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High mobility group box 1 protein (HMGB1) is gaining recognition as the mechanism of fatality from influenza. No work to date has addressed the role of high mobility group box 1 protein (HMGB1) in influenza, the parallel being that in other severe proinflammatory cytokine syndromes (e.g., sepsis and malaria) levels of circulating HMGB1 are elevated and may correlate with death. Using a commercially available ELISA for HMGB1, we found that HMGB1 was not increased in the plasma of influenza virus-infected mice (A/Japan/305/57) on day 7 post infection, about the time of peak mortality, and peak levels of HMGB1 in the plasma did not occur until relatively late in infection, on day 9 post infection. In keeping with the late peak of HMGB1 being unassociated with mortality, administration of ethyl pyruvate, which inhibits active secretion but not passive release of HMGB1, to influenza virus-infected mice, did not affect their survival. Further work is required to determine whether influenza virus infection induces passive release of HMGB1, and whether HMGB1 neutralization with a specific Ab would improve survival. The Journal of Immunology, 2008, 181: 1454–1459.

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 × 10^7 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++ and Mg++ (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.), 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 100 × 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 mice were bled from the tail vein on days 2, 5, 6, 7, and 8 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 Centrifor filters (YM-100; Millipore) to concentrate proteins and remove complexes >100 kDa, as has been described (1, 12, 13). For comparison, recombinant human HMGB1 (Sigma-Aldrich) was diluted in centrifroned pooled normal human serum. Proteins were separated by SDS-PAGE (4–16% acrylamide gel; NuSep) and transferred to polyvinylidene fluoride membranes. Membranes were washed as before, and proteins detected using ECL reagents (Millipore). For the time course of systemic HMGB1 production, 30 μl of plasma were transferred to each well of Immobilon P membranes (Millipore) and then blocked with 5% BSA in PBS, for 2 h at room temperature. Abs against HMGB1 were detected with IgG HRP conjugate (Sigma-Aldrich) at 1/12,000 in blocking buffer. 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 1/50,000 in blocking buffer. Membranes were washed as before, and proteins detected using ECL reagents (Millipore).

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 μl/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 (p-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-phenylene-diamine, 200 μmol 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 0.05% 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. Naive mice bled from the tail vein had a resting level of HMGB1 of 130.48 ± 19.78 (n = 3; mean ± SEM).

FIGURE 2. A, HMGB1 levels in the local vs systemic plasma of A/Japan/305/57 (H2N2) influenza virus–infected mice. The solid line shows results from repeated tail bleeds (n = 11 on days 2 and 5, n = 9 on day 6, n = 4 on day 7, n = 2 on day 8; systemic HMGB1) and the broken line shows results from mice bled from the chest cavity (n = 5 mice per time point; local HMGB1). (B) Accompanying systemic and (C) local anti-HMGB1 autoantibody levels. Results are plotted as mean ± SEM.

FIGURE 3. Individual plasma HMGB1 levels and mortality in mice infected with A/Japan/305/57 (H2N2) influenza virus. After bleeding mice on day 6 p.i. from the tail vein, mice were monitored until death. Results are pooled from three experiments (n = 48). Groups are “Postbleed” (death immediately after tail bleed), “D7” (death 1 day after tail bleed), “D8–9” (death 2–3 days after tail bleed), “D10–12” (death 4–5 days after tail bleed), and “Survived” (survivors).

FIGURE 4. Effect of ethyl pyruvate on the survival of mice infected with A/Japan/305/57 (H2N2) influenza virus. A, Mice were given 40, 80, or 120 mg/kg ethyl pyruvate in PBS, daily from days 4 to 10 (n = 9 mice for vehicle, n = 10 for 40 mg/kg, n = 9 for 80 mg/kg, and n = 9 for 120 mg/kg groups). B, Administration of 40 mg/kg ethyl pyruvate in Ringer’s solution (n = 15 mice per group) or in C, propylene glycol, daily from days 4 to 10 (n = 12 mice per group).
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. 5A). 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).

**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 hypotokemia 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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**FIGURE 5.** (a) Systemic production of HMGB1 following LPS treatment. Circulating HMGB1 (solid line) vs HMGB1 autoantibodies (dashed line) are plotted for comparison (\( n = 3 \) mice per time point). (b) Validation of HMGB1 Western blot, showing that recombinant human HMGB1 (30 kDa) was detected by the HMGB1 mAb. Two concentrations of HMGB1 (50 and 100 ng/ml) are shown. (c) Detection of HMGB1 in the plasma of naive mice (LPS 0 h, mouse 1 and 2) and at 2 h post-LPS (LPS 2 h, mouse 1 and 2), and the correlation with ELISA-detected HMGB1 in the same samples.

(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 (Log rank 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 1 and 2, and the correlation with ELISA-detected HMGB1 in the same samples.
reduces mortality. It warrants noting that glycyrrhizinic 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 Urbonaviciute 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.

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Disclosures
The authors have no financial conflict of interest.

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