 h either in the presence or absence of the three FMDV peptides encoded in the vaccine (1 μg/ml each) (Borrego et al., 2006). As expected, pCMV-BTT did not induce detectable responses before FMDV challenge (Borrego et al., 2006). Conversely, a strong positive response was observed in two of the four mice immunized with pCMV-APCH1BTT (mices 1 and 3 within the group), with both $CD4^+$ and $CD8^+$ T-cells specifically expressing IFNγ in response to ex vivo stimulation (Fig. 1). The presence of overlapping $CD8^+$ and $CD4^+$ T-cell epitopes in FMDV has been recently reported (Guzman et al., 2010) and might also explain the induction of specific $CD8^+$-T cell responses after immunization with pCMV-APCH1BTT. Finally, the xenoreactive potential of the 1F12 Balb/c mouse monoclonal antibody (Bullido et al., 1997; Brodersen et al., 1998), from which the APCH1 was derived (Gil et al., 2011), might also help to explain its capability to recognize the MHC II from 50% of the pCMV-APCH1BTT immunized outbred non-syngeneic swiss mice. These results add new evidence to the recently described immunopotentiation effect of APCH1 in mice both after subunit vaccination (Gil et al., 2011) and after DNA immunization (Argilaguet et al., 2011), confirming the potential of targeting vaccine-encoded antigens to professional antigen presenting cells (APCs) with specific ScFvs (Demangel et al., 2005; Gil et al., 2011; Nchinda et al., 2008).

In contrast to the potent cellular responses induced after immunization with pCMV-APCH1BTT, no specific antibody responses, neither by ELISA nor by neutralization, were detected in sera from any of the immunized animals (not shown).

To evaluate the protective capacity of pCMV-APCH1BTT in swine, four Landrace × Large White pigs (animals 1–4, weighing approximately 20 kg) were immunized with 3 doses of the plasmid (400 μg each) every 2 weeks (following an optimized DNA immunization protocol adapted to pigs). Two additional animals (pigs 5 and 6) received the plasmid pCMV-APCH1 as negative controls for the assay. Fifteen days after receiving the last DNA dose, all animals were needle-challenged in the coronary band with 10^4 pfu of FMDV C-S8c1. Progression of disease was evaluated daily using a score based on a semi-quantitative rating of clinical signs (fever, vesicle formation and size). The disease outcome in control pigs 5 and 6 was as expected for the C-S8c1 virus dose used for challenge (Cubillos et al., 2008). Pigs 2 and 4, showed no significant signs of disease during the 10 days monitoring period while pigs 1 and 3, showed a delay in disease onset, a shorter period of acute

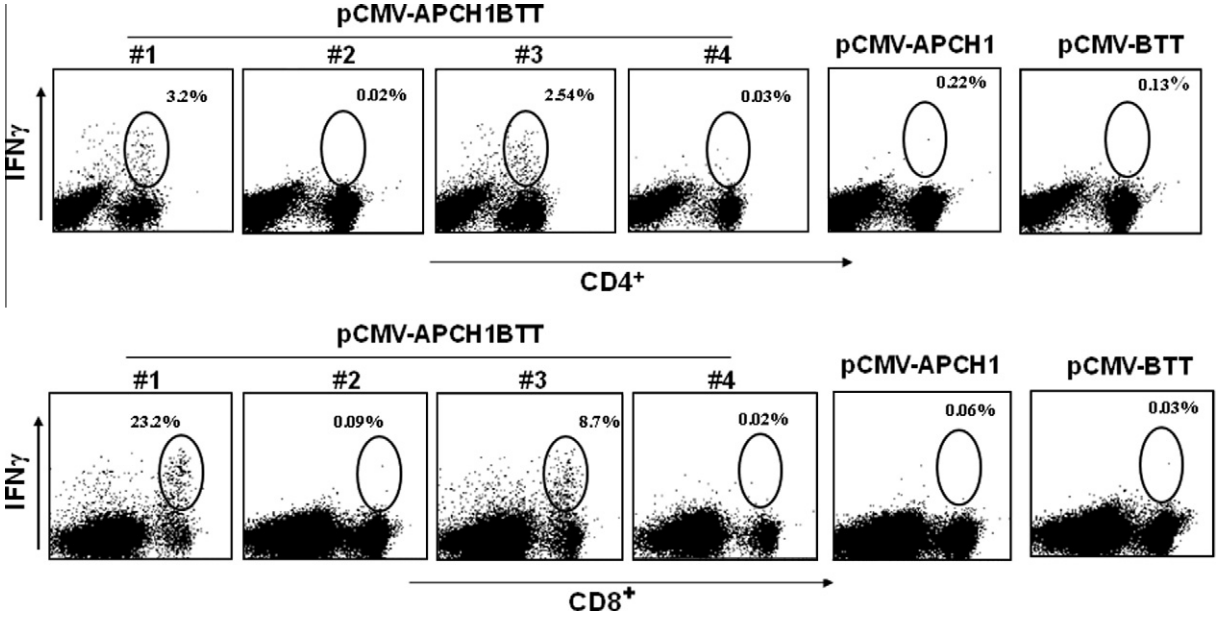
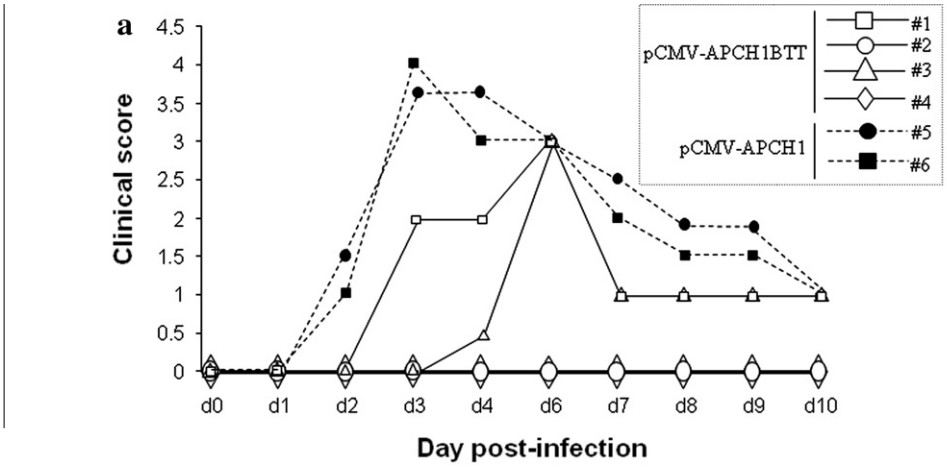


Fig. 1. pCMV-APCH1BTT induces specific T-cell responses in mice. Splenocytes from mice immunized with pCMV-APCH1BTT, pCMV-APCH1 or pCMV-BTT were *in vitro* stimulated with or without a mixture of the three specific FMDV synthetic peptides (1 μg/ml of each one), corresponding to those encoded in the DNA vaccine (B, T3A and TVP4). Values correspond to the percentage of peptide-specific $CD4^+$ (upper panel) or $CD8^+$ (lower panel) T-cells expressing IFN gamma (after subtracting the values obtained in cells incubated with medium alone). Results from only one mouse either immunized with either pCMV-BTT or with pCMV-APCH1 are shown to illustrate the lack of detectable specific responses observed in these two animal groups (undistinguishable between mice within the group).



b

Vaccines	Viremia[1]		Viral RNA in (N/P) swabs[2]		NSP seroc.[3]
	d2	d3	d2	d3	d10
pCMV-APCH1BTT #1	+	++	-/-	-/+	2.8
pCMV-APCH1BTT #2	-	-	-/-	-/-	0.5
pCMV-APCH1BTT #3	+	++	-/-	-/-	2.8
pCMV-APCH1BTT #4	-	-	-/-	-/-	1.2
pCMV-APCH1 #5	++	++	+/+	+/+	4.4
pCMV-APCH1 #6	++	++	+/+	+/+	4.8

Fig. 2. pCMV-APCH1BTT protects pigs from FMDV infection. (a) FMD clinical score. Semi-quantitative rating of FMD clinical signs (from 0 to 4), including: anorexia, fever, limping and vesicle formation (number, size and body localization). (b) Virus detection and seroconversion in DNA-immunized pigs after FMDV-challenge. [1]Results for both RT-PCR and isolation on cell culture are represented as: (−): negative for both techniques; (+): negative for the isolation technique but positive by RT-PCR; (++): positive for both techniques. [2]Results of conventional RT-PCR targeted to 3D are expressed as + (positive) or − (negative) for each one of the two swab samples (N:nasal/P:pharyngeal) analyzed. [3]Results expressed as the OD_{450} ratio of the corresponding serum from day 10 versus that of the pre-immune serum (day 0), assayed at a dilution 1/150 in a 3ABC-ELISA (duplicate). Values below 1.5 were considered as negative for the assay.

disease and small-sized vesicles that only appeared on the feet and tongue, when compared with those found in the control pigs (Fig. 2a).

Viral load in serum and swab samples collected at days 2 and 3 pc was analyzed by virus isolation and/or by RT-PCR targeted to FMDV 3D polymerase sequences (Rodriguez Pulido et al., 2009). In agreement with the lack of signs of disease, no virus could be detected in nasal and pharyngeal samples collected at different times post-FMDV challenge (pc) from pigs 2 and 4. Remarkably, viral RNA was only detected in pig 1, restricted to one of the four swab

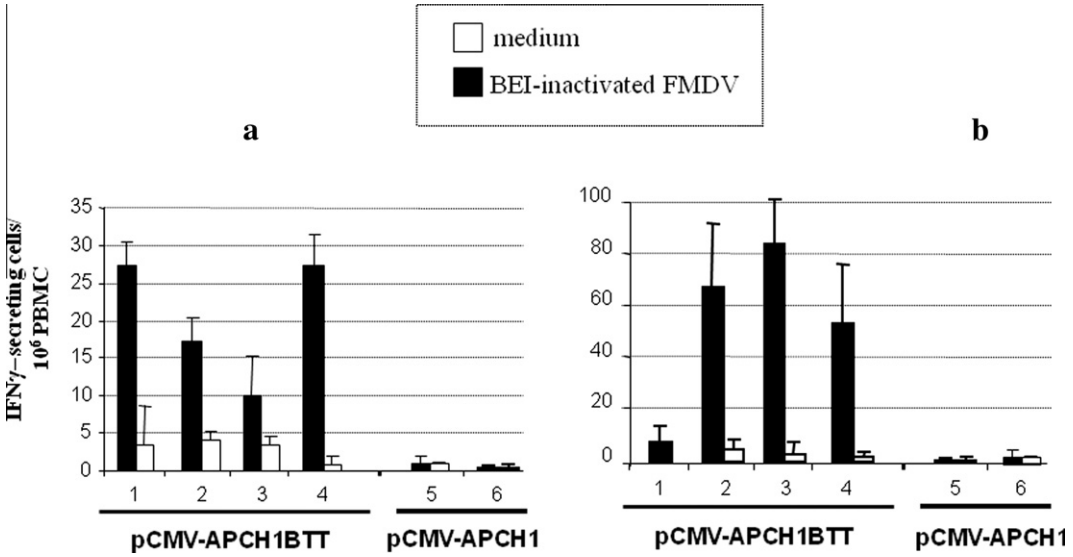


Fig. 3. Detection of IFNγ by ELISPOT in DNA-immunized pigs. Average number of IFN-gamma producing cells per 10^6 peripheral blood mononuclear cells (PBMCs) upon *in vitro* stimulation with: 10^5 pfu/ml of BEI-inactivated FMDV Cs8c1 (black bars) or medium alone (white bars) are shown. Standard deviation bars correspond to three replicates. Panel a: prior to viral challenge; panel b: at day 10 after viral challenge.

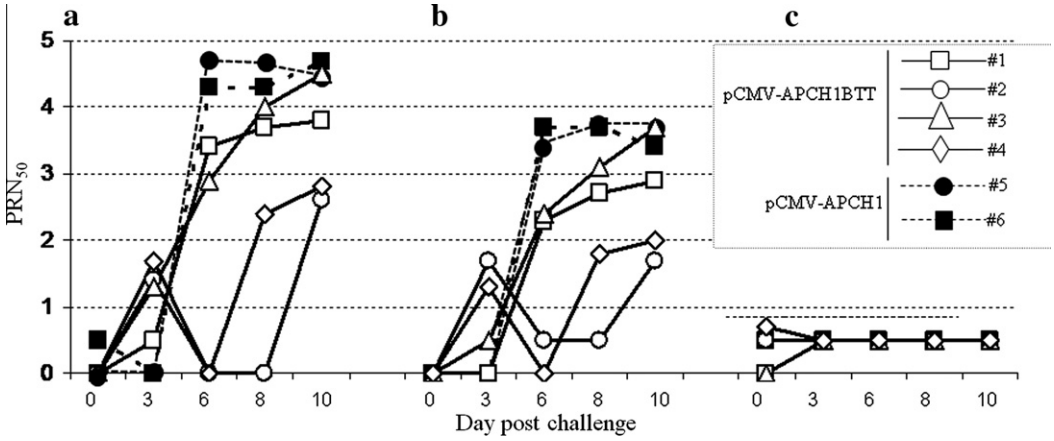


Fig. 4. Kinetics of seroneutralization in DNA-immunized pigs after viral challenge. Results are represented as PRN50, i.e., dilution of serum (log10) causing a reduction of 50% in the number of PFU in a plaque-reduction assay (21). Samples assayed were collected at day 43 post immunization (corresponding to day 0 relative to viral challenge), and at days 3, 6, 8 and 10 pc. Viral reductions are represented relative to the viral titers obtained with preimmune sera (collected at day 0 of the experiment, prior to DNA immunization). The viruses used in the assays were: FMDV C-S8 (panel a), FMDV C3Arg (panel b) and FMDV O1 Campos (panel c). Results are the means of at least two independent experiments.

samples analyzed, suggesting a limited viral replication at least at the mucosal level (Fig. 2b). Whether this limited amount of virus could prevent in-contact transmission remains to be studied. The capacity of FMDV vaccines to control nasal shedding is important, since airborne excretion from infected animals is one of the main routes for virus transmission (Alexandersen and Mowat, 2005). Due to the fact that most quantitative data about FMDV in contact transmission comes from work recently reported in cattle infected with type O FMDV (Charleston et al., 2011), no further conclusions could be derived from our pig-experiment, especially taking into account the limited number of animals used.

Finally, the reduction in viral replication observed in pigs 1 and 3 was consistent with the low levels of seroconversion to the non-structural 3ABC FMDV polyprotein (Rodríguez et al., 1994) observed in the sera of these animals at day 10 pc, a time when control pigs 5 and 6 showed high antibody titers against these FMDV antigens. Consistent with the lack of viremia and nasal shedding, no antibodies against 3ABC were detected in sera from pigs 2 and 4 (Fig. 2b).

Since levels of neutralizing antibodies correlate with protection against FMDV (McCullough, 2004; van Bekkum, 1969), we analyzed the neutralizing activity in serum samples collected prior to viral challenge by a plaque reduction assay (Mateu et al., 1988). As previously found for mice, neither total anti-FMDV antibodies by ELISA (not shown) nor neutralizing antibodies were detected in any of the animals immunized with pCMV-APCH1BTT at the time of challenge (see Fig. 4a, day 0). These results strongly suggest that the protection afforded by pCMV-APCH1BTT was, at least, partially mediated by cellular immunity.

Supporting this hypothesis, all pigs that received pCMV-APCH1BTT showed specific T cell-responses before FMDV-challenge (Fig. 3a), detectable by IFNγ-ELISPOT (Diaz and Mateu, 2005), even after the administration of only one vaccine dose in some of the animals (data not shown). Interestingly enough, the only pig from the pCMV-APCH1BTT group that showed detectable pharyngeal virus shedding (pig 1), did not show specific T-cell responses upon *in vitro* stimulation with BEI-inactivated FMDV (Fig. 3b).

In contrast to the role played by neutralizing antibodies, little is known about the cellular mechanisms involved in protection against FMDV. Lessons learned while using emergency vaccines, clearly demonstrated the relevance that innate immune responses can play in protection against FMDV, even in the absence of detectable antibodies (Barnard et al., 2005). Furthermore, the potential antiviral activity of IFNγ against FMDV, both *in vitro* and *in vivo*, has already been reported (Eble et al., 2006; Moraes et al., 2007; Parida et al., 2006; Zhang et al., 2002).

Despite the lack of detection of neutralizing antibodies before challenge, pigs immunized with pCMV-APCH1BTT developed significant neutralizing antibody titers earlier than control pigs after FMDV infection (Fig. 4a). Thus, neutralizing antibodies were detected in control pigs 5 and 6 from day 6 pc, while in pigs 2, 3 and 4 they were detected (PRN50 between 1.0 and 2.0) as soon as day 3 pc. As observed with T-cell responses, pig 1 was the only immunized animal that did not show a clear acceleration of the induction of neutralizing antibodies (Fig. 4a). This activity was serotype specific since both C-S8c1 and FMDV C3 Argentina – a type C isolate of a different subtype but with antigenic resemblance to the strain used for challenge (Mateu et al., 1988) – were efficiently neutralized by sera taken from the immunized animals after FMDV infection (Fig. 4b), while type O FMDV O1-Campos (Fig. 4c) and type A FMDV A5-Westerwald (data not shown) were not neutralized. The bimodal induction of neutralizing antibodies observed in pigs 2 and 4, correlated with the lack of viremia found in these two animals. Lack of detection of neutralizing activity in sera at day 6 pc might reflect an efficient blocking of FMDV early after the infection followed by the efficient clearance of the immunocomplexes from the blood stream, therefore helping to explain the full protection observed in pigs 2 and 4. The mechanisms behind these effects are currently being studied. From our results, no antibody-mediated protection should be expected against any of the seven major regional pools of FMDV currently in circulation (Domenech et al., 2010), as none of them belong to the C-subtype. However, the picture might be different regarding T-cell cross-protection. Thus, preliminary results from our laboratory point towards the highly conserved 3A peptide as being the main T-cell determinant included within the pCMV-APCH1BTT (data not shown), albeit further analyses are needed to identify both the exact nature of the T-cell responses induced and the peptide/s presented.

In summary, the results presented here show that half of the pigs vaccinated with pCMV-APCH1BTT were fully protected against FMDV challenge, while the other half remained partially protected. Full protection correlated with the induction of specific IFNγ-secreting T-cells, detectable prior to FMDV-challenge, together with a rapid development of neutralizing antibodies after viral challenge. These results confirm recent data obtained in our laboratory (Argilaguet et al., 2011) and make us optimistic about

the future use of APCH1 as a genetic adjuvant in DNA immunization protocols. We are currently extending these studies in two complementary directions, aiming at improving the vaccine coverage: including additional FMDV-B and T-cell determinants with protective potential in our DNA vaccines and trying to improve the induction of both neutralizing antibodies and cellular responses by prime-boost strategies, similar to those currently used to improve the protection afforded against human and animal diseases, including FMD (Li et al., 2008). Both the durability and the cross-protective ability of our DNA vaccines will be compared to the traditional inactivated FMD vaccines.

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