

Characterization of an enhanced antigenic change in the pandemic 2009 H1N1 influenza virus haemagglutinin

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Murine hybridomas producing neutralizing mAbs specific to the pandemic influenza virus A/California/07/2009 haemagglutinin (HA) were isolated. These antibodies recognized at least two different but overlapping new epitopes that were conserved in the HA of most Spanish pandemic isolates. However, one of these isolates (A/Extremadura/RR6530/2010) lacked reactivity with the mAbs and carried two unique mutations in the HA head (S88Y and K136N) that were required simultaneously to eliminate reactivity with the murine antibodies. This unusual requirement directly illustrates the phenomenon of enhanced antigenic change proposed previously for the accumulation of simultaneous amino acid substitutions at antigenic sites of the influenza A virus HA during virus evolution (Shih *et al.*, *Proc Natl Acad Sci USA*, **104**, 6283–6288, 2007). The changes found in the A/Extremadura/RR6530/2010 HA were not found in escape mutants selected *in vitro* with one of the mAbs, which contained instead nearby single amino acid changes in the HA head. Thus, either single or double point mutations may similarly alter epitopes of the new antigenic site identified in this work in the 2009 H1N1 pandemic virus HA. Moreover, this site is relevant for the human antibody response, as shown by competition of mAbs and human post-infection sera for virus binding. The results are discussed in the context of the HA antigenic structure and challenges posed for identification of sequence changes with possible antigenic impact during virus surveillance.

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INTRODUCTION

Influenza viruses (family *Orthomyxoviridae*) have two major surface glycoproteins inserted into the viral envelope, the receptor-binding haemagglutinin (HA) and the receptor-destroying enzyme neuraminidase (NA) (for a review of structural and biological properties, see Gamblin & Skehel, 2010). The most effective neutralizing antibodies are directed against the HA molecule, interfering with

receptor binding. Protection against influenza infections correlates with high levels of circulating neutralizing antibodies (Couch & Kasel, 1983).

Antigenic differences have been used to classify influenza type A viruses into 16 HA and 9 NA subtypes (Wright *et al.*, 2007). Recently new HA (H17 and H18) and NA subtypes (NA10 and N11) were identified by sequence analysis of influenza viruses isolated from bats (Tong *et al.*, 2012, 2013). Only viruses of the H1N1, H2N2 or H3N2 subtype have circulated in humans, although sporadic infections with viruses of other subtypes have been reported (Koopmans *et al.*, 2004; Uyeki & Cox, 2013; WHO, 2011). Introduction into the human population of a new subtype leads to major

The GenBank/EMBL/DDBJ accession numbers for the HA sequences determined in this study are: KF860844 (A/Castille La Mancha/4430/2009), KF860845 (A/Castille La Mancha/5911/2009) and KF860846 (A/Extremadura/RR6530/2010).

antigenic changes (antigenic shift) of circulating viruses, resulting in high infection rates (pandemic) of the immunologically naïve population. When influenza viruses evolve in humans, minor antigenic changes accumulate within subtypes (antigenic drift) as a result of the immune pressure enforced by neutralizing antibodies present in a human population previously infected with related viruses (Wright *et al.*, 2007). Antigenic drift leads to yearly (seasonal) epidemics of influenza infections in the winter months and accumulation of amino acid changes in the immunodominant globular head of the HA homotrimer, near the receptor (sialic acid)-binding site (Knossow & Skehel, 2006). This antigenic drift determines the requirement for vaccine updates.

The first influenza pandemic of the 21st century emerged in 2009 and quickly spread worldwide, causing mostly mild symptoms but also a number of severe cases in young adults without underlying disease (Bautista *et al.*, 2010). The virus, named A(H1N1)pdm09, was a reassortant containing sequences of avian, swine and human influenza viruses (Garten *et al.*, 2009; Smith *et al.*, 2009). The HA sequence of A(H1N1)pdm09 was found to be closely related to the current H1N1 subtype swine viruses, circulating in pigs at least since the 1930s without drifting significantly (Garten *et al.*, 2009; Smith *et al.*, 2009), and related to the human H1N1 that caused the 1918 Spanish influenza pandemic (Masoodi *et al.*, 2012). In contrast, the HAs of human H1N1 viruses causing seasonal outbreaks after 1977 but before 2009 had diverged extensively from the earliest 1918 H1N1 virus (Tumpey *et al.*, 2004). These results can explain the reported presence of antibodies that neutralize pandemic A(H1N1)pdm09 virus in individuals born prior to 1918 but their absence in younger subjects (Hancock *et al.*, 2009; Itoh *et al.*, 2009). In fact, some human mAbs directed against the 1918 HA, obtained using blood cells from survivors of the 1918 influenza pandemic (Yu *et al.*, 2008), were found to neutralize a prototypic virus, A/California/04/2009 (A/Cal/07/09), of the A(H1N1)pdm09 pandemic (Krause *et al.*, 2010; Xu *et al.*, 2010).

While developing reagents to study and characterize the HA of A(H1N1)pdm09 viruses, we obtained four murine mAbs that bound and neutralized pandemic viruses isolated in Spain between 2009 and 2011 but failed to react with seasonal H1N1 viruses isolated before 2009. However, the four antibodies failed to react with a Spanish strain, A/Extremadura/RR6530/2010 (A/Extr/6530/10), isolated from a patient during the pandemic wave. Sequence comparison identified three amino acid changes in the HA head that were unique to this virus. Introduction of these changes in a cloned HA revealed that two were simultaneously required for loss of reactivity with the noted mAbs. In contrast, escape mutants selected with one of the mAbs contained single amino acid substitutions in nearby positions of the HA head. These results provide structural insights about an enhanced antigenic change in the pandemic influenza HA at a site shown to be relevant for the human antibody response.

RESULTS

Isolation and characterization of neutralizing mAbs specific for the influenza HA of pandemic H1N1 viruses

Four hybridomas (named α -HACal/1–4) that produced neutralizing mAbs specific for the influenza HA of the pandemic 2009 virus prototype, A/Cal/07/09 were obtained as described in Methods. The HA specificity of these antibodies was confirmed by ELISA reactivity with extracts of cells infected with a recombinant vaccinia virus expressing the A/Cal/07/09 HA antigen (see below). ELISA binding also showed that the four mAbs recognized a panel of pandemic viruses isolated in Spain between 2009 and 2011 [exemplified in Fig. 1a by the /Castille La Mancha/4430/2009 (A/CLM/4430/09) and A/Castille La Mancha/5911/2009 (A/CLM/5911/09) strains], but failed to react with another virus (A/Extr/6530/10) isolated also in Spain at about the same time (Fig. 1a). The four mAbs also did not react with seasonal H1N1 viruses isolated before 2009, illustrated in Fig. 1(a) by the H1N1 seasonal A/New Caledonia/20/1999 (A/New Cal/20/99). The latter virus did not bind antibodies of a rabbit hyperimmune serum raised against A/Cal/07/09 HA, which in contrast reacted with the HA of all pandemic H1N1 isolates, including A/Extr/6530/10 (Fig. 1a, right panel).

In agreement with the binding results, the antibodies α -HACal/1–4 neutralized the infectivity of the prototype A/Cal/07/09 strain as well as the Spanish isolates A/CLM/4430/09 and A/CLM/5911/09. However, they failed to neutralize A/Extr/6530/10 and A/New Cal/20/99, even at the highest concentration tested (Fig. 1b).

As the four mAbs of Fig. 1 competed with each other for antigen binding (not shown) and behaved similarly in the ELISA binding and in the neutralization test, and the four hybridomas were obtained from splenocytes of a single mouse, it was conceivable that they were derived from related lymphocyte clones. Indeed, sequencing of the first 420 nt of their heavy chain variable regions indicated that mAbs α -HACal/1 and α -HACal/2 were highly related but had four nucleotide differences, two of them being translated into amino acid changes. Likewise, mAbs α -HACal/3 and α -HACal/4 were highly related in this region but differed in 13 nt and 8 aa (not shown). Additionally, reactivity with isotype-specific reagents showed that mAbs α -HACal/1 and α -HACal/2 were IgG1, whereas mAbs α -HACal/3 and α -HACal/4 were IgG2a (not shown). Therefore, the four hybridomas must have been derived from at least two distinct clones that produced antibodies of similar but not identical specificities.

Identification of mutations in the A/Extr/6530/10 HA that eliminate reactivity with the murine mAbs

Sequence comparison of the A/Cal/07/09 HA with contemporary Spanish isolates showed a limited number of amino acid changes. Most of these changes were shared

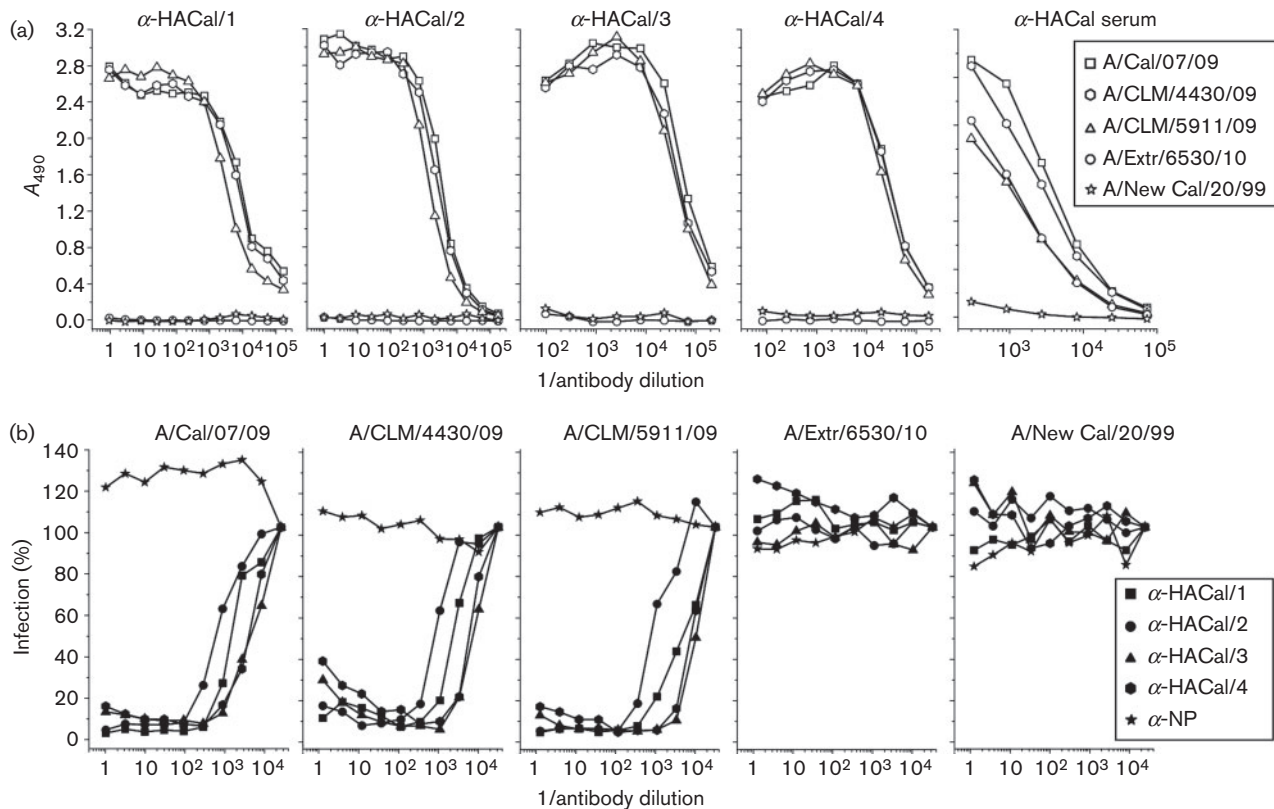


Fig. 1. ELISA binding and neutralization of A(H1N1)pdm09 pandemic viruses by murine mAbs. (a) Serial dilutions of culture supernatants (x -axis) containing the mAbs (α -HACal/1–4) as indicated were tested for binding by ELISA [measured as absorbance at 490 nm (A_{490})] to extracts of Madin–Darby canine kidney cells infected with the indicated viruses. A/Cal/07/09 is the pandemic prototype and A/New Cal/20/99 is a seasonal H1N1 vaccine strain. α -HACal is a control hyperimmune serum raised in rabbits inoculated with a recombinant vaccinia virus expressing the A/Cal/07/09 HA, as described in Methods. (b) Serial dilutions of culture supernatants containing the indicated antibodies were tested in a microneutralization assay with a fixed amount of the indicated viruses. α -NP is an anti-nucleoprotein (NP) mAb (M58/p44/E) described previously (Sánchez-Fauquier *et al.*, 1987), used as a non-neutralizing control. The amount of viral antigen produced 24 h after infection was estimated by ELISA, as described in Methods, and is shown as a percentage of each virus infection in the absence of antibody.

by multiple Spanish isolates (shown in Fig. 2 for three of these), but A/Extr/6530/10 had three unique amino acid changes, S88Y, K136N and H155Y (Fig. 2, ovals) which were not present in any of the other viruses. Furthermore, these three changes were located in the HA1 subunit of the HA molecule, which normally bears the sequence changes that impact on HA antigenicity (Knossow & Skehel, 2006).

To identify which of the three amino acid changes unique to A/Extr/6530/10 HA was responsible for the loss of reactivity with the indicated mAbs, the A/Cal/07/09 HA gene was cloned into the pRB21 plasmid, and mutants containing the S88Y, K136N and H155Y changes either individually or in double and triple combinations were made. The corresponding HA proteins were transiently expressed in A549 cells, which were first infected with the vTF7-3 vaccinia virus (expressing T7 RNA polymerase) and then transfected with the pRB21 plasmids encoding

either WT A/Cal/07/09 or mutant HAs. These proteins were visualized by immunofluorescence 24 h after transfection. None of the individual mutations S88Y, K136N or H155Y eliminated the reactivity with any of the four mAbs, as shown in Fig. 3 for the α -HACal/2 and α -HACal/3 antibodies. However, the combination of S88Y plus K136N, either as a double mutant or as a triple mutant with H155Y, abolished the mAbs reactivity, whilst still retaining reactivity with a hyperimmune rabbit serum raised against purified A/Cal/07/09 virus. Three main conclusions can be drawn from the results of Fig. 3: (i) none of the individual changes, S88Y, K136N or H155Y, was sufficient to eliminate the reactivity of the mAbs with A/Cal/07/09 HA; (ii) the amino acid changes S88Y and K136N were needed simultaneously to inhibit binding of the mAbs to A/Cal/07/09 HA; and (iii) the H155Y substitution, either individually or in combination with the change S88Y or K136N, did not influence the reactivity of A/Cal/07/09 HA with the mAbs.

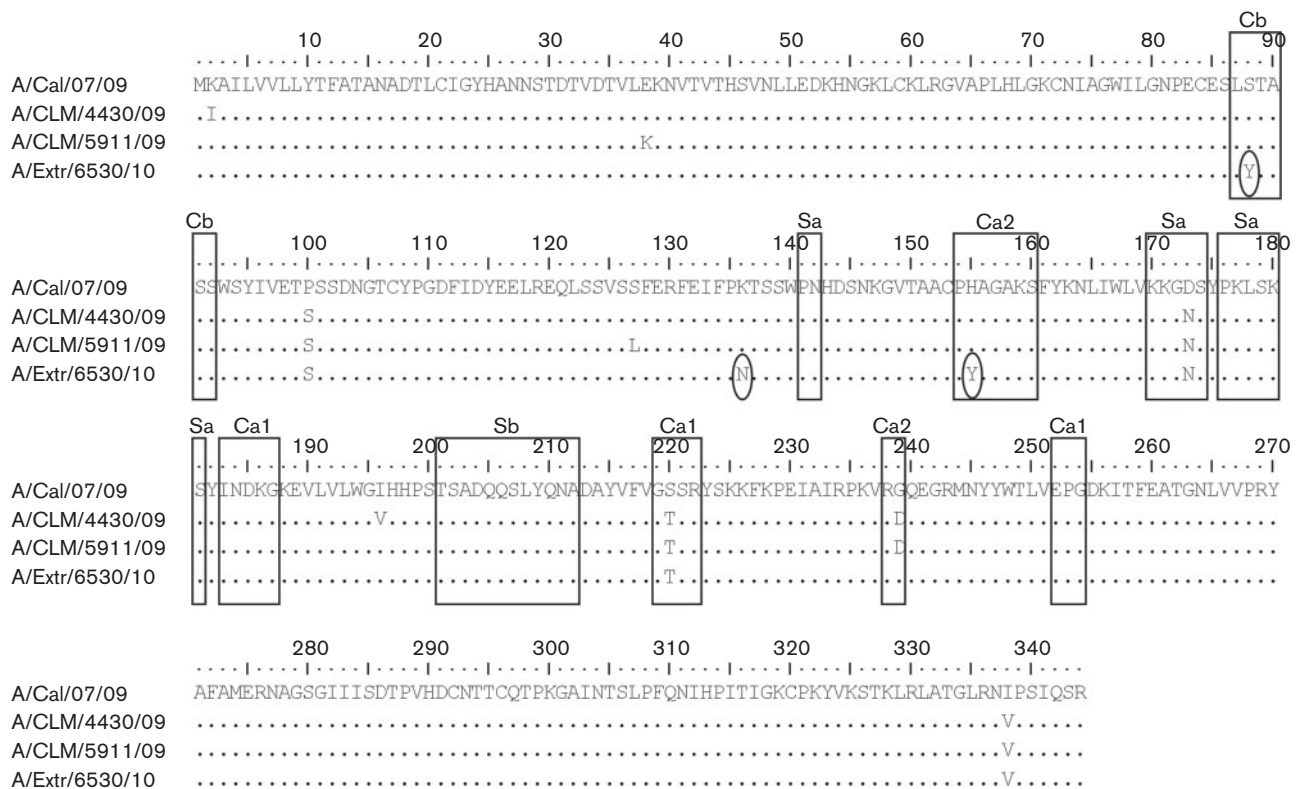


Fig. 2. HA sequence alignment of the pandemic viruses indicated. The HA numbering system used in this study starts with the first Met residue of the polypeptide chain. For comparison, the first residue using the H1 numbering proposed by Caton *et al.* (1982) is Asp18. Only the amino acid sequence of the HA1 subunit is shown, as no relevant changes were found in the HA2 subunit. The GenBank accession numbers of the entire HA sequences are: A/Extr/6530/10, KF860846; A/CLM/5911/09, KF860845; A/CLM/4430/09, KF860844. Residues of the antigenic sites (Ca1, Ca2, Cb, Sa and Sb) identified in the A/PR/8/34 HA by Caton *et al.* (1982) are boxed. The amino acid changes unique in A/Extr/6530/10 are enclosed in ovals. The A/Cal/07/09 HA nucleotide sequence published in GenBank (FJ966974) differs from ours at two positions, A517G and A716G, which are translated into amino acid differences N173D and D239G, respectively.

When the reverse substitutions Y88S, N136K and Y155H were introduced either individually or in combination as two or three changes in the A/Extr/6530/10 HA, the results obtained confirmed that Y88 and N136 were required simultaneously to block binding of the four mAbs (not shown). Thus, the additional amino acid changes that distinguish A/Extr/6530/10 from A/Cal/07/09 HA did not influence recognition of the respective antigens by the mAbs.

Antibody α -HACal/2 selects escape mutants with single amino acid changes in the A/Cal/07/09 HA head

The majority of influenza virus escape mutants selected with HA-specific mAbs bear single amino acid sequence changes that obliterate the corresponding epitopes (Caton *et al.*, 1982; Wiley *et al.*, 1981). It was thus unusual that the A/Extr/6530/10 HA required two simultaneous changes for the loss of mAb epitopes. To test whether these epitopes have structural constraints that prevented selection and/or survival of mutants with single amino acid changes, the

prototype A/Cal/07/09 virus was plaque titrated in the presence of an excess of mAb α -HACal/2. The virus titre was reduced by 5 log units in the presence of the antibody, but several plaques that grew at low virus dilutions in the presence of mAb α -HACal/2 were isolated and confirmed to be resistant by replaques in the presence of that antibody. Sequence analysis revealed one of the following single changes in the HA of different escape mutants: G80K, T89K or K163E (Fig. 4, insert). Strikingly, the mutant R1 (change T89K) was fully resistant to neutralization by the four α -CalHA/1 to -4 antibodies, similar to the resistance conferred by the double amino acid change S88Y/K136N, unique to A/Extr/6530/10 (Fig. 4a). In contrast, the other two mutants (changes G80K or K163E) were neutralized by α -HACal/3 and -4, although at slightly higher concentrations than A/Cal/07/09, but were still resistant to neutralization with α -HACal/1 and -2 antibodies. The neutralization results correlated with the ELISA binding of the four antibodies to the different viruses (Fig. 4b). Thus, α -HACal/3 and -4 were still able to bind to HA mutants R6 and R12, although with lower

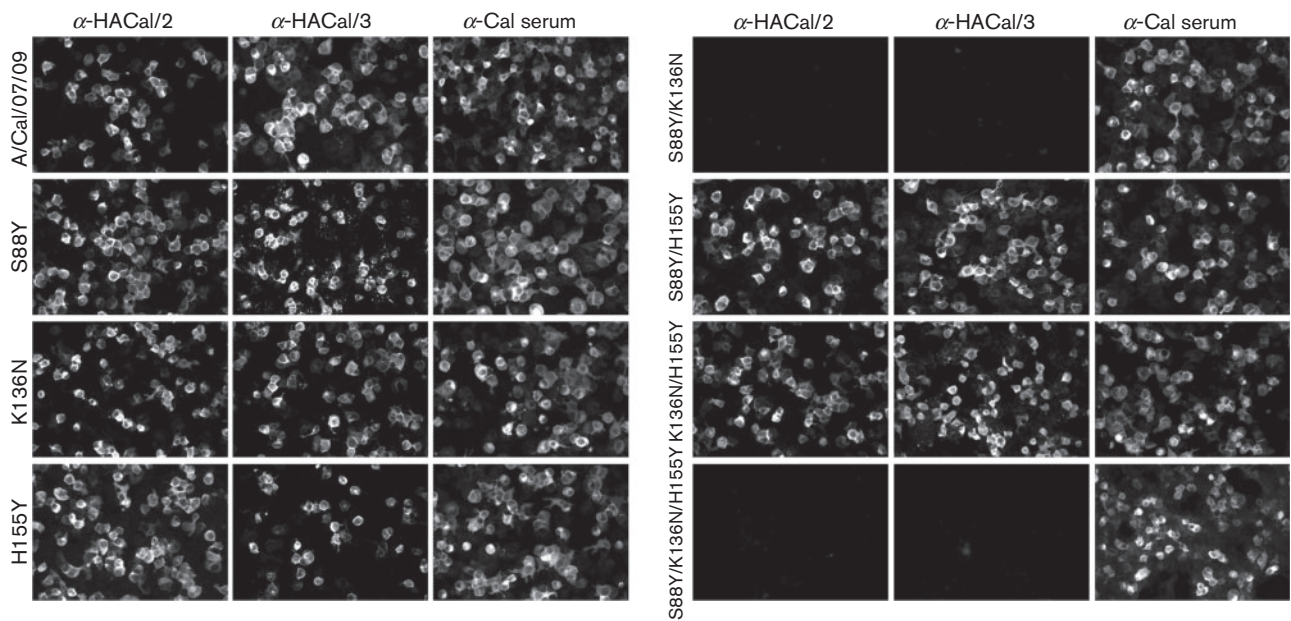


Fig. 3. Immunofluorescence of transfected cells expressing the WT or mutant HAs. A549 cells were infected at an m.o.i. of 10 with vaccinia virus vTF7-3, which expresses T7 RNA polymerase. Following infection, the cells were transfected with pRB21 plasmids bearing the genes of either the WT A/Cal/07/09 HA or the single, double or triple mutants indicated. At 24 h post-transfection, the cells were fixed and immunostained with the antibodies indicated. α -Cal serum is a hyperimmune serum raised in rabbits against purified A/Cal/07/09 virus.

strength than to A/Cal/07/09 HA. These results reinforced the idea that at least two different but overlapping epitopes are being recognized by the four mAbs and that in this case neutralization is directly related to antibody avidity.

Fig. 5 shows the location in the A/Cal/07/09 HA trimer of the amino acid changes selected in escape mutants and those of the A/Extr/6530/10 virus relevant for mAb reactivity. All the changes are in the HA head. The two unique changes in A/Extr/6530/10 that suppressed reactivity with the mAbs α -HACal/1–4 are partially buried. This finding may explain the simultaneous requirement for S88Y and K136N for loss of reactivity with the mAbs, as individual amino acid changes in partially buried sites may not be sufficient to block interaction with mAbs. The change T89K selected in one of the escape mutants distorts a surface bulge of the HA head. This major structural change might explain the high impact of the T89K mutation on binding of the four mAbs to the HA molecule. In contrast, the other two escape mutant changes (G80E and K163E) are in residues less exposed to the solvent, which might explain their limited effect on binding of the α -HACal/3 and -4 antibodies (Fig. 4).

The α -HACal/2 epitope is relevant in the human antibody response

As the mAbs described here were of murine origin, it was important to explore the relevance of their epitopes for the human immune response. To this aim, post-infection sera

of individuals that were diagnosed as positive for infection with A(H1N1)pdm09 viruses by PCR of respiratory specimens were used to compete with the binding of mAb α -HACal/2 to purified A/Cal/07/09 virus. Fig. 6(a) shows that most sera competed with this binding with different strengths. The strength of competition roughly reflected the serum neutralizing titre (compare Fig. 6a and b). It is worth noting that the neutralizing strength and competition capacity of human sera range from very low titres, i.e. inhibited infectivity only at very low dilutions (e.g. 400a and 571c), to sera that inhibited virus infectivity almost as effectively as the hyperimmune α -HACal rabbit serum described in Fig. 1 (e.g. 1402, 5146 and 5429).

DISCUSSION

The four mAbs described in this study recognize new neutralizing epitopes in the HA molecule of A(H1N1)pdm09 pandemic viruses that are altered in the A/Extr/6530/10 Spanish strain. Sequence comparison identified three unique amino acid differences that distinguished A/Extr/6530/10 from other pandemic isolates that reacted with the mAbs. Site-directed mutagenesis of the A/Cal/07/09 HA demonstrated that the S88Y and K136N changes were required simultaneously for loss of antibody binding, whereas the third change (H155Y) was irrelevant for antibody recognition. The first two changes are in residues partially buried in the HA head (Fig. 5). The single change K136N has been reported previously by O'Donnell *et al.* (2012) (K123N with

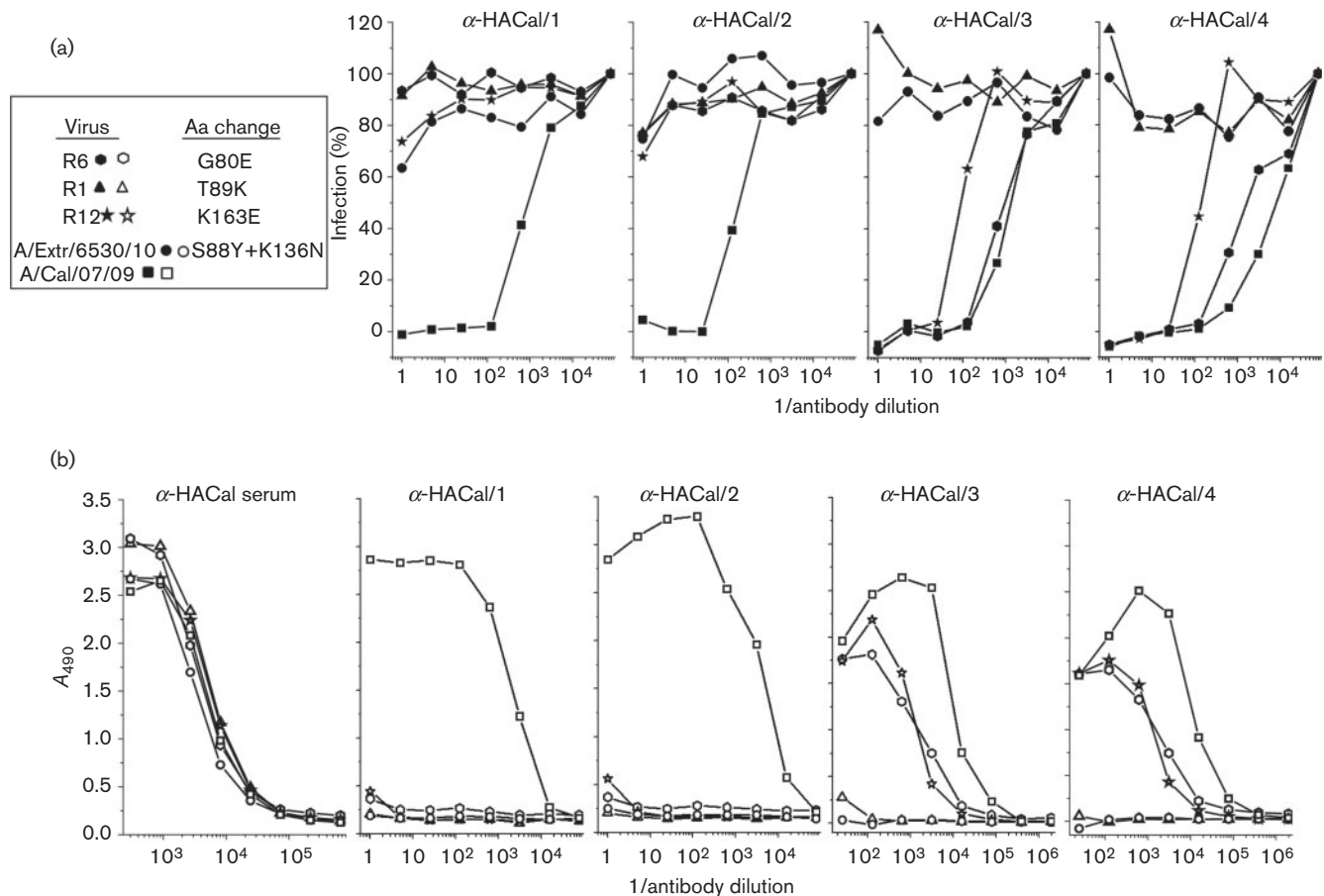


Fig. 4. A/Cal/07/09 escape mutants selected with mAb α -HACal/2. (a) The insert shows the amino acid changes selected in escape mutants R1, R6 and R12 obtained with mAb α -HACal/2. Also included are the unique sequences in A/Extr/6530/10 HA. Each graph shows the microneutralization of viruses from the insert with the indicated mAbs. Results are presented as in Fig. 1. (b) ELISA binding results of the indicated antibodies with extracts of MDCK cells infected with the different viruses. α -HACal is the same hyperimmune control serum as in Fig. 1. Closed symbols are used for viruses in the microneutralization test and the same open symbols are used for viruses in the ELISA (see insert).

their numbering system) in an escape mutant of A/Cal/07/09 resistant to the human mAb EM4C04. These authors argued that, as K136N introduces a new potential glycosylation site in the virus HA, the loss of reactivity with EM4C04 antibody might be a consequence of this new glycosylation. Whilst this might be true for their antibody, it is not the case for the murine mAbs described in the present study, as none of the mAbs lost reactivity with the single mutant K136N. Therefore, the most likely explanation for the loss of the murine mAb epitopes in A/Extr/6530/10 is that the simultaneous changes S88Y and K136N substantially altered the local structure of the influenza HA at the site of antibody binding.

It is doubtful that antibody-driven immune pressure played a role in the selection of the A/Extr/6530/10 virus, as it was obtained from a 20-year-old male unlikely to have been exposed previously to a virus related to the A(H1N1)pdm09 pandemic. It seems also that the pair of

changes S88Y and K136N (unique to A/Extr/6530/10) does not confer a selective advantage for propagation in humans, as this unique combination of changes has not been found in more than 800 Spanish pandemic viruses sequenced to date. Furthermore, A/Extr/6530/10 virus was indistinguishable from other H1N1 pandemic viruses in standard haemagglutination inhibition assays done with either reference antisera raised against the current vaccine virus A/Cal/7/2009 (reference sheep antiserum lot 58842141, WHO Influenza Reagent kit; Centers for Disease Control and Prevention, GA, USA) or post-infection ferret antiserum obtained from the WHO Collaborating Centre (European Region, London, UK) (not shown). A/Extr/6530/10 virus was also similar to other pandemic virus in both ELISA binding (Figs 1a and 4b) and neutralization (not shown) with the rabbit α -HACal serum reported here. Thus, the unique changes in the A/Extr/6530/10 HA did not have an overall impact on HA antigenicity but rather had a restricted influence on the

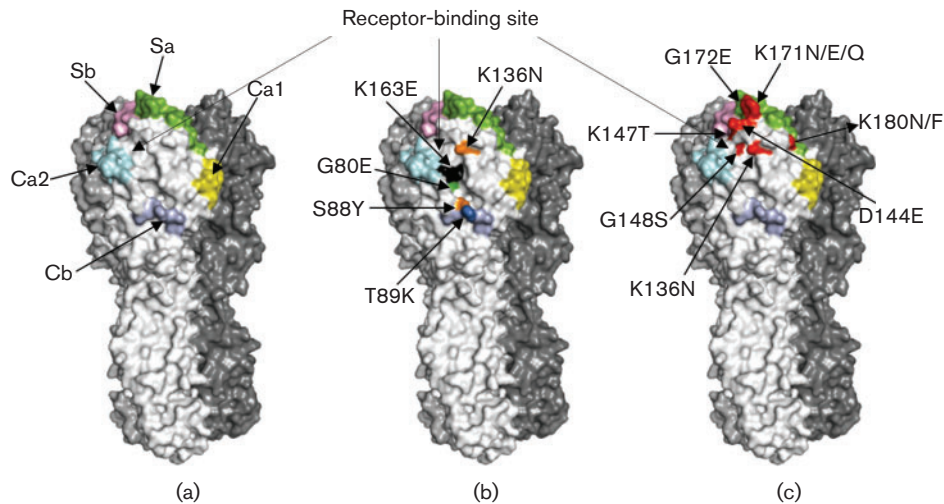


Fig. 5. Location of amino acid changes in the three-dimensional HA structure. (a) The HA trimer was built with PyMol using PDB 3AL4 (Zhang *et al.*, 2010). The previously identified antigenic sites of the H1 subtype indicated in Fig. 2 are highlighted with different colours in the front subunit. (b) Residues with changes described in the current study are indicated with different colours in the front subunit. (c) The location of changes in escape mutants of A/Cal/07/09 reported by other laboratories (Krause *et al.*, 2010; Manicassamy *et al.*, 2010; O'Donnell *et al.*, 2012) are shown in red.

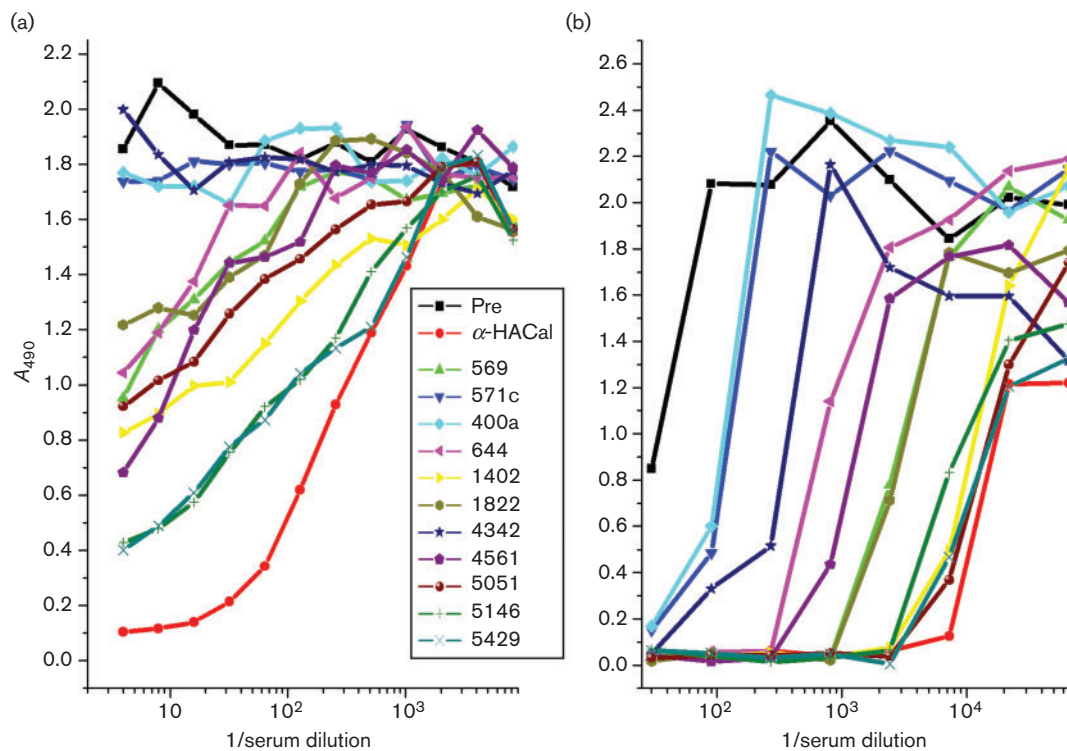


Fig. 6. Competition of mAb α -HACal/2 binding by human post-infection sera. (a) Serial dilutions of the human sera indicated by numbers in the insert were incubated with a non-saturating (pre-titrated) amount of mAb α -HACal/2 before being added to microtitre plates coated with an extract of cells infected with A/Cal/07/09 virus. ELISA was then carried out with murine-specific reagents to assess mAb binding. (b) Serial dilutions of the same sera were tested for inhibition of A/Cal/07/09 infectivity in the microneutralization assay described in Methods. Pre, pre-immune serum of the same rabbit used to obtain the hyperimmune α -HACal serum shown in Fig. 1.

epitopes recognized by mAbs α -HACal/1 to -4. Nevertheless, the results reported here emphasize that sequence changes in HA that may not have a direct effect on antigenicity could predispose to antigenic changes upon incorporation of accompanying mutations.

The simultaneous fixation of two or more amino acid changes was noted when a large number of HA sequences from natural isolates of the H3 subtype were analysed in the context of antigenic changes accumulated over a period of 37 years (Shih *et al.*, 2007). Several situations were found in which individual mutations were not enough to confer an antigenic transition but combinations of two or more changes were fixed together and impacted on HA antigenicity (Shih *et al.*, 2007; Smith *et al.*, 2004). Whether the simultaneous fixation of mutations was related to enhancement of antigenic drift, compensatory mutations or hitchhiking could not be discerned. In this sense, the situation found in A/Extr/6530/10 is a direct example of the phenomenon of enhanced antigenic change in influenza virus HA, as loss of antibody binding was observed in transfected cells where compensatory or hitchhiking mutations are unlikely to play any role.

Nonetheless, a recent publication by Koel *et al.* (2013) demonstrated that a limited number of mutations near the receptor-binding site of the influenza HA have a major effect on antigenic drift, even if accompanied by other amino acid changes. Whether or not there is an analogous hierarchy in the unique amino acid changes of A/Extr/6530/10 HA remains to be determined.

In contrast to the situation found in the A/Extr/6530/10 virus, *in vitro* selected escape mutants of A/Cal/07/09 contained single amino acid substitutions that eliminated the epitope recognized by the mAb used in their selection (α -HACal/2). One of the mutations (T89K) was in a surface knob of the HA head and was sufficient to suppress reactivity with the four murine mAbs. The other two changes (G80E and K163E) altered residues that were only partially exposed to solvent, explaining the incomplete loss of the α -HACal/3 and -4 epitopes (Fig. 4). In any case, the escape mutant behaviour rules out any inability of A/Cal/07/09 to change epitopes by single point mutations at the site described here. It may be that selective pressures *in vitro* and *in vivo* differently influence the type of escape mutations preferred in either environment.

The mutations in A/Extr/6530/10 and in the escape mutants of Fig. 4 delineated an antigenic site in the pandemic HA molecule different from those identified by other laboratories with either human (Krause *et al.*, 2010; O'Donnell *et al.*, 2012; Xu *et al.*, 2010) or murine (Manicassamy *et al.*, 2010) mAbs (Fig. 5). The amino acid changes selected in escape mutants of pandemic A/Cal/09 viruses by other groups are mostly located towards the tip of the HA molecule, near the receptor-binding site. In contrast, the mutations reported here were further down the HA head and overlapped only partially with the antigenic sites identified previously in H1N1 seasonal

viruses isolated before 2009 (Brownlee & Fodor, 2001; Caton *et al.*, 1982) (see Figs 2 and 5).

It might be argued that HA epitopes recognized by murine mAbs could be immunologically silent in humans. However, the results of Fig. 6 demonstrated that human sera contain antibodies that compete for antigen binding with the murine mAbs. The competition strength of each human serum reflected its neutralizing activity, suggesting quantitative differences in the level of competing antibodies in different sera, rather than differences in specificities. A similar range of competition strengths has been reported for human sera and murine mAbs specific for the HA of the H3N2 subtype (Wang *et al.*, 1986).

In summary, our results provide new information about the antigenic properties of the A(H1N1)pdm09 HA and possible sequence changes and mechanisms that may lead to alterations of HA epitopes. These results should be borne in mind while doing virus surveillance or studies of virus evolution.

METHODS

Ethics statement. The National Influenza Center in Madrid, Spain (which belongs to Instituto de Salud Carlos III) and other regional laboratories from different Spanish regions, constitute the ReLEG network included in the Spanish Influenza Surveillance System, which monitors the circulation of influenza viruses as part of the country-wide surveillance. This study has been developed within this activity, which was approved by the institutional review board at the Instituto de Salud Carlos III.

All procedures that required the use of animals complied with Spanish and European legislation concerning vivisection and the use of genetically modified organisms, and protocols were approved by the 'Comité de Ética de la Investigación y de Bienestar Animal' of Instituto de Salud Carlos III (CBA PA 19_2012). In particular, we followed the guidelines included in the current Spanish legislation on protection for animals used in research and other scientific aims: RD 1201/2005, 10 October and the current European Union Directive 86/609/CEE, DOCE 12.12.86 (N.L358/1 to N.L358/28) on protection for animals used in experimentation and other scientific aims.

Cells and viruses. The Madin-Darby canine kidney (MDCK), CV-1 and A549 cell lines were obtained from the American Type Culture Collection. They were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 U penicillin ml⁻¹, 200 µg streptomycin ml⁻¹, 4 mM glutamine and 10% FCS (DMEM10).

The prototype A/Cal/07/09 influenza virus was used throughout this study. It was grown in MDCK cells with serum-free DMEM supplemented with 2.5 µg trypsin (Sigma) ml⁻¹. Clinical samples were collected in 3 ml transport medium (MEM supplemented with 200 U penicillin ml⁻¹, 200 µg streptomycin ml⁻¹, 200 U mycostatin ml⁻¹ and 0.25% FCS). MDCK monolayers growing in 5 cm² tubes (Fisher) were inoculated with 200 µl homogenized clinical samples and incubated with serum-free DMEM plus trypsin until a cytopathic effect was evident. Supernatants were harvested and used to infect new cultures to obtain high-titre virus stocks. Virus titration was done by infecting MDCK cells with serial dilutions of the virus stock. After 1 h adsorption, the medium and unadsorbed virus were removed and replaced with DMEM with 2.5 µg trypsin ml⁻¹, 80 µg DEAE-dextran ml⁻¹ and 0.7% agar. After 2–3 days, plaques were

visualized by fixing the monolayers with 10% formaldehyde in PBS and staining with 0.1% crystal violet in 20% methanol.

Virus purification and RNA sequencing. Culture supernatants of MDCK-infected cells were clarified by centrifugation at low speed and then loaded on to a 33–50% sucrose step gradient in TNE buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 5 mM EDTA) and centrifuged for 1 h at 40 000 r.p.m. and 4 °C in a SW41 rotor. The 33–50% interphase was collected, diluted in TNE, pelleted through a cushion of 33% sucrose in TNE for 1 h at 40 000 r.p.m. and 4 °C in a SW41 rotor and finally resuspended in TNE. Total RNA was isolated from the pellet using RNeasy isolation reagent (Qiagen) following the manufacturer's instructions. Library preparation was performed using an Illumina mRNA sequence sample preparation kit and sequencing was carried out as described previously (Rodríguez *et al.*, 2013). The most abundant nucleotide in each position was considered the 'consensus' sequence.

Mouse immunization and isolation of mAbs. Clarified culture supernatant of A/Cal/07/09-infected MDCK cells was used to infect BALB/c mice intranasally (1.5×10^4 p.f.u. in 50 µl PBS per mouse). After 2 weeks, mice received an intraperitoneal boost of purified UV-inactivated virus (150 µg viral protein) in PBS and 2 weeks later a final intravenous boost of 200 µg virus protein in PBS. The following day, splenocytes were isolated and fused to Sp2-0 myeloma cells as described previously (García-Barreno *et al.*, 1989) but using ClonaCell-HY PEG solution (StemCell Technologies) as the fusing agent. Hybridomas were selected in ClonaCell-HY liquid HAT medium and subcloned and stabilized in ClonaCell-HY Hybridoma Growth Medium E by standard procedures. Hybridoma supernatants were tested for antibodies reacting with viral antigens by ELISA, using purified A/Cal/07/09 virus as antigen, and those that were positive were retested against an extract of CV-1 cells infected with a recombinant vaccinia virus expressing Cal/07/09 HA (see below).

ELISA-positive supernatants were tested for blocking infectivity in a microneutralization test, carried out as follows: MDCK cells growing in 96-well plates were infected at an m.o.i. of 0.1 in the presence of serial dilutions of hybridoma supernatants. After 24 h, the medium was removed, and after washing with PBS, the cells were fixed with cold 80% acetone in PBS. After air drying, the amount of viral antigen in each well was quantified by ELISA with a mixture of mAbs M58/p44/E and M3, specific for viral nucleoprotein and matrix protein, respectively (Sánchez-Fauquier *et al.*, 1987).

Cloning, expression and mutagenesis of influenza HA. RNA was extracted from MDCK cells infected with A/Cal/07/09 virus by the Trizol method. The HA gene was amplified by reverse transcription-PCR using primers HA-Cal/1-22/EcoRI⁺ and HA-Cal/1681-1701/Xma⁻ (sequences available on request). The amplified DNA was digested with EcoRI and XmaI and ligated to pRB21 plasmid (Blasco & Moss, 1995) digested with the same enzymes. The resulting plasmid was used to obtain a recombinant vaccinia virus expressing the A/Cal/07/09 HA antigen, as described previously (Bembridge *et al.*, 1998). This virus was used to infect CV-1 cells from which cell extracts were made to confirm the specificity of the mAbs described above. Additionally, the recombinant vaccinia virus expressing A/Cal/07/09 HA was inoculated into rabbits to obtain α-HA-specific polyclonal serum, as described elsewhere (Magro *et al.*, 2012).

HA mutants containing the amino acid changes indicated in the figures were made using the pRB21/HA plasmid and a QuikChange directed mutagenesis kit (Stratagene), as recommended by the manufacturer. Transient expression of the WT and mutant HAs was assessed in A549 cells. These cells were infected with the recombinant vaccinia virus vTF7-3 (m.o.i. 10), which expresses the T7 polymerase, 1 h before transfection with pRB21 plasmids carrying either the WT or the mutant HA genes, using Lipofectamine 2000

(Invitrogen). After 24 h, the cells were fixed with cold methanol for 5 min, followed by 30 s with acetone. After air drying, the cells were incubated with the antibodies indicated in the figures and visualized with FITC-labelled secondary antibodies.

Isolation of escape mutants. Serial dilutions of A/Cal/07/09 were used to infect monolayers of MDCK cells growing in 24-well plates, in either the absence or presence of hybridoma supernatant containing α-HA-Cal/2 mAb. After virus adsorption, the monolayers were washed with serum-free medium and overlaid with 0.7% soft agar to allow plaques to develop for 3 days. The plates were visually inspected and several plaques were picked in 250 µl serum-free medium from wells with the highest virus dilution grown in the presence of mAb. These plaque-purified viruses were titrated again in the absence or presence of mAb to confirm resistance to neutralization, and grown in MDCK cells for sequence analysis and further characterization.

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