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The effects of intravenous anaesthetic agents on human neutrophil chemiluminescence

Intact neutrophil function is essential for the defence against infection. Any alteration in neutrophil function, which decreases their ability to phagocytose and kill bacteria, might contribute to mortality and morbidity. We investigated the effects of clinical concentrations of thiopentone, Alfathesin, methohexitone, morphine, lidocaine and diazepam on the microbicidal oxidative function of human neutrophils. The oxidative activity was assessed utilizing the technique of chemiluminescence, which is a measure of free radical generation. Thiopentone and Alfathesin produced a significant dose dependent depression in chemiluminescence. There was a 27 per cent reduction in activity with thiopentone $5 \mu\text{g}\cdot\text{ml}^{-1}$, a concentration equivalent to the free plasma concentration achieved following an anaesthetizing dose of thiopentone. There was a 55 per cent reduction in chemiluminescence at an alphaxalone concentration of $1.25 \mu\text{g}\cdot\text{ml}^{-1}$, a concentration equivalent to the free plasma level obtained after induction of Alfathesin anaesthesia. The effect of thiopentone and Alfathesin was reversed by cell washing. Methohexitone, morphine, diazepam, and lidocaine caused no significant reduction in chemiluminescence over the dose ranges studied.

These observations indicate that thiopentone and Alfathesin can adversely affect leucocyte function in vitro and, therefore, may contribute to impaired host resist-

ance in the perioperative period and in the intensive care unit.

Key words

ANAESTHETICS, INTRAVENOUS: thiopentone, Alfathesin, methohexitone, morphine, diazepam.
ANAESTHETICS, LOCAL: lidocaine. BACTERIA: bioluminescence.

Normal leucocyte function is important in the immunologic defence against infection. Previous studies have shown that inhalational anaesthetic agents depress the ability of neutrophils to ingest and kill bacteria and that thiopentone and Alfathesin depress phagocytosis.¹⁻³ As bacterial killing is the most important function of the neutrophil, any interference with it could have serious clinical consequences in terms of promoting sepsis. We have, therefore, investigated the effects of intravenous anaesthetics used in the operating room and the intensive care unit on the killing mechanism.

A major mechanism of killing invading bacteria by neutrophils is the production of excited oxygen radicals. These are produced following attachment to specific receptors on the neutrophil surface of opsonized bacteria. The radicals produced cause destruction of the bacterial cell wall and thus cell death. Associated with the return of the radicals to the ground state is the release of photons resulting in the phenomenon of chemiluminescence. Quantitation of the light emission by suitably stimulated neutrophils can be used as an index of the free radical generation and thus an indirect index of the ability of neutrophils to kill bacteria.^{4,5} This study investigates the effects of varying clinical concentrations of thiopentone, methohexitone, Alfathesin,

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morphine, diazepam and lidocaine on the chemiluminescence of human neutrophils.

Methods

Preparation of human neutrophils and zymosan activation

Ten ml of heparinized blood and 20 ml of clotted blood were obtained from healthy human volunteers. The neutrophils were obtained by centrifugation of the heparinized blood with 150 mol. wt. dextran at 350 rpm for 10 min. The cells were washed twice with phosphate buffered saline (PBS). The cell concentration was adjusted to 500,000 cells per ml, viability was confirmed with trypan blue stain and a differential count was done to confirm the presence of greater than 70 per cent neutrophils.

The serum obtained from the subjects was incubated with 10 mg·ml⁻¹ of zymosan (Sigma Chemical Company, St. Louis, Missouri) at 37° C for 30 min, then centrifuged and resuspended in PBS in a concentration of 10 mg·ml⁻¹.

Preparation of agents and exposure of cells to drugs

Dilutions of thiopentone (10–200 micrograms per ml), Alfathesin (0.1–5 micrograms per ml of alphaxalone), methohexitone (1.25–20 micrograms per ml), morphine sulfate (1.0–50 micrograms per ml), diazepam (0.1–5.0 micrograms per ml), and lidocaine (1.0–20 micrograms per ml) were prepared in Hank's balanced salt solution (HBSS) and the pH confirmed to be between 7.2 and 7.4.

One ml of test solution was incubated with 1 ml of cell suspension (500,000 cells) for 1 hour at 37° C. One ml of cell suspension with 1 ml of HBSS was used as a control solution.

Chemiluminescent assay

Following incubation, 100 microlitres of Luminol (Sigma Chemicals) in DMSO (2 mg·ml⁻¹) were added to the test vials and chemiluminescence was measured after neutrophil activation with 200 microlitres of the previously opsonized zymosan (10 mg·ml⁻¹). The light emission was then measured using a Beckman Liquid Scintillation Counter (Model 230), in the in-coincidence mode and the counts were measured for 0.1 minutes every 10 min

for one hour. All assays were done in triplicate. Use of the in-coincidence mode ensured a low background activity before stimulation and avoided the need for dark adaption of the counting vials. Basal background activity was measured with the Scintillation Counter in the out-of-coincidence mode in order to increase the sensitivity of the measurement.

Reversibility of inhibition

To determine whether the depressant effect of Alfathesin and thiopentone on chemiluminescence was reversible, neutrophil/test drug suspensions were incubated for one hour at 37° C and then washed twice by gentle centrifugation at 1500 rpm with PBS. Cell concentrations were adjusted to 5×10^5 and chemiluminescence was reexamined. Cells incubated with PBS and then similarly washed were used as controls. Cell counts were done before and immediately after incubation but before washing so as to exclude a cytolytic effect.

The data were recorded in counts per minute (CPM), and analyzed by a two-way analysis of variance of basal activity, peak activity and the area under the time/activity curve. The area under the curve was calculated by numerical integration using Simpson's formula.

Results

Normal chemiluminescence

The normal control response curve shows peak activity at 20–30 min following stimulation with zymosan (Figure 1). Basal background activity, both in and out-of-coincidence, was always less than 100 CPM and was unaffected by the presence of any of the drugs studied.

Thiopentone

The effect of thiopentone was studied using neutrophils from seven healthy subjects. The curves of light emission for each of the concentrations studied (Figure 1) follow the control curve closely but were depressed in a dose dependent fashion. The peak activity and the area under the curve showed a significant depression ($p < 0.01$) at all concentrations of thiopentone studied (Figures 1 and 2 and Table I). Specifically, there was a 27 per cent reduction in peak counts with 5 micrograms per ml, a level comparable to the free plasma concentration obtained following an anaesthetizing dose of thiopentone.⁶

FIGURE 1 The effect of thiopentone on the chemiluminescence of human neutrophils. Chemiluminescence is shown as counts per minute (CPM). Each point is the mean $\pm$ S.D.

Alfathesin

The effect of Alfathesin was also examined in seven healthy subjects. There was a similar dose dependent depression of chemiluminescence at all concentrations studied ($p < 0.01$) with the exception of 0.05 micrograms per ml of alphaxalone (Figure 3 and Table I). There was a 55 per cent reduction in chemiluminescence at a concentration of 1.25 micrograms per ml, a level comparable to the free plasma concentration achieved following the induction of Alfathesin anaesthesia.⁷

Methohexitone, morphine, diazepam, lidocaine

There was no significant depression of chemilumi-

TABLE Effect of thiopentone and Alfathesin on the area under the chemiluminescence curve

Thiopentone		Alfathesin	
Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	% of control activity	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)	% of control activity
5	71*	0.05	92 n.s.
10	56*	0.25	67*
25	37*	0.50	62*
50	22*	1.25	45*
100	10*	2.50	33*

Control - no drug added.

n.s. - not significant ($p > 0.05$).

* $p < 0.01$.

nescence over the dose ranges studied (Figure 4). In view of this, only three subjects were studied with each drug. These dose ranges were chosen to include blood concentrations expected with normal therapeutic doses used in the intensive care unit and the operating room.⁸⁻¹¹

Reversibility of inhibition

Neutrophils from seven normal subjects were studied with each drug. Washing of the cells removed the inhibitory effects of thiopentone and Alfathesin. Cell counts before and after incubation were unchanged.

Discussion

This study demonstrates that neutrophils from healthy humans, when incubated with clinical

FIGURE 2 The effect of thiopentone on peak chemiluminescence is shown as a function of drug concentration. Each point is the mean $\pm$ S.E.M.

FIGURE 3 The effect of Alfathesin on peak chemiluminescence is shown as a function of drug concentration. Each point is the mean $\pm$ S.E.M.

FIGURE 4 The effect of methohexitone, lidocaine, diazepam and morphine on the peak chemiluminescence. Each point is the mean $\pm$ S.E.M.

concentrations of thiopentone and Alfathesin, have a reduced ability to produce the oxygen radicals necessary for bacterial killing. The reduction was dose dependent but could be reversed by washing the cells. A similar depression in function could not be demonstrated with methohexitone, diazepam, morphine or lidocaine.

The interaction between bacteria and neutrophils that ultimately leads to bacterial destruction involves a number of complex steps. The bacterium or foreign protein is first coated (opsonized) by specific antibody or complement to facilitate recognition by the neutrophils. This is followed by ingestion (phagocytosis) which is an active process that includes the use of the contractile proteins actin and myosin. Once the phagocytic vacuole is within the leucocyte, cytoplasmic granules discharge their hydrolytic and proteolytic enzymes into it. Of more importance than the latter, is oxidative killing.^{12,13} The same stimuli that result in phagocytosis and degranulation also stimulate an intense burst of oxidative metabolism within the neutrophil. This results in the production of excited oxygen radicals (superoxide, hydroxyl radical, and hydrogen peroxide) within the vacuole. The production and activity of these substances is dependent, in part, upon NADPH oxidase located in the plasma membrane and myeloperoxidase located in the granules. The radicals destroy the bacteria by oxidation and/or halogenation. The relaxation of the excited oxygen radical to the ground state is associated with the emission of light and the phenomenon of chemilu-

minescence. The *in vitro* addition of luminol amplifies the light output, and thus, increases the sensitivity of the test.

The relationship of chemiluminescence to microbicidal activity has been the subject of recent research.^{4,5} The use of chemical and enzyme inhibitors of oxygen radical reactions in *in vitro* bactericidal assays significantly reduces human neutrophil bacterial killing.¹⁴ Welch has shown that the killing of suitable opsonized bacteria correlates with the extent of the chemiluminescence.⁵ Further evidence for the role of leucocytic oxidative metabolism comes from the study of patients with chronic granulomatous disease.¹³ This is an inherited disorder characterized by recurrent, severe, pyogenic infections. While phagocytic activity and granule content are normal, leucocytes from these patients exhibit no chemiluminescence. Our demonstration of a reduction in chemiluminescence by thiopentone and Alfathesin suggests that these two agents reduce neutrophilic microbicidal activity.

The mechanism producing the depression in chemiluminescence was not directly investigated in this study. The depression in activity was dose dependent and the kinetics of the depression suggest that there was a global reduction in excited oxygen radical production rather than a reduction in a specific radical, as this leads to a change in the contour of the chemiluminescence curve.⁴ Thiopentone, Alfathesin, diazepam, lidocaine, morphine and methohexitone have all been shown to reduce phagocytosis while only thiopentone and

Alfathesin reduce chemiluminescence.³ The latter drugs may exert their action by either interfering with the events leading to the production of excited oxygen radicals or by scavenging the radicals. The difference in effect between thiopentone and methohexitone may be explained by the fact that thiopentone is a good free radical scavenger while methohexitone is not.¹⁵

Methohexitone, diazepam, morphine and lidocaine were also studied because these drugs are commonly given either as bolus injections or by continuous infusion, in the operating room and intensive care unit. Lidocaine had no depressant effect on chemiluminescence in the dose ranges studied. This is of interest because lidocaine is a membrane stabilizer and may be expected to influence membrane related events.¹⁶ Previous studies of the effect of lidocaine on alveolar macrophages have shown a reduction in superoxide anion production.¹⁷ This disparity may reflect the difference in the cell types studied. Morphine, diazepam and methohexitone did not produce a statistically significant reduction in chemiluminescence at therapeutic levels but depression was apparent at the higher concentrations studied.

The cells were incubated with the drugs for one hour. Our findings may, therefore, not be relevant to the clinical situation in which a single injection of drug is given. However, all the drugs studied may be given, in the operating room or in the intensive care unit, by continuous infusion or by intermittent injections so as to maintain a therapeutic blood level. In this situation, our results are germane. The depressant effects of thiopentone and Alfathesin were easily reversed by washing the cells. Clinically, one would expect the cells to return to normal function when the drug concentration had dropped to a low enough level. This reversibility might explain some of the previous discrepancies among studies of depression in neutrophil function.¹⁸ Cells from anaesthetized patients are usually washed during separation and this may remove the anaesthetic and thus reverse its effects on the cells. All our studies were done in an aqueous medium using drug concentrations equivalent to the free plasma levels that might be found clinically.⁶⁻¹¹ It is generally believed that the protein bound drug does not actively participate in pharmacological effects.

The possibility that anaesthesia might increase the chances of bacterial infection has been sug-

gested for many years. In 1911, Graham found a significant inhibition of phagocytosis of streptococci by leucocytes in the presence of ether, and Hamburger made a similar observation with chloroform.^{19,20} Over the last decade, there have been numerous *in vitro* and *in vivo* studies of the effects of anaesthetics, with and without surgery, on chemotactic migration and phagocytosis of human leucocytes.^{3,18} Despite the lack of uniformity in the study designs, the consensus is that anaesthesia diminishes these functions. Bacterial killing is a major function of the neutrophil. Welch has recently demonstrated that halothane inhibits neutrophil bacterial killing, and animal models have suggested that infection and mortality are worsened by anaesthesia and surgery.^{1,21} The depression of neutrophil excited oxygen radical generation by thiopentone and Alfathesin reflects a depression in cellular competence which might contribute to impaired host resistance in the postoperative period and in the intensive care unit.

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Résumé

Il est essentiel dans la défense contre l'infection de maintenir intacte la fonction des leucocytes neutrophiles. Toute altération de cette fonction, en diminuant la capacité de phagocytes et de tuer les bactéries, peut contribuer à la mortalité et à la morbidité. Nous avons étudié les effets de concentrations cliniques de thiopental, alphathésin, méthohexital, morphine, xylocaïne et de diazépam sur la fonction oxydative bactéricide des neutrophiles humains. L'activité bactéricide fut évaluée en utilisant la chemoluminescence qui est une mesure de la génération de radicaux libres. Le thiopental et l'alphathésin produisent une diminution significative et proportionnelle à la dose de la chemoluminescence. Il y a eu une réduction de 27 pour cent avec le thiopental $5 \mu\text{g}\cdot\text{ml}^{-1}$, (concentration équivalente à la concentration libre plasmatique obtenue à la suite d'une dose anesthésique de thiopental). Il y a eu une réduction de 55 pour cent de la chemoluminescence avec une concentration d'alphaxalone de $1.25 \mu\text{g}\cdot\text{ml}^{-1}$, (concentration équivalente à la concentration plasmatique libre obtenue après induction à l'alphathésin). Les effets du thiopental et de l'alphathésin ont été annulés après lavage des cellules. Le méthohexital, la morphine, le diazépam et la xylocaïne n'ont pas provoqué de réduction significative de chemoluminescence dans l'échelle des doses étudiées.

Ces observations indiquent que le thiopental et l'alphathésin peuvent atteindre la fonction leucocytaire in-vitro et de ce fait, peuvent contribuer à une résistance amoindrie de l'hôte durant la période péri-opératoire et le séjour aux soins intensifs.