Inhibition of luminol-dependent chemiluminescence of human granulocytes by low doses of inorganic mercury

A.H.M. Al-Hashimi,[1] F.H. Mohammad[2] and E.A. Abbass Al-Krawi[1]

تثبيط اللمعان الكيميائي المعتمد على اللومينول في الخلايا البشرية المحبة بإضافة جرعات قليلة من الزئبق غير العضوي

علي حسين محمد الهاشمي وفائق حسين محمد وابتهال علي عباس الكروي

خلاصة: أحدثت إضافة كلوريد الزئبق في الزجاج إلى الخلايا البشرية المحبة في الدم الكامل، تأثيراً مثبطاً واضحاً للمعان الكيميائي المعتمد على اللومينول، والمحدث ببلورات سلفات الباريوم المضافة إلى معلّق لهـذه الخلايـا. وكـان هـذا الأثر معتمـداً على الجرعة والزمن عند استخدام سلفات الباريوم لتنبيه هبّة الأكسـدة في الخلايا المحبة. ومـن ناحية أخرى فإن الحضانة بأعلى تركيز استُعمل من كلوريد الزئبق (10 ميليمـول في اللتر) لم تؤد إلى انفـاح أغشـية الخلايا المحبة. كما أن تأثير كلوريد الزئبق على الخلايا المحبة كـان غـير عكـوس بعد غسل هـذه الخلايـا بمحلول ملحي مـدروء بالفسفات. و لم يؤثـر كلوريد الزئبـق في اللمعـان الكيميائي النـاتج عنـد استثارة اللومينـول بالمـاء الأكسيجيني المؤكسِد في وسط خال من الخلايا. إن هذه النتائج لتوحي بأن بعض السمية الـتي يتسـم بـها كلوريد الزئبق، قد تكون أكبر مما يتوسط في إحداثه تأثيره على الجهاز المناعي البلعمي.

ABSTRACT HgCl$_2$, added *in vitro* to human granulocytes in whole blood, caused a marked inhibitory effect on the luminol-dependent chemiluminescence induced by BaSO$_4$ crystals in suspension of these cells. The effect was both dose- and time-dependent when BaSO$_4$ was used to stimulate the oxidative burst in granulocytes. Incubation with the highest concentration of HgCl$_2$ used (10 mmol/L), however, did not cause disruption of the membranes of granulocytes. The effect of HgCl$_2$ on the granulocytes was irreversible following washing of the HgCl$_2$-treated cells with phosphate buffered saline. HgCl$_2$ did not affect chemiluminescence produced when luminol was excited by oxidative hydrogen peroxide in a cell-free medium. These results suggest that some of the toxicity of HgCl$_2$ may be greater than mediated by an action on the phagocytic immune system.

Inhibition de la chimioluminescence associée au luminol des granulocytes humains par de faibles doses de mercure inorganique

RESUME Le chlorure mercurique (HgCl$_2$) ajouté in vitro aux granulocytes humains dans le sang total a eu un effet inhibiteur prononcé sur la chimioluminescence associée au luminol induite par des cristaux de sulfate de baryum (BaSO$_4$) dans une suspension de ces cellules. Cet effet dépendait à la fois de la dose et du temps lorsque BaSO$_4$ était utilisé pour la stimulation du métabolisme oxydatif dans les granulocytes. Toutefois, l'incubation en présence de la plus forte concentration de HgCl$_2$ utilisée (10 mmol/l) n'a pas entraîné de rupture des membranes des granulocytes. L'effet de HgCl$_2$ sur les granulocytes a été irréversible après lavage des cellules traitées au HgCl$_2$ avec une solution salée tamponnée de phosphate. HgCl$_2$ n'a pas affecté la chimioluminescence produite lorsque le luminol était excité par le peroxyde d'hydrogène dans un milieu ne contenant pas de cellules. Ces résultats donnent à penser qu'une partie de la toxicité de HgCl$_2$ peut être plus importante que celle résultant de ses effets sur le système immunitaire phagocytaire.

[1]*Department of Physiology;* [3]*Department of Pharmacology, College of Medicine, University of Basra, Basra, Iraq.*
[2]*Department of Physiology, Saddam Medical College, Baghdad, Iraq.*

Introduction

The major physical forms of mercury to which humans are exposed are mercury vapour and ointment that has an extremely high mercury content [1,2]. Radioactive mercury has also been used as a tracer in diagnostic medicine [3–6] and in amalgam dental fillings [7].

Inorganic mercury may cause immunologically mediated disease as well as specific manifestation of the autoimmune response [8,9]. Mercurial ions have been reported to interact and exhibit a high affinity for thiol groups, and may severely disturb many metabolic functions in the cells and inhibit cellular respiration [10]. This leads to increased cellular concentrations of glutothione and increased release of glutothione into extracellular levels [11,12].

The production of reactive oxygen metabolites by granulocytes plays a key role in a host defence against invading microorganisms and foreign bodies [13]. The ability of granulocytes to kill bacterial organisms by a process of phagocytosis respiratory burst is related, in part, to their capacity to generate several reactive oxygen species (ROS) [14]. These ROS include superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen [15]. The term respiratory burst refers to a coordinated series of metabolic events that takes place when phagocytes are exposed to appropriate stimuli [14]. This group of events underlies all oxygen-dependent killing by phagocytes and a sharp increase in oxygen uptake occurs upon stimulation [16]. The potent ROS generated by phagocytosis is capable of oxidizing luminol (chemiluminescence indicator), and chemiluminescence light bursts are produced [17]. This technique of luminol-amplified chemiluminescence is a sensitive system, permitting

the use of less than 10^4 phagocytes per assay [18].

Luminol can react with the ROS generated during phagocytosis to produce an excited intermediate state that emits light upon returning to the ground state. The luminol-amplified chemiluminescence activity can be simplified by the formula [19]:

$$\text{Luminol} + \text{ROS} \xrightarrow{\text{peroxide catalyst}} N_2 + \text{amino-phthalate ion} + \text{light}$$
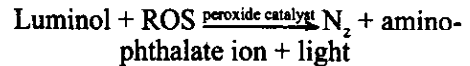
The purpose of our work was to study the effect of low doses of $HgCl_2$ (mercury chloride) on the induced functional activity of granulocytes in whole blood. This mirrors the *in vivo* situation, since granulocyte function and metabolism are greatly influenced by the presence of other cells, mediators generated by immune complexes and components of the complement system [20].

Materials and methods

Luminol (5-amino-2, 4-phthalazinedion, a cyclic hydrazide) was dissolved in dimethylsulfoxide to give a concentration of 0.01 M. This stock solution was further diluted in normal phosphate buffered saline to 0.00001 M prior to use.

In order to activate granulocytes to burst, a medium of the following composition (mM) was used: 165 sodium chloride, 15 Tris hydrochloric acid, 2.25 $BaSO_4$ (barium sulfate) (pH = 8). The $BaSO_4$ in this medium was in a suspended form.

Venous blood samples were obtained from healthy adults (5% sodium citrate as anti-coagulant was used) and then kept at 37 °C until the start of the assay (usually chemiluminescence was measured within 1 hour). The number of cells was estimated by means of thin film techniques.

The effect of $HgCl_2$ (10 mmol/L) on the viability of granulocytes was tested at intervals of 0, 15, 30, 45 and 60 minutes following incubation at 37 °C. The percentage of viable cells was estimated by a trypan blue exclusion method.

This was carried out using the principle of oxidation of luminol by reactive oxygen metabolic species produced during phagocytosis in phagocytic cells [21,22]. Luminol-dependent chemiluminescence (CL) in stimulated granulocytes in whole blood was measured in an ultra-high-sensitive photon counting system [23]. The reaction mixture consisted of 1 mL of CL-inducer plus 100 mL (0.2 N) sodium hydroxide and 100 mL of luminol. To this mixture, 100 mL of whole blood was added and agitated in the measuring cuvette of the photon counting system, where the temperature was kept at 37 °C. The resulting light output was recorded continuously until the CL peaked and demonstrated a definite decline. All results were taken in relative arbitrary units and corrected to the same number of the granulocytes.

The effect of $HgCl_2$ on the luminol-dependent CL was determined by incubating blood cells with various concentrations of $HgCl_2$ at 37 °C for different time intervals prior to stimulation with $BaSO_4$ suspension. Luminol-induced CL was also measured for whole blood incubated with the $HgCl_2$ before and after washing away the $HgCl_2$ with phosphate buffered saline. The results were estimated at a peak high of the chemiluminescence kinetics curve and corrected for the same number of granulocytes cells in all tests and control experiments.

The $HgCl_2$ in the concentration used did not induce CL from luminol when added to unstimulated granulocytes in whole blood. In order to exclude the possibility that the effect of the $HgCl_2$ takes place entirely

within the fluid in which the granulocytes were suspended, the $HgCl_2$ was added to luminol and hydrogen peroxide and CL was measured.

The results of CL in the area under the kinetics curve were estimated. The results were analysed for significance using the Student t-test and results were considered significant when $P \leq 0.05$.

Results

The effect of $HgCl_2$ on luminol-dependent CL of granulocytes in whole blood is shown in Tables 1-4.

Table 1 shows the effect of $HgCl_2$ at various concentrations on CL of granulocytes.

Table 2 shows the effect of $HgCl_2$ at various concentrations on CL of granulocytes in whole blood stimulated with $BaSO_4$ in suspension at different time intervals of incubation.

Table 1 **Effect of various concentrations of HgCl₂ on granulocytes stimulated by BaSO₄ in suspension**

HgCl₂ concentration (mmol/L)	Chemiluminescence (arbitrary units)[a]	% Chemiluminescence Inhibition
Control	1077.3 ± 36	–
0.5	951.5 ± 31	15.02
1.0	821.7 ± 17	23.73
5.0	547.1 ± 19	49.22
10	211.3 ± 11	80.39

[a]Data are expressed as mean ± standard error of the mean of at least 5 observations
Measurements were made at 37 °C for 30 minutes.
P < 0.05 in comparison with controls

Table 2 Effect of HgCl$_2$ on chemiluminescence (CL) of granulocytes in whole blood stimulated with BaSO$_4$ in suspension at different time intervals of incubation

| HgCl$_2$ concentration (mmol/L) | Incubation time (minutes) | | | | | |
| | 15 | | 30 | | 60 | |
	CL[a]	% CL inhibition	CL[a]	% CL inhibition	CL[a]	% CL inhibition
Control	887 ± 35	–	967 ± 27	–	919 ± 43	–
5	577 ± 19	34.9	479 ± 33	50.5	379 ± 15	58.8
10	305 ± 23	65.6	209 ± 17	78.3	113 ± 13	87.7

[a]*Data are expressed as mean ± standard error of the mean of at least 5 observations*
Measurements were made at 37 °C for 30 minutes
P < 0.05 in comparison with controls

Table 3 Influence of HgCl$_2$ (10 mmol/L) on the viability of granulocytes following incubation at different time intervals at 37 °C

Incubation time (minutes)	% viability
0	100
15	97
30	96
45	95
60	95

Table 4 Effect of washing HgCl$_2$ with phosphate buffered saline from the incubation medium on the luminol-dependent CL of granulocytes

Treatment	HgCl$_2$ (10 mmol/L)	CL (arbitrary units)[a]
Not washed (control)	–	1011.31 ± 53
Not washed	+	434.61 ± 51
Washed (control)	–	828.63 ± 67
Washed	+	414.01 ± 53

[a]*Data are expressed as mean ± standard error of the mean of at least 5 observations*
Incubation was for 30 minutes at 37 °C
pH of the medium was adjusted to 7.4 with phosphate buffered saline
P < 0.05 in comparison with controls
CL = chemiluminescence

Table 3 shows the influence of HgCl$_2$ (10 mmol/L) on the viability of granulocytes following incubation at different time intervals at 37 °C.

Table 4 shows the effect of washing HgCl$_2$ with phosphate buffered saline from the incubation medium on the luminol-dependent CL of granulocytes.

Discussion

In recent years, CL has emerged as an important tool in the assessment of the oxidative burst of granulocytes. The technique involves the use of luminol to increase the amount of measurable light emitted due to liberation of oxygen metabolites during phagocytosis [24–27].

We found that HgCl$_2$ had a marked inhibitory effect on the luminol-dependent CL of granulocytes in whole blood of hu-

mans. This inhibitory effect was both dose- and time-dependent when $BaSO_4$ crystals in suspension were used to stimulate the oxidative burst of granulocytes. The range of doses used was 0–10 mmol/L with incubation time periods of 15, 30, 45 and 60 minutes. The kinetics of the chemiluminescence response were significantly different from the controls ($P < 0.05$) at different incubation periods at doses used, as shown in Tables 1 and 2.

The availability of the cells was not significantly changed following incubation at different time intervals with the $HgCl_2$ (Table 3). This result indicates that the inhibitory effects of $HgCl_2$ on the CL of stimulated cells was not due to direct lysis of the cell membrane (morphological shape of the cells and the number did not change with different amounts of incubation). The $HgCl_2$ by itself did not influence the CL state, which was evident from the control experiments.

Since $HgCl_2$ induces an autoimmune syndrome [10], it may be that it had a disruptive effect on granulocyte function [20]. It has been demonstrated that inorganic mercury causes autoimmunity syndrome characterized by production of antinucleolar antibodies and increases in serum IgG and IgE [8]. Since granulocytes functional activity is influenced by the immune complex [20,29], $HgCl_2$ might affect its functional activity [29]. The viability studies that we carried out showed that $HgCl_2$ did not have any significant effect on the integrity of the membranes of the granulocytes.

Removal of the $HgCl_2$ by washing from an incubation medium containing the granulocytes did not result in the elimination of the effect of the $HgCl_2$ which indicates irreversibility of its action as shown in Table 4. Furthermore, incubating the test sample with a thiol compound (cysteine) had no effect on the $HgCl_2$ inhibition of respiration of these cells, again indicating irreversibility of its action. In addition, the $HgCl_2$ did not excite luminol in a cell-free medium. These results suggest that the effect of $HgCl_2$ is not taking place entirely within the fluid in which the cells are suspended.

The inhibitory effect could be due to interference with $BaSO_4$ binding to membrane receptors on granulocytes as a result of rapid interaction of mercury ions with the membrane sulfhydral groups.

In the intact cell, however, the actual effect of mercury ions is determined by the distribution and accessibility of various sensitive sites, especially the sites on the membrane sulfhydral groups. Following the diffusion of mercury ions, the kinetics function and permeability of cation transport may be affected and this results in the inhibition of the CL signal [30]. On the other hand, the $HgCl_2$ might have interfered with the generation of oxygen reactive species by granulocytes through a mechanism that cannot be explained on the basis of the present results. Recently, it has been suggested that mercury ions may affect the role of the reactive oxygen intermediates generated by phagocytic cells, and cause a disruption of the redox status of the cells [28].

Conclusions

Our study suggests that the inhibitory effect produced by inorganic $HgCl_2$ on the luminol-dependent CL of granulocytes *in vitro* may provide an explanation, at least in part, for some of the toxic effects of inorganic mercury. This suggests that reactive oxygen intermediates may have a role in the genotoxicity of mercury ions and the disruption of the redox status of the granulocytes.

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