Immune function of bovine leukocytes after in vitro exposure to selected heavy metals

Sylvain De Guise, DMV, PhD; Jacques Bernier, PhD; Patrice Lapierre, MSc; Marc M. Dufresne, MSc;
Pascal Dubreuil, DMV, PhD; Michel Fournier, PhD

Objective—To study effects of in vitro exposure of bovine leukocytes to mercury, cadmium, and lead on phagocytosis, natural killer cell activity, and lymphocyte proliferation.

Sample Population—Leukocytes from 6 nonpregnant Holstein heifers.

Procedure—Leukocytes were exposed in vitro to the aforementioned metals, and leukocyte functions were assessed.

Results—Phagocytosis was suppressed by 10⁻⁶ to 10⁻³ M CdCl₂ and by 10⁻⁴ and 10⁻³ M HgCl₂ but not 10⁻¹ M HgCl₂ or 10⁻⁴ to 10⁻³ M PbCl₂. Spontaneous and concanavalin A- or phytohemagglutinin-stimulated proliferation of metal-treated bovine blood mononuclear cells was not significantly different from that of non-treated control cells, except for enhanced spontaneous proliferation in response to 10⁻⁴ M HgCl₂. When proliferation was expressed as a stimulation index, a dose-dependent increase of spontaneous proliferation was observed in response to exposure to HgCl₂ and PbCl₂. Compared with response to 10⁻⁴ or 10⁻³ M CdCl₂, reductio of mitogen-induced and spontaneous proliferation was observed on exposure to 10⁻⁴ M CdCl₂. Natural killer cell activity against yeast artificial chromosome target cells, evaluated by flow cytometry, was decreased only in cells exposed to 10⁻⁴ M HgCl₂.

Conclusion and Clinical Relevance—Bovine leukocytes are susceptible to the immunomodulatory effects of in vitro exposure to heavy metals at concentrations equal to or higher than those at which similar effects are seen for leukocytes from most other animal species for which data are available for comparison. Exception is phagocytosis, which is severely affected by low concentrations of CdCl₂ and HgCl₂ in cattle. Reduction of defense mechanisms on exposure to metals could lead to increased susceptibility to potential pathogens. (Am J Vet Res 2000;61:339–344)

Because of the lack of oral discrimination of and tendency to chew or lick foreign objects and their propensity to drink used motor oil, cows are particularly prone to encounter heavy metals. Lead is perhaps the most consistently important poison encountered in farm animals, especially cattle. The disease may be acute or subacute depending on the dose ingested, with signs mainly of neurologic dysfunction.¹,² Paint, batteries, and used oils are the usual sources of lead for cows.¹,² Although long-term ingestion of lead causes it to accumulate in tissues of ruminants, it is not considered a cumulative poison in these species.²

Mercury compounds are cumulative poisons, but clinical signs of toxocerosis observed in animals usually are acute and include gastroenteritis and renal and nervous system dysfunction.² Mercury poisoning is becoming increasingly important in farm animals,¹ because problems in animals are often caused by the organic salts, which are applied to seed grains as an antifungal agent.¹,²

Cadmium poisoning has been reported in cattle, mostly by accidental ingestion of farm chemicals, but increasing interest has been given to this element as an environmental pollutant.¹ Lesions included keratinization and degenerative changes in most organs.¹ Nevertheless, to the authors’ knowledge, long-term effects of heavy metal exposure on immune functions, studied in laboratory animals¹ have not been investigated in cows. Potential effects of heavy metal exposure on acquired and innate immunity is clinically relevant given the central role of those defense mechanisms in protection against potential pathogens. The objective of the study reported here was to determine effects of in vitro exposure of bovine leukocytes to mercury, cadmium, and lead on 3 functions of the immune system: phagocytosis, lymphocyte proliferation, and natural killer (NK) cell activity.

Materials and Methods

Animals—Six nonpregnant purebred Holstein heifers, 9 to 16 months old and kept indoors since birth, were studied. Heifers were housed in a tie barn and fed hay, grain, and hay silage.

Blood sample collection and isolation of leukocytes and mononuclear cells—Blood samples were collected from coccygeal vessels into heparinized tubes.³ The blood was then transported at 20 to 22 C (room temperature) to the laboratory within 1 to 2 hours.

For assessment of lymphocyte viability and proliferation, and for evaluation of NK activity, the blood was diluted in an equal volume of Hank’s balanced salt solution (HBSS)³ and blood mononuclear cells (BMC) were isolated by use of density gradient centrifugation on Ficoll® for 30 minutes at 750 × g. The BMC were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml; complete RPMI medium) and washed 3 times; viability was assessed by addition of acridin orange-propidium iodide, followed by viewing of the cells by use of fluorescence microscopy. Viability of

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From TOXEN, UQAM, CP 8888, Succursale A, Montréal, Québec, Canada H3C 3P8 (De Guise, Bernier, Lapierre, Dufresne, Fournier); and Faculté de Médecine Vétérinaire, Université de Montréal, CP 5000, St-Hyacinthe, Québec, Canada J2S 7C6 (Dubreuil). Dr. De Guise's present address is Department of Pathobiology, University of Connecticut, Storrs, CT 06269. Supported in part by the US Environmental Protection Agency and the Canadian Network for Toxicology Centers. Dr. De Guise was supported by a fellowship from the Medical Research Council of Canada.

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Address correspondence to Dr. De Guise.
bovine lymphocytes also was assessed after a 66-hour incubation with different concentrations of the various metals, using propidium iodide labeling and flow cytometry.

For evaluation of phagocytosis, erythrocytes were lysed by addition of NH₄Cl. Briefly, 45 ml of NH₄Cl was added to 5-ml tubes of blood, and after 3 minutes, the tubes were centrifuged for 10 minutes at 250 × g. The leukocytes were washed twice in HBSS and resuspended in complete RPMI medium, then viability was assessed by use of acridin orange-propidium iodide and fluorescence microscopy.

**Lymphocyte proliferation**—For evaluation of lymphocytes proliferation, lymphocytes were exposed to 0, 10⁻⁵, 10⁻⁶, and 10⁻⁷ M HgCl₂, CdCl₂, and to 0, 10⁻⁴, 10⁻⁵, and 10⁻⁶ M PbCl₂ in flat-bottomed 96-well plates. The cells were either stimulated with concanavalin A (conA; 1 µg/ml) or phytohemagglutinin (PHA; 1 µg/ml), or were cultured without mitogen. Each assay consisted of triplicate samples with 2 × 10⁵ cells/well in a total volume of 200 µl. Lymphocyte proliferation was assessed, using thymidine incorporation as described. Briefly, the cells were cultured for 48 hours at 37 C and 5% CO₂, then were pulsed for 18 hours by addition of 0.5 µCi of

<table>
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<td>6</td>
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*Viability was evaluated by use of flow cytometry after labeling with propidium iodide.† Significantly (P < 0.05) different from that of unexposed control cells.

conA = Concanavalin A. PHA = Phytohemagglutinin.
[H]thymidine (6.7 Ci/mmol) to each well. Cells were then filtered using a cell harvester, and radioactivity was evaluated. Results are expressed in disintegrations per minute (dpm).

Natural killer cell activity—A murine lymphoma cell line, yeast artificial chromosome (YAC) cells, were used as target cells for flow cytometric evaluation of NK cell activity; the procedure used was modified from that reported by Chang et al. Target cells were washed once in complete RPMI medium, and centrifugation was adjusted to 10⁶ cells/ml. One milliliter of the suspension was forcefully added to 10 µl of a 3 mM solution of the lipophilic carbocyanine membrane dye 3,3'-dioctadecyloxacarbocyanine perchlorate, dissolved in dimethyl sulfoxide (DMSO). The YAC cells were then incubated for 20 minutes at 37 C in 5% CO₂, followed by 2 washes in complete RPMI medium, then resuspension in complete RPMI medium at a concentration of 10⁶ cells/ml.

Effector cells (10⁵ BMC) were exposed in duplicate to 0, 10⁻⁶, 10⁻⁷, and 10⁻⁸ M HgCl₂ and CdCl₂, and to 0, 10⁻⁶, 10⁻⁷, and 10⁻⁸ M PbCl₂ for 3 hours in 12 X 75-mm polystyrene round-bottom tubes at 37 C with 5% CO₂. The tubes were then washed twice in complete RPMI medium and resuspended in 1 ml of complete RPMI medium. One hundred microliters of labeled target cell suspension was then added to each tube to obtain a 100:1 effector-to-target ratio, and to tubes without effector cells to assess spontaneous cell mortality. The tubes were centrifuged at 300 × g for 30 seconds, then were incubated at 37 C in 5% CO₂ for 120 minutes, after which they were centrifuged again for 10 minutes at 250 × g, the supernatant was discarded, and the cells were resuspended in 300 µl of a solution of propidium iodide (200 µg/ml). The tubes were then placed on ice and analyzed by flow cytometry.

Bovine BMC were distinguished from the YAC target cells on the basis of fluorescence at 530 nm (FL1) and were excluded from acquisition, using an electronic gate. The viability of the YAC cells was assessed on the basis of fluorescence at 630 nm (FL3). Duplicates were averaged, and NK cell activity was calculated as the difference between mortality of the YAC cells incubated with effector cells and spontaneous mortality of the same target cells (incubated without effector cells).

Phagocytosis—For evaluation of phagocytosis, the cell concentration was adjusted to 2.0 × 10⁶ leukocytes/ml. Volumes (0.7 ml) of the cell suspensions were exposed to 0, 10⁻⁴, 10⁻⁵, and 10⁻⁶ M HgCl₂ and CdCl₂, and to 0, 10⁻⁴, 10⁻⁵, and 10⁻⁶ M PbCl₂ for 3 hours in 12 X 75-mm polystyrene round-bottom tubes at 37 C with 5% CO₂. The tubes were then transferred to a 37 C water bath in which tubes were kept in motion, and phagocytosis was evaluated, using flow cytometry as described. Briefly, 1-µm diameter fluorescent latex beads were added to the cell suspensions to obtain a concentration of 100 beads/leukocyte. After 60 minutes, 500 µl of each cell suspension was layered on a cushion of 3% bovine serum albumin gradient and centrifuged at 150 × g for 6 minutes at 4 C. The supernatant containing the free beads was withdrawn, and 1 ml of an isotonic solution containing 0.9% formalin was added to the remaining cells. Fluorescence of approximately 10,000 leukocytes was read within 36 hours after fixation, using the aforementioned flow cytometer that was adjusted by use of a threshold on forward scatter to avoid contamination with cell debris or erythrocytes. Analysis was performed on neutrophils that were discerned from other leukocytes on the basis of relative size and complexity, using an electronic gate with the help of computer software. Phagocytosis was evaluated as the percentage of the neutrophils that had phagocytized 3 or more beads, and as mean fluorescence of the neutrophils, which accounts for mean number of beads phagocytized by neutrophils.

Statistical analyses—For each analysis, triplicates were averaged, and the mean and SD were evaluated for values from the various animals and experiments. A Student t-test (P < 0.05 for significance) was used to compare each concentration of metal with the control (without metal) for viability, NK activity, and phagocytosis or with the other concentrations of metal for lymphocyte proliferation.

Results

Leukocyte viability—Leukocyte viability after preparation procedures and immediately before use in the assays was always > 95%. Lymphocyte viability after a 66-hour incubation with the various concentrations of the different metals (same procedures as in proliferation assay) was determined (Table 1). Lymphocyte viability was significantly different from that of control cells incubated without metals only when they were exposed to 10⁻⁷ M HgCl₂ with PHA and without mitogen, and to 10⁻⁶ M CdCl₂ with PHA. In all the instances where there was a significant difference, the viability of the cells incubated with metals was higher than that of control cells incubated without metal.

Lymphocyte proliferation—Proliferation of bovine BMC stimulated with conA or PHA or not stimulated with mitogens was reported as stimulation index (counts [dpm] of cells exposed to metals per counts [dpm] of unexposed controls; Fig 1). Proliferation of bovine BMC cultured without mitogen

![Figure 2: Natural killer activity (difference between mortality of yeast artificial chromosome target cells incubated with and without bovine BMC) previously exposed to HgCl₂, CdCl₂, or PbCl₂. See Figure 1 for key.](image-url)
in the presence of $10^{-5}$ M HgCl$_2$ was significantly higher than that of cells cultured in the presence of $10^{-6}$ M HgCl$_2$, which was in turn significantly higher than that of cells cultured in the presence of $10^{-7}$ M HgCl$_2$. Proliferation of bovine BMC cultured without mitogen in the presence of $10^{-4}$ M PbCl$_2$ was significantly higher than that of cells cultured in the presence of $10^{-5}$ M PbCl$_2$, which in turn was significantly higher than that of cells cultured in the presence of $10^{-6}$ M PbCl$_2$. At the concentrations studied, HgCl$_2$ and PbCl$_2$ had no effect on proliferation of mitogen-stimulated cells.

Proliferation of bovine BMC cultured with conA or PHA or without mitogen in the presence of $10^{-5}$ M CdCl$_2$ was significantly reduced, compared with that of cells cultured in the presence of $10^{-6}$ or $10^{-7}$ M CdCl$_2$.

**Natural killer cell activity**—The NK cell activity was evaluated as the difference between mortality of YAC target cells incubated with and without bovine BMC. The effects of in vitro exposure to heavy metals on NK cell activity of bovine PBMC were determined (Fig 2). Exposure to $10^{-5}$ M HgCl$_2$ significantly reduced NK cell activity to 61.4% of that of control cells, whereas NK cell activity did not differ significantly from that of controls when cells were incubated with $10^{-6}$ or $10^{-7}$ M HgCl$_2$. Exposure to CdCl$_2$ and PbCl$_2$ did not significantly affect NK cell activity at the concentrations tested.
Phagocytosis—Two variables were examined to evaluate phagocytosis: percentage of neutrophils that had phagocytized 3 or more beads, which accounts for the percentage of beads involved in the process of phagocytosis, and mean fluorescence of the neutrophils, which accounts for the mean number of beads phagocytized by each neutrophil. The effects of in vitro exposure to heavy metals on phagocytosis of bovine neutrophils (2 variables) was determined (Fig 3). Exposure to 10⁻⁶ and 10⁻⁵M HgCl₂ significantly reduced the percentage of neutrophils that had phagocytized 3 or more beads to 24 and 63% of that of controls, respectively, and significantly reduced mean fluorescence to 31 and 51% of that of controls, respectively. These values did not differ significantly from control values when cells were incubated with 10⁻⁶M HgCl₂. Exposure to 10⁻⁷, 10⁻⁶ and 10⁻⁵M CdCl₂ significantly reduced the percentage of neutrophils that had phagocytized 3 or more beads to 24 and 63% of that of controls, respectively, and significantly reduced mean fluorescence to 46, 63, and 64% of that of controls, respectively. Lead chloride did not significantly affect phagocytosis at the concentrations tested.

Discussion

The effects of exposure to various heavy metals on different immune functions of bovine leukocytes were evaluated in vitro. When the effect of exposure to the various heavy metals was evaluated as a proliferation index, compared with that of unexposed cells, differences were found among the various concentrations of HgCl₂, CdCl₂, and PbCl₂. Use of a stimulation index allows detection of direct effects of a xenobiotic that are not masked by interindividual variability that is frequently observed in lymphocyte proliferative response.

Effects of exposure to metals when cells were stimulated with concanavalin A were similar to those observed when cells were stimulated with PHA. Concanavalin A and PHA induce a strong stimulus for proliferation, and such a strong stimulus did not allow measurement of subtle differences caused by exposure to HgCl₂ and PbCl₂. Unstimulated cells were more sensitive to exposure to metals, and differences in the stimulation index were observed between the various concentrations of the 3 metals tested. Reduction of proliferation observed with the highest concentration of CdCl₂ cannot be attributed to a difference in cell viability, which is either not significantly different (concA and no mitogen) or significantly higher (PHA) than that of unexposed control cells. On the other hand, the increased proliferation of unstimulated cells incubated with the highest concentration of HgCl₂ could be attributed to a significantly higher viability of those cells, compared with that of unexposed control cells. Evaluation of a function (cell proliferation) appears more sensitive than measurement of cell viability to determine the effects of incubation with metals.

Among the different concentrations of the various metals tested, only exposure to 10⁻⁵M HgCl₂ reduced NK cell activity of bovine BMC. Exposure to HgCl₂ and CdCl₂ reduced phagocytosis in a dose-dependent manner. The percentage of neutrophils that had phagocytized 3 or more beads and mean fluorescence of the neutrophils were affected, and the degree to which these 2 parameters were affected by different concentrations of the various metals was in the same range. Suppression of phagocytosis was more severe in response to 10⁻⁴M HgCl₂ than with 10⁻³M CdCl₂, but CdCl₂ affected phagocytosis at lower concentrations (10⁻⁶M) than HgCl₂.

Effects of in vitro exposure to heavy metals have been studied in other species. Spontaneous proliferation of mouse splenocytes and lymph node cells was increased by 10⁻⁴M HgCl₂ as was the case with bovine BMC. Concanavalin A and PHA-stimulated bovine BMC were less sensitive to the immunomodulatory effects of HgCl₂ than were mouse splenocytes, which were affected by concentrations of 10⁻⁰ to 10⁻⁴M. Concentrations of CdCl₂ that induced immunomodulatory effects on proliferation of concA or PHA-stimulated lymphocytes in various species varied with studies and species. Human BMC were affected by 10⁻⁶ to 10⁻⁴M and 3 X 10⁻⁷ to 10⁻³M, and rat splenocytes had effects at 5 X 10⁻⁴ to 5 X 10⁻³M, and mouse T-cell hybridomas were affected by 10⁻⁴M but not by 10⁻³M. Sensitivity of concA- or PHA-stimulated bovine BMC to immunomodulatory effects of CdCl₂ is in the same range as that of rat splenocytes, between that of human BMC and mouse T-cell hybridomas. Lead chloride did not affect the proliferation of mitogen-stimulated bovine BMC as for mouse T lymphocytes cultured with 10⁻³or 10⁻⁴M PbCl₂. Nevertheless, PbCl₂ increased spontaneous proliferation of bovine BMC similar to that for human BMC cultured with 10⁻⁴M PbCl₂. Overall, susceptibility of bovine lymphocytes to in vitro exposure to heavy metals is observed at metal concentrations higher or in the range of those found to affect lymphocyte proliferation in laboratory animals and human beings. Nevertheless, the amplitude of the effects observed in bovids was generally rather small.

In our study, NK cell activity was reduced by exposure to 10⁻⁴M HgCl₂. Suppression of NK cell activity has already been documented in mouse and rat following in vivo exposure to mercury, either directly or through the placenta or milk, but similar effects after in vitro exposure to HgCl₂ has not been documented. Natural killer cell activity was not affected by in vitro exposure to CdCl₂ in our study, but it was either reduced or augmented after in vivo exposure to various doses of CdCl₂ in mice, using different routes of exposure. Natural killer cell activity was also reduced after in vitro exposure of human BMC to CdCl₂. The NK cell activity was not affected by exposure to PbCl₂ in our study similar to that for laboratory animals. The NK cell activity of BMC was then less sensitive to the in vitro effects of CdCl₂ in bovine than in human cells.

Phagocytosis of bovine neutrophils was severely affected by in vitro exposure to small concentrations of HgCl₂ and CdCl₂. To our knowledge, comparable data for effects of in vitro exposure to heavy metals on phagocytosis in any other species are not available. Nevertheless, results of in vitro experiments indicated that phagocytosis of mouse peritoneal macrophages was either increased or decreased after oral exposure to CdCl₂. Mice of strain B6C3F1 seemed sensitive to lower doses of CdCl₂ (increased phagocytosis at 10, 50, and 250 ppm) than did CD-1 mice (decreased
phagocytosis at 300 ppm, but not 3 or 30 ppm), though the effects were different.

These data suggest that concentrations of metals required to affect phagocytosis in cattle are lower than those required to affect lymphocyte proliferation and NK activity. This finding is of clinical relevance in view of the numerous pyogenic microorganisms found in the immediate environment of cows. In addition, the various defense mechanisms studied were unequally affected by the different metals; for example, CdCl₂ affected phagocytosis at the lowest dose, whereas HgCl₂ induced more severe suppression of phagocytosis at concentrations of 10⁻³M. Also, 10⁻³M HgCl₂ affected NK cell activity and lymphocyte proliferation (when expressed as dpm), but CdCl₂ was the only metal that modified the proliferative response of conA- and PHA-stimulated lymphocytes (expressed as a stimulation index). Although lead had no effect on phagocytosis, NK cell activity, and proliferation of mitogen-stimulated PBMC, it had marked effect on spontaneous proliferation of BMC.

The exact in vivo importance of the changes reported in this in vitro study is hard to assess. Although reduction of phagocytosis may have an obvious immediate consequence, reduction of NK cell activity which is a key mechanism in the initial defense against viral diseases, or a change in the ability of either quiescent or stimulated lymphocytes to proliferate, may create a substantial weakness in the delicate balance that exists between a functional immune system and potential pathogens. An apparently small defect in immune responsiveness may provide an opportunity for a pathogen to invade an organ system and induce disease. Our data provide a basis for the understanding of subclinical manifestations in cattle that may follow exposure to heavy metals.

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