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Effects of Glucan on Immunosuppressive Actions of Mercury

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ABSTRACT Global cycling of mercury results in the presence of mercury salts in the environment. The well-established negative effects of mercury on the immune system led us to the study whether natural immunomodulator glucan can overcome the immunosuppressive effects of mercury. Two types of mercury, thimerosal and mercury acetate, were administered in a dose of 2–8 mg/L of drinking water to mice. After 2 weeks, all mice exhibited profound suppression of both cellular (phagocytosis, natural killer cell activity, mitogen-induced proliferation, and expression of CD markers) and humoral (antibody formation and secretion of interleukin-6, interleukin-12, and interferon-γ) responses. The mice were then fed with a diet containing a standard dose of glucan. Our results showed that simultaneous treatment with mercury and glucan resulted in significantly lower immunotoxic effects of mercury, which suggests that glucans can be successfully used as a natural remedy of low-level exposure to mercury.

KEY WORDS: • *glucan* • *immune system* • *immunosuppression* • *mercury* • *phagocytosis*

INTRODUCTION

FOR A LONG TIME, THIMEROSAL has been used as a wound disinfectant and a preservative in medical preparation, including human vaccines. Recently, concerns regarding the immunosuppressive effects of low-level exposure to mercury raised the question of thimerosal safety.[1,2] Thimerosal contains an organic ethylmercury with similar biological properties as the well-known immunotoxic methylmercury.[3,4] However, recent studies have shown that the effects on the immune system might be different.[5]

Additional studies showed that exposure to most mercury compounds, including mercuric chloride, resulted in cell toxicity[6] and immunosuppression[7] regardless of exposure duration.[8]

β1,3-Glucans are structurally complex homopolymers of glucose, usually isolated from yeast, fungi, wheat, and seaweed. β1,3-Glucan's role as a biologically active immunomodulator has been well documented for over 40 years. Interest in the immunomodulatory properties of polysaccharides was initially raised after experiments indicated that a crude yeast cell preparation stimulated macrophages via activation of the complement system.[9] Further work identified the immunomodulatory active component as β1,3-glucan.[10] Numerous studies (currently more than 4,000 publications) have subsequently shown that β1,3-glucans, either particulate or soluble, exhibit immunostimulating properties that include antibacterial and antitumor activities.[11,12] Studies showing the strong potential of glucans to help overcome immunosuppressive effects of factors, such as irradiation or chemotherapy,[13,14] led us to the hypothesis evaluated in this article. The aims of the present study are to compare immunosuppression caused by either organic (thimerosal) or inorganic (mercury acetate) mercury and to show if this suppression can be reversed by glucan.

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MATERIALS AND METHODS

Materials

RPMI 1640 medium, Iscoves's modified Dulbecco's medium, sodium citrate, antibiotics, sodium azide, thimerosal, mercury acetate, bovine serum albumin, Wright stain, *Limulus* lysate test E-TOXATE, Freund's adjuvant, ovalbumin, lipopolysaccharide, and concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT, USA). β-1,3-Glucan (#300) was purchased from Transfer Point (Columbia, SC, USA), NOW BETA glucan from NOW FOODS (Bloomingdale, IL, USA), Glucagel T from GraceLinc (Christchurch, New Zealand), and Epicor from Embria Health Sciences (Ankeny, IA, USA).

Animals

Female, 6–10-week-old BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All animal work was done according to the University of Louisville Institutional Animal Care and Use Committee protocol. Animals were sacrificed by CO_2 asphyxiation.

Cells

The human monocytoid cell line U937 and the mouse macrophage cell line RAW 264 were purchased from the American Tissue Culture Collection (Manassas, VA, USA). The murine tumor cell line YAC-1 was provided by Dr. Julie Djeu of the Moffitt Cancer Research Center (Tampa, FL, USA). Cells were maintained in RMPI 1640 medium supplemented with 10% FCS, 2 m*M* glutamine, and antibiotics.

Treatment

The samples were collected after a 14-day feeding with glucan-containing diet (100 μg/day) and/or mercury acetate (8 mg/L of drinking water) or thimerosal (2 mg/L of drink-L of drinking water), respectively. All diets (Laboratory Rodent Diet 5001 alone or enhanced with glucan) were formulated and prepared by Purina (Richmond, IN, USA). Diet ingredients for all groups were identical except for the addition of glucan.

Antibodies

For fluorescence staining, the following antibodies were used: anti-mouse CD4, CD8, and CD19, conjugated with fluorescein isothiocyanate, which were purchased from Biosource (Camarillo, CA, USA).

Flow cytometry

Cells were stained with monoclonal antibodies on ice in 12-×75-mm glass tubes using standard techniques. Pellets of 5×10^5 cells were incubated with 10 μL of fluorescein isothiocyanate-labeled antibodies (1–20 μg/mL in phosphate-buffered saline [PBS]) for 30 minutes on ice. After washing with cold PBS, the cells were resuspended in PBS containing 1% bovine serum albumin and 10 m*M* sodium azide. Flow cytometry was performed with a FACScan™ (Becton Dickinson, San Jose, CA) flow cytometer, and the data from over 10,000 cells or samples were analyzed.

Phagocytosis

The technique using phagocytosis of synthetic polymeric microspheres was described earlier.[15,16] In brief, peritoneal cells were incubated with 0.05 mL of 2-hydroxyethyl methacrylate particles (5×10^8/mL). The test tubes were incubated at 37°C for 60 minutes, with intermittent shaking. Smears were stained with Wright stain. The cells with three or more 2-hydroxyethyl methacrylate particles were considered positive. The same smears were also used for evaluation of cell types.

Natural killer (NK) cell assay

Spleen cells were isolated from the spleen of mice by standard methods. Cell suspension was generated by pressing minced spleen against the bottom of a Petri dish containing PBS. After elimination of erythrocytes by a 10-second incubation in distilled water and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6/mL; 0.1 mL per well) in V-shaped 96-well microplates were incubated with glucan (2 μg/mL) for 30 minutes at 37°C and then washed three times with RPMI 1640 medium. After washing, 50 μL of target cell line YAC-1 were used at three different concentrations of target cells so the final effector-target ratio was 10:1, 50:1, and 100:1. After the plates were spun at 250 *g* for 5 minutes, they were incubated for 4 hours at 37°C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Madison, WI, USA) according to the manufacturer's instructions. In brief, 10 μL of lysis solution was added into appropriate control wells 45 minutes before the end of incubation. The next step was to spin the plates at 250 *g* for 5 minutes, followed by transferring 50 μL of supernatant into flat-bottomed, 96-well microplates. After 50 μL of reconstituted substrate was added into each well, plates were covered and incubated for 30 minutes at room temperature at dark. The optical density was determined by using an STL enzyme-linked immunosorbent assay (ELISA) reader (Tecan U.S., Research Triangle Park, NC, USA) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

$$\text{Percentage specific killing (\% cytotoxicity)} = 100\times([\text{OD}_{492}\ \text{experimental} - \text{OD}_{492}\ \text{spontaneous}]/[\text{OD}_{492}\ \text{maximum} - \text{OD}_{492}\ \text{spontaneous}])$$

as described in the manufacturer's instructions, where OD_{492} is the optical density at 492 nm, spontaneous release was target cells incubated with medium alone, and maximum release was that obtained from target cells lysed with the solution provided in the kit.

In vitro *cytotoxicity*

For growth experiments, cells growing in normal culture media were trypsinized and washed six times in Iscoves's modified Dulbecco's medium supplemented with glutamine, antibiotics, and 5% FCS. Cells were seeded in 96-well tissue culture plate at a density of 5×10^4 cells/mL (150 μL per well) in the presence of glucan (1 μg/mL) in triplicates and incubated for 24 hours. After the glucan was washed off, various doses of mercury diluted in RPMI 1640 medium were added to the wells. After 2 days in culture, proliferation was evaluated using the Biotrak cell proliferation ELISA system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to instructions given by the manufacturer.

Mitogenic response assay

For the mitogenic response assay, splenic lymphocytes (5×10^5 per well) were cocultured with either 10 μg of concanavalin A or 1 μg of lipopolysaccharide (LPS) in 200 μL of RMPI 1640 plus 10% FCS for 72 hours. The proliferation was evaluated using the Biotrak cell proliferation ELISA system.

Evaluation of interleukin (IL)-6, IL-12, and interferon-γ (IFN-γ) production

Purified spleen cells (2×10^6/mL in RPMI 1640 medium with 5% FCS) were added into wells of a 24-well tissue culture plate. After the addition of 5 μg of concanavalin A into positive control wells, cells were incubated for 72 hours in a humidified incubator (37°C, 5% CO_2). At the end point of incubation, supernatants were collected, filtered (pore size, 0.45 μm), and tested for the presence of IL-6, IL-12, or IFN-γ. Levels of the individual cytokines were measured using a Quantikine mouse kit (R&D Systems, Minneapolis, MN, USA).

Antibody formation

Formation of antibodies was evaluated using ovalbumin as an antigen. Mice were injected twice (2 weeks apart) with 100 μg of albumin, and the serum was collected 7 days after last injection. Level of specific antibodies against ovalbumin was detected by ELISA. As the positive control, Freund's adjuvant was used.

Statistics

Student's *t* test was used to statistically analyze the data.

RESULTS

The direct cytotoxicity of mercury compounds was tested on two different human cell lines, RAW 264 and U937. F1 ▶ Figure 1 shows that of the two tested compounds, thimerosal is the more toxic one, with 80% growth inhibition at the 0.1 μg dose. When the cells were treated with both mercury compounds and glucan, significantly lower growth inhibition was observed, particularly in the case of thimerosal (Fig. 1B).

Phagocytosis is one of the biological activities traditionally connected with effects of immunomodulators, including glucans. Therefore, we started our study by measuring the effects of mercury on phagocytic activity of blood neutro- F2 ▶ phils. Figure 2 shows that only glucans #300 and NOW BETA exhibit significant stimulation of phagocytosis. The 14-day exposure to Hg compounds strongly depressed the phagocytic activity, which was restored by 71.5% in the case of Hg acetate and 63.5% in the case of thimerosal.

Production of cytokines is among the valuable indicators of the immune activities. Therefore, we compared the effects of tested glucans on the secretion of IL-6, IL-12, and IFN-γ by spleen cells isolated from Hg- and/or glucan-treated mice. The cytokine production was measured after a 72-hour *in vitro* incubation of cells in the presence of concanavalin A. Again, in untreated mice, the same two glucans increased the IL-6 production. In Hg-suppressed animals, glucans #300, NOW BETA, and Glucagel showed a sup- F3 ▶ portive effect, with #300 being superior (Fig. 3). A similar situation was found in the case of IL-12, where, in each experiment, all tested glucans exhibited significant stimu- F4 ▶ lation of IL-12 production (Fig. 4). A different situation was observed when we measured production of IFN-γ. In control

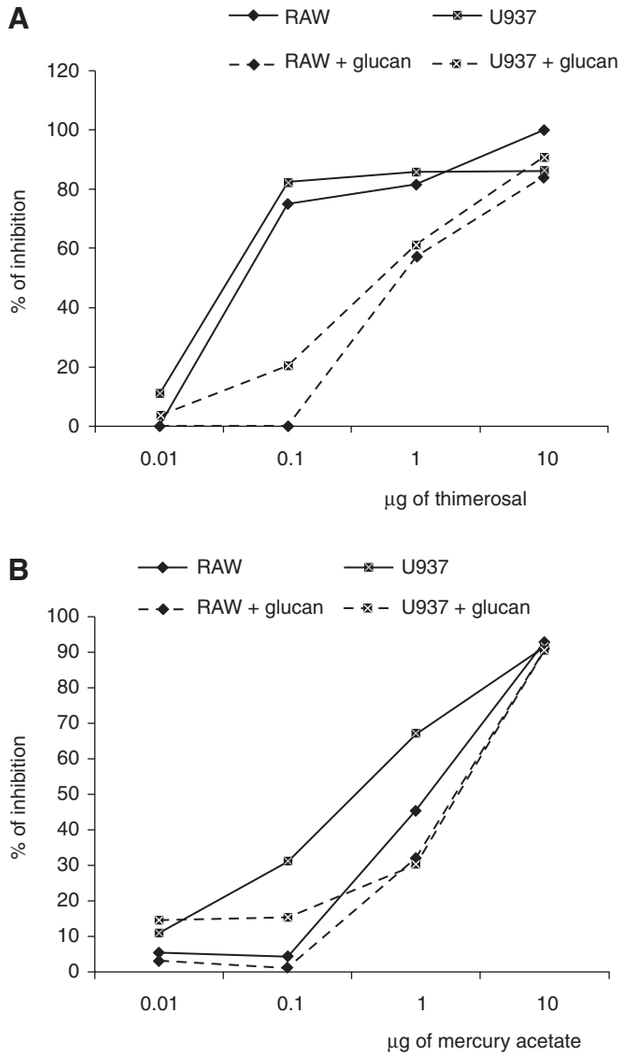

FIG. 1. Glucan #300 lowers the inhibition of proliferation caused by (**A**) thimerosal or (**B**) mercury acetate. Results represent mean values from three independent experiments with two cell lines.

mice, all glucans showed only a small, insignificant increase of production. In Hg acetate-induced suppression, only #300 showed improvement, whereas in thimerosal-induced suppression, all glucans showed significant, albeit small, improvement of IFN-γ secretion (Fig. 5). ◀ F5

We then measured the proliferation of T and B lymphocytes after a mitogenic stimulation. In concanavalin-A stimulated splenocytes, the individual glucans had no effect on the proliferation of T lymphocytes. In spleens obtained from Hg-treated mice, only glucan #300 increased the proliferation of T cells (Fig. 6), but the proliferation remained ◀ F6 lower than in untreated mice. LPS-stimulated proliferation of B lymphocytes proved to be a similar situation (Fig. 7). ◀ F7

Next, we compared the effects of tested glucans on the expression of several membrane markers on splenocytes. At the end of experiments, spleen cells were isolated, and the

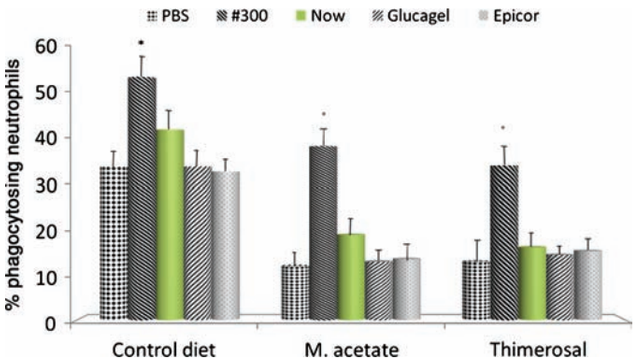

FIG. 2. Effect of Hg compounds and different glucan samples on phagocytosis of synthetic microspheres by peripheral blood granulocytes. Data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level. M. acetate, mercury acetate. Color images available online at www.liebertonline.com/jmf

surface expression of CD4, CD8, and CD19 was evaluated by flow cytometry. The results, summarized in Figure 8, demonstrated that two glucans—NOW BETA glucan and glucan #300—significantly increased the migration of CD4-positive T lymphocytes, but none of the glucans returned the Hg-depressed numbers to the normal values. In the case of $CD8^+$ lymphocytes, glucan #300 and Glucagel decreased their numbers in control mice. The return to the normal values in Hg-treated mice was significant only with regard to thimerosal and Glucagel. A dissimilar situation was observed in the case of $CD19^+$ cells. None of the glucans had any effect on control mice; however, all returned the depressed numbers of $CD19^+$ cells to normal.

We then focused on the use of glucan as an adjuvant. As an experimental model, we used immunization with ovalbumin. Glucans were applied together with two intraperitoneal doses of antigen, and a commonly used Freund's adjuvant was used as additional positive control. The results (Fig. 9) showed that both mercury acetate and thimerosal significantly lowered the antibody response, which none of the glucans managed to return to the normal values. However, in mice simultaneously treated with Hg compounds and glucan, the observed decrease of antibody production was lower when compared to a 85–97% decrease in the case of mercury acetate and thimerosal, respectively.

The final experiments were focused on NK cells. All glucans (with the exception of Glucagel) significantly increased the activity of NK cells in control mice. In Hg-treated animals, all tested glucans increased the depressed NK cells activity to almost normal levels, but there were significant differences among individual glucans (Fig. 10). Only one of the three tested effector-target ratios is shown. The results, however, of the 10:1 and 100:1 ratio were identical (data not shown).

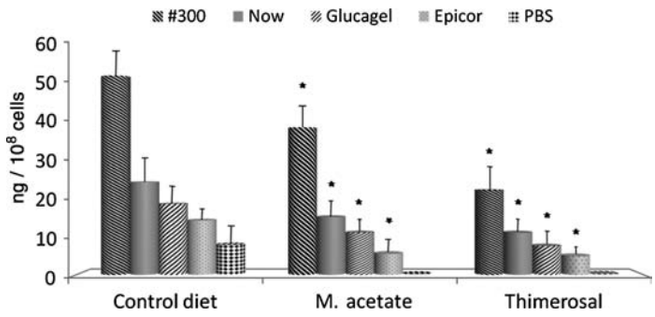

FIG. 4. Effect of Hg compounds and different glucan samples on secretion of IL-12 by concanavalin A-stimulated splenocytes. Data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level. M. acetate, mercury acetate.

DISCUSSION

For a long time, thimerosal has been used as a wound disinfectant and a preservative in vaccines.[17] However,

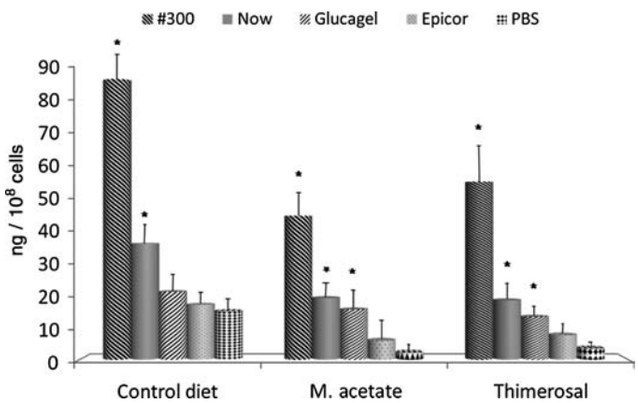

FIG. 3. Effect of Hg compounds and different glucan samples on secretion of IL-6 by concanavalin A-stimulated splenocytes. Data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level. M. acetate, mercury acetate.

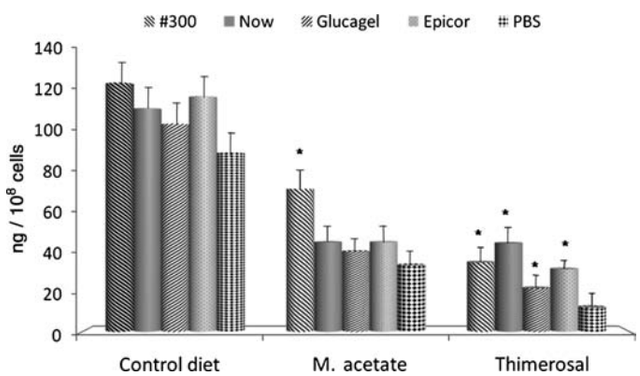

FIG. 5. Effect of Hg compounds and different glucan samples on secretion of IFN-γ by concanavalin A-stimulated splenocytes. Data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level. M. acetate, mercury acetate.

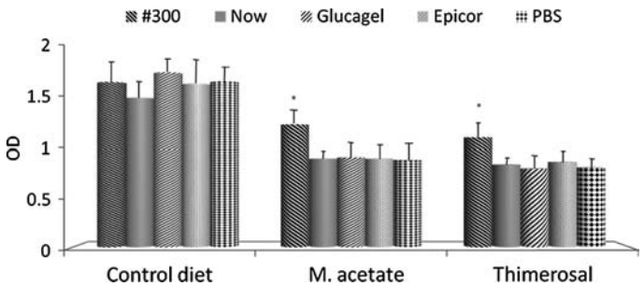

FIG. 6. Effect of Hg compounds and different glucan samples on concanavalin A-induced proliferation of T lymphocytes. Data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level. M. acetate, mercury acetate.

studies performed during the past several decades clearly established the immunosuppressive effects of various types of mercury.[5–7]

Immunodepression was originally connected more with organic methyl mercury,[18] and, only later did reports show that inorganic mercury caused similar problems.[19] Therefore, we used two different compounds, mercury acetate and thimerosal.

β-D-Glucans belong to a group of natural, physiologically active compounds, generally called biological response modifiers. The best-known effects of β-glucans consist of the augmentation of phagocytosis of professional phagocytes—granulocytes, monocytes, macrophages, and dendritic cells. Activation of macrophages and other professional phagocytes represents an element of more complicated processes, when mediator molecules secreted by them (such as IL-1, IL-9, or tumor necrosis factor-α) initiate inflammation reactions. Additional studies established that glucans also stimulate the humoral branch of immune reactions.[14,20] As glucans were repeatedly shown to overcome the immunosuppression, caused by either irradiation or chemotherapy,[13,14] we elected to test the hypothesis that orally administered glucans can help to overcome Hg-induced immunosuppression.

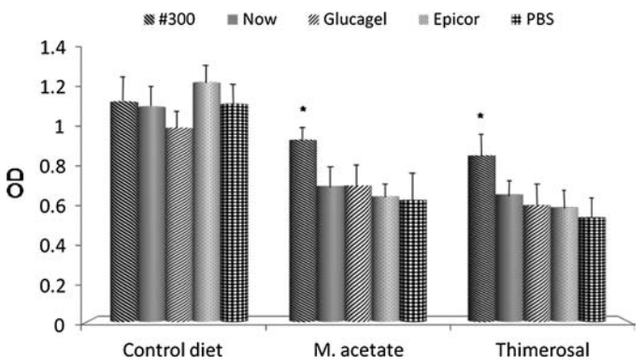

FIG. 7. Effect of Hg compounds and different glucan samples on LPS-induced proliferation of B lymphocytes. Data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level.

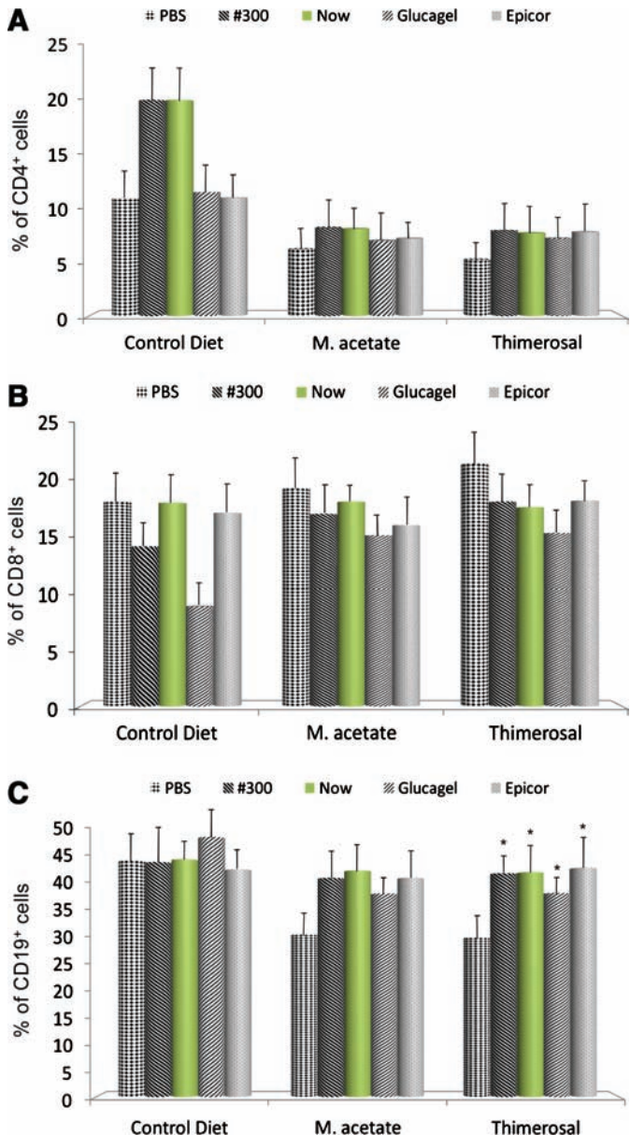

FIG. 8. Effect of Hg compounds and different glucans on expression of (**A**) CD4, (**B**) CD8, and (**C**) CD19 marker by spleen cells. The cells from three mice at each time interval were examined, and data are mean ± SD values. *Represents significant differences between control and experimental samples at the $P \leq .05$ level. M. acetate, mercury acetate. Color images available online at www.liebertonline.com/jmf

The rationale for the choice of glucans parallels what was stated in our previous article.[20] We chose four glucans that are widely sold and available in the United States, Europe, and the Far East, representing grain-, mushroom-, and yeast-derived glucans in partly soluble and insoluble form. In brief, #300 is insoluble yeast-derived glucan, Glucagel is barley-derived glucan, NOW BETA is a mixture of both insoluble glucans from yeast and soluble glucans from mushrooms, and Epicor is a partly soluble yeast-derived glucan. By this choice, we covered all basic types of glucans.

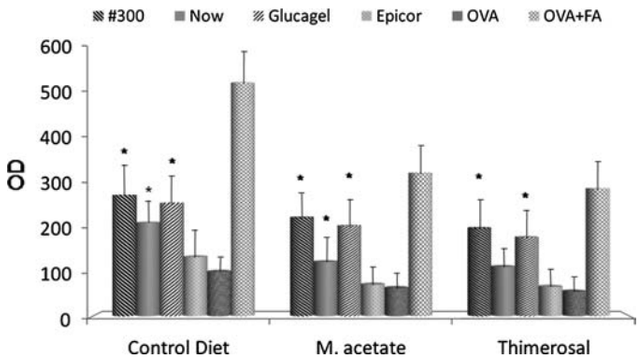

FIG. 9. Effects of 14 days of oral delivery of Hg compounds and/or tested glucans on formation of antibodies against ovalbumin (OVA). Mice were injected twice (2 weeks apart) with antigen, and the serum was collected 7 days after the last injection. The level of specific antibodies against ovalbumin was detected by ELISA. As the positive control, Freund's adjuvant was used. Data are mean ± SD values. *Represents significant differences between control (OVA alone) and samples at the $P \leq .05$ level. M. acetate, mercury acetate.

Two weeks of a daily dose of mercury acetate corresponding to approximately 800 μg of mercury/kg (or 200 μg of mercury/kg in case of thimerosal) induced a systemic suppression of all tested immune reactions, from cellular (phagocytosis, NK cell activity, mitogen-induced proliferation, and expression of CD markers) to humoral immunity (antibody formation and secretion of IL-6, IL-12, and IFN-γ).

Studies of the simultaneous adminstration of mercury compounds and glucans started with changes in direct toxicity of mercury. In both cases, glucans significantly lowered the toxicity. As the direct toxic effects of mercury compounds are hypothesed to be caused by apoptosis,[6] the well-known inhibition of apoptosis caused by glucan[14] might be the explanation.

Glucans are generally considered to be potent stimulators of cellular immunity, with macrophages and neutrophils

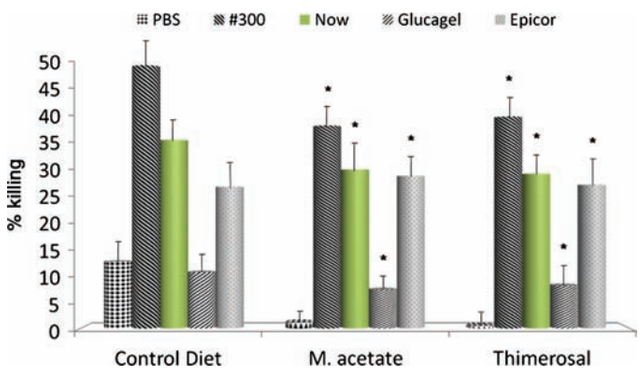

FIG. 10. Evaluation of NK cell cytotoxicity of YAC-1 cells. Different ratios of NK cells to YAC-1 cells were tested for cytotoxicity in the Hg compound- and/or β-glucan-treated mice. Only the 50:1 ratio is shown here. Data are mean ± SD values from three experiments. The differences were significant at the $P \leq .05$ level at all three effector to target cell ratios. M. acetate, mercury acetate. Color images available online at www.liebertonline.com/jmf

being the most important targets. Not surprisingly, we started our evaluation of glucan activities by phagocytosis. We used 2-hydroxyethyl methacrylate particles, which have only a slight negative charge and thus do not nonspecifically adhere to the cell surface. This guarantees that only phagocytosing cells will engulf these particles, and it significantly lowers the chance of false negativity.[15] Our previous investigation showed that whereas most of the tested glucans stimulated phagocytosis of synthetic microspheres (with exception of Glucagel T), the highest effects were obtained with glucan #300.[20] In this study, glucans #300 and NOW BETA significantly restored the suppressed ability of blood neutrophils to engulf synthetic beads.

In addition to the direct effect on various cells of the immune system, the immunostimulating action of β-glucans is caused by potentiation of a synthesis and release of several cytokines such as tumor necrosis factor-α, IFN-γ, IL-1, and IL-2. This cytokine-stimulating activity is dependent on the triple helix conformation.[21] The only glucan without a trace of pro-inflammatory cytokine stimulation is PGG-glucan.[22] We focused on the stimulation of IL-6, IL-12, and IFN-γ production by spleen cells *in vitro* and found that three glucans strongly reversed the inhibition of IL-6 and IL-12 production caused by Hg compounds. In the case of IFN-γ secretion, #300 partly reversed the effects of Hg acetate, whereas they all partly reversed the thimerodal-induced suppression.

These effects were observed for all additional tests, ranging from mitogen-induced proliferation of T and B lymphocytes up to antibody response and NK cell activity. In all cases, we found significant suppression by 14 days of feeding with Hg compounds, which mostly confirmed previous data obtained by other groups.[7] In all cases, we found that this immunosuppression was partly reversed by glucans. In addition, yeast-derived glucan #300 was consistently the most active glucan.

Regarding the mechanisms by which glucan reverses Hg-mediated immunosuppression, the answer remains unclear. In addition to direct stimulation of cells via Dectin-1 and CR3 receptors (for review, see Vetvicka *et al.*[23]), glucans are known to alter some important genes and their transcription factors.[24] Moreover, because Hg causes inflammation and oxidative stress,[25] intracellular mechanisms that involve antioxidant processes might be assumed[26] as well.

In conclusion, we report, for the first time, that mercury-caused immunosuppression can be, at least partly, restored by orally administered glucans. From our data one can imagine that glucan can be used for prophylactic in mercury poisoning. The clinical importance of this observation clearly deserves further studies.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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AU1: Reference addition ok?