Alum, an aluminum-based adjuvant, induces Sjögren's syndrome-like disorder in mice

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Abstract

Objective

Adjuvant-induced innate immune responses have been suspected to play a role in the initiation of certain autoimmune disorders. This study investigates the role of alum, an aluminum-based adjuvant in the induction of Sjögren's syndrome-like disorder in mice.

Methods

Inbred, female New Zealand Mixed (NZM) 2758 strain of mice were injected with alum. Control mice were treated similarly with PBS. The mice were monitored for salivary gland dysfunction by measuring pilocarpine-induced salivation. Presence of lymphocytic infiltrates within the submandibular glands was studied by histopathology. Autoantibodies to Ro and La proteins were analysed by ELISA and the presence of anti-nuclear antibodies (ANA) was analysed by indirect immunofluorescence.

Results

By eight weeks after treatment, the saliva production in the alum-treated mice was significantly decreased in comparison to the PBS-treated mice. This functional loss persisted till the termination of experiments at 20 wks. The incidence and severity of sialoadenitis was significantly higher in the alum-treated mice. Although there were no differences in the levels of anti-Ro/La autoantibodies in sera of alum and PBS-treated groups, the alum group showed higher ANA reactivity.

Conclusion

In the NZM2758 mice, alum induces a Sjögren's syndrome-like disorder that is characterised by chronic salivary gland dysfunction and the presence of lymphocytic infiltrates within the salivary glands. Thus, the potential of aluminum-based adjuvants for induction of autoimmunity should be closely monitored in individuals genetically susceptible to developing autoimmune disorders.

Key words

adjuvants, sialoadenitis, Sjögren's syndrome

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Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder characterised by lymphocytic infiltration in the exocrine glands, loss of salivary and/or lacrimal gland function and the presence of circulating autoantibodies (1). While role of adaptive immunity has been extensively investigated in SS, it is now clear that activation of innate immunity is also important for SS pathogenesis (2). Using mouse models, we have previously reported the role of innate immunity, specifically the type I interferon pathway in SS (3-5). Treatment of NZB/W F1 mice with incomplete Freund's adjuvant (IFA) led to an accelerated SS-like disease with increased infiltration of type I IFN producing plasmacytoid dendritic cells in the salivary glands (3). A similar exacerbation of SS was seen in mice treated repeatedly with poly(I:C), a potent inducer of type I interferon (4). Furthermore, genetic ablation of type I interferon signaling in the B6.*Aec1.Aec2* mice, a spontaneous mouse model of SS, resulted in a complete protection from the disease (5). All these studies support the essential role for innate immune activation and the type I IFN pathway in the disease process.

Another pathway for activation of innate immunity involves the Nucleotide-binding oligomerisation domain (NOD) like receptors (NLRs) (6). Aluminum-based adjuvants, such as alum, can stimulate innate immunity through NOD-like receptor pyrin containing domain 3 (NLRP3) which associates with Caspase-1 to form an inflammasome complex (7). The NLRP3 inflammasome regulates cleavage of pro-inflammatory cytokines like IL-1β, IL-18 and IL-33 into their active forms, thereby activating multiple inflammatory processes. However, whether inflammasome activation can initiate SS is not known. Thus, in this study, we investigated the effects of alum, on development of SS-like disease in female NZM2758 mice. The NZM2758 mouse is an inbred strain generated by in-breeding of the NZB and NZW mice (8). These mice do not spontaneously develop SS-like disease. However, they are genetically susceptible and develop reduced salivary gland function and sialoadenitis following injection with IFA (9).

Materials and methods

Mice and experimental design

All procedures were in accordance to NIH guidelines for humane use of laboratory animals and were approved by the Institutional Animal Care and Use Committee. NZM2758 mice were generated in a breeder colony and housed under specific pathogen free conditions. Eight- to ten-week old female mice were injected subcutaneously with a 50% alum (Pierce Chemical Company, IL, USA) suspension mixed with phosphate buffered saline (0.1ml/mouse). Additional intraperitoneal injections of alum (0.05ml/mouse) were given 4 wks and 8 wks later. Age-matched control groups were injected similarly with PBS alone. Pilocarpine-induced saliva production was measured at different time points as described previously (4).Mice were euthanised at 20 wks of age and salivary glands collected in 10% buffered formalin for histopathologic analysis.

Histopathologic analysis of salivary glands

Submandibular salivary glands (SMG) were embedded in paraffin blocks and sections stained with haematoxylin and eosin using standard procedures. Three micron sections were cut from each block and two sections approximately 50 microns apart were evaluated for severity of sialoadenitis as previously described (3).

Analysis of serum autoantibodies

Sera from terminal bleeds were used for all autoantibody analyses. Serum antibodies to Ro60 and La antigens were measured in an ELISA described previously (3), except plates were coated with purified recombinant Ro60 and La expressed as fusion proteins with maltose binding protein. MBP alone was used as a control. Anti-nuclear antibodies were measured by indirect immunofluorescence staining on HeLa cells grown on coverslips using standard protocols (5). Intensity of staining was scored by an observer blinded to experimental details.

Statistical methods

Mann-Whitney non-parametric *t*-test, paired *t*-test, and One-way ANOVA with Bonferroni post-test for multiple comparisons were used to calculate *p*-values as indicated. A confidence level of 95% was used for all tests and analyses were carried out using Graph Pad Prism 6.0 software.

Results

The NZM inbred strains of mice carry genes that render them susceptible to different autoimmune diseases such as lupus (NZM2328, NZM2410) and Sjögren's syndrome (NZM2758) (8, 9). For the present study, NZM2758 female mice were injected either with alum or PBS. Pilocarpine-induced saliva production was measured at 8 wks after the first injection. Figure 1A shows that in comparison with PBS-treated control mice, just two injections with alum induced a significant drop ($p<0.0001$) in the mean saliva volume. To further investigate the kinetics and persistence of functional loss, additional cohorts of mice were injected with alum and PBS at 0 wks, 4 wks and 8 wks. At 8 wks after treatment, the mean saliva volume in alum-treated group was significantly ($p=0.0103$) reduced by 27%, compared to mean saliva volume on day 0 (Fig. 1B). This drop in function persisted at 13 wks ($p=0.003$) and at 20 wks ($p=0.0005$), when the experiment was terminated. In contrast, the PBS-treated mice did not show any statistically significant changes in saliva production over time. Thus, alum induces a persistent drop in saliva production in the NZM2758 mice.

SMG were harvested from mice after 20 wks of treatment and evaluated for development of inflammatory infiltrates. Representative photomicrographs are shown in Figure 2. Only one out of four mice injected with PBS showed the presence of a small inflammatory focus in the SMG. In contrast, all five mice injected with alum developed multiple inflammatory foci of different sizes. The larger infiltrates were predominantly located in the peri-vascular or peri-ductal regions (arrows), although smaller foci were also seen surrounding secretory acini (arrowheads). The severity of sialoadenitis was scored by an observer blinded to experimental details. As shown in Figure 2, mice treated with alum developed significantly higher ($p=0.012$) inflammation of the SMG.

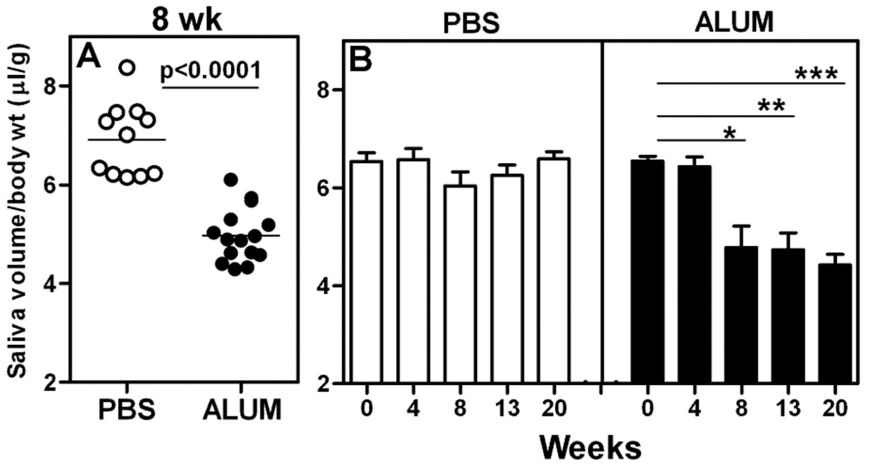

Fig. 1. Alum injection leads to loss of salivary gland function in NZM2758 mice. **A**. The mean saliva level in alum-treated NZM2758 mice (●, n=15) is significantly lower ($p<0.0001$; Mann-Whitney test) than that in PBS- (○, n=11) treated group. Each data point represents one mouse. **B**. In an additional cohort, mice were injected with PBS (n=4) or Alum (n=5) at 0, 4, and 8wk time points and saliva was measured at indicated time points. Compared to day 0, in alum-injected mice, a significant drop in saliva production was seen at 8, 13 and 20 wks. Statistical analyses were done by paired t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Analyses by One-way ANOVA with Bonferroni post-test for multiple comparisons showed that the saliva production in the alum-treated mice was significantly lower than the age-matched PBS-treated group. Data are mean ± SEM saliva volumes for each group.

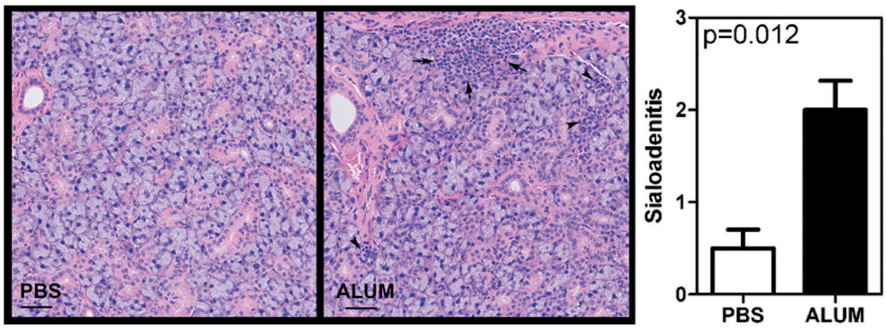

Fig. 2. Alum-injected mice show increased sialoadenitis. Representative photomicrographs of SMG gland sections stained with H & E are shown. Arrows indicate large, and arrowheads indicate small inflammatory foci in alum-injected mice. Scale bar is 50µm. The severity of sialoadenitis was scored on a scale of 0 (no inflammation) to 5 (severe inflammation and gland destruction) and data are represented as mean ± SEM severity score for each group. Alum-injected mice have a significantly ($p=0.012$) higher disease score compared to PBS-injected mice Mann-Whitney test was used for the statistical analysis.

Presence of circulating autoantibodies to Ro and La proteins is a hallmark of SS (1). Therefore, terminal bleeds were studied for reactivity to Ro60 and La expressed as fusion proteins with MBP. As shown in Figure 3, alum failed to exacerbate the anti-Ro/La autoantibody response.

Anti-nuclear antibodies were studied by indirect immunofluorescence using HeLa cells as substrates (Fig. 4). The ANA reactivity in alum group was higher than that in the PBS group. NZM2758 are autoimmune prone mice and develop a low level of circulating autoantibodies with age. Thus, detection of some ANA reactivity in the PBS-treated group is not surprising. However, collectively these data demonstrate that alum exacerbates autoimmune responses in NZM2758 mice.

Discussion

In the present study, we show that alum treatment of NZM2758 mice, leads to reduced salivary gland function, in-

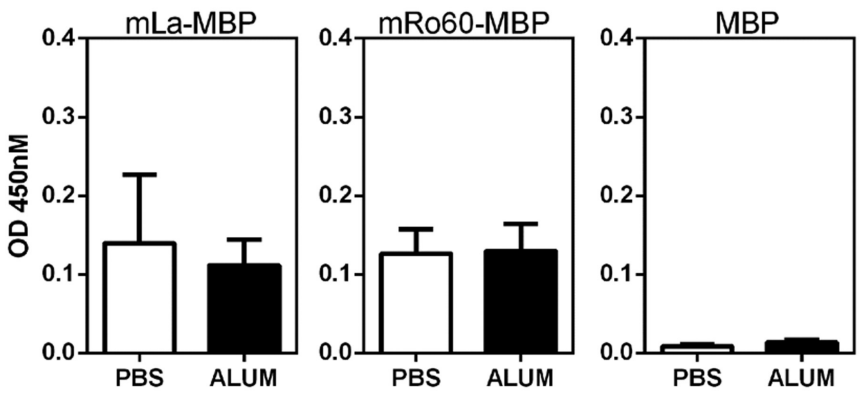

Fig. 3. Exacerbation of SS-like disease with alum injections is not associated with increase in circulating autoantibodies to mouse La and Ro60 proteins. ELISA plates were coated with recombinant mLa-MBP, mRo60-MBP or MBP alone. Sera from terminal bleeds were tested at a 1:100 dilution and bound IgG antibodies were detected. Data are shown as mean ± SEM of absorbance at 450nM.

creased SMG inflammation and ANA production. This study is the first report demonstrating induction of SS-like disorder in a genetically susceptible mouse strain by an aluminum-based adjuvant, alum.

Alum is responsible for the activation of inflammasome pathway leading to the production of pro-inflammatory cytokines like IL-1β and IL-18. Although increased levels of IL-1β (10) and IL-18 (11) has been reported in SS patients, the role of inflammasome components in pathogenesis of SS is only recently being investigated. Baldini et al show that the expression of $P2X_7$ receptor ($P2X_7R$), is increased in salivary glands of SS patients (12). The $P2X_7R$ is involved in the activation of NLRP3 inflammasome. A concomitant increase in the gene expression of inflammasome components (NLRP3, ASC and caspase-1) in these patients is suggestive of their involvement in disease process. Interestingly, another recent study also suggests that $P2X_7R$ polymorphisms might be involved in SS pathogenesis (13). These reports and the data from our investigation suggests that inflammasome activation plays an important role in the induction and progression of SS.

A surprising observation in the present study was that alum did not induce an exacerbation of anti-Ro/La autoantibody response. Moreover, the reactivity to recombinant Ro and La antigens was not very robust, in comparison to our previous study of IFA treatment of NZB/W F1 mice (3). These data suggest a dissociation between circulating autoantibodies to Ro and La from other SS characteristics in alum-treated NZM2758 mice. However, the ANA data suggests the possibility that exacerbation of autoimmune responses in this mouse following alum injections may target other antigens. The precise mechanisms responsible for increased sialoadenitis and salivary gland dysfunction in this model are not known. They might involve all or some of the pro-inflammatory mediators induced by activation of inflammasome as well as localised apoptosis in the salivary glands and chemokine production leading to inflammatory cell infiltration. Clearly, the alum-NZM2758 model will be valuable for evaluating the role of different components of the inflammasome pathway in SS pathogenesis.

Alum is commonly used in animals and humans. It has a well-established record of safety and efficacy as an adjuvant. However, autoimmune/autoinflammatory syndrome induced by adjuvant (ASIA) has been recently described in humans (14). Shoenfeld and colleagues suggest that alum or other

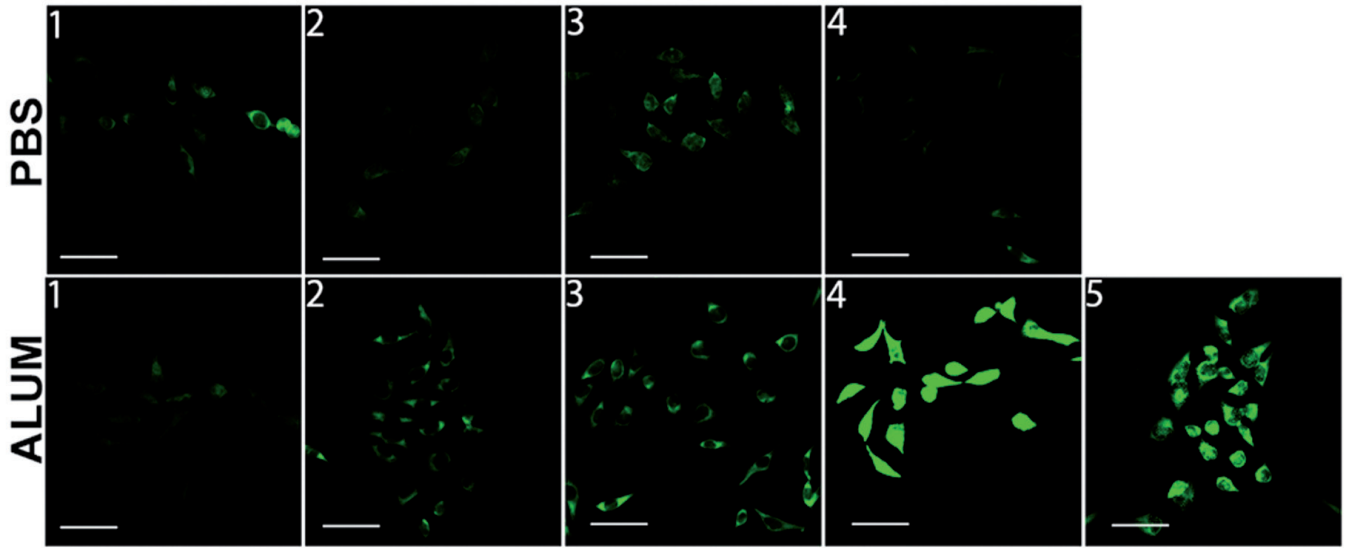

Fig. 4. Comparison of ANA reactivity between alum- and PBS-treated-mice show higher ANA in the alum group. HeLa cells coated coverslips were used as substrate for indirect immunofluorescence. All sera were used at 1:50 dilution and bound IgG antibodies were revealed by goat anti-FITC conjugate. The numbers indicate individual mice. Scale bar is 20μm.

stimulators of innate immunity may trigger different clinical entities including macrophagic myofascitits, the gulf war syndrome, siliconosis, and post-vaccination phenomenon. The recent reports of adjuvant mediated acceleration of autoimmunity in spontaneous murine models of SLE (9, 15)and this study with alum, support the hypothesis that activation of innate and adaptive immunity by adjuvants can initiate and exacerbate autoimmunity in genetically susceptible individuals.

However, there are major differences between administration of alum in mice and in humans (15). Firstly, the dose in mice is significantly higher than in humans. Secondly, the route of administration in mice is subcutaneous and intraperitoneal, while in humans, it is intramuscular. These factors may influence the immune responses and add to the complexity of translating the observations from rodents directly to humans. Nevertheless, the studies in autoimmune prone mice clearly demonstrate the importance of investigating possible side effects of adjuvants, especially in individuals who repeatedly receive vaccinations in a short period of time. The study also underscores the need for developing newer and safer adjuvants for human use.

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