Systemic Release of High Mobility Group Box 1 Protein during Severe Murine Influenza

Lisa M. Alleva, Alison C. Budd and Ian A. Clark

*J Immunol* 2008; 181:1454-1459; doi: 10.4049/jimmunol.181.2.1454
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High mobility group box 1 protein (HMGB1) was first shown in 1999 to have an additional function as a late mediator of endotoxin lethality (1). Since then, it has attracted considerable attention as a protein with inflammatory cytokine activity, whether actively secreted from immune cells (2, 3) or passively released from necrotic cells (4). There is an expanding literature on the involvement of HMGB1 in the pathogenesis of infectious diseases. HMGB1 potentiates the pathology of sepsis in animal models (1, 5) because it further amplifies inflammation via its cellular receptors, the receptor for advanced glycation end products (6, 7) and TLRs 2 and 4 (8). It has been suggested that HMGB1 is an important therapeutic target in human sepsis (9–11), although correlation of disease severity with serum HMGB1 concentration has proven difficult (12–14). Therapeutic interventions in animal sepsis models, such as administration of ethyl pyruvate (15) or anti-HMGB1 Abs (16, 17), save a significant proportion of animals, indicating that HMGB1 contributes to sepsis-induced mortality.

As in sepsis, recent results reported in humans and monkeys with severe influenza are consistent with excessive proinflammatory cytokine responses being the cause of death (18, 19). What is not known is how HMGB1 might contribute to the ensuing “cytokine storm,” given its ability to amplify and prolong inflammation. With this in mind, we investigated the role of circulating HMGB1 during severe influenza using a murine model, A/Japan/305/57 (H2N2) influenza virus, which caused the 1957 influenza pandemic, in BALB/c mice. Using a commercially available ELISA for HMGB1, we found that circulating levels of HMGB1 were not elevated at the time of peak mortality and, instead, peaked relatively late in infection on day 9. Treatment with ethyl pyruvate to reduce active secretion of HMGB1 did not reduce mortality, and thus the question of the role of passively released HMGB1 during influenza remains to be addressed.

Materials and Methods

Mice

BALB/c mice were obtained through the Animal Services Division at the Australian National University, Canberra, Australia. Mice were provided with food and water ad libitum and housed in Physical Containment Level 2-designated housing, maintained at 21°C. At the time of writing, this level of containment was appropriate for H2N2 influenza, according to Australian biosafety standards (AS/NZS 2243.3:2002). All experiments were approved by the Australian National University Animal Ethics Committee.

Influenza virus stocks and infection of mice

Stocks of non-mouse-adapted A/Japan/305/57 influenza virus (H2N2) were grown in embryonated eggs. Virus-containing allantoic fluid was harvested and stored in aliquots at −80°C. Virus content was determined by hemagglutination using chicken erythrocytes. The stock virus titer used was 1.6 × 10^5 hemagglutination units (HAU) per ml of allantoic fluid (HAU/ml). Female BALB/c mice, 6 wk old, were anesthetized with 250 μl avertin via i.p. injection, and infected with A/Japan/305/57 influenza virus via intranasal instillation of 32 μl of virus-containing allantoic fluid, equivalent to 50 HAU. Body weights were monitored throughout infection. Mice that did not lose >10% of their uninfected body weight were excluded as uninfected.

Treatment with ethyl pyruvate

Ethyl pyruvate was used to inhibit release of HMGB1 into the circulation, as has been described previously (15). Ethyl pyruvate (Sigma-Aldrich) was added to the vehicle to deliver either 40, 80, or 120 mg/kg to each mouse in an injection volume of 250 μl (except when the vehicle was propylene glycol, in which case 10 μl was administered) and mice were injected i.p. Four different vehicles were tried—a commercial Ringer’s solution ( Baxter), Dulbecco’s PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (ICN Biomedicals), endotoxin-free saline (Sigma-Aldrich), and propylene glycol (MP Biomedicals). Ethyl pyruvate was given either daily, from days 4 to 10 post infection (p.i.) as we have done previously for the drug gemfibrozil (20), or prophylactically, from 1 h before infection, and then daily, until day 10 p.i.
**LPS treatment of mice**

Male BALB/c mice, 8 wk old, were injected i.p. with 10 mg/kg LPS (Escherichia coli 0111:B4), as described in Ref. 1, and plasma collected at 0, 2, 4, 6, 16, and 24 h post-LPS treatment for measurement of HMGB1 by ELISA. Blood was taken from the axillary artery, and plasma collected and stored as described below. Serum samples were collected by allowing the blood to clot and then centrifuging at 1000 × g, 4°C, 15 min.

**HMGB1 measurement by ELISA**

Plasma HMGB1 was measured using a commercially available ELISA (Shino-Test Corporation; distributed by IBL, Hamburg) according to the manufacturer’s directions. For the time course of systemic HMGB1 production, 50 mice were infected with influenza virus and randomly sorted into cages at the time of infection, so that blood could be collected at each time point from the survivors. Mice were bled out from the axillary artery under anesthesia, and blood collected into tubes containing 5 μl of heparin (DBL, Heparin Injection BP; Mayne Pharma). Plasma was collected after centrifugation at 1000 × g, 4°C, 15 min, and stored at −80°C until analysis.

For analysis of HMGB1 levels in combination with mortality, three separate experiments were performed. In two experiments, mice were infected with influenza virus and bled from the tail vein on day 6 p.i. In the third experiment, mice were infected with influenza virus and bled from the tail vein on days 2, 5, 6, 7, and 8 p.i. Mice were warmed under a heat lamp and then tail blood harvested by removing the tip of the tail with a scalpel blade. Drops of blood were collected onto a microscope slide, and 45 μl pipetted into tubes containing 5 μl of heparin. Plasma was collected and stored as described above. When multiple tail bleeds were performed, mice were injected i.p. with 200 μl of warm endotoxin-free saline after collection of tail blood to compensate for lost fluids.

In a further experiment, mice were killed by cervical dislocation and a local blood sample taken. To do this, the rib cage was opened and the blood that had pooled in the chest cavity around the lungs was removed. Plasma was collected and stored as described above.

**HMGB1 measurement by Western blot**

Plasma or serum from LPS-treated mice (100 μl) was spun through Centricon filters (YM-100; Millipore) to concentrate samples and remove complexes >100 kDa, as has been described (1, 12, 13). For comparison, recombinant human HMGB1 (Sigma-Aldrich) was diluted in centricron-pooled normal human serum. Proteins were separated by SDS-PAGE (4–20% NuBlu Tris-glycine gels; NuSep) and transferred to polyvinylidene difluoride membranes overnight at 4°C. All incubations were subsequently conducted at room temperature. Membranes were blocked with 1% BSA in blocking buffer (Medical and Biological Laboratories) applied for 2 h at 0.25 μg/ml in blocking buffer (21). Membranes were washed in four changes of PBS plus 0.05% Tween 20 over 60 min, and then incubated for 1 h with anti-mouse IgG HRP conjugate (Sigma-Aldrich) at 1/12,000 in blocking buffer. Membranes were washed as before, and proteins detected using ECL reagents (Sigma-Aldrich).

**ELISA for anti-HMGB1 autoantibodies**

Autoantibodies against HMGB1 were assayed by ELISA as described by Ref. 21 with some modifications. In brief, MaxiSorp 96-well plates (Nunc) were coated with 50 μg/well recombinant HMGB1 (Sigma-Aldrich) at 2 μg/ml in PBS. After overnight incubation at 4°C, plates were washed three times and then blocked with 5% BSA in PBS, for 2 h at room temperature. After 5 washes, plasma samples were added at 100 μl/well, 1/50 dilution, in 5% BSA in PBS, and incubated overnight at 4°C. As a negative control, some wells contained normal human plasma at a 1/50 dilution in 5% BSA, 100 μl/well, and some wells had only 5% BSA. Plates were washed five times before adding 100 μl/well HRP-conjugated goat anti-mouse IgG (γ-chain specific, Sigma-Aldrich) at 1/1,000 in PBS, and incubated for 1 h at room temperature. Abs against HMGB1 were detected with O-phenylenediamine, 200 μmole per tablet (Sigma-Aldrich); 1 tablet per 9 ml substrate buffer (0.05 M citric acid, 0.05 M sodium phosphate buffer (pH 5.0), and 0.03% hydrogen peroxide). After 30 min, the reaction was stopped with 2.5 M sulfuric acid, and the absorbance read at 490 nm. Owing to a lack of reference sera only the absorbances, corrected by subtracting the average of the negative control absorbances, are reported.

**Statistical analysis**

All analyses were performed using GraphPad Prism 4, except for t tests (two-tailed, unequal variance), which were performed using Excel.

**Results**

(i) Systemic levels of HMGB1 during A/Japan/305/57 (H2N2) influenza virus infection in mice

In influenza virus-infected mice, the plasma concentration of ELISA-detected HMGB1 did not change significantly until day 9 p.i., when it increased compared with uninfected mice (t test p = 0.01, Fig. 1A). This peak of HMGB1 release into the circulation occurred after most mice had died, as shown by the accompanying survival plot (Fig. 1B). In all experiments, most deaths occurred on the morning of day 7, which was a reliable indicator of a normal course of infection. Intriguingly, uninfected mice consistently displayed “high” resting levels of plasma HMGB1 (around 100 ng/ml; Figs. 1A and 2A). H&E-stained sections of lung taken from mice whose plasma HMGB1 levels were assayed in Fig. 1A appeared normal on day 0 and showed no evidence of a cellular infiltrate, which might indicate a pre-existing condition. In comparison, by day 6 p.i., marked cellular infiltrate was evident (data not shown).

(ii) Variation in local vs systemic levels of plasma HMGB1

We noted that when blood was collected from the tail vein (Fig. 2A, solid line), the pattern of HMGB1 concentration over time was similar to that observed in blood collected from the axillary artery (Fig. 1A). In contrast, chest cavity blood contained significantly more HMGB1 on day 7 p.i. (Fig. 2A, broken line, t test p = 0.0088). When autoantibodies against HMGB1 were examined in both tail blood and chest cavity blood we noted a consistent decline in autoantibody presence on day 7 p.i. but the pattern of autoantibody production was different between the two sites (Fig. 2B tail blood, Fig. 2C chest cavity blood).

(iii) Systemic HMGB1 levels and survival

Because the results presented in Fig. 1A reflect circulating HMGB1 levels only in survivors at each time point, we sought to...
assess whether circulating HMGB1 levels could be correlated with mortality. Most mice died on day 7 p.i. (Fig. 1B), so we determined HMGB1 levels in tail blood collected 1 day before death, on day 6 p.i. The results shown in Fig. 3 are pooled from three separate experiments (n = 48 day 6 p.i. samples), as described in Materials and Methods. Fig. 3 plots the plasma HMGB1 level on day 6 p.i., vs the day each mouse died, to determine whether day 6 p.i. levels of HMGB1 were predictive of death. While mice that died shortly after bleeding on day 6 p.i. appeared to have higher levels of HMGB1 in the circulation, the medians of these groups were not significantly different (one-way ANOVA p = 0.1334). Again, most deaths (15) occurred on day 7 p.i., vs four deaths immediately after bleeding, 10 on days 8 and 9 p.i., and 8 on days 10, 11, and 12 p.i. Naïve mice bled from the tail vein had a resting level of HMGB1 of 130.48 ± 19.78 (n = 3; mean ± SEM).
Ringer’s solution (Log rank test \( p \leq 0.027 \)) nor at 40 mg/kg when diluted in A after the onset of symptoms (days 4–10 inclusive) at 40, 80, or 120 mg/kg, did not increase the survival of mice when administered daily.

Ethyl pyruvate, which inhibits the active secretion of HMGB1, did not significantly affect mortality from influenza (21, 22).

We repeated the LPS experiment published previously (1) but used ELISA as our method for detection of HMGB1. We found that ELISA-detected HMGB1 increased 2 h post-LPS injection compared with naive mice (\( t \) test \( p = 0.023 \)) but was not significantly different from naive values at 4, 6, 16, or 24 h post-LPS (Fig. 4A). Since the previous study determined HMGB1 concentrations in serum rather than plasma, we also compared HMGB1 levels in both at about the time of the reported HMGB1 peak, 16 h post-LPS treatment (1), and found no difference (serum 157.15 ± 35.05, plasma 172.06 ± 38.10 mean ± SEM) \( t \) test \( p = 0.79 \). When we compared HMGB1 levels at 96 h post-LPS treatment in both serum and plasma, there was again no significant difference between serum (125.06 ± 18.74) and plasma (172.42 ± 5.70) HMGB1 measurements (mean ± SEM, \( t \) test \( p = 0.11 \)). We note that our measurements tended to be higher in plasma than in serum, as has been previously reported (21).

Autoantibodies declined sharply at 16 h \( (t \) test \( p = 0.027 \)) compared with naive mice and remained low at 24 h post-LPS \( (t \) test \( p = 0.014 \)) but were not significantly different from naive levels at any other time points (Fig. 5A). It should be noted that, in our hands, the dose of LPS used (10 mg/kg) was not lethal. We confirmed that under our conditions, we could detect recombinant HMGB1 via Western blot (Fig. 5B) and found we could again detect HMGB1 in the plasma of naive mice (Fig. 5C).

(iv) Treatment with ethyl pyruvate solution does not affect mortality from influenza

Ethyl pyruvate, which inhibits the active secretion of HMGB1 (15), did not increase the survival of mice when administered daily after the onset of symptoms (days 4–10 inclusive) at 40, 80, or 120 mg/kg diluted in PBS (Fig. 4A), nor at 40 mg/kg when diluted in Ringer’s solution \( (t \) test \( p = 0.4391 \); Fig. 4B). In addition, we observed that ethyl pyruvate (40 mg/kg) given prophylactically (in Ringer’s solution) 1 h before infection and then daily until day 10 did not confer a survival advantage (data not shown). This lack of effect on survival was also observed using saline as the vehicle (data not shown). Because ethyl pyruvate might be labile in aqueous solutions under our experimental conditions, we also administered ethyl pyruvate (40 mg/kg) in propylene glycol from days 4–10 inclusive, which may have worsened survival. Median time to death was 10 days for the vehicle group and 7.5 days for the ethyl pyruvate group, although this apparent effect was again not significant (Log rank test \( p = 0.3280 \); Fig. 4C).

(v) Measurement of HMGB1 by ELISA during LPS toxicity

We repeated the LPS experiment published previously (1) but used ELISA as our method for detection of HMGB1. We found that ELISA-detected HMGB1 increased 2 h post-LPS injection compared with naive mice \( (t \) test \( p = 0.023 \)) but was not significantly different from naive values at 4, 6, 16, or 24 h post-LPS (Fig. 4A).

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Discussion

As has been suggested (22), it is crucial to determine the dynamics of production of HMGB1 during severe influenza infections, to assess its potential as a therapeutic target among the spectrum of other inflammatory mediators and targets. We began this study predicting that, as for other markers of inflammation, HMGB1 was likely to be elevated during severe murine influenza, in that hypercytokinemia is associated with severe seasonal influenza (23), and is most pronounced in fatal H5N1 infections in people (18, 24, 25) and 1918 pandemic influenza infections in macaque monkeys (19). However, using a commercially available assay for HMGB1, we found that circulating levels of HMGB1 were not increased on day 7 p.i. when most mice died but, rather, peaked relatively late in infection on day 9 p.i. (Figs. 1 and 3). In keeping with this observation, a recent study of over 200 patients with sepsis showed that serum levels of HMGB1 did not predict mortality, and, in fact, serum levels of HMGB1 were found to be lower in patients with severe organ dysfunction (14). These results were gained using both Western blot and the commercially available ELISA.

Our results showing HMGB1 levels are elevated in chest cavity blood on day 7 p.i. (Fig. 2A) support the idea that passively released HMGB1 could contribute to influenza-induced mortality, as has been postulated (26). Local production of HMGB1 via passive release is possible during influenza infections, because other viruses are known to induce HMGB1 leak from infected cells (27–29). Furthermore, our results using ethyl pyruvate to reduce active secretion of HMGB1 support the notion that active secretion of HMGB1 might occur too late in influenza virus infections to significantly affect mortality (Fig. 4). Because our study did not address the contribution of passively released HMGB1 to the pathology of influenza in mice, further work is required to determine the level of passively released HMGB1, and whether its neutralization...
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reduces mortality. It warrants noting that glycyrrhizic acid, an active component of licorice root that binds to and inactivates HMGB1 (30), reduces the pathology of influenza A virus (H2N2) infections, (31) although these effects are, at least in part, due to the documented antiviral effects of this agent (31, 32).

A new report has described the existence of autoantibodies against HMGB1 in the circulation of healthy adults (21), in addition to their production during chronic inflammatory disorders (33–35). We therefore concurrently analyzed autoantibodies against HMGB1 in our samples. Because most mice died at the time when plasma HMGB1 was low, HMGB1 was plausibly being released from cells and bound to cellular receptors, removing it from the circulation and rendering it undetectable. Another possibility was that anti-HMGB1 autoantibodies were binding most of the released HMGB1, which would explain the consistent decline in autoantibody concentrations we observed on day 7 p.i. (Fig. 2). When Urbanoviciute and others (21) attempted to identify the binding partners of HMGB1, they found that, in addition to complexes with an apparent molecular mass of 130 kDa (HMGB1 complexed with predominantly IgG1), there were others of lower molecular mass. Other molecules present in the circulation, such as soluble thrombomodulin (36) or soluble receptor for advanced glycation end products (37, 38), also bind to HMGB1 and decrease its biological activity. In any event, the ability of HMGB1 to bind to many molecules at present complicates its accurate measurement. This requires further study with a view to standardization, so that researchers working in this field adopt a universal form of measurement.

When we used the commercial ELISA to attempt to replicate a previously published experiment (1), we saw a peak in HMGB1 as soon as 2 h post-LPS treatment (Fig. 5A), different from the prior study that showed a peak at 16 h post-LPS. At 16 h post-LPS, the concentration of autoantibodies detected in the circulation significantly decreased compared with levels in naive mice. This relative disappearance of autoantibodies from the circulation could mean that autoantibodies are again binding to any HMGB1 then present. Questions remain about whether or not the Centricon filtration step, used to clear samples of macromolecular complexes, would clear autoantibody-HMGB1 complexes (or other HMGB1 complexes), and whether the commercial ELISA detects free HMGB1 as well as HMGB1 complexed with other molecules. A recent human study showed that the pattern of results acquired by Western blot could be verified using the commercial ELISA (14). Our own Western blot results using a commercially available anti-HMGB1 mAb show that plasma HMGB1 levels in naive mice correlated with those detected by ELISA, but at 2 h post-LPS they appeared not to (Fig. 5C). This may reflect as yet unidentified differences in levels of molecules that compete for binding sites on HMGB1 in humans compared with mice. In addition, HMGB1 interacts with molecules such as IL-1β (39), plausibly forming complexes after LPS administration that might prevent its accurate measurement. We were careful to determine the level of ELISA-detected HMGB1 in both serum and plasma at the time of the reported peak, 16 h, and found no difference in HMGB1 levels. Others have noted that plasma HMGB1 measurements tended to be higher than corresponding serum measurements (21), and, while HMGB1 levels were not significantly different between these samples, we did note the same trend toward higher HMGB1 levels in plasma.

We consistently observed that basal levels of HMGB1 were higher using ELISA detection (Figs. 1A, 2A, and 5) than in studies using Western blot (see Ref. 1 for example). We examined H&E-stained lung sections from the mice used to generate Fig. 1A and observed that lung morphology was normal in uninfected mice. As expected, a marked cellular infiltrate was present 6 days after influenza virus infection, indicating that pre-existing inflammation could not have been the cause of the high HMGB1 levels in naive mice. The reported “unspecific background” in Western blots using the Centricon method has been corrected by subtracting the mean value of controls from all patient samples (13), yet it is not clear whether this background was indeed HMGB1 or not. Our Western blot results, derived from a method using Centricon filtration, show that HMGB1 was still detectable in the plasma of naive mice (Fig. 5C). HMGB1 has been previously reported in the serum of some healthy patients, confirmed by Western blot using a protocol that did not include a Centricon step (21).

In summary, we have found that in a mouse model of influenza that results in 70–80% mortality, systemic HMGB1 production did not correlate with death and therapeutic intervention to reduce active secretion of HMGB1 did not reduce disease. Noteworthy was the finding that chest cavity blood contained significantly more HMGB1 on day 7 p.i., the time of peak mortality. Targeting passively released HMGB1 remains a plausible therapeutic target in severe influenza, and studies should be extended to H5N1 to determine the extent and duration of production of HMGB1 during lethal influenza infections. We anticipate that future studies will also address the problems that inevitably arise in regards to the accurate measurement of HMGB1, including further comparisons between the commercial ELISA and semiquantitative Western blotting in animal models of disease, as well as further analysis of the role of autoantibodies against HMGB1.

Acknowledgments
We thank Prof. Arno Mullbacher for provision of A/Japan/305/57 (H2N2) stocks and animal facilities.

Disclosures
The authors have no financial conflict of interest.

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