Molecular basis of the behavior of hepatitis A virus exposed to high hydrostatic pressure

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Foodborne hepatitis A outbreaks may be prevented by subjecting foods at risk of virus contamination to moderate treatments of high hydrostatic pressure (HHP). A pretreatment promoting hepatitis A virus (HAV) capsid-folding changes enhances the virucidal effect of HHP indicating that its efficacy depends on capsid conformation. HAV populations enriched in immature capsids (125S provirions) are more resistant to HHP, suggesting that mature capsids (150S virions) are more susceptible to this treatment. Additionally, the MAb K24F2 epitope contained in the immunodominant site is a key factor for the resistance to HHP. Changes in capsid-folding inducing a loss of recognition by MAb K24F2 render more susceptible conformations independently of the origin of such changes. Accordingly, codon usage-associated folding changes and changes stimulated by pH-dependent breathings, provided they confer a loss of recognition by MAb K24F2, induce a higher susceptibility to HHP. In conclusion, resistance of HAV to HHP treatments may be explained by a low proportion of 150S particles combined with a good accessibility of the epitope contained in the immunodominant site close to the fivefold axis.

Keywords: Hepatitis A, HAV, codon usage, capsid-folding, capsid breathing, high hydrostatic pressure
Foodborne hepatitis A outbreaks in western countries are increasingly described as being associated with food imports from endemic areas. The implicated foods include shellfish (1, 2), green onions (3, 4), dried tomatoes (5) and berries (6-8) among others. Three very recent outbreaks, which occurred in 2013-2014, are of particular interest. The first of these outbreaks occurred during 2013 in four European Nordic countries (Denmark, Finland, Norway and Sweden), affected 103 patients and was associated with the consumption of frozen strawberries from Egypt (9, 10). The second also occurred in 2013 across nine USA states (Arizona, California, Colorado, Hawaii, New Hampshire, New Mexico, Nevada, Utah, and Wisconsin) affecting 154 patients and was caused by the consumption of contaminated pomegranate seeds from Turkey (http://www.cdc.gov/hepatitis/Outbreaks/2013/A1b-03-31/). The third outbreak was a very long-lasting one (January 2013- May 2014), affecting eleven European countries (Bulgaria, Denmark, France, Germany, Ireland, Italy, The Netherlands, Norway, Poland, Sweden and United Kingdom) with over 1300 patients (http://www.efsa.europa.eu/en/supporting/pub/581e.htm). Frozen strawberries, likely produced inside Europe, were the food associated with this outbreak.

In the present context of global food trade the implementation of procedures for the inactivation of viruses in food are required. However, hepatitis A virus (HAV) is a highly stable virus even under extreme conditions such as high temperatures (11) or very low pH (12). However, its sensitivity to genome-target affecting treatments such as UVC-light is much higher than to high temperatures and low pH (13). This behavior suggests that the highly resistant phenotype of HAV is mainly due to an extremely cohesive capsid. Recently, we have proposed that capsid-folding in HAV is codon usage-dependent and controlled by the kinetics of translation (14), and that a fine combination of codons pairing with abundant and non-abundant tRNAs is required to
get a controlled ribosome pace (rapid translation of codons pairing with abundant tRNAs and slow translation of codons pairing with non-abundant tRNAs), which in turn is necessary for a proper folding of a highly cohesive capsid. HAV has a highly deoptimized codon usage, with respect to that of the cell host, which may be the result of its inability to shut down the cellular protein synthesis and its unfair competition for tRNAs (14). Inhibition of cellular mRNA synthesis may be achieved by treating cells with actinomycin D, which specifically inhibits the cellular DNA-dependent RNA polymerases with no effect on viral RNA-dependent RNA polymerases. Under these conditions, tRNA pools available for HAV translation are modified (14, 15).

Adaptation of HAV to actinomycin D-induced cellular shut-off resulted in the selection of mutant virus populations with subtle capsid-folding changes, induced by adjustments of their codon usage, which have profound influences in capsid biological functions such as cell binding and genome uncoating (15). These newly generated capsids showed a completely different phenotype and became highly sensitive to high temperature, low pH and bile salts (15).

In recent years, high hydrostatic pressure (HHP) has been proposed as an emerging technology to inactivate viruses in food (16) and in the particular case of HAV, the literature show that is quite resistant to this treatment, generally requiring pressures above 400-MPa for at least 5 min to get a complete inactivation (17). Additionally, it has been shown that inactivation is strongly influenced by temperature, pH and salt concentration, with higher efficiencies at acid pH and lower efficiencies at increasing salt concentration (18, 19), implying that HHP may be a good candidate technique for berries, tomatoes, pomegranate seeds and green onions due to their natural low pH. However, despite all these practical and valuable data on HAV inactivation by HHP, the molecular basis underlying the inactivation process remains unknown. In the
in their capsid-folding, to elucidate the mechanism of HHP inactivation.

METHODS

Virus and Cells

Five HAV populations derived from the cell-adapted pHM175 43c strain were used throughout this study (15): L0 (parental type), F0.05A (population adapted to low levels of cellular shut-off), F0.05LA (population long-adapted to low levels of cellular shut-off), F0.2A (population adapted to high levels of cellular shut-off) and F0.2LA (population long-adapted to high levels of cellular shut-off). The physical, antigenic and biological properties of these virus populations have been previously described (15).

Virus stocks of these populations were titrated (TCID$_{50}$) in the FRhK-4 cell line as previously described (15).

Production of concentrated viral stocks

Suspensions with high virus titers were used in the ELISA tests and in the sucrose gradients. Concentrated viral stocks were obtained as previously described (15). At 5-6 d post-infection, FRhK-4 infected cells (m.o.i. of 1) from a T-175 flask were trypsin-treated, collected by low-speed centrifugation 1,000 x g, resuspended in 500 µl of NT buffer (0.1 M NaCl, 10 mM Tris-HCl, 1% NP-40, pH 7.4) and incubated for 30 min at room temperature. These lysed cell suspensions were centrifuged at 1,700 x g for 5 min and the supernatants again centrifuged at 13,000 x g for 5 min. Viruses recovered in the
supernatants were subjected to three sonication cycles of 30 s at 60 W in the presence of 0.4% SDS. Populations F0.05A and F0.05LA on the one hand and F0.2A and F0.2LA on the other were grown in the presence of 0.05 µg/ml and 0.2 µg/ml of actinomycin D, respectively, to induce low and high levels of cellular shut-off.

**HAV Antigenic Structure Determination**

The antigenic structure of capsids from the different virus populations as well as from capsids of the parental-type at different pH was analyzed through the recognition with MAbs K24F2, K34C8 and H7C27. K24F2 and K34C8 MAbs are directed against the immunodominant site (20) and H7C27 MAb recognizes the glycophorin-A binding site (13, 21). While K24F2 and H7C27 epitopes are present in the protomers, procapsids, and capsids, the K34C8 epitope is present only in procapsids and capsids (22). All MAbs were used at the highest dilution yielding HAV recognition. For the recognition with each individual MAb, a sandwich ELISA was performed (23) in which particles were captured by a convalescent-phase serum and detected with MAbs. Mock-infected FRhK-4 cell extracts were used as negative controls. Normalization of the added antigen was made through the infectious titer (average of 1.5 x 10⁶ TCID₅₀ per well).

**Determination of the relative proportion of pentamers, procapsids and capsids**

The morphogenesis pathway of the different populations was analyzed as previously described (24, 25). Five-hundred microliters of concentrated viral stocks were layered onto a 15 to 45% sucrose gradient in TNMg buffer (20 mM Tris-HCl, 10 mM NaCl, and...
50 mM MgCl₂, pH 6.7) and spun at 205,000 x g for 165 min. Fractions of 500 µl were collected and the presence of HAV antigenic material and refraction indexes were determined for each fraction. The different fractions containing subviral (14S pentamers) and viral particles (70S procapsids, 125S immature capsids and 150S mature capsids) were identified by a sandwich ELISA, essentially as above described, using MAb K24F2, in the final detection step. The sum of the antigenicity of the different fractions belonging to 14S, 70S, 125S and 150S peaks was figured in each of three different stocks submitted to a sucrose gradient separation for each population and the average and standard error were obtained.

High Hydrostatic Pressure (HHP) Treatment

The different virus populations were submitted to three different high hydrostatic pressures (150, 300 and 400-MPa) for 1 min using the Thiot ingenierie-Hyperbaric unit (Breteieux, France - Burgos, Spain). This unit is a 2-L single-chamber which uses di-2-ethylhexyl azelate as pressure-transmitting fluid. Pressure come up rate was on average 220 MPa/min and pressure release was almost immediate. Samples were introduced into the unit at 7°C and temperature was monitored at the end of the process, ranging from 10 to 15°C.

Some experiments with L0 population were performed on acid-modified capsids. With this aim, the pH of virus suspensions was lowered to 5.5 and 5.0 and the efficacy of a 1- min 400-MPa treatment compared with that of capsids at pH 7.0.
Pulsed-Light (PL) treatment

Virus populations were also treated with pulsed light using the Pulsed Light TECUM unit (Claranor, Manosque, France) under a regime of 20 pulses of 300 ms and 1 Hz and an intensity of 1 J/cm². The light frequencies were 15% UVC (200-280nm), 50% visible (280-780nm) and 35% proximal IR (780-1100nm). Samples were introduced at 4°C and temperature was monitored at the end of the process, ranging from 35°C to 40°C.

Statistical analysis

Statistical differences in the inactivation of the different virus populations were assessed by using the ANOVA test. Particular differences between pairs of populations were assessed using the Student t-test (unpaired). Differences in the MAb recognition of the different populations as well as of the L0 population pretreated at pH 6.0, 5.5 and 5.0 and in the efficiency of capsid maturation and capsid formation were similarly analyzed. Results are expressed as the mean ± standard error of at least three independent experiments. Correlation analyses between the level of inactivation at 400-MPa treatment and the percent of recognition by the three MAbs were performed, including all different capsid conformations (L0, F0.5A, F0.5LA, F0.2A, F0.2LA, L0 pH 5.5 and L0 pH 5.0).
RESULTS

HAV inactivation by HHP depends on capsid-folding

Five HAV populations differing in their capsid-folding were comparatively submitted to 150, 300 and 400-MPa treatments for 1 min. A 150-MPa treatment was ineffective in inactivating any of the five populations tested (Fig. 1), and only in the case of population F0.2LA a small, but significant (p<0.05), reduction of 0.22±0.21 log_{10} TCID_{50} was observed. A 300-MPa treatment allowed significant (p<0.05) reductions of 0.28±0.06 and 1.03±0.25 log_{10} TCID_{50} for the F0.05LA and F0.2LA populations, respectively (Fig. 1). Three significantly different (p<0.05) behaviors were observed at 400-MPa: L0 population showed a reduction of 1.34±0.38 log_{10} TCID_{50}, the group of F0.05A, F0.05LA and F0.2A populations showed reductions of 2.78±0.18, 2.97±0.09 and 3.19±0.21 TCID_{50}, respectively, and population F0.2LA a reduction of 4.37±0.05 log_{10} TCID_{50} (Fig. 1 and Table 1). Consequently, L0 and F0.2LA were the most and least resistant populations, respectively, as revealed by statistical analyses (p<0.05). Additionally, F0.05LA population, on the one hand, and F0.05A and F0.2A populations, on the other, showed intermediate behaviors between L0 and F0.2LA (Fig. 1). In contrast, a PL treatment of 20 J/cm^{2} was highly effective in inactivating all HAV populations (Table 1). This was not an unexpected result since the major difference between these populations relies on their capsid-folding and while HHP supposedly affects capsids, PL is essentially directed to the nucleic acid.

In an attempt to elucidate the different inactivation patterns observed after exposure to HHP, the particle/subparticle composition of the populations was investigated through the study of the morphogenesis pathway. The main difference found was at the capsid maturation step (Fig. 2A) which involves a change in the...
sedimentation coefficient from 125S to 150S. A significant increase (p<0.05) in the efficiency of maturation was observed in all populations with changes in codon usage (Fig. 2A and Fig. 2D-G), respect to the parental L0 type (Fig. 2A and Fig. 2C) suggesting that mature 150S particles are more susceptible to HHP. Another additional difference was that the efficiency of capsid (70S+125S+150S) formation from pentamers (14S) was significantly lower (p<0.05) in the population F0.05LA (Fig. 2B and Fig. 2D-G) compared to any other population.

Given the direct influence of capsid-folding on HHP it may be assumed that capsid pretreatments inducing changes in its conformation may alter the efficacy of HHP treatments of the parental type HAV. It has been previously described that lowering the pH to 5.5 induces changes in the capacity of HAV to interact with the glycoporphin-A present on the human erythrocyte membrane (21) which in turn indicates capsid conformational changes. Consequently, the pH of the parental type L0 population was adjusted to 5.5 and 5.0 previously to a 400-MPa treatment. Interestingly, a change in pH from 7.0 to 5.5 induced a drastic and statistically (p<0.05) significant increase in the TCID_{50} log_{10} reduction (Fig. 3) from 1.34± 0.38 at pH 7.0 to 5.15±0.11 at pH 5.5 (Fig. 3). A further decrease down to pH 5.0 significantly (p<0.05) increased the TCID_{50} log_{10} reduction after the HHP treatment to 5.84±0.09 (Fig. 3).

Antigenic structure as a measure of capsid-folding changes

HAV populations F0.05A, F0.05LA, F0.2A and F0.2LA differ in their recognition patterns by MAbs H7C27, K34C8 and K24F2 compared to the L0 parental type (15). Population F0.05A showed only subtle changes in its antigenic structure with the most
relevant change being a 21% reduction of recognition by MAb K24F2 (Fig. 4). In contrast, the rest of populations showed much more drastic changes (Fig. 4), mainly characterized by a higher accessibility of the H7C27 (F0.2A > F0.05LA > F0.2LA > F0.05A = L0) and K34C8 (F0.05LA > F0.2A > F0.02LA > F0.05A = L0) epitopes, and a lower accessibility of the K24F2 epitope (F0.05LA < F0.2LA < F0.05A < F0.2A = L0).

Capsid conformation changes induced at pH 5.5 and pH 5.0 were more homogeneous and characterized by significant decreases of recognition by all MAbs (Fig. 5). No significant changes in the antigenic structure were observed at pH 6.0 (Fig. 5). However, at pH 5.5 a significantly (p<0.05) lower accessibility of the different epitopes was observed (H7C27 < K34C8 < K24F2) as well as at pH 5.0 (K24F2 < H7C27 = K34C8).

**Recognition of the K24F2 epitope as a measure of the susceptibility to HHP**

The above described results on susceptibility to HHP showed that populations F0.2LA and F0.05LA were the most sensitive (Fig. 1). Antigenically, these two populations shared the greatest inaccessibility of the K24F2 epitope site (Fig. 4). Similarly, capsid conformations at acid pH showed a linear decrease of the recognition by the MAb K24F2 (Fig. 5) which is associated with increases in their inactivation by HHP (Fig. 3). To statistically confirm this observation, analyses of correlation between the log_{10} TCID_{50} reductions induced by a 400-MPa treatment for 1 min and the percent of recognition by each of the MAb, were performed. All capsid changes, either induced by codon usage-derived changes or by lowering the pH, were included in the analyses.
significant negative correlation (-0.81; \( r^2=0.65 \)) exists between the inactivation by the HHP treatment and the recognition by the K24F2 MAb (Fig. 6). In contrast, no significant correlations were observed (Fig. 6) between the HHP inactivation and the recognition with MAb H7C27 (-0.51; \( r^2=0.26 \)) or K34C8 (-0.37; \( r^2=0.14 \)).

**DISCUSSION**

High hydrostatic pressure (HHP) has proven to be a promising emerging technology to inactivate pathogenic microorganisms in food (16, 19, 26, 27). Its efficiency is highly variable and depends on the specific microorganism (16). Focusing on viruses and particularly on the Picornaviridae family, into which HAV belongs, the susceptibility to HHP is in general low. Regarding the behavior of picornaviruses exposed to HHP are sorted into highly resistant, including poliovirus (28), Aichivirus and coxsackievirus B5 (29), moderately resistant, including paraechovirus 1, coxsackievirus A9 (29) and hepatitis A virus (30, 31) and highly sensitive such as foot-and-mouth disease virus (32). These widely variable resistance thresholds are unexpected keeping in mind that, coxsackievirus A9, coxsackievirus B5 and poliovirus belong to the same genera Enterovirus, with the former two into the same Enterovirus B species and the third into Enterovirus C species (33). Consequently, these data reveal that susceptibility to HHP may depend on small differences among highly structurally-related viruses. Another intriguing finding from these studies is the lower HHP resistance of HAV compared to other picornaviruses such as poliovirus, since under other hostile conditions such as low pH or high temperatures, it is remarkably more resistant (11, 12, 34). The mechanism of inactivation of HAV using HHP has not been clearly elucidated, although it has been
suggested to be related with the induction of capsid conformation changes preventing
the cell entry process rather than the complete capsid disruption (17).

The present study attempts to provide some insights into such a mechanism,
HAV populations differing in their capsid-folding versus the parental type were
comparatively analyzed after exposure to several HHP and pulsed-light (PL) treatments.
The inactivation of populations with codon usage-associated changes of capsid-folding
(F0.05A, F0.05LA, F0.2A and F0.2LA) through a PL treatment, whose target is mainly
the nucleic acid, revealed no differences among them. In contrast, these same
populations show differential responses after exposure to HHP inactivation. Altogether
these results confirm capsid-folding as the key factor of the sensitivity to HHP.

Overall, populations F0.05A, F0.05LA, F0.2A and F0.2LA showed a higher
susceptibility to HHP than the L0 parental type. Interestingly, a significant structural
feature of these populations was their higher yield of mature (150S) particles, in
contrast to the L0 parental type, suggesting that the mature capsids may be more
susceptible to HHP than the immature capsids. The main difference between the mature
virion and the immature provirion of HAV has been proposed to rely on a protracted
autocatalytic VP0 cleavage into VP4 and VP2 (35, 36). Although the immature (125S)
particles have been described to be less infectious (35), they could act as a reservoir
which will be more relevant in the L0 parental-type. Additionally, the different
populations could be gradually sorted from quite resistant to highly susceptible: the L0
parental type population was the most resistant, populations F0.05A and F0.2A were
significantly less resistant, population F0.05LA was a little bit more susceptible than the
latter group and population F0.2LA was the most susceptible. Structurally, capsids must
be cohesive enough to protect the genome but flexible enough to allow its release upon infection. Thus, the very high susceptibility to HHP of population F0.2LA, could be anticipated due to its very low uncoating time, 8 versus 18 hours for a 50% uncoating, compared to the L0 population (15). However, population F0.2A, with an even shorter 50% uncoating time (around 3 hours), had a more resistant phenotype than F0.2LA, which indicates that HHP susceptibility prediction is rather difficult. The explanation to this intriguing behavior must rely on the different capsid-folding of these two populations as revealed by their antigenic structure and by their cell-binding efficiency, which is much lower in the F0.2A population (15). Another difficult to explain point is the differential behavior of the F0.05LA population at moderate treatment of 300-MPa compared to the F0.05A and F0.2A populations which shared the same inactivation level at 400-MPa. One plausible reason could derive from the low efficiency of procapsid formation from pentamers which could suggest a less stable folding. The critical role of the ratio of mature (150S virions) vs immature capsids (125S virions) in HHP susceptibility may have relevant implications for food safety, provided that in patients’ stool, the 125S immature virions were predominant over the 150S mature virions. Data on HAV sedimentation coefficients in feces are scarce, since stool is not usually collected. However, reports on HAV purification from feces in CsCl gradients predominantly describe particles with a density equivalent to 150S virions (reviewed in 37). On the other hand it should be mentioned that this ratio in cell culture-adapted strains is highly variable and may depend on the strain, the cells and the time post-infection, with higher 150S/125S ratios at shorter times (37, 38).

The critical role of capsid conformation was further explored by inducing pH-dependent capsid “breathings” of the L0 parental population. While at pH 6.0 the
antigenic structure of the capsids remained unaltered, at pH 5.5 it was dramatically modified, as previously described (21), and even more at pH 5.0. These changes in the antigenic structure correlated with increases in the susceptibility to HHP. The enhancement of HAV inactivation by HHP at acid pH has been previously described (18), and it was postulated to be related with the induction of changes of capsid conformation. The present work provides scientific evidence supporting this hypothesis.

Changes produced either during the initial folding of the HAV structural polyprotein, through modifications of the genomic composition controlling the speed of translation, or playing with the “breathing” flexibility of the already folded capsids, render different conformations which show different susceptibility to HPP treatments. Independent of how they were originated, these different capsid conformations differ in the accessibility of the K24F2 MAb epitope with the lower its accessibility the higher the capsid susceptibility to HHP.

In conclusion, the ratio between mature and immature capsids in combination with the accessibility of the immunodominant site near the five-fold axis, are envisaged to direct the susceptibility of HAV to HHP.

Acknowledgements

This work was supported in part by Spanish Ministry of Economy project BIO2011-23461 and project 2009SGR00024 and Biotechnology Reference Network (XRB) from the Generalitat de Catalunya.
Lucia D'Andrea and Francisco J. Pérez-Rodríguez were recipients of fellowships from the Spanish Ministries of External Affairs and Cooperation and of Science and Education, respectively.
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FIG. 1. Survival curves of HAV after exposure to 150 to 400-MPa High Hydrostatic Pressure (HHP) for 1 min. Five HAV populations, derived from the cell-adapted pHM175 43c strain (15), were tested: L0 (parental type), F0.05A (population adapted to low levels of cellular shut-off), F0.05LA (population long-adapted to low levels of cellular shut-off), F0.2A (population adapted to high levels of cellular shut-off) and F0.2LA (population long-adapted to high levels of cellular shut-off). Figures represent the mean ± standard error of the log_{10} N_t/N_0 from three independent assays, where N_t is the titer after treatment and N_0 the titer before treatment.

FIG. 2. Relative yield of pentamers, procapsids and capsids in different HAV populations. Concentrated viral stocks were layered onto a 15 to 45% sucrose gradient and spun at 205,000 X g for 165 min. The presence of HAV antigenic material (14S pentamers, 70S empty procapsids, 125S immature capsids and 150S mature capsids) was determined by a sandwich ELISA using MAb K2-4F2 in the final detection step. Populations studied are the same described in Fig. 1. In panel A, the relative proportion of mature capsids versus immature procapsids is depicted. In panel B, the relative proportion of viral particles, including mature capsids, immature capsids and empty procapsids, versus subviral pentamers is depicted. Panels C through G show typical sucrose gradients of the antigenic yields for each studied population. In panels A and B, figures represent the mean ± standard error of three independent sucrose gradients. Statistically significant differences (p<0.05) between pairs of populations are indicated by different letters; populations sharing a letter are not significantly different.
FIG. 3. Survival curves of neutral and acid-modified capsids of the HAV parental-type (L0), after exposure for 1 min to 400-MPa. Figures represent the mean ± standard error of the log_{10} N_t/N_0 from three independent assays, where N_t is the titer after treatment and N_0 the titer before treatment.

FIG. 4. Recognition of the different HAV populations by monoclonal antibodies H7C27, K34C8, and K24F2. Populations studied are the same described in Fig. 1. Values represent the mean ± standard error of ELISA absorbance values of three different virus stocks. Statistically significant differences (P< 0.001) between pairs of populations are indicated by different letters.

FIG. 5. Recognition of neutral and acid-modified capsids of the HAV parental-type (L0) by monoclonal antibodies H7C27, K34C8, and K24F2. Values represent the mean ± standard error of ELISA absorbance values of three different virus stocks. Statistically significant differences (P< 0.05) between pairs of populations are indicated by different letters.

FIG. 6. Analysis of correlation between the levels of inactivation of different capsid conformations of HAV after exposure for 1 min to 400-MPa and their recognition by monoclonal antibodies H7C27, K34C8, and K24F2.
Table 1. Inactivation of different Hepatitis A virus (HAV) populations by High Hydrostatic pressure (HHP) or Pulsed Light (PL) treatments.

<table>
<thead>
<tr>
<th>Populations</th>
<th>HHP (400-MPa) log₁₀ (Nₜ/N₀) TCID₅₀</th>
<th>PL (20 J/cm²) log₁₀ (Nₜ/N₀) TCID₅₀</th>
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<td>L₀</td>
<td>-1.34 ± 0.38ᵃ</td>
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<tr>
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