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Original Article

Potency under pressure: the impact of hydrostatic pressure on antigenic properties of influenza virus hemagglutinin

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Background Influenza vaccines are effective in protecting against illness and death caused by this seasonal pathogen. The potency of influenza vaccines is measured by single radial immunodiffusion (SRID) assay that quantifies antigenic forms of hemagglutinin (HA). Hydrostatic pressure results in loss of binding of influenza virus to red blood cells, but it is not known whether this infers loss of potency.

Objectives Our goal was to determine the impact of pressure on HA antigenic structure.

Methods Viruses included in the 2010–2011 trivalent influenza vaccine were subjected to increasing number of cycles at 35 000 psi in a barocycler, and the impact of this treatment measured by determining hemagglutination units (HAU) and potency. Potency was assessed by SRID and immunogenicity in mice.

Results After 25 cycles of pressure, the potency measured by SRID assay was below the limit of quantification for the H1N1 and B viruses used in our study, while the H3N2 component retained some potency that was lost after 50 pressure cycles. Pressure treatment also resulted in loss of HAU, but this did not strictly correlate with the potency value. Curiously, loss of potency was abrogated when influenza A, but not B, antigens were exposed to pressure in chicken egg allantoic fluid. Protection against pressure appeared to be mediated by specific interactions because addition of bovine serum albumin did not have the same effect.

Conclusions Our results show that pressure-induced loss of potency is strain dependent and suggests that pressure treatment may be useful for identifying vaccine formulations that improve HA stability.

Keywords Hemagglutinin, hydrostatic pressure, immunogenicity, influenza, potency.

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Introduction

Influenza continues to pose a threat to human health on a seasonal basis, with an average of 25 470 influenza-associated respiratory and circulatory deaths each year.[1] Pandemic outbreaks occur when an antigenically unique virus, such as the swine-origin strain in 2009, infects and causes disease in an immunologically naïve population. Immunity is largely attributed to antibodies specific for hemagglutinin (HA), the predominant glycoprotein on the virus surface that is responsible for binding to cellular sialic acid-containing receptors. HA is therefore the primary antigen contained in licensed inactivated influenza vaccines, with vaccine efficacy correlating with HA-inhibition (HI) titers.[2,3]

Until the early 1980s, influenza vaccine potency was measured by its ability to agglutinate chicken cells as this functional property of HA is indicative of its native conformation. This assay, however, cannot be used to differentiate between influenza A and B viruses, or between H1N1 and H3N2 subtypes that are included in the trivalent vaccine, and this measure of agglutination did not always correlate with the vaccine's immunogenicity in man.[4] This test was replaced by the single radial immunodiffusion (SRID) assay.[5] This assay is specific for virus type and subtype, using sheep antiserum specific for native HA in agar to precipitate HA that is present in its native conformation. The assay is stability indicating, because denatured HA does not precipitate with this antiserum.

Denaturation of HA resulting in a decreased SRID measurement can be achieved by heat treatment,[6] freeze-thawing,[7] and acidification.[8] It is well recognized that acidification is required for virus entry, with pH approximately 5·5 resulting in conformational change within the late endosome, exposing a fusogenic peptide that facilitates fusion of viral and endosomal membranes to permit entry of the viral genome into the cytoplasm of the infected cell.[9] Gaspar *et al.*, demonstrated a similar change in conformation when virus is treated with hydrostatic pressure at neutral pH, showing fusogenic properties of HA are increased after pressure treatment.[10] Importantly, these authors demonstrate a large decrease in the infectivity of Sindbis, loss in hemagglutination of influenza, and suggest exposure to pressure may be a useful method to inactivate whole virus for vaccine production. While Sindbis and influenza viruses are enveloped, others have reported inactivation of a non-enveloped virus, foot-and-mouth disease virus,[11] after treatment with pressure. In these reports, it is implied that immunogenic properties are not impacted by this treatment, but antibody responses to specific viral proteins after vaccination with live and inactivated virus preparations were not compared.

The extent of pressure-induced denaturation of HA to generate its fusogenic form is not known, and therefore, it is not known whether the antigenic structure of HA is retained following pressure treatment. In this report, we examine the impact of hydrostatic pressure on the antigenic form of HA from each of the viruses included in the 2011/2012 seasonal trivalent influenza vaccine, A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008.

Materials and methods

Antigens

Virus reference antigens of the following strains were obtained from the Office of Product Quality and Control, CBER: H1N1 virus, A/California/7/2009 X-179A (A/CA/09), H3N2 virus, A/Victoria/210/2009 X-183 (A/VI/09), B/Florida/04/2006 (B/FL/06), and B/Brisbane/60/2008 (B/BR/08). Each lyophilized reference antigen was stored at −20°C and reconstituted within 24 hour of use. Recombinant HA (rHA) from each virus was purchased from Protein Science Incorporated (Meridan, CT), and stored at 4°C. Live virus was prepared by inoculation of 9–11-day-old embryonated eggs, incubation of the eggs at 33°C, and harvesting allantoic fluid 60-72 hour later. After pelleting cellular debris by centrifugation, the allantoic fluid was aliquoted and stored at −80°C.

Barocycler treatments

Samples (700–750 μl) were aliquoted into 1·5-ml pressure cycling technology (PCT) pulse tubes. Each treatment consisted of a different number of cycles at ambient temperature in which 35 000 psi was applied for 8 seconds followed by 7 seconds at atmospheric pressure in a Barocycler NEP3229 (Pressure BioSciences Inc., South Easton, MA, USA). Samples were kept on ice until assays were conducted on the same day.

Mouse experiments

Mouse experiments were conducted according to a protocol approved by the institutional Animal Care and Use Committee and followed federal guidelines. Female BALB/c mice (5/group) approximately 8 weeks old were immunized intramuscularly (50 μl/dose) with antigens diluted to approximately 30 μg/ml in PBS-0·1% DOC or PBS-0·1% DOC-50% allantoic fluid. Antigen in each diluent was treated with either 0 or 99 cycles of pressure. Three weeks after a primary dose, the mice were boosted in the same way, with antigen prepared in the same manner. Serum was obtained from tail bleeds collected 3 weeks after each dose.

Quantitative assays

Protein: Protein concentration was determined following the manufacturer's instructions using a Pierce BCA kit (Pierce, Rockford, IL, USA).

Hemagglutination: Hemagglutination assays followed a standard procedure using equal volumes (50 μl) of twofold serial dilutions of sample and 0·5% turkey red blood cells (RBC). Forty-five minutes after mixing equal volumes (50 μl) of sample dilutions and RBC in a round-bottom plate, hemagglutination units (HAU) were read as the inverse of the last dilution with hemagglutination.

NA activity: Twofold serial dilutions of samples in PBS were made in black 96-well plates (50 μl/well), and an equal volume of 100 μM MU-NANA (Sigma, St Louis, MO) added. After 1 hour incubation at 37°C, 100 μl stop solution, 0·1 M glycine, pH 10·7, 25% EtOH, was added and fluorescence read on a Victor V plate reader (Perkin Elmer, Shelton, CT) with excitation at 355 nm, and emission at 460 nm. The relative fluorescence units (RFU) were reported at the same dilution for all samples in a group.

Virus infectivity: A 50% tissue culture infectious dose ($TCID_{50}$) assay was performed. Briefly, MDCK cells were plated into 96-well flat-bottom plates at 5×10^4/well in Iscove's serum-free medium. Twenty-four hour later, the medium was removed, and 100 μl of quadruplicate replicates of 10-fold serial sample dilutions added to each plate. An equal volume of serum-free medium containing 1 μg/ml TPCK-treated trypsin was added, and the plates were incubated for 3 days at 37°C. Medium from each well (25 μl) was transferred to a round-bottom plate to which 25 μl PBS was added. A 50 μl volume of 0·5% turkey RBC was added, the contents of each well mixed by gentle shaking and hemagglutination read 45 minutes later. Wells in which

hemagglutination was observed were recorded as containing infectious virus, and the $TCID_{50}$ calculated by method of Reed and Muench.[12]

HA potency: A published SRID method[13] was followed, with some modifications. Briefly, 1% agarose gels were prepared in phosphate-buffered saline, pH 7·2 and placed in a water bath to equilibrate to 50°C before adding the appropriate amount of HA-specific sheep antiserum (CBER, FDA). After gentle mixing, the gel was poured onto GelBond film (Cambrex, East Rutherford, NJ, USA) and allowed to solidify. Equally spaced 4-mm wells were punched into the gel. Reference antigens (CBER, FDA) and samples were reconstituted in PBS containing 1% Zwittergent 3-14 (Calbiochem, San Diego, CA, USA), and incubated at room temperature for 30 minutes. Several dilutions of the reference antigens were made in the same buffer spanning 8–35 μg/ml HA concentrations. Reference dilution and samples were loaded into wells on replicate gels (20 μl/well) and incubated in a sealed, humid chamber at room temperature for 18–24 hour. Gels were then washed in saline and rinsed in water before drying at 40°C. Gels were stained with 0·5% Coomassie Brilliant Blue for 10 minutes, destained and then dried prior to measuring the diameter of precipitant rings using an Immunolab scanner. Antigen potency was computed from the reference antigen linear dose–response curve. The test validity was based on correlation coefficient (r) and equality of slopes (t) between test and reference antigen.

Hemagglutination inhibition (HAI) titers: HAI titers were measured as previously described.[14] Briefly, serum was treated with receptor-destroying enzyme and then heat-inactivated. Serial dilutions (25 μl) were mixed with an equal volume of virus (4 HAU) and 50 μl 0·5% turkey red blood cells, and agglutination read after 45 minutes. The reciprocal of the last dilution of serum that completely inhibited agglutination was recorded as the HI titer.

Results

Reduction in infectivity of influenza virus correlates with loss of receptor binding

To verify the impact of hydrostatic pressure on HA structure shown by others[10] and examine changes in virus infectivity, live A/CA/09 and B/BR/08 viruses were treated for 0, 25, 50, or 99 cycles of pressure at 35 000 psi. The infectious dose ($TCID_{50}$) of A/CA/09 was reduced 1000-fold (3 $\log_{10}$) after 99 cycles. Loss of infectivity did not correspond to protein adsorption to tubes during barocycler treatment – the protein concentration in A/CA/09 samples without pressure treatment was 604 ug/ml, and after 99 cycles of treatment, 580 μg/ml, only 3% less. Reduction in $TCID_{50}$ titer correlated with HA's capacity to bind to receptors on turkey red blood cells (Table 1). The impact of pressure on virus infectivity and receptor binding (as measured by hemagglutination) was greater for A/CA/09 (H1N1) than for B/BR/08; the latter virus had less than 10-fold reduction in infectivity after 99 cycles of pressure (Table 1). Together, these results support the idea that loss of infectivity resulted from the inability of HA to bind to host cell receptors.

Table 1. Impact of pressure on hemagglutination and infectivity of live virus preparations

	A/CA/09		B/BR/08	
Cycles of pressure	Log_{10} $TCID_{50}$/ml (±SD)	HAU	Log_{10} $TCID_{50}$/ml (±SD)	HAU
0	6·5 ± 0	2560	5·8 ± 0	128
25	4·5 ± 0·2	640	5·8 ± 0	128
50	5·5 ± 0·1	640	5·9 ± 0·1	128
99	3·5 ± 0	80	5·0 ± 0	128

Treatment with hydrostatic pressure alters the antigenic form of HA

As the amount of HA in live virus preparations is usually below the level of SRID quantitation, concentrated A/CA/09 (H1N1), A/VI/09 (H3N2), and B/BR/08 whole virus preparations were used to determine the impact of pressure on potency. These preparations were lyophilized, inactivated, whole virus preparations made for use as reference antigens in SRID assays. Initial test results showed a significant loss of protein after barocycler treatment of these purified virus preparations due to adsorption to the tubes. We therefore tested conditions to reduce this protein adsorption. Addition of 1% deoxycholate (DOC) prevented adsorption of A/VI/09 reference antigen during pressure treatment, with no significant reduction of protein concentration following 99 cycles of pressure treatment (148 μg/ml compared with initial concentration of 150 μg/ml) compared to approximately 35% loss of protein in the absence of this detergent (80 μg/ml following 99 cycles compared with an initial concentration of 123 μg/ml (Table 2). A similar reduction was not

Table 2. Impact of pressure on protein concentration, hemagglutination, and potency of A/VI/09 (H3N2)

Number of cycles	Protein (μg/ml)		HAU ($\times 10^{-2}$)		SRID (μg/ml)	
		+DOC		+DOC		+DOC
0	123	150	2048	512	38·2	34·4
25	104	147	512	8	20·6	21·3
50	103	147	256	8	<8	<8
99	80	148	16	8	<8	<8

evident for virus that was not purified, possibly because the greater total protein concentration of allantoic fluid (>2000 μg/ml) saturated the tubes without significant impact on overall concentration. Purified virus preparations were therefore resuspended in 1% DOC for all subsequent pressure tests. The addition of DOC destabilized the HA to some degree, resulting in fourfold lower hemagglutinating units (HAU) even before treatment with pressure (shown as 0 cycles in Table 2), as well as a greater loss of HAU in samples containing DOC after pressure treatment. For example, after 25 cycles, there was a fourfold and 64-fold loss of HAU for A/VI/09 without and with DOC, respectively. The addition of DOC did not result in a large difference in potency – the amount of antigenic HA measured by SRID in samples with DOC added was similar to the sample without DOC, and for this H3N2 antigen, a similar decrease in potency after pressure treatment was recorded for samples with and without DOC (Table 2).

The impact of pressure on potency was then compared between H1N1 (A/CA/09), H3N2 (A/VI/09) and B (B/BR/08) viruses, each of the antigens included in the 2010/2011 vaccine formulation. There were strain-specific differences in ability of each virus preparation to agglutinate turkey RBC. While the potency of all preparations before treatment was approximately 30 ug/ml, A/VI/09 agglutinated RBC to high titer, B/BR/08 had lower HAU, and no HAU were measured for the A/CA/09 preparation (Table 3). As indicated by the experiments we described earlier in this report, this comparison provided additional evidence that the absolute HAU titer was not predictive of potency as measured by SRID.

Decreases in HA potency were measured by SRID following pressure treatment of all of the viruses tested (Table 3). The sensitivity of each virus to pressure-induced changes in HA functional properties appeared to be strain specific, with greater retention of potency of the H3N2 virus. However, potency and hemagglutination was significantly reduced by 99 cycles of pressure for all 3 viruses. The change in protein concentration under these conditions was minimal (after 99 pressure cycles, protein concentration of each strain was >97% of the control preparation), demonstrating that the decreases in functional attributes were not due to loss of protein content, but rather, due to a change in protein structure. As pointed out previously, HAU and potency of each of the preparations used in this study were not reflective of one another, and therefore, it was not surprising that pressure-induced reductions in potency and HAU were not equivalent – only one-third of A/VI/09 potency was lost, but HAU was reduced 64-fold after 25 pressure cycles of A/VI/09.

The tetrameric form of neuraminidase (NA) is essential for its enzyme activity,[15] and retention of the native conformation correlates with induction of functional antibodies.[16] Unlike HA, the functional property of NA was retained even after pressure treatment, with retention of enzyme activity even after 99 cycles at 35 000 psi for A/VI/09 and B/BR/08, and only modest reduction in enzyme activity of A/CA/09 at this highest number of pressure cycles (Table 4). This was somewhat surprising considering that the stability of NA is tenuous.[17] The pressure-induced change in structure was therefore specific to HA.

Table 3. Impact of pressure on potency and hemagglutination of A/CA/09 (H1N1), A/VI/09 (H3N2), and B/BR/08

	Whole inactivated virus*		
Number of pressure cycles	**H1N1**	**H3N2**	**B**
Potency (μg/ml HA)			
0	29·4	34·4	32·5
25	<8	21·3	<8
50	<8	<8	<8
99	<8	<8	<8
HAU ($\times 10^{-2}$)			
0	<2	512	64
25	<2	8	8
50	<2	8	<2
99	<2	8	<2

*Each antigen (lyophilized whole virus) was solubilized in PBS-1% DOC.

Allantoic fluid stabilizes the antigenic structure of HA in influenza A viruses

In an attempt to understand why pressure did not completely inactivate live influenza virus (Table 1), as the live virus used in these experiments was suspended in allantoic fluid, we tested whether pressure-induced changes were dependent on total protein concentration of the solution or perhaps due to specific interactions with components of allantoic fluid. A purified preparation of A/VI/09 was resuspended in diluent (PBS-1% DOC), or diluent containing bovine serum albumin (BSA), or diluent with addition of allantoic fluid from healthy 10-day-old embryonated eggs. The total protein concentration of each virus suspension was approximately 120 μg/ml, 4 mg/ml, and 2·2 mg/ml, respectively. Our results showed that without applying pressure, both the high concentration of BSA and allantoic fluid interfered with hemagglutination but not potency allowing full reactivity with antibodies used in the SRID assay (Figure 1, without pressure). The preparation containing BSA lost potency and

Table 4. Neuraminidase activity of whole virus samples exposed to increasing cycles of hydrostatic pressure

	Neuraminidase activity (Relative fluorescence units × 10^{-3}) ±SD		
Number of cycles	**A/CA/09 (1/40)***	**A/VI/09 (1/1600)***	**B/BR/08 (1/16)***
0	490 ± 41	568 ± 29	549 ± 59
25	408 ± 9	613 ± 34	548 ± 3
50	518 ± 11	641 ± 30	559 ± 20
99	318 ± 7	682 ± 36	542 ± 38

*Results are for the dilution of sample shown in parentheses.

HAU after 99 cycles of pressure. However, addition of allantoic fluid to A/VI/09 fully prevented loss of potency without retaining hemagglutinating activity (Figure 1). We next evaluated whether this protection by allantoic fluid was also applicable to H1N1 and B antigens (Table 5). This was indeed also the case for A/CA/09, in which potency was completely retained when the virus was resuspended in diluent containing allantoic fluid (Figure 2 and Table 5). This antigen was also partially stabilized by the addition of BSA, suggesting buffering against pressure by the greater protein concentration. In contrast to influenza A viruses, the potency of neither B/FL/06 nor B/BR/08 (Tablel 5) was stabilized by addition of allantoic fluid, suggesting that stabilization of HA's antigenic structure by allantoic fluid is specific for influenza A viruses. The B/FL/06 preparation had exceptionally high levels of HAU that were not completely eliminated by pressure treatment. Despite this residual hemagglutinating activity and some protection against the impact of pressure on HAU by allantoic fluid (16-fold greater HAU for sample in diluent containing allantoic fluid compared to diluent alone), the potency of B/FL/06 was below the limit of quantification following pressure treatment when the diluent contained allantoic fluid.

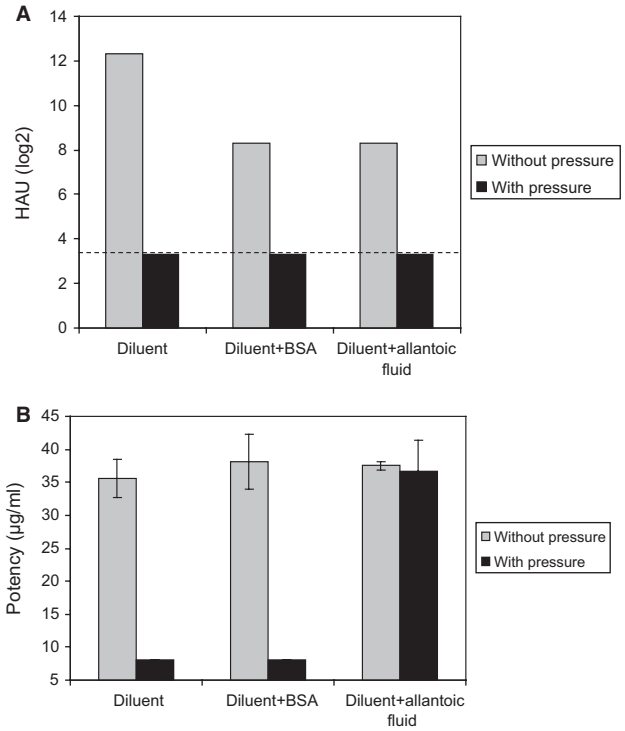

Figure 1. Allantoic fluid protects the antigenic structure of A/VI/09's HA from pressure. Inactivated whole virus was resuspended in diluent (PBS containing 1%DOC), diluent containing 4 mg/ml BSA, or diluent mixed 1:1 with allantoic fluid. Hemagglutinating units (A) and potency (B) of samples without pressure treatment, or after 99 cycles of 35 kpsi pressure, were measured, as described in Materials and methods. Error bars show standard deviation (SD) for potency measured by SRID assay, and a dashed line shows the limit of assay quantitation.

While addition of allantoic fluid maintained the potency of influenza A H1N1 and H3N2 antigens as measured by the SRID assay, hemagglutination activity was not preserved. This suggests that in the presence of allantoic fluid, pressure resulted in a modification of HA structure that retained its antigenic structure, but prevented accessibility of the receptor-binding domain of HA1 to glycoproteins on the red blood cell surface. As intact HA trimers have been proposed as necessary for induction of HAI titers,[18] we verified that the potency measured by SRID was indeed reflective of its immunogenicity. Mice were immunized with A/CA/09 that had been pressure-treated in the presence or absence of allantoic fluid. Three weeks after the first immunization, robust HAI titers were measured against A/CA/09 for mice immunized with untreated antigen in diluent alone, as well as diluent containing allantoic fluid. HAI titers were below the level of quantitation for antigen that had been subjected to pressure treatment without allantoic fluid, whereas addition of allantoic fluid resulted in retention of the immunogenicity of pressure-treated virus. The geometric mean HAI titers of this latter group was not significantly less than titers measured for mice immunized with untreated antigen (Figure 3A), reflecting the SRID potency results. HAI titers were increased after a second dose in all groups, suggesting that small amounts of antigenic HA are present even when the antigen is treated with pressure in the absence of allantoic fluid (Figure 3B).

Discussion

Our results show that the antigenic structure of HA is changed by pressure treatment. The reduction in antigenic form and protection of loss in potency by allantoic fluid is dependent on virus strain. These observations are consistent with data showing HA is present in a metastable state,

Table 5. Impact of allantoic fluid on pressure-induced changes of hemagglutination and potency of influenza A (H1N1 and H3N2) and B viruses*

		HAU				SRID (μg/ml)±SD			
		Type A		Type B		Type A		Type B	
Protein added to diluent†	Number of cycles	CA/09	VI/09	FL/06	BR/08	CA/09	VI/09	FL/06	BR/08
None	0	1280	5120	10240	10240	28·0 ± 2·0	35·6 ± 2·9	40·0 ± 2·0	32·5 ± 0·3
None	99	<20	<20	320	320	<8	<8	<8	<8
BSA	0	320	320	2560	ND	33·2 ± 2·9	38·1 ± 4·2	39·9 ± 4·0	ND
BSA	99	<20	<20	160	ND	17·8 ± 1·9	<8	<8	ND
Allantoic fluid	0	2560	320	20480	5120	32·6 ± 0·3	37·5 ± 0·6	38·2 ± 3·0	15·8 ± 0·2
Allantoic fluid	99	<20	<20	5120	80	29·2 ± 3·0	36·6 ± 4·7	<8	<8

ND, not done.
*Results are shown for A/CA/09 (H1N1), A/VI/09 (H3N2), B/FL/06 (B/Yamagata lineage) and B/BR/08 (B/Victoria lineage) whole virus preparations.
†Each virus preparation was resuspended in PBS-1% DOC containing either no additional source of protein, BSA, or allantoic fluid. The total protein concentration of each virus suspension was approximately 120 μg/ml, 4 mg/ml, and 2·2 mg/ml, respectively.

allowing conformational changes that are an essential element of viral entry. These changes include folding back of HA1 of the cleaved HA_0 molecule, and exposure of the fusogenic peptide in acidic endosomes.[19] Our results as well as those of Gaspar *et al.*,[10] suggest that acidification is not absolutely necessary for structural changes that result in loss of hemagglutination and increase in fusogenic activity – hydrostatic pressure at neutral pH results in a similar functional change.

Hydrostatic pressure has been a useful tool to investigate protein structure, because tertiary and quaternary structures are highly dependent on specific areas of hydration on the molecule surface and also hydrophobic, water-excluded cavities within the molecule.[20] Studies of hydrostatic pressure-induced changes in purified proteins and viruses have facilitated an understanding of protein–DNA recognition and virus assembly,[21] contributed to an understanding of the formation of protein aggregates that play a role in Parkinson's disease[22] and transmissible spongiform encephalopathies.[23] Importantly, these studies allowed identification of compounds that inhibit protein aggregation, providing drug candidates to prevent or treat these diseases. Our results demonstrating that addition of allantoic fluid protects hemagglutinins of influenza A but not B viruses from pressure-induced loss in potency suggest that specific interactions between molecules contained in allantoic fluid and HA preserve its antigenic structure. Given that aggregates can form when molecules are exposed to pressure,[23] one possibility is that addition of allantoic fluid prevents the formation of large complexes that limit migration of HA through the agarose used in the potency assay. If this were the explanation, we would expect that a pressure-treated influenza sample would retain immunogenicity in mice – this was not the case. Our opinion is therefore that in the presence of allantoic fluid, pressure induces the fusion-active form of HA, resulting in loss of hemagglutination, but that the native structure of HA is preserved, perhaps through binding of specific glycoconjugates within the allantoic fluid of chicken eggs that bind to the HA of influenza A, but not influenza B viruses. Birds are not a natural host of influenza B viruses, and therefore, this may reflect differences in receptor binding that support replication of influenza A viruses in avian species. Further studies are needed to determine whether the difference in the protective capacity of allantoic fluid is observed more generally for larger numbers of influenza A and B viruses, and to identify components of allantoic fluid that contribute to the protection of HA's antigenic structure.

Our results show discordance between hemagglutination and potency assay results, and while pressure impacts both, the changes we measured were often independent of one another. This is not surprising considering the interactions between HA and receptors on red blood cells and between HA and specific antibodies are fundamentally different. Alternate potency assays are currently being considered for influenza vaccines because of the lengthy time needed to prepare reagents for SRID analysis. Careful thought should be given to avoid the use of assays that depend on HA's receptor-binding activity to capture or detect antigen because, as our results demonstrate, this is not always indicative of the immunogenic form of HA.

There are several industrial and research applications for hydrostatic pressure technology. It is used in the food industry to inactivate adventitious agents,[24] and has been studied as a method to inactivate norovirus,[25] hepatitis A virus,[26] simian immunodeficiency virus,[27] human immunodeficiency virus[28] and highly pathogenic avian influenza virus.[29] Pressure-inactivated vesicular stomatitis virus,[30]

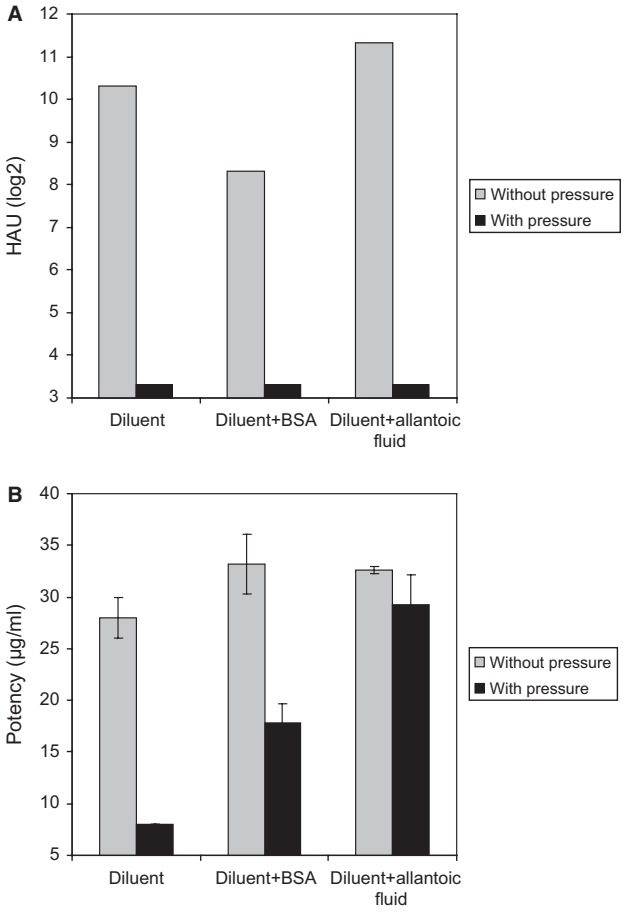

Figure 2. Allantoic fluid protects the antigenic structure of A/CA/09's HA from pressure. Inactivated whole virus was resuspended in diluent (PBS containing 1%DOC), diluent containing BSA, or diluent mixed 1:1 with allantoic fluid. Hemagglutinating units (A) and potency (B) of samples without pressure treatment, or after 99 cycles of 35 kpsi pressure, were measured, as described in Materials and methods. Error bars show standard deviation (SD) for potency measured by SRID assay.

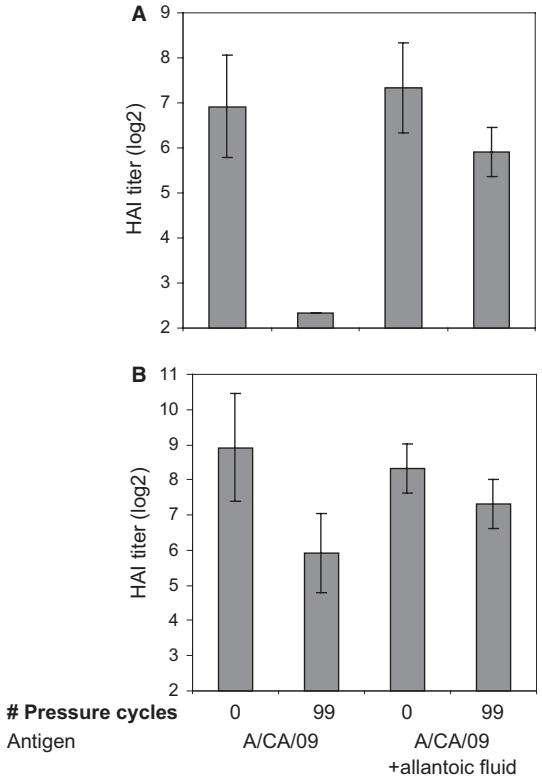

Figure 3. Geometric mean HAI titers against A/CA/09 after (A) primary and (B) secondary immunization of mice (n = 5) with A/CA/09 that had been treated with either 0 or 99 cycles of 35 kpsi pressure. Standard deviation (SD) is shown by error bars.

rotavirus,[31] foot-and-mouth disease virus,[11] chicken infectious bursal disease virus,[32] and yellow fever virus,[33] have been tested as potential vaccines. While multiple pressure-induced changes could contribute to virus inactivation, our data suggest that disassembly of oligomers or changes in conformation of receptor-binding domains explain the lack of infectivity. For example, inactivation of influenza is likely due to induction of the fusion-active state, resulting in a loss in binding to receptors.[10] In the case of chicken infectious bursal disease virus, the antigenic structure of the pressure-treated virus was reported as intact.[32] This may not be the case for all antigens; in fact, the impact of pressure on the potency has not been reported for many viral vaccine candidates, including simian immunodeficiency virus[27] and HIV-1,[28] and a detrimental effect of pressure on antigenic structure may explain the low levels of neutralizing antibodies generated in response to pressure-inactivated yellow fever virus.[33] Our data show that the antigenic form of HA and subsequent antibody response to HA is significantly reduced by pressure, suggesting that inactivation of influenza virus with pressure is unlikely to provide a suitable influenza vaccine candidate. Hydrostatic pressure technology does, however, provide a tool to identify buffer conditions or molecules that stabilize HA. Our data suggest that components of allantoic fluid protect the antigenic structure of HA without improving hemagglutination; further studies are needed to understand these results fully and to discover the molecular interactions that contribute to this observation.

In summary, we show that hydrostatic pressure changes the conformation of HA, resulting in loss of reactivity with antibodies generated against the native molecule. Not only is there a change in antigenic form, HA can no longer bind to receptors, shown as a loss of hemagglutination, although these measures are often independent of one another. Inclusion of allantoic fluid protected hemagglutinins of the influenza A (A/CA/09 and A/VI/09), but not B (B/FL/06 and B/BR/08), viruses from pressure-induced changes in structure, preserving antigenic structure in the absence of HA's ability to agglutinate red blood cells. Further studies are needed to determine whether this difference is observed more generally for larger numbers of influenza A and B

viruses. Our data support the use of hydrostatic pressure technology as a tool to examine HA stability. As shown by inclusion of allantoic fluid, subjecting antigens to pressure may also provide the means to identify buffer conditions that improve stability of the antigen, and may therefore be helpful in generating formulations that extend vaccine shelf-life.

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