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Parenteral fluids do not affect pulmonary immune responses to influenza or susceptibility to secondary bacterial pneumonia in mice

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Animal models of viral respiratory disease often use weight loss as a marker of disease severity; however, this may relate to dehydration and malnutrition that would be corrected clinically. We tested whether parenteral fluid therapy improved weight loss from influenza infection. BALB/c and C57BL/6 mice were infected with A/X31 (H3N2) influenza and randomized to intraperitoneal fluid therapy. Blood glucose was also measured post-viral infection on day 3 and 6 in BALB/c mice and on day 6 in C57BL/6 mice. Parenteral fluids did not alter weight loss or the immunological response to infection, and glucose levels were not abnormal.

Keywords Glucose, influenza, rehydration.

Introduction

In murine respiratory virus models, weight loss is used as a systemic correlate of disease severity;[1–3] however, it is unknown whether this weight loss relates to dehydration or even malnutrition (evidenced by hypoglycemia). Indeed, a frequent criticism of animal infection models is that dehydration and hypoglycemia frequently complicate them,[4] compromising the extrapolation of their findings to clinical situations (where these variables are addressed). Furthermore, it is unclear whether the lack of resuscitation may exaggerate the benefits of immunomodulating therapies in mouse models.[1–3] Herein, we wished to probe whether parenteral fluids could alter weight loss, immunity, or pathophysiology in the murine influenza infection model.

Methods

The study protocol was approved by the Home Office (UK) and conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986. Adult (8–12 week) female BALB/c or C57BL/6 mice (Harlan, London, UK) were intranasally (i.n.) administered sterile PBS or

3.7×10^3 plaque-forming units of A/X31 (H3N2) influenza under isoflurane anesthesia. In a further cohort, BALB/c mice were infected with 4.9×10^3 plaque-forming units of influenza. Influenza X31 is a reassortant strain containing the genes of internal and non-structural proteins of A/Puerto Rico/8/34 (H1N1).

Weight was measured daily following infection. On day three after influenza infection, mice typically start to lose weight, at this point, we began 20 ml/kg intraperitoneal fluid administration daily for four days with compound sodium lactate (CSL), normal saline (NS), or sham injection. A further group received 20 ml/kg CSL twice daily (2×20 ml/kg) for four days. These fluid bolus strategies were based on current clinical practice.[5] For BALB/c mice, on day three and six, blood glucose measurements were taken by tail prick (25G needle) and measured by a clinical grade monitor (Optium exceed; Abbott, Maidenhead, UK). Blood glucose was also measured in C57BL/6 mice on day six post-influenza infection.

In further cohorts, BALB/c and C57BL/6 mice were infected with influenza and rehydrated with fluid as above and susceptibility to bacterial infection was tested. On day 7 following influenza infection, mice ($n = 8$ per group) were infected (i.n.) with *Streptococcus pneumoniae* (serotype 2),
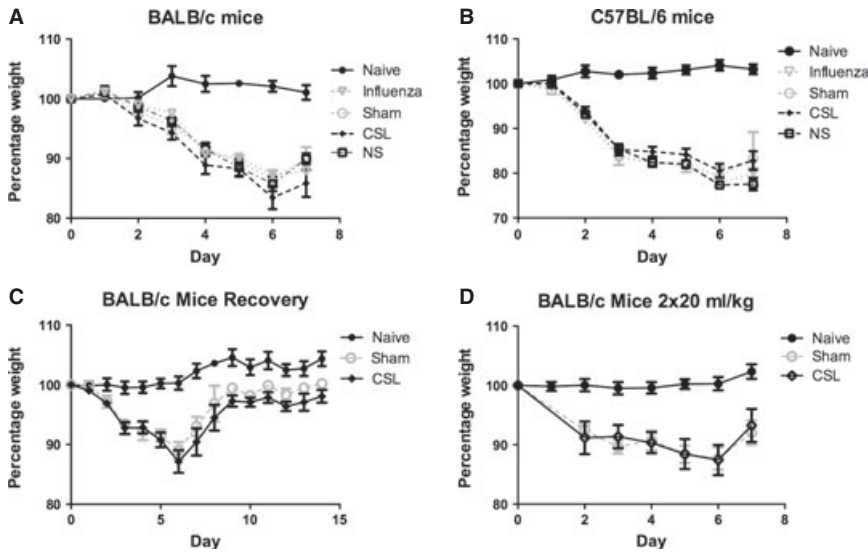
Figure 1. Adult BALB/c (A) or C57BL6 mice (B) were infected with 3.7×10^3 plaque-forming units of X31 (H3N2) influenza via the intranasal route and randomized to intraperitoneal parenteral fluids with 20 ml/kg compound sodium lactate (CSL), normal saline (NS), or sham (control injections but no fluid administered) starting on day three following infection and continued for four days (n = 8/group). An influenza-infected group was also included that did not receive injections (influenza) as were naïve (uninfected) mice (n = 8/group). (C) BALB/c were followed up to day 14 following infection with 3.7×10^3 plaque-forming units of X31 (H3N2) influenza (n = 5/group). (D) BALB/c mice were infected with 4.9×10^3 plaque-forming units of X31 (H3N2) influenza and rehydrated with 2×20 ml/kg CSL per day and followed for 7 days.

Table 1. Influenza infection induces weight loss but not hypoglycemia

	Naive	Influenza	Control	CSL	NS
BALB/c					
Starting weight (g)	19·4 (0·4)	19·6 (0·7)	19·9 (1·1)	19·9 (0·8)	19·8 (0·6)
Weight loss on day 6 (g)	+0·2 (0·4)	−2·5 (0·8)*	−2·5 (0·9)*	−3·3 (1·0)*	−2·8 (0·7)*
Glucose on day 3 (mM)	7·9 (1·0)	9·2 (5·2)	9·8 (2·0)	8·8 (2·6)	NT
Glucose on day 6 (mM)	7·2 (0·4)	6·8 (1·7)	7·3 (0·8)	6·9 (0·5)	7·2 (0·6)
C57BL/6					
Starting weight (g)	18·4 (0·8)	19·5 (0·5)	19·0 (0·5)	19·1 (0·7)	18·5 (1·3)
Weight change on day 6 (g)	+0·6 (0·5)	−4·3 (0·6)*	−4·0 (0·8)*	−3·5 (1·1)*	−4·2 (0·5)*
Glucose on day 6 (mM)	9·8 (2·0)	7·0 (1·7)	8·8 (1·3)	10·0 (1·5)	7·9 (1·3)

NT, not tested; CSL, compound sodium lactate; NS, normal saline.
The data are presented as mean (standard deviation). Weight loss and glycemic load are unaffected by rehydration.
*P < 0·05 versus naïve.

strain D39 (NCTC 7466; National Collection of Type Cultures, London, UK). BALB/c mice were infected with 1×10^6 bacterial colony-forming units (CFUs), and C57BL/6 mice were infected with 1×10^4 CFUs of *S. pneumoniae* based on pilot studies indicating differing vulnerabilities to secondary bacterial infection. For the survival analysis, death was predefined as consistent with Home Office rules limiting the severity of animal illness. Three or more of the following criteria necessitated that the animal was culled: piloerection, increased docility or aggression, immobility, hunched posture, sunken eyes, respiratory distress, dehydration, and loss of more than 25% of body weight. A further cohort of BALB/c mice (n = 5 per group) were sacrificed 48 hours after bacterial infection and blood, lung, and nasal wash cultured for bacterial CFUs. Lung and bronchoalveolar lavage (airway) samples from mice were also taken for flow cytometric (FCM) analysis.

Mice were sacrificed by administration of 3 mg of pentobarbitone and then exsanguination via the femoral artery. BAL fluid, lung tissue, and serum were harvested as previously reported.[1] BAL fluid was obtained by inflation of the lung four times with 1·5 ml 5 mM EDTA in MEM via an intratracheal cannula. Hundred microliters was used for bacterial CFU counts and the remainder centrifuged and the supernatant stored at −80°C. Lung tissue was disrupted through a 100-μM sieve (BD labware, Franklin Lakes, NJ, USA) with 100 μl, then set aside for bacterial CFU counts. The remaining suspension was centrifuged and the red blood cells lysed by adding ACK buffer (0·15 M ammonium chloride, 1 M potassium hydrogen
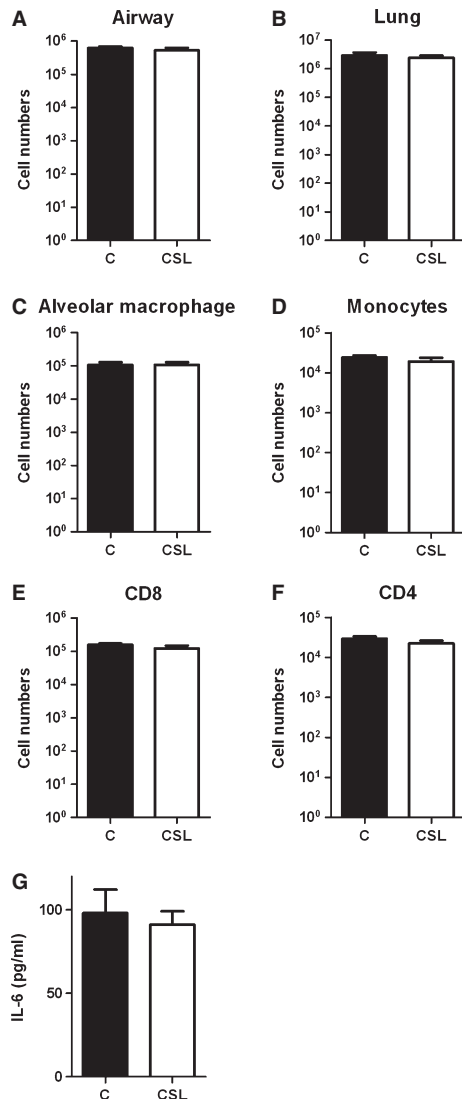
Figure 2. Immune cell recruitment and IL-6 levels on day 7 following influenza infection. Adult BALB/c mice were infected with 4.9×10^3 plaque-forming units of X31 (H3N2) influenza and treated with 2×20 ml/kg CSL per day for 4 days. On day 7, cellular recruitment to the airway and lung was assessed. (A) Total airway counts, (B) total lung counts, airway numbers of (C) alveolar macrophage, (D) monocytes, (E) CD8 lymphocytes, and (F) CD4 lymphocytes. Airway IL-6 levels were also measured by ELISA (G).

carbonate, and 0.01 mM EDTA, pH 7.2) and washed with RPMI containing 10% fetal calf serum (R10F). BAL and lung cell viability was assessed by trypan blue exclusion and cells resuspended in R10F containing 2 mM L-glutamine at 1×10^6 cells/ml. Cells were selected by forward/side scatter and the following surface markers: alveolar macrophage (CD11c high, CD11b high), macrophage/monocyte (CD11c low, CD11b high, Ly6G low), neutrophil (CD11c low, CD11b high, Ly6G high), CD4-positive

lymphocyte (CD4 high, CD3 high, CD8 negative), and CD8-positive lymphocyte (CD4 negative, CD3 high, CD8 high). Additionally, myeloid cells were costained for cell markers including CD200 receptor (CD200R) and OX40L (critical receptors involved in the anti- and pro-inflammatory response, respectively[1,3,6]). CD200R signaling acts to negatively regulate innate immune cell function and the clearance of influenza virus.[1] OX40L is expressed by antigen-presenting cells and acts as a costimulatory molecule to potentiate the immune response.[3] Myeloid cells were also stained for Toll-like receptor-2 (TLR-2)[8] and MARCO as they are important receptors for the detection and clearance of bacteria such as *S. pneumoniae*.[1,6,7] The class A scavenger receptor MARCO is important for the phagocytosis of unopsonized *S. pneumoniae*. TLR-2 is a pathogen recognition receptor that binds to peptidoglycan in the cell wall of *S. pneumoniae*. We have recently shown that CD200R knockout mice do not develop secondary bacterial complications as the heightened immune response in these mice clears the inflammation quicker and hence limits collateral damage.[9] Lymphocytes were also stained for OX40 and CD69. OX40 is a costimulatory molecule that activates OX40L signaling in innate cells and stimulates increased T-cell cytokine production and cell survival.[3] CD69 is an early activation marker on T cells, involved in cell proliferation and as a regulator of cytokine production.[10] Cells were stained for surface markers in PBS containing 0.1% sodium azide and 1% BSA (PBA) for 30 minutes at 4°C and fixed with 2% PFA. All antibodies were purchased from BD Pharmingen (Heidelberg, Germany). Cells were then washed in PBA, data acquired on a BD FACS LSR II and 30 000 lymphocyte or myeloid events analyzed with the FlowJo analysis program. *Streptococcus pneumoniae* titer was determined by serial dilution in PBS of 20-µl aliquots from single-cell suspensions, plating on Columbia agar supplemented with 5% defibrinated horse blood and incubated overnight at 37°C. The total CFUs per tissue were determined manually by counting bacterial colonies (number of colonies x dilution factor × original cell suspension volume). Cytokine quantification of bronchoalveolar lavage samples was performed by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

GRAPHPAD PRISM software was used for all statistical calculations. Analysis of variance followed by *post hoc* Tukey test was used to compare weight loss, cell recruitment, and geomean. Kruskal–Wallis test was used to compare bacterial load data and log-rank Mantel–Cox test to determine significance in survival curves. Categorical differences in survival were analyzed by Fisher's exact test. On the weight charts, data represent the mean ± standard error of the mean (SEM), in the table, data represent the

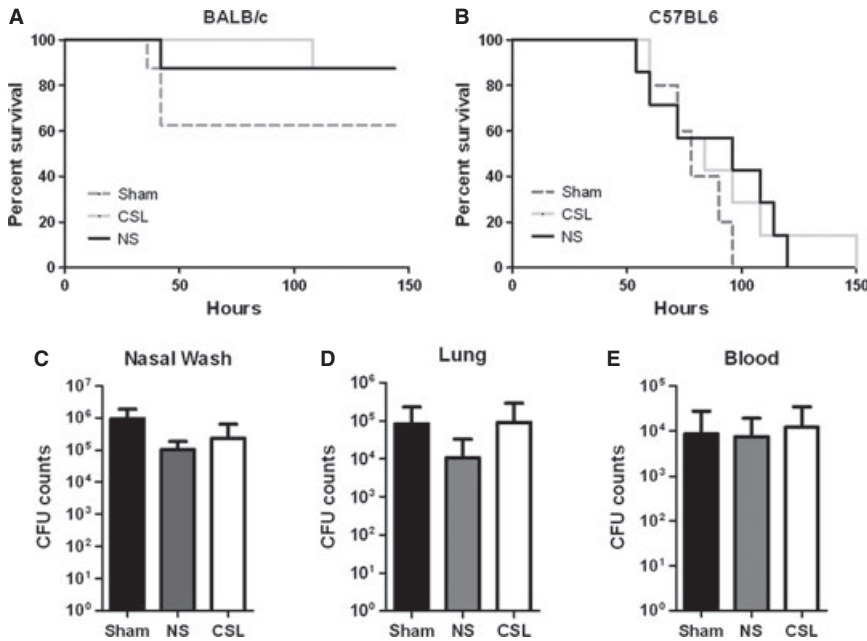**Figure 3.** Mice were infected with 3.7×10^3 plaque-forming units of X31 (H3N2) influenza and rehydrated between day 3 and 7 or not with 20 ml/kg compound sodium lactate (CSL) or normal saline (NS). Controls received sham injections with no fluids administered. On day seven, (A) BALB/c mice were challenged with 1×10^6 *Streptococcus pneumoniae* and (B) C57BL/6 mice with 1×10^4 *S. pneumoniae*. For the survival experiments, naïve and influenza alone groups were also included (but incurred no mortality). Survival following bacterial challenge was unaffected by rehydration in either species. In BALB/c mice, bacterial colony-forming unit counts in the (C) nasal wash, (D) lung, and (E) blood were measured. Rehydration with 20 ml/kg CSL or NS during the influenza did not affect susceptibility to bacterial superinfection compared to sham.

mean ± standard deviation (SD). $P < 0.05$ was considered significant.

Results

In BALB/c (Figure 1A) and C57BL/6 (Figure 1B) mice, influenza induced significant weight loss ($P < 0.05$ versus naive group) that was unaffected by rehydration with 20 ml/kg CSL or NS (Table 1). Follow-up to day 14 in BALB/c mice given 20 ml/kg CSL showed the intervention did not affect recovery of weight loss (Figure 1C; $P < 0.05$ versus control group). Further experiments in BALB/c mice showed that twice-daily 20 ml/kg CSL up to day 7 did not alter weight loss induced by intranasal infection of 3.7×10^3 plaque-forming units of A/X31 (H3N2) influenza ($P > 0.05$ versus sham control; data not shown). Rehydration with 2×20 ml/kg CSL did not affect weight loss from an increased influenza dose of 4.9×10^3 plaque forming units Figure 1D. Blood glucose levels were unaffected by rehydration on day three or day six of influenza infection in BALB/c mice (Table 1). A similar lack of effect was confirmed in C57BL/6 mice on day six (despite approximately 4 g of weight loss at this time).

Influenza-induced pulmonary (BAL or lung) cell recruitment (total and subset numbers) and activation status were also unaffected on day 7 and 14 postinfection by rehydration with 20 ml/kg CSL ($P < 0.05$ versus sham control; data not shown). Administration of 2×20 ml/kg CSL did not affect cell recruitment ($P > 0.05$ versus sham control; Figure 2) or activation status ($P > 0.05$ versus sham control; data not shown) and airway interleukin-6

($P > 0.05$ versus sham control; Figure 2) in response to influenza.

Parenteral fluid rehydration during influenza did not affect the subsequent susceptibility to secondary bacterial pneumonia in BALB/c ($P = 0.39$) or C57BL/6 mice ($P = 0.8$) (Figure 3A,B). Comparison of the control groups shows evidence of the different susceptibility to bacterial superinfection in C57BL/6 and BALB/c mice. 100% (8/8) of C57BL/6 mice died versus 38% of BALB/c mice (3/8) ($P = 0.02$ by Fischer's exact test) despite C57BL/6 mice receiving a 100-fold lower bacterial dose. In further rehydration experiments in BALB/c mice, the bacterial CFU counts did not differ in the blood, lung, or nasal wash ($P > 0.05$ versus control; Figure 3C–E) nor were there differences in cell recruitment following secondary bacterial pneumonia ($P > 0.05$ versus control; Figure 4).

Discussion

As parenteral fluid administration did not affect immunity or pathophysiology during A/X31 influenza infection in two murine strains, we conclude that significant dehydration is not a confounder of disease severity in our model. Likewise, we did not observe hypoglycemia during the influenza infection, another important confounder in animal infection models.[4] Our model has some important caveats that need mentioning. We chose to administer the fluids via intraperitoneal rather than intravenous injection due to ease of administration and to avoid acute hemodynamic changes. Despite administration of up to 3.2 g of fluid over 4 days (in the 2×20 ml/kg rehydration group), the mice did not gain
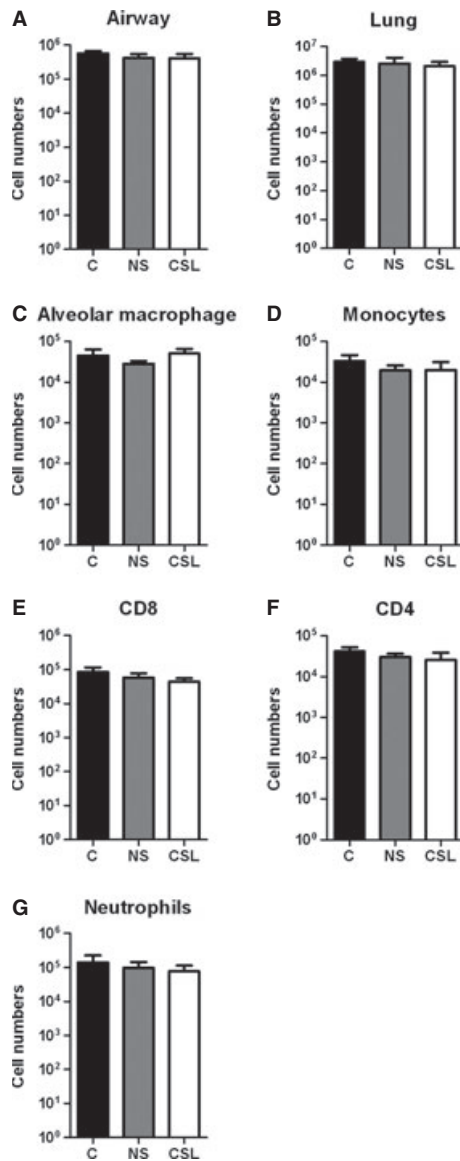
Figure 4. Immune cell recruitment at 48 hours following bacterial superinfection is unaffected by rehydration during influenza. Cellular recruitment to the airway and lung was assessed. (A) Total airway counts, (B) total lung counts, airway numbers of (C) alveolar macrophage, (D) monocytes, (E) CD8 lymphocytes, (F) CD4 lymphocytes, and (G) neutrophils.

weight, and therefore, we chose not to change the method of administration especially as absorption occurs rapidly via the intraperitoneal route.

Our data suggest that dehydration and hypoglycemia do not confound this disease model. Hence, immunomodulatory advances made in these murine models[1–3] may be more easily translated into the clinical domain paving the way for testing other therapies.[11–15] However, our findings do not negate the importance of testing these variables in other species, especially larger animal models, where immune reactions are often different.[14]

Acknowledgements

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