Antioxidants inhibit mercuric chloride-induced early vasculitis

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Keywords: antibodies and autoimmunity, cytokines, in vivo animal model, mast cells

Abstract

In the Brown Norway (BN) rat, mercuric chloride (HgCl2) induces a T_h2-dominated autoimmune syndrome which includes an early phase of mast cell-dependent vasculitis. We have shown in vitro that oxidative stress up-regulates IL-4 in mast cells and predisposes to degranulation. The aim of this study was to determine whether administration of antioxidants inhibits HgCl2-induced early vasculitis in vivo, and, if so, to examine whether modulation of the oxidative/antioxidative balance influences IgE and IL-4 expression by mast cells in situ. Groups of rats were given HgCl2 + saline, HgCl2 + N-acetyl-L-cysteine (NAC), saline + saline or saline + NAC respectively and blood was taken and animals killed 48 h later. NAC significantly reduced both HgCl2-induced early vasculitis and HgCl2-enhanced IgE expression on mast cells with a trend to a decrease in HgCl2-enhanced IL-4 expression in these cells. In addition, there was an increased rat mast cell protease (RMCP) II concentration in the serum after HgCl2 injection and the elevated levels of RMCP II stimulated by HgCl2 were totally abolished by the administration NAC in the HgCl2 + NAC group. However, there was no significant change in serum total IgE concentrations between the HgCl2 + saline group and the HgCl2 + NAC group. The non-sulphydryl-containing antioxidants desferrioxamine and pyruvate demonstrated a similar effect in inhibiting HgCl2-induced early vasculitis. Our data show that administration of an antioxidant to BN rats reduces HgCl2-induced early vasculitis, suggesting that oxidative stress plays a role in the pathogenesis of HgCl2-induced early vasculitis. This finding may have implications for the understanding of the initiation in this experimental model of T_h2 cell-driven autoimmunity and possibly of analogous human diseases.

Introduction

In the Brown Norway (BN) rats chemical compounds such as mercuric chloride (HgCl2), d-penicillamine or gold salts induce a T_h2-dominated autoimmune syndrome, characterized by the production of various autoantibodies (1,2), a significant increase in serum IgE concentration, tissue injury in the form of a narcotizing vasculitis especially affecting the caecum and an inflammatory polyarthritis (3,4). The induction of a necrotizing vasculitis and anti-neutrophil cytoplasm antibody (ANCA) of the anti-myeloperoxidase (MPO) type in BN rats is analogous to human systemic vasculitis, despite the different distribution of vasculitis (2,5). The presence of both anti-MPO ANCA and elevated IgE are features of Churg–Strauss vasculitis (6). The presence of salivary gland infiltrates has parallels with the exocrine predilection of Sjögren's syndrome (7). The autoimmune syndrome is known to be T cell dependent (8) and the marked increase in IgE concentration implicates the T_h2 subset, as isotype switching to IgE production is dependent on IL-4 (at least in the mouse), a major cytokine product of T_h2 cells (9).

Although vasculitis peaks at the same time as the serological response ~2 weeks after the administration of HgCl2, significant vasculitis occurs in the caecum within 24–48 h of an injection of HgCl2, a time at which there is no significant T cell infiltrate (10). Our work has demonstrated a role for mast cells in this early vasculitis (11). Our previous studies have also shown that IL-4 mRNA expression was up-regulated in the caecum within 24 h of an injection of HgCl2 in vivo (10).

In rats, two subtypes of mast cells have been identified based on differences in tissue distribution, biochemical phenotype and histochemical characteristics (12). Connective...
tissue mast cells contain the rat mast cell proteases (RMCP)-1, -5, -6 and -7, and carboxypeptidase A (13), while mucosal mast cells express RMCP-2 (or II), -8, -9 and -10 (12,13). Both populations synthesize and secrete chemokines and cytokines (12), including IL-4. Mast cells are known to be essential resident effector cells in the initiation of allergic responses (12). There is increasing evidence that mast cells may also function as immunoregulatory cells (14,15) and that mast cell-derived cytokines, in particular IL-4, are capable of polarizing T cells to differentiate to the Th2 subset (16). In mast cell-derived cytokines, in particular IL-4, are capable of responses (12). There is increasing evidence that mast cells are essential resident effector cells in the initiation of allergic cytokines (12), including IL-4. Mast cells are known to be mast cells express RMCP-2 (or II), -8, -9 and -10 (12,13). Tissue mast cells contain the rat mast cell proteases (RMCP)-2, 8, 9, and 10 (12,13). Most recently, we have shown that HgCl2 enhances both IL-4 mRNA and protein expression in RBL-2H3 cells in vitro, that oxidative stress by H2O2 mimics the effects of HgCl2 in enhancing IL-4 promoter activity and translation, and that antioxidants ( sulphhydril and non-sulphhydril containing) diminish the HgCl2-induced enhancement of IL-4 (21). These data suggest a novel mechanism of IL-4 transcriptional up-regulation by oxidative stress. We therefore propose the hypothesis that up-regulation of IL-4 and sensitization for mediator release in mast cells, caused by production of intracellular ROS, contributes to HgCl2-induced autoimmunity.

The aim of this study was to test this hypothesis by determining whether administration of antioxidants to BN rats inhibits HgCl2-induced early vasculitis in vivo, and, if so, to examine whether modulation of oxidative/antioxidative balance influences IgE and IL-4 expression by mast cells in situ. Although we had previously shown that certain antioxidants can diminish HgCl2-induced expression of IL-4 at the protein level, as a prelude to the present study we wished to find out whether this was also true for N-acetyl-l-cysteine (NAC), the principle antioxidant we planned to use in vivo.

Methods

Cells and reagents
The rat mast cell line RBL-2H3 was obtained from ATCC (Rockville, MD). The cells were cultured as described previously (21). HgCl2, pyruvate and NAC were purchased from Sigma (Poole, UK). NAC used in vivo was purchased from Evans Medical (Leatherhead, UK), while desferrioxamine was from Ciba (Horsham, UK). Mouse anti-rat IgE and IL-4 mAb were purchased from Serotec (Oxford, UK), as were isotype-matched control antibodies.

Treatment of RBL-2H3 cells with NAC and HgCl2
The day before the experiment, RBL-2H3 cells were removed by trypsinization and seeded to new flasks at a cell density of 2×10^6/20 ml of medium. The cells were incubated with or without NAC (2×10^{-2} M) for 1 h and then cultured in the presence or absence of HgCl2 (10^{-3} M) for 48 h. The same amount of NAC was also added 24 h after addition of HgCl2 in one of the flasks.

Treatment of rats with antioxidants and HgCl2
Male BN rats (250–400 g) were purchased from Harlan-Olac (Bicester, UK) and used in age-matched groups. In one group (HgCl2 + NAC group), rats were given NAC dissolved in drinking water at a concentration of 1 g/l 2 days before and during the experimental period, and injected i.p. with 1 g/kg body wt of NAC solution at ~2, 6, 24 and 30 h with respect to injection of HgCl2. A second group of animals (HgCl2 + saline group) was given tap water and injected i.p. with equal volumes of saline at the above time points. All rats were given HgCl2 s.c. 1 mg/kg at 0 h. In one experiment, a third group of rats (saline + saline group) was injected (i.p. and s.c.) with saline and served as normal controls. A fourth group of rats (saline + NAC group) was given the same dose schedule of NAC as the HgCl2 + NAC group and a single injection of saline (s.c.) at 0 h. In addition, the non-sulphhydril containing antioxidants desferrioxamine (22) and pyruvate (23) were given in a separate experiment. One group (HgCl2 + desferrioxamine group) of rats were injected (i.p.) with 0.4 g/kg body weight of desferrioxamine solution and another group of rats (HgCl2 + pyruvate group) was injected (i.p) with 0.4 g/kg of pyruvate at ~2, 0, 6, 24, 27 and 30 h. In the HgCl2 + pyruvate group, rats were given pyruvate in drinking water at a concentration of 2 g/l at 2 h before and during the experimental period. Two additional groups of rats (HgCl2 + saline group and HgCl2 + NAC group) were also included in the experiment.

Blood samples were taken from tail vessels at various time points, and serum was stored at −20°C for measurement of RMCP II and IgE concentrations. Animals were killed and scored for macroscopic evidence of vasculitis at 48 h after the start of HgCl2 by an experienced observer blinded to treatment group. The total macroscopic vasculitis score was the sum of both serosal and mucosal score for each rat (22). The scoring system has been shown to correlate well with histological vasculitis scores (24). Biopsies were taken from caecal tissues, and fixed for microscopic vasculitis scores and other histological analysis. Coded histological sections were examined by an experienced histopathologist (D. R. T.).

Histology

Cytospin preparations of RBL-2H3 cells were fixed in alcohol and endogenous peroxidase production blocked with methanol/H2O2. Antigen retrieval was effected by microwave treatment for 10 min in citrate buffer. The mouse mAb against rat IL-4 (Serotec) at 1:100 dilution in 1% BSA was applied to the cells, and a DAKO Envision kit (monoclonal) provided enhancement and the visual marker (diamino-leuzidine). The slides were lightly counterstained with Mayer’s haematoxylin.

Directed biopsies from rat caecal mucosa were fixed in formalin and/or Carnoy’s fluid prior to paraffin embedding. Sections were cut at 5 μm and stained with haematoxylin & eosin, toluidine blue and choloracetate esterase. The immunoperoxidase technique was used on tissue specimens fixed in
Carnoy’s fluid without antigen retrieval. The animal research kit (ARK) from Dako was employed. Surface IgE+ cells were identified using a mouse anti-rat IgE heavy chain mAb (MCA 193; Serotec). For IL-4 staining, the mouse anti-rat IL-4 mAb (Serotec) was used.

**Serum RMCP II and total IgE analysis**

The rat RMCP II ELISA kit was purchased from Moredun Scientific (Edinburgh, UK) and used according to the manufacturer’s instructions. Total IgE was also measured by ELISA (4).

**Statistical analysis**

Data are expressed as mean ± SEM or median and analyzed using repeated measures analysis of variance or the non-parametric Mann-Whitney U-test and Kruskal-Wallis H-test as appropriate. Two-tailed P values <0.05 were considered significant.

**Results**

**NAC down-regulates HgCl₂-enhanced IL-4 production in RBL-2H3 cells**

Although some basal expression of IL-4 was detectable in RBL-2H3 cells (Fig. 1A), the level of the protein was considerably enhanced after HgCl₂ treatment for 2 days (Fig. 1B). The enhancement of IL-4 protein expression by HgCl₂ was inhibited by adding NAC (Fig. 1C).

**Antioxidants reduce HgCl₂-induced early vasculitis in BN rats**

Administration of HgCl₂ to BN rats (n = 12) induced macroscopic vasculitis in 11 of 12 rats, with a median total vasculitis score of 3.5. Animals (n = 12) treated with both HgCl₂ and NAC showed a decrease in the incidence (four out of 12) and the median total vasculitis score (median = 0) (Fig. 2A). There was a significant difference (P = 0.0006, Mann-Whitney U-test) between the severity of macroscopic vasculitis in the two groups. In a separate experiment, rats (n = 10) given saline i.p. and s.c. had no macroscopic vasculitis (data not shown). Microscopic vasculitis score from directed caecal biopsies confirmed the above findings: median score HgCl₂ + saline = 4; HgCl₂ + NAC =1 (P = 0.0003, Mann-Whitney U-test). These results were confirmed in three further experiments.

Figure 2(B) shows that treatment of the rats with either desferrioxamine or pyruvate decreases the incidence and the median total vasculitis score induced by HgCl₂ injection. These results were confirmed in two separate experiments (including the one shown). Data pooled from the two identical experiments revealed a significant difference in total macroscopic vasculitis score, both between the HgCl₂ + desferrioxamine group and the HgCl₂ + saline group (Kruskal-Wallis, P < 0.0001), and between the HgCl₂ + pyruvate group and the HgCl₂ + saline group (P = 0.0043).

**NAC influences HgCl₂-enhanced IgE and IL-4 expression on mast cells in vivo**

Figure 3(A and B) shows typical IgE+ and IL-4+ mast cell staining respectively, while Table 1 shows the numbers of IgE+ and IL-4+ mast cells in the submucosa of caecums from BN rats given different treatments. Treatment of BN rats with HgCl₂ (HgCl₂ + saline group) significantly increased the number of IgE+ mast cells in vivo when compared to controls (saline + saline group) at 48 h (P = 0.0084, Kruskal-Wallis adjusted for ties). Treatment of rats with both HgCl₂ and NAC (HgCl₂ + NAC group) significantly decreased the increased number of IgE+ mast cells produced by HgCl₂ (P = 0.0286).

Treatment of BN rats with HgCl₂ (HgCl₂ + saline group) significantly increased the number of submucosal mast cells...
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Fig. 2. Early macroscopic vasculitis scores (48 h). (A) BN rats were treated with HgCl₂ + saline or HgCl₂ + NAC. Results are representative of four separate experiments. (B) Rats were treated with HgCl₂ + saline, HgCl₂ + desferrioxamine, HgCl₂ + pyruvate or HgCl₂ + NAC. Results are representative of two independent experiments. Bars indicate the median. Black dots indicate scores for individual rats.

expressing IL-4 when compared with controls (Kruskal–Wallis \( P = 0.0186 \)) (Table 1). The increase in the number of IL-4⁺ mast cells seen in BN rats treated with HgCl₂ was decreased by administration of NAC, although there were no quantitative data available to support this observation. There was no obvious lymphocyte infiltration or IL-4⁺ T cells present in the tissues of the different groups.

**NAC influences serum RMCP II concentration**

In BN rats, there was an increased RMCP II concentration in the serum after HgCl₂ injection, reaching a peak at 48 h in the HgCl₂ + saline group (\( P = 0.013 \), repeated measures analyses of variance) (Fig. 4A). The elevated levels of RMCP II stimulated by HgCl₂ were totally abolished by the administration of NAC in the HgCl₂ + NAC group. Moreover, the serum levels of RMCP II in the BN rats after HgCl₂ injection were significantly lower than the levels before HgCl₂ administration in the HgCl₂ + NAC group (\( P = 0.001 \)). In a separate experiment, the serum levels of RMCP II in the rats (saline + NAC group) significantly decreased after NAC administration (\( P = 0.001 \)) (Fig. 4A). These indicate that NAC decreases the basal level of serum RMCP II in BN rats.

**IgE concentrations**

As a possible indicator of T_h2 cell involvement in HgCl₂-induced early vasculitis, serum total IgE concentrations were measured. There was no significant change in IgE concentrations between the HgCl₂ + saline group and the HgCl₂ + NAC group, although the basal level of IgE concentration is lower in the HgCl₂ + NAC group than that in the HgCl₂ + saline group (Fig. 4B). In a separate experiment, no significant changes were observed in the saline + NAC group (Fig. 4B).

**Discussion**

We have previously reported that exposure of the mast cell line RBL-2H3 to HgCl₂ enhances both IL-4 mRNA and protein expression, and that antioxidants diminish the HgCl₂-induced enhancement of IL-4 in vitro (21). In the present study, we demonstrate that the antioxidants NAC, desferrioxamine and pyruvate can reduce HgCl₂-induced early vasculitis in BN rats in vivo, and that NAC influences HgCl₂-enhanced IgE and IL-4 expression on mast cells in situ. We have also shown that NAC decreases serum RMCP II concentration. The effectiveness of the non-sulphhydryl containing antioxidants suggests that these effects are not due to the chelation of Hg²⁺ by NAC. This study therefore provides evidence to support our hypothesis that oxidative stress plays a role in the up-regulation of IL-4 and sensitization for mediator release in mast cells in vivo and contributes to HgCl₂-induced early vasculitis. The study also demonstrates the potential of antioxidants in the treatment of vasculitis.

NAC is a precursor of intracellular glutathione which provides a major cellular defence against oxidative stress (25). Desferrioxamine is capable of inhibiting the Fenton reaction and hence prevents H₂O₂ from converting into a highly reactive hydroxyl radical (22), whereas pyruvate reduces H₂O₂ to water (23). NAC has recently been shown to prevent the reduction in renal function induced by an oxidative radiographic contrast agent (26). In vitro, NAC is capable of reducing HgCl₂-enhanced IL-4 mRNA (21) and protein expression in the mast cell line RBL-2H3. In vivo, HgCl₂-induced early vasculitis is markedly reduced by administration
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Fig. 3. Immunoperoxidase staining with IgE or IL-4 mAb. (A) Caecal submucosal mast cells stained for IgE showing a roughly linear arrangement along a blood vessel (original magnification: ×400). (B) A corresponding section from the same block of tissue showing mast cells stained for IL-4 in a corresponding location to the previous illustration (original magnification: ×400). (C) Caecal submucosa from a rat stimulated with HgCl₂ containing a group of mast cells (one partly degranulated) identified using anti-IL-4 antibody (original magnification: ×200). (D) Caecal submucosa containing two mast cells identified with anti-IL-4 antibody from a rat treated with saline only (original magnification: ×200).

Table 1. Numbers of IgE⁺ and IL-4⁺ mast cells per 10 high power fields in submucosa of caecum at 48 h from BN rats given various treatments

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Number of mast cells</th>
<th>IgE⁺</th>
<th>IL-4⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>HgCl₂ + saline</td>
<td>10</td>
<td>14.10 ± 6.99</td>
<td>11.00 ± 6.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.10 ± 5.11&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>HgCl₂ + NAC</td>
<td>8</td>
<td>12.75 ± 6.10</td>
<td>4.50 ± 3.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.00 ± 5.29&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Saline + saline</td>
<td>10</td>
<td>9.10 ± 4.61</td>
<td>3.40 ± 2.20</td>
<td>7.50 ± 4.50</td>
</tr>
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</table>

<sup>a</sup>All values are expressed as mean ± SEM. In each instance, values are from the analyses of the same area that contained IgE⁺ or IL-4⁺ mast cells. All statistics represent the Kruskal-Wallis H-test adjusted for ties.

<sup>b</sup><sub>P</sub> = 0.0084 versus saline + saline.
<sup>c</sup><sub>P</sub> = 0.0286 versus HgCl₂ + saline.
<sup>d</sup><sub>P</sub> = 0.0186 versus saline + saline.
<sup>e</sup><sub>P</sub> > 0.05 versus HgCl₂ + saline or versus saline + saline.

of the antioxidants. We had previously found that treatment of BN rats with agents that stabilize mast cells, but not with an antibody to the αβ TCR, significantly ameliorates the early vasculitis (11). These results suggest that the mast cell plays a role in this early phase of vasculitis. Our present results have shown that the reduction in early vasculitis caused by NAC is accompanied by in vivo evidence of mast cell inhibition (inhibition of the increase in IgE binding to mast cells produced by HgCl₂ and decreased RMCP II concentration); at the same time there is no significant T cell infiltrate or increase in serum IgE concentration. These data strongly suggest that NAC reduces the early vasculitis, at least in part, through inhibition of mast cell function in situ. The mechanism by which NAC inhibits the basal level of RMCP II is unknown. One speculative possibility is that exogenous addition of an antioxidant might block ROS produced during normal cellular metabolism which...
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Fig. 4. Mean serum RMCP II concentration (A) and mean serum total IgE levels (B) in BN rats treated with HgCl₂ + saline (●), HgCl₂ + NAC (▲) and saline + NAC (▼). Groups of rats (HgCl₂ + NAC group and saline + NAC group) were given NAC (1 g/l) in drinking water 2 days before and during the experiment period, and injected (i.p.) with 1 g/kg body weight of NAC solution at –2, 6, 24, and 30 h. All rats except for those in the saline + NAC group were injected with HgCl₂ (s.c., 1 mg/kg) at 0 h. Results for the saline + NAC group are obtained from a separate experiment. Error bars indicate the SEM.

might, in turn, inhibit their function in cell signalling and regulation in mast cells (27).

The main pathway of mast cell activation is IgE dependent. This activation is initiated through the cross-linking of the high-affinity IgE receptor, FcεRI, on the mast cell membrane, which results in the degranulation of the mast cell and the release of mediators (12). It has been shown that IgE-dependent mast cell function requires mast cells to bind IgE via the FcεRI expressed on the surface of the cells (28). Our data showing that HgCl₂ increases the number of IgE⁺ mast cells and that NAC inhibits this increase indicate that the oxidative/antioxidative balance might be one of the factors modulating IgE-dependent activation.

Recent studies by other groups have demonstrated that IgE itself can enhance the IgE-binding ability of mast cells from rodents and human (29,30), and that IgE increases the ability of mast cells to release preformed mediators or cytokines in response to challenge with specific antigen or anti-IgE antibody (31); in particular, there is a striking effect on the ability of cells to secrete IL-4. Moreover, it has also been shown that IL-4 itself, or in synergy with IgE, co-enhanced FcεRI expression and IgE-dependent mediator secretion in mast cells (29,30). In contrast, there was no or very little pre-loaded IgE on FcεRI of peritoneal mast cells derived from IL-4-deficient mice (29). This indicates that IL-4 has the potential to enhance mast cell activation via control of FcεRI expression and IgE binding. Therefore, our finding that NAC inhibited HgCl₂-enhanced IgE binding on mast cells with a trend to a decrease in IL-4 expression in these cells, may, at least in part, be explained via an effect of mast cell-derived IL-4 on IgE-binding capacity in the microenvironment.

IL-4 expression by mast cells is transcriptionally regulated. However, the mechanisms used by mast cells and Th₂ cells are different. IL-4 gene expression in T lymphocytes is more strictly controlled than in mast cells (32). However, mast cells can release pre-stored IL-4 within minutes of stimulation and start to synthesize more hours after the stimulation. Thus, it is possible that mast cells could be activated and provide an initial source of IL-4 which could induce or expand T cells for an effective Th₂ response. In our present study, a trend to a decrease in HgCl₂-enhanced IL-4 expression in mast cells produced by the antioxidant NAC was recorded. We do not know at this stage whether the decreased amount of IL-4 is above or below the threshold required for initiation or amplification of an effective Th₂ response.

In conclusion, our data demonstrate that administration of antioxidants to BN rats reduces HgCl₂-induced early vasculitis, suggesting that oxidative stress plays a role in the pathogenesis of the early vasculitis. This finding may have implications for the understanding of the initiation of this experimental model of Th₂ cell-driven autoimmunity and possibly of analogous human diseases.

Acknowledgements

This work was supported in part by grants from the Wellcome Trust UK and the Special Trustees of St George’s Hospital Medical School, UK.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANCA</td>
<td>anti-neutrophil cytoplasm antibody</td>
</tr>
<tr>
<td>BN</td>
<td>Brown Norway</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>mercuric chloride</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>RMCP</td>
<td>rat mast cell protease</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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</table>

References

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