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SHORT COMMUNICATIONS

Chlorphentermine-induced phospholipidosis in rat alveolar macrophages—luminol-dependent chemiluminescence

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The alveolar macrophage (AM) functions as the principal component in the pulmonary defense against inhaled particles and microbes [1, 2]. As a result, agents that affect AM function may increase the susceptibility of the lungs to damage or infection [3, 4].

The chronic administration of a number of cationic, amphiphilic drugs leads to a lysosomally derived phospholipid storage disorder in the AMs of several species of laboratory animals [5, 6]. As the induction of phospholipidosis proceeds, the cells increase in size and undergo striking morphological changes intracellularly [6–8]. These ultrastructural changes have been described extensively; little information exists, however, concerning the effects of this disorder on cellular function.

We have found that changes occur in certain oxidative processes of AMs of rats treated with one such drug, chlorphentermine, when these cells are challenged with zymosan particles *in vitro* [9]. Among these is the generation of chemiluminescence (CL), or light emission. In response to particulate challenge, AMs release reactive forms of oxygen including superoxide anion and hydrogen peroxide [10]. In addition, the hydroxyl radical, which can be formed from the interaction of these two species, is detectable after the challenge of AMs with zymosan [11]. These oxidizing species appear to function in the killing of invading pathogens [12]. With zymosan particles and certain bacteria, some or all of these species interact directly with the challenging material with this interaction giving rise to the CL [13, 14]. Other particles that stimulate the cells to release such reactive species, however, fail to elicit CL because they are not oxidizable like zymosan. Luminol (3-aminophthalhydrazide), a cyclic hydrazide, chemiluminescences upon reaction with oxidizing species [15, 16]. The use of luminol-dependent CL has gained popularity as a method for studying oxidant production by polymorphonuclear leukocytes (PMNs) [17, 18] and AMs [16, 19] during challenge with certain non-oxidizable particulate and soluble stimulants. In addition to allowing CL detection during stimulation with a variety of agents, one advantage of using luminol is that it is possible to monitor oxidant production at rest as well as in the presence of stimulants. This allows the determination of relative increases in oxidant production during stimulation. In this report, AMs were collected from control rats and rats treated with chlorphentermine; the luminol-dependent CL was measured at rest and during exposure to latex beads.

Chlorphentermine administration. Male, Long Evans hooded rats (Charles River, Wilmington, MA) were injected i.p. with chlorphentermine hydrochloride (30 mg/kg in 0.9% NaCl) 5 days per week for 4 weeks. Control rats received an equal volume of vehicle [0.9% (w/v) NaCl]. Since chlorphentermine is an anorectic drug, treated rats failed to gain weight as rapidly as controls; therefore sufficient food was withheld from the controls so that the weight gains were similar for both groups. We have shown that this dosing regimen leads to the induction of phospholipidosis, with isolated AMs containing eighteen times as much phospholipid as control cells [20].

Recovery of alveolar macrophages. Twenty-four hours after the last injection, rats were anesthetized with pentobarbital (100 mg/kg) and killed by severing the abdominal aorta. The trachea was exposed and cannulated with polyethylene tubing. The lungs were then lavaged with 4–5 ml aliquots of calcium- and magnesium-free Hanks' balanced salt solution, pH 7.4, at 37°. Following collection of about 80 ml, the lavage fluid was centrifuged at 250 *g* for 10 min to collect the AMs. The cells were washed and resuspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.4, containing 5.5 mM glucose. Histological examination revealed that, in both preparations, 85–90 per cent of the cells were mononuclear cells with characteristics of AMs. Less than 1 per cent of the cells were identified as PMNs. Cell counts were performed using a hemocytometer. For most experiments, either two to three control rats or one treated rat was needed to provide enough cells.

Reagents. Luminol (3-aminophthalhydrazide) (Aldrich Chemical Co., Milwaukee, WI) was dissolved in dimethylsulfoxide at a concentration of 10 mg/ml. This solution was diluted to a final luminol stock concentration of 0.001 mg/ml with PBS prior to use. Latex beads (styrene butadiene—0.527 ± 0.012 μm diameter; Dow Diagnostics, Dow Chemical, Indianapolis, IN) were used as the challenging particles.

Measurement of CL. Alveolar macrophage CL was measured in an ambient temperature Packard model 2002 liquid scintillation counter in the tritium channel operated in the out-of-coincidence mode. All experiments were conducted in a final volume of 4 ml. Cells (8×10^6) were preincubated for 10 min at 37° in dark-adapted polyethylene vials (Packard Instrument Co., Downers Grove, IL). A 0.2 ml aliquot of the luminol stock was added (to give a final concentration of 2.8×10^{-7} M), and the CL measured for 0.5 min. At 15 min, the latex was added to give a particle-to-cell ratio of about 200 to 1; this ratio was found to be on the linear portion of the CL dose–response curve for latex. Lesser quantities of latex gave an amount of CL too small to follow for 30 min, and greater quantities tended to quench the CL due to increasing turbidity in the incubation solution. For measurement of resting CL, the latex beads were omitted from the vial. In some experiments, superoxide dismutase (SOD) (Type I, from bovine blood, 2900 units/mg protein, Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 0.1 mg/ml just before luminol was added.

CL was monitored for 0.5 min at 1 min and at 5 min after the addition of latex and then at 5-min intervals thereafter for a total of 30 min. The vials were maintained at 37° when not counting. Both resting and latex-stimulated CL were calculated as counts per 0.5 min minus background (vials containing cells with no luminol or latex). Cells alone gave no CL above that obtained with empty vials.

This interval counting method gave a temporal profile of CL. Since counts were measured for only 0.5 min in every 5-min period, this procedure precluded determination of total counts produced during the test period. To estimate the continuous CL over the 30 min, a straight line regression was calculated on the data from 0 to 1 min, and a polynomial regression was calculated using the data obtained by interval counting from 1 to 30 min. The data for resting CL from treated cells were fitted to a 5^{th} degree polynomial, while the data for resting CL from control AMs and the data for latex-stimulated CL for both cell types were fitted to a 4^{th} degree polynomial. From these

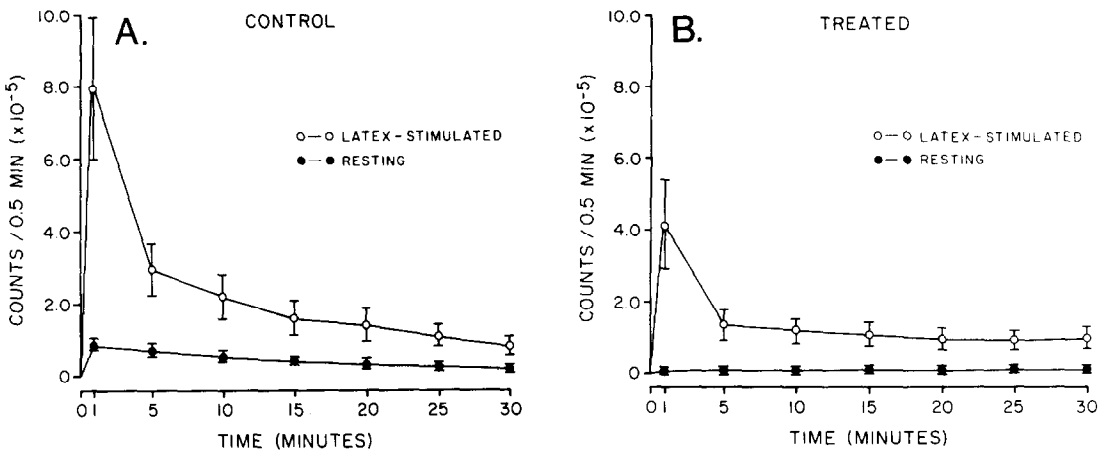

Fig. 1. Temporal chemiluminescence curves. (A) Alveolar macrophages from control rats. (B) Alveolar macrophages from chlorphentermine-treated rats. Because of the very low resting level of CL for the cells from chlorphentermine-treated rats, the abscissae on the graphs have been lowered slightly for clarity of data presentation. Values are means ± S.E.; N = ten experiments.

regression equations, curves were drawn and counts obtained for every 0.15-min interval from 0 to 30 min. By summing these data the total counts (cumulative CL) generated in 30 min were then estimated.

The CL responses of AMs from control and chlorphentermine-treated rats were clearly different (Fig. 1, panels A and B). Perhaps the most striking difference between the two cell types was in the resting CL which was much lower from the lipidotic AMs. The CL from control cells showed a progressive decrease as the experiment continued (89,100 ± 24,300 counts/0.5 min at 1 min vs 26,300 ± 5,800 counts/0.5 min at 30 min; means ± S.E.), whereas the resting CL from lipidotic cells remained rather constant (11,600 ± 4,200 counts/0.5 min at 1 min vs 10,400 ± 3,200 counts/0.5 min at 30 min). The estimated cumulative counts generated during rest in 30 min are shown in Table 1.

The temporal CL response of both cell types in the presence of latex was similar, although both showed a large variation at each time point (Fig. 1); both showed a peak response almost immediately after the addition of latex. In some experiments, the CL response was monitored every 0.75 min to determine more precisely when the peak response occurred. For both cell types, the peak CL occurred within 1 to 2 min after additon of latex. The CL then dropped off very rapidly, with the control cells dropping somewhat more rapidly than the lipidotic ones. Because this initial burst of oxidant release occurred so rapidly after exposure to zymosan, it is probable that this CL resulted from the initial contact of latex beads with the cell membrane rather than from the entire phagocytic process itself. By estimating the cumulative counts, it was found that the control AMs generated more CL than the lipidotic AMs during latex-challenge (Table 1). When latex alone was added to cells, no CL resulted, thus illustrating the requirement for luminol for the detection of CL. Neither luminol nor latex alone nor in combination gave CL above background in the absence of cells. When cells were heated in a boiling water bath for 20 min, neither the addition of luminol nor luminol plus latex resulted in CL. Therefore, the CL observed from both cell types is due to specific cellular function.

The results shown in Fig. 1 and Table 1 indicate that following the induction of phospholipidosis by chlorphentermine, AMs generated less CL and, therefore, fewer oxidizing species when challenged with latex. If the latex-stimulated data are presented as the percentage of resting CL (Fig. 2), the lipidotic cells show a much greater increase relative to resting levels than do control AMs. Therefore, although these unusual cells appeared unable to generate as strong a CL response as control cells, it was because their resting level was so low. In fact, the lipidotic cells responded more vigorously than controls to the latex beads if the data are evaluated as shown in Fig. 2

It is believed that the oxidation of luminol by AMs occurs via the superoxide anion [16] or, possibly, a product derived from it. To evaluate the role of superoxide anion in the luminol-dependent CL produced by both cell-types, experiments were conducted with superoxide dismutase (SOD) present at a concentration of 0.1 mg/ml (Fig. 3). This is the enzyme that destroys this highly reactive species. In both resting and latex-stimulated states, the CL from lipidotic cells was inhibited less than controls by SOD. This was true even when the SOD level was increased to 0.4 mg/ml in the incubation containing lipidotic cells. The fact that SOD inhibited the CL from lipidotic AMs to a lesser extent than it inhibited controls indicates that the oxidant species or processes responsible for this phenomenon are partially different for both types of AMs.

Using zymosan as the challenging particle in a non-luminol-containing system, we found contrasting results when compared to the present study [9]. With zymosan, the CL response from lipidotic AMs was slower in developing and was increased in magnitude when compared with that of the controls. It appears that different results may be obtained when the stimulant or the source of the CL (oxidation of the particle or oxidation of luminol) is varied.

Using zymosan alone [9], there was no way to monitor resting CL, and hence it was not possible to determine the relative increase in oxidant release as a result of particle challenge. Using the luminol-dependent system as in the present report permits both the resting CL and the relative changes in response to the stimulant to be monitored. In addition, there is the advantage of being able to study the responses of abnormal cells to a wide range of particles since the CL is not dependent on the oxidation of the particle as is the case with zymosan [14].

Table 1. Estimated total chemiluminescence

Macrophages	Resting	Latex-stimulated
	(counts/30 min)*	
Control	3,040,000 ± 830,000	12,960,000 ± 3,470,000
Lipidotic	667,000 ± 223,000†	7,490,000 ± 2,330,000†

* Mean ± S.E., N = 10.
† $P < 0.05$, Mann–Whitney Rank Test [21].

Although this luminol-dependent chemiluminescent system revealed changes in the response of the AMs to latex beads once phospholipidosis had been induced, it is not possible with this information to know whether an impairment in the ability of the cells to defend the lungs had occurred. Further work, such as the direct measurement of microbial killing, is necessary to understand more completely the effects of this disorder on AM function. If the correlation between CL and cell function is found to be valid for AMs in the same way as for PMNs, however, this technique may prove to be valuable in assessing the effect of chlorphentermine and other chemicals on this important cell.

Since many of the drugs that induce phospholipidosis [22] are used chronically by humans, studies such as the present one may prove useful in assessing their effects on pulmonary defense processes.

In summary, a luminol-dependent chemiluminescent assay was used to monitor oxidant release from AMs following induction of phospholipidosis by administration of chlorphentermine to rats. The lipidotic AMs generated much lower levels of CL at rest than did control AMs. Both types of AMs responded rapidly upon exposure to latex beads, generating maximum CL within 1–2 min. Control AMs generated more CL than lipidotic AMs over a 30-min exposure period; lipidotic cells, however, showed a greater increase above resting CL levels. Superoxide dismutase inhibited CL from lipidotic AMs to a lesser extent than from control AMs during rest and in the presence of latex beads, indicating that the oxidizing species responsible for CL generation may differ somewhat for both cell types. Why the induction of this disorder should lead to such changes in oxidant generation is unknown at present.

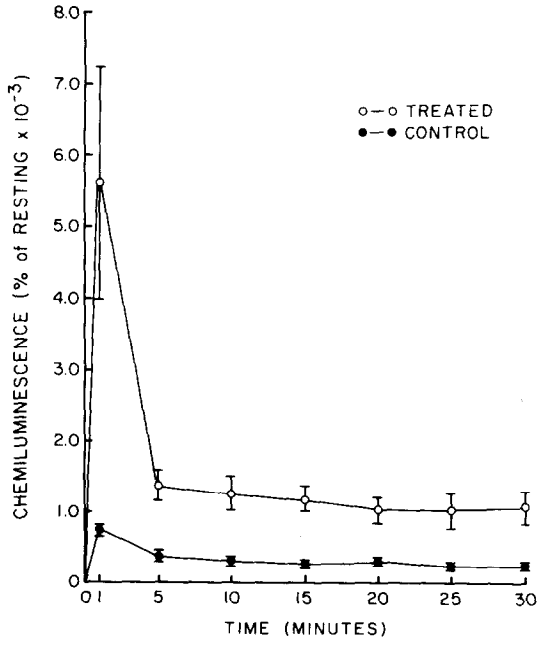

Fig. 2. Relative increase in chemiluminescence following challenge with latex beads. Values are the percentages of resting chemiluminescence following challenge of alveolar macrophages from control or chlorphentermine-treated rats with latex beads. Values are means ± S.E.; N = 10.

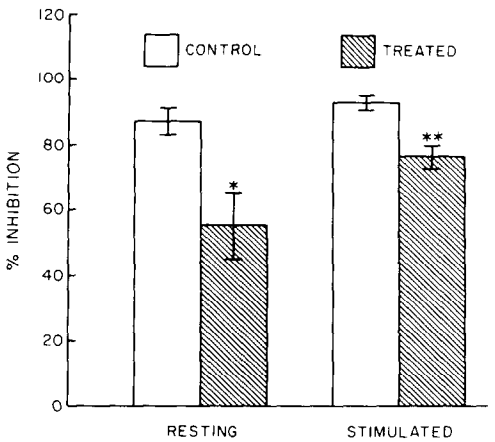

Fig. 3. Effect of superoxide dismutase (SOD) on chemiluminescence needs a period after chemiluminescence. SOD (0.1 mg/ml) was added to the incubation mixtures containing AMs from either control or chlorphentermine-treated rats, and the percent inhibition of resting and latex-stimulated CL was determined at each time shown in Fig. 1. The values from each time point were averaged to give a per cent inhibition for each experiment. The data shown are the means ± S.E. with N = 5–6. Key: (*) $P < 0.05$ and (**) $P < 0.005$ using Student's *t*-test.

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Interaction between arsenic and alloxan-induced diabetes—effects on rat urinary enzyme levels*

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General kidney dysfunction has been detected previously in patients afflicted with pathological renal conditions by monitoring the presence of aberrant urinary enzyme patterns [1–4]. The microvascular changes associated with diabetes mellitus result in clinically important effects on the kidneys as well as on other organs. Diabetic patients have various degrees of nephropathy and of elevated urinary enzyme excretion, when compared to non-diabetic subjects [5, 6]. Increased urinary excretion of lyosomal enzymes is presumed to reflect secondary renal tubular involvement and to serve as a sensitive indicator of the onset of diabetic nephropathy [7]. In addition, experimental animals given either nephrotoxins [8–11] or an inducer of diabetes [12] have been found to produce elevated urinary enzyme levels. Enzymes with characteristic subcellular locations may be selected to indicate the site of the primary event and to determine the extent to which the various cellular compartments are involved [9].

The effects of environmental agents on urinary enzyme levels in diabetes have not been studied. Some environmental agents that accumulate in the kidney, however, also produce effects on normal renal function [13]. Identification of an interaction between the renal lesions of diabetes and exposure to an environmental toxin is potentially significant with respect to the interpretation of clinical findings and assessment of renal damage. The alteration of urinary enzyme levels is used in this study as a convenient, non-invasive, non-destructive method for assessing such an interaction.

This investigation is focused on the interaction between an oral exposure to chronic low doses of an environmental nephrotoxin and the diabetic state. The model system studied is the alloxan-diabetic rat with previous exposure to low levels of sodium arsenate (As^{5+}) in the drinking water. Alloxan has been shown previously to produce damage specifically in the insulin-secreting β-cells present in the pancreas of laboratory animals [14, 15]. The inability of the affected islet tissue to secrete insulin provides a model for inducing experimental diabetes and studying the ontogeny of the diabetic state. If the dosage of alloxan is higher than that required to damage the β-cells, definite effects may be observed on other tissues [16–19]. The levels of several key lysosomal enzymes (acid phosphatase and *N*-acetyl-β-D-glucosaminidase) and non-lysosomal enzymes (alkaline phosphatase, lactate dehydrogenase and L-glutamate oxaloacetate transaminase) from these animals were mon-

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