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Low level exposure to inorganic mercury interferes with B cell receptor signaling in transitional type 1 B cells

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ABSTRACT

Mercury (Hg) has been implicated as a factor contributing to autoimmune disease in animal models and humans. However the mechanism by which this occurs has remained elusive. Since the discovery of B cells it has been appreciated by immunologists that during the normal course of B cell development, some immature B cells must be generated that produce immunoglobulin reactive to self-antigens (auto-antibodies). However in the course of normal development, the vast majority of immature auto-reactive B cells are prevented from maturing by processes collectively known as tolerance. Autoimmune disease arises when these mechanisms of tolerance are disrupted. In the B cell compartment, it is firmly established that tolerance depends in part upon negative selection of self-reactive immature (transitional type 1) B cells. In these cells negative selection depends upon signals generated by the B Cell Receptor (BCR), in the sense that those T1 B cells who's BCRs most strongly bind to, and so generate the strongest signals to self-antigens are neutralized. In this report we have utilized multicolor phosphoflow cytometry to show that in immature T1 B cells Hg attenuates signal generation by the BCR through mechanisms that may involve Lyn, a key tyrosine kinase in the BCR signal transduction pathway. We suggest that exposure to low, environmentally relevant levels of Hg, disrupts tolerance by interfering with BCR signaling in immature B cells, potentially leading to the appearance of mature auto-reactive B cells which have the ability to contribute to auto-immune disease.

1. Introduction

Although the etiologies of most autoimmune diseases (ADs) are unknown, it is clear that they are multifactorial involving both genetic and environmental drivers. Generally, an immune response to self-antigens is held in check by processes collectively known as tolerance. Autoimmune disease arises when normal tolerogenic mechanisms are disrupted, either by genetic abnormalities, environmental insults, or a combination of both. However while much progress has been made in elucidating underlying genetic lesions that contribute to the loss of tolerance to self-antigens, in comparison little is known mechanistically regarding how environmental factors drive the immune system to AD. One reason for this imbalance is that historically it has been difficult to unambiguously link exposure to specific environmental factors with the onset of autoimmune diseases.

One of the exceptions to this is the heavy metal mercury (Hg). The strongest association between Hg exposure and AD is found in animal models. Studies in rats and mice show that low level Hg^{2+} triggers a systemic lupus erythematosus (SLE)-like disorder: Hg induced autoimmune disease (HgIA) (reviewed in (Bagenstose et al., 1999; Druet, 1995; Pollard and Hultman, 1997; Rowley and Monestier, 2005)). In humans epidemiological studies clearly indicate that exposure to Hg at levels that are common within current occupational settings, or in environments which have been contaminated with Hg as a result of illegal artisanal gold mining operations, contribute to immune system dysfunction and autoimmune disease (Cooper et al., 2004; Dahlgren et al., 2007; Dantas and Queiroz, 1997; Mayes, 1999; Queiroz and Dantas, 1997a; Queiroz and Dantas, 1997b; Silbergeld et al., 2005). Significantly, a more recent epidemiological study has now shown that exposure to Hg at lower levels associated with typical environmental exposures outside of the workplace, and which have been perceived to be non-toxic, is correlated with the increased titers of auto-antibodies to double stranded DNA, a well-established autoimmune marker (Somers et al., 2015).

Signaling through the B cell receptor (BCR) complex is a major determinant that normally shapes the immune repertoire so as to establish tolerance to self-antigens. Immature B cells migrate into the spleen from the bone marrow, where they are initially referred to as type 1 (T1) B cells. In the spleen T1 B cells differentiate into type 2 transitional B cells (T2 B cells), and finally T2 B cells differentiate into mature B cells (Chung et al., 2003; Loder et al., 1999). T1 and T2 B cells reside in

Abbreviations: Hg, mercury; AD, autoimmune disease; BCR, B cell receptor; T1, transitional type 1 B cell.

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a milieu of self-antigens, and due to the random nature of immunoglobulin genetic rearrangements during development, many T1 B cells initially express receptors which strongly recognize self-antigens. However the T1 to T2 transition depends upon BCR signal strength in the sense that T1 cells expressing BCRs with the highest affinity to encountered (mainly self) antigens are negatively selected. In this way the mature B cell repertoire is shaped to be poorly responsive to self-antigens, in that the most auto-reactive T1 B cell clones are eliminated by apoptosis, or else become anergic, before the clones can differentiate into T2 B cells (Chung et al., 2003; Loder et al., 1999).

Considering the importance of BCR signaling in the establishment of tolerance, it is not surprising that genetic abnormalities associated with key proteins within the BCR signaling pathway are linked with autoimmune disease (Cambier, 2013). Because of the similarity of HgIA to systemic lupus erythematosus (SLE), and the essential role played by autoreactive B cells in the pathology of a variety of autoimmune diseases (Yanaba et al., 2008), especially SLE (Grimaldi et al., 2005), some time ago we hypothesized that the association of Hg with autoimmunity might likewise be in part explained by Hg negatively impinging upon the BCR signaling pathway, so as to disrupt the function of one or more key elements, thereby compromising tolerance. Using the WEHI 231 B cell line, an *in vitro* model of immature B cells (Warner and Scott, 1988), we initially showed that low levels of Hg do indeed interfere with BCR function in a dose dependent manner (McCabe et al., 1999). BCR signaling is mediated by protein tyrosine phosphorylation events, and in particular activation of the ERK tyrosine kinase by tyrosine phosphorylation is an important signaling intermediary in the BCR signaling pathway (Dal Porto et al., 2004). We have also previously shown that low levels of Hg^{2+} alter B cell tyrosine phosphorylation events in a dose-dependent manner (Rosenspire et al., 1998). We subsequently showed that in WEHI 231, as well as in primary splenic B cells, that Hg exposure was associated with attenuation of ERK activity as assessed by ERK tyrosine phosphorylation. However at low levels Hg did not seem to act directly on ERK, but rather upstream of the kinase (McCabe et al., 2007). In any event, the idea that Hg could be associated with autoimmunity through interference with BCR signaling at the T1:T2 checkpoint seemed plausible.

The Src family tyrosine kinase Lyn is well known to be a critical regulatory component of the BCR signaling pathway upstream of ERK (Lowell, 2004; Xu et al., 2005). So this view was further reinforced when we used mass spectrometry to identify Lyn as the most significant phosphoprotein regulatory node affected in Hg^{2+} burdened B cells. Furthermore we found that several phospho-sites on Lyn were affected by Hg^{2+}, but the carboxyl terminal dominant negative regulatory site was the most sensitive (Caruso et al., 2014).

We recently examined BCR signaling in Hg exposed WEHI-231 cells utilizing phospho-flow cytometry (Irish et al., 2006b; Krutzik et al., 2004) where we demonstrated the utility of the technique in examining the BCR signaling pathway in *in vitro* Hg burdened B cells (Gill et al., 2014). In this report we have utilized phospho-flow cytometry to directly analyze BCR signaling in primary mouse splenic T1 B cells which have been exposed *ex vivo* to similar low cellular burdens of Hg^{2+}. We have found that in both instances ERK as well as upstream elements of the BCR signaling pathway, including phosphorylation of the immune tyrosine activation motif (ITAM) of the BCR co-receptor CD79a and activation of the tyrosine Syk are attenuated during signaling. Furthermore, we have found that phosphorylation of the Lyn C terminal dominant negative regulatory tyrosine, in response to BCR activation is also attenuated in Hg burdened T1 B cells.

2. Materials and methods

2.1. Experimental animals

Seven week old female BALB/c mice were ordered from Jackson Laboratories (Bar Harbor, ME). Mice were allowed to acclimate for one week after arrival at Wayne State University. The animals were housed under conventional conditions and given water and rodent laboratory chow (Ralston Purina, St. Louis, MO) *ad libitum*. The Wayne State University animal care program is AAALAC accredited and all experimental procedures received Institutional Animal Care and Use Committee approval. All animal care and treatment procedures were in compliance with the "Guiding Principles in the Care and Use of Animals" (DHEW Publication, NIH80-23).

2.2. Lymphocyte isolation

Mice were euthanized by CO_2 narcosis followed by cervical dislocation. After euthanasia, spleens were removed, cleaned of fat and connective tissue and placed in cold RPMI buffered with 20 mM Hepes (ThermoFisher, Waltham MA). Cell suspensions were created by passing through a 70 um nylon screen and rinsing with additional RPMI. Splenocytes were pelleted at 900 $\times g$ for 6 min and suspended in cold RPMI. Splenocytes were then further purified by density gradient centrifugation utilizing Lymphocyte separation medium (ThermoFisher, Waltham MA). Cells were resuspended in serum-free RPMI 1640 supplemented with 20 mM HEPES.

2.2.1. Ex vivo exposure regimens. Half of the purified splenocytes were treated with 5 μM $HgCl_2$ (in H_2O) for 10 min, while the untreated cells served as controls. All cells were then treated with the identical dose of goat anti-mouse immunoglobulin (MP Biomedicals, Solon, OH) to initiate BCR signaling for timed periods. To stop BCR signaling, the cells were treated with an equal volume of Fix/Permeabilization Buffer containing 4% paraformaldehyde, 0.1% saponin and 0.01% HEPES in Dulbecco's Phosphate buffer (DPBS) at pH 7.4. Cells were then incubated for 10 min at 37 °C. Cells were washed in Permeabilization/Wash (P/W) buffer containing 0.1% saponin, 0.1% BSA, 0.01 M HEPES and 0.1% sodium azide in DPBS at pH 7.4. Nonspecific antibody binding was blocked by incubating the fixed cells in P/W buffer containing 10% normal mouse serum and 10% normal rat serum for 10 min at room temperature. Cells were washed in P/W buffer and then stained for flow cytometry.

2.2.2. Antibodies and lymphocyte staining for flow cytometry. The following monoclonal fluorochrome conjugated antibodies were used: CD45R (clone) conjugated to APC-CY7, CD24 (clone M1/69) conjugated to PE-CY7, phospho-ERK1/2 (pT202/pY204) (clone 20A) conjugated to PE, ZAP70 (PY319)/Syk(PY352) (clone 17A/P-ZAP70) conjugated to Alexa Fluor 647, IgD (clone 11-26c.2a) conjugated to APC. Antibodies were obtained from BD, Franklin Lakes NJ. Anti-CD21/CD35 (clone 7E9) conjugated to Pacific blue from Biolegend, San Diego, CA. Anti-Lyn (phospho Y507) (rabbit polyclonal) from Abcam, Cambridge, MA. Anti-phospho-CD79a (Tyr 182) (rabbit polyclonal) from Cell Signaling Technology, Danvers, MA. Goat anti-rabbit IgG conjugated to Alexa Flour 488 from Invitrogen/ThermoFisher, Waltham, MA. Goat anti-mouse IgM conjugated to biotin from Jackson ImmunoResearch Laboratories, West Grove, PA. Biotin was detected by Pacific Orange conjugated streptavidin obtained from Invitrogen/Thermo Fisher, Waltham, MA. Cells were stained with a cocktail of the above described labeled antibodies in P/W buffer. Cells were then washed and stained with Pacific Orange conjugated streptavidin and/or goat anti-rabbit IgG conjugated to Alexa Fluor 488. Labeled cells were then examined on a Cyan (Beckman/Coulter) flow cytometer. Flow cytometry results were analyzed utilizing Summit software (Beckman/Coulter).

2.2.3. Presentation of results. Results are presented as the mean fluorescent index (MFI) for the indicated cell population, where MFI is defined as the mean value of the fluorescence intensity for the population of interest. At least 10,000 events (cells) were examined for each data point. Each experiment was independently replicated multiple times, with the number replicates indicated in the figure legend.