Anesthesiology
60:57–60, 1984

In Vitro *Effect of Benzodiazepines on Polymorphonuclear Leukocyte Oxidative Activity*

G. Goldfarb, M.D.,* J. Belghiti, M.D.,† H. Gautero,‡ P. Boivin, M.D.§

The effect of three benzodiazepine compounds, diazepam, flunitrazepam, and clorazepate, on oxidative activity of human polymorphonuclear leukocyte (PMN) was investigated. The oxidative activity of zymosan-stimulated PMN in the presence of three concentrations (10, 20, and 40 μg/ml) of these compounds was measured polarographically. In addition, zymosan-stimulated nitroblue tetrazolium (NBT) reduction was measured in the presence of various concentrations of flunitrazepam. All three compounds inhibited oxygen consumption of the PMN. The extent of inhibition was linear with respect to log-concentrations; oxygen consumption was reduced 50% for concentrations of diazepam, flunitrazepam, and clorazepate of 13 μg/ml, 56 μg/ml, and 285μg/ml, respectively. In addition 30% and 100% inhibition of NBT reduction by flunitrazepam were observed at respective concentrations of 10 μg/ml and 60 μg/ml. The clinical relevance of these findings remains to be determined. (Key words: Anesthetics, intravenous: benzodiazepines. Blood: neutrophils; microbicidal function.)

IT HAS BEEN COMMONLY SPECULATED that anesthetic agents may modify the immune status of humans and play a role in the occurrence of postoperative infections. The response to an invading microorganism depends on various factors such as cell-mediated immunity, humoral immunity, and neutrophil function. Polymorphonuclear leukocytes (PMN) provide an essential defense against invading bacteria. This defense has two phases: first chemotaxis, when the PMN is attracted toward the invading microorganism, and secondly, phagocytosis, when the microorganism is incorporated and killed by the PMN. This killing is associated with metabolic activation of the PMN, which can be evaluated by measuring biologic parameters such as oxygen consumption, nitroblue tetrazolium (NBT) reduction, and iodination.[1] Thus, the bactericidal power of PMN can be evaluated precisely, *in vitro*. This study was undertaken to determine the effects of three benzodiazepine compounds, diazepam, flunitrazepam, and clorazepate, on the metabolic activation of the PMN in the presence of an activating substance, zymosan.

Methods

Zymosan A from *Saccharomyces cerevisiae* and nitroblue tetrazolium (NBT) were obtained from Sigma Chemical Co, St. Louis, Missouri. Oxygen tension was determined polarographically using a Gilson oxygraph equipped with a Clark electrode. Human PMN were isolated from heparinized venous blood by sedimentation of the erythrocytes using Dextran as previously described.[1] The final pellet was resuspended in calcium-free Krebs–Ringer phosphate buffer (KRP) *p*H 7.40 containing 5.5 mM glucose and 10% AB serum. Isolated cells were adjusted to a concentration of 10^7 PMN/ml. Aliquots of this suspension were used in each experiment.

Oxygen consumption of zymosan-stimulated PMN was measured polarographically according to Kvarstein.[2] The incubation medium was composed of KRP, 5.5 mM glucose, 10% AB serum, and 10^6 PMN/ml. The results are expressed in nanomoles of oxygen consumed per minute per 10^6 PMN at 37° (nmol · min^{-1} · 10^{-6} PMN). Zymosan-stimulated NBT reduction was measured according to Baehner and Nathan,[3] except that cyanide was omitted. The results are expressed in nanomoles NBT reduced per minute per 10^6 PMN. The incubation time was 15 min at 37°.

The benzodiazepine compounds, diazepam, clorazepate, and flunitrazepam, were diluted in solvent. These solutions containing various concentrations of benzodiazepines derivatives (10, 20, and 40 μg/ml) were added to KRP containing about 10^7 PMN/ml. After 15 min of incubation, the PMN were centrifuged, washed, resuspended in KRP, and counted again. Control PMN were treated identically, except benzodiazepine compounds were not present in the solution. In order to verify the correlation between oxygen consumption and NBT reduction,[1] zymosan-stimulated NBT reduction was measured in the presence of solutions containing 5, 10, 20, 40, and 60 μg/ml of flunitrazepam. Three measurements were carried out at each concentration. Means, standard errors, linear regression, and correlation coefficient were calculated for each compound. Wilcoxon signed rank test

* Assistant of Anesthesiology, UER Xavier Bichat.
† Assistant of Surgery, UER Xavier Bichat.
‡ Research Assistant, INSERM U160, UER Xavier Bichat.
§ Professor of Hematology, INSERM U160, UER Xavier Bichat.
Received from the Département D'anesthésiologie, the Département de Chirurgie, and INSERM U160, Hôpital Beaujon Clichy, France. Accepted for publication July 12, 1983.
Address reprint requests to Dr. Goldfarb: Département D'anesthésiologie, Hôpital Beaujon, 92118 Clichy, France.

TABLE 1. Comparative Studies of PMN Zymosan-stimulated Oxygen Consumption under Control Conditions and in the Presence of Various Concentrations of Three Benzodiazepine Compounds, Flunitrazepam, Diazepam, and Clorazepate after a 15-min Preincubation Time (Results Shown Represent Mean ± SEM of Three Separate Experiments)

	Oxygen Consumption in nmol·min^{-1}·10^{-6} PMN (mean ± SEM)				
	Control	10 μg·ml^{-1}	20 μg·ml^{-1}	40 μg·ml^{-1}	Average Consumption*
Flunitrazepam	9.6 ± 0.3	6.9 ± 0.5	6.1 ± 0.5	5.2 ± 0.3	6.1 ± 0.3†
Diazepam	9.0 ± 0.2	4.9 ± 0.3	3.8 ± 0.3	2.6 ± 0.2	3.7 ± 0.3†
Clorazepate	10.4 ± 1.4	8.1 ± 1.2	7.8 ± 1.1	6.9 ± 1.1	7.7 ± 0.6†

* Average consumption is the mean of all the oxygen consumptions observed in the presence of each compound.

† $P < 0.01$, Wilcoxon signed rank test.

was used to compare oxygen consumption or NBT reduction in the presence or absence of each compound.

Results

Effects of Benzodiazepine Compounds on PMN Zymosan-stimulated Oxygen Consumption

Diazepam, clorazepate, and flunitrazepam show an inhibitory effect ($P < 0.01$) on PMN oxygen consumption (table 1). The data shown in figure 1 reveal a negative correlation ($P < 0.01$) between the oxygen consumptions and the log concentrations for each compound; the oxygen consumption decreases in the presence of increasing concentration of the drugs. As reflected by the ID_{50} (the concentration of the drug that reduces the oxygen consumption associated with phagocytosis by 50%), diazepam is the most potent inhibitory drug (ID_{50} = 13 μg/ml) followed by flunitrazepam (ID_{50} = 56 μg/ml) and clorazepate (ID_{50} = 285 μg/ml).

Effects of Flunitrazepam on NBT Reduction

Flunitrazepam has a potent inhibitory effect ($P < 0.01$) on NBT reduction (fig. 2). The latter is reduced by 30% in the presence of 10 μg/ml flunitrazepam. At a higher concentration (60 μg/ml), NBT reduction is inhibited completely.

Discussion

The possibility that anesthetic agents may alter PMN function has been under consideration for the past 70 years[4]; but since anesthesia in humans is associated with surgery, its specific action on PMN function is difficult to establish. *In vitro* studies have shown that many anesthetic agents may decrease PMN chemotaxis. Such an effect can be observed with diazepam at concentrations ranging from 4 μg per ml to 200 μg per ml[5]; at a concentration of 10 μg per ml, chemotaxis is decreased 40%.[6] Other studies have reported similar effects with halothane,[7] althesin,[7], ketamine,[6] thiopental,[6,7] and nitrous

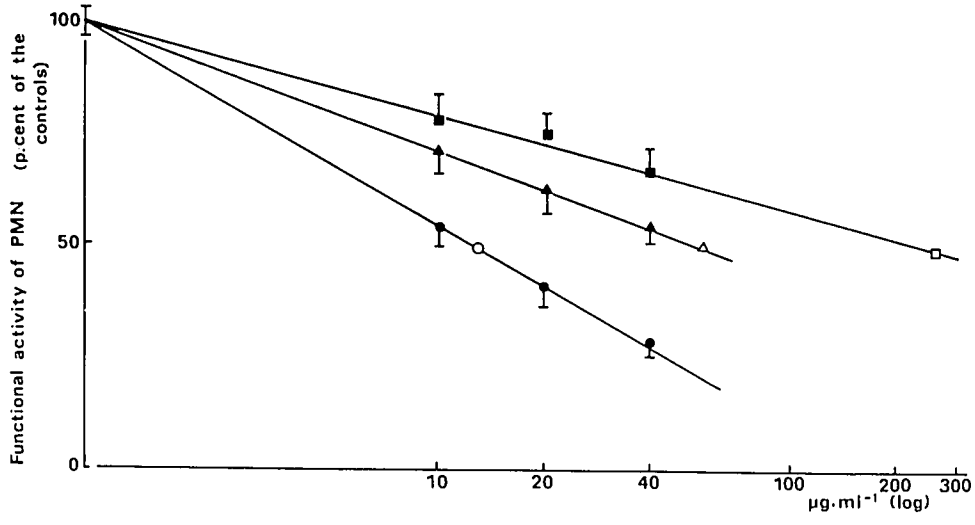

FIG. 1. Effects of various concentrations of flunitrazepam (▲), diazepam (●), and clorazepate (■) on PMN zymosan-stimulated oxygen consumption measured after a 15-min preincubation time. Values of the control zymosan-stimulated oxygen consumption for flunitrazepam, diazepam, and clorazepate were (±SEM) 9.6 ± 0.3, 9.0 ± 0.2, and 10.4 ± 1.4 nmol·min^{-1}·10^{-6} PMN, respectively. Estimated values of ID_{50} for flunitrazepam (△), diazepam (○), and clorazepate (□) were 56, 13, and 285 μg·ml^{-1}, respectively. Bars show mean ± SEM of three separate experiments.

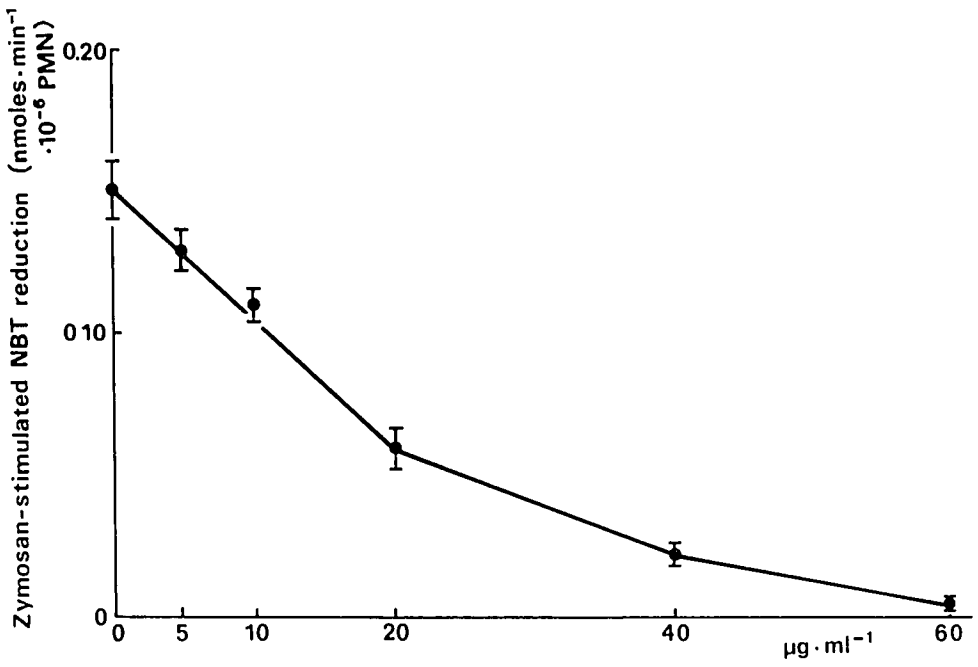

FIG. 2. Effects of various concentrations of flunitrazepam on PMN zymosan-stimulated NBT reduction measured after a 15-min preincubation time. Value of the control PMN zymosan-stimulated NBT reduction was (±SEM) 0.15 ± 0.01 nmol·min^{-1}·10^{-6} PMN. Bars show mean ± 2 SEM of three separate experiments.

oxide.[8] *In vitro* studies of phagocytosis have yielded contradictory effects for halothane; some workers reported no inhibition,[9,10] while others reported an effect.[11] This discrepancy could be related to the sensitivity of the different methods used in these studies. Enflurane is reported to inhibit the PMN function,[12] while nitrous oxide shows no effect.[10,11]

In our study, we found that flunitrazepam, diazepam, and clorazepate inhibit *in vitro* the oxidative activity of PMN. Diazepam and flunitrazepam seem to have far more potent inhibitory effects than does clorazepate. This effect could be related to their structures; clorazepate is less hydrophobic than the two other compounds. It cannot be determined if the effect results from a PMN membrane perturbation or from a direct inactivation of the oxydative enzymes by the compounds.

The effect of a drug on PMN depends on two factors inversely correlated: its concentration and its incubation time.[13] In clinical practice, there is no obvious correlation between the plasma level and the clinical effect of benzodiazepines.[14] Thus, there is a wide range of administered doses for premedication and general anesthesia. Single doses, usually given intramuscularly for premedication,[14] diazepam 10 mg, clorazepate 20 mg, flunitrazepam 2 mg result in plasma levels of 200–400 ng/ml,[15] 700 ng/ml,[16] and 25 ng/ml,[17] respectively. A single intravenous bolus of diazepam 20 mg results in a plasma level of 1,200 ng/ml,[18] whereas repeated boluses result in a plasma level up to 5,000 ng/ml.[19] These benzodiazepines are detectable in the plasma of patients for several hours to several days after administration.[14,15,19,20] During *in vitro* studies, the viability of PMN does not allow such an incubation time; in the present study the incubation time was 15 min. Thus, in order to try to correlate the *in vitro* effect of the drugs and their possible clinical effect, it is reasonable to study *in vitro* higher concentrations than those observed in clinical practice. However, it is still difficult to predict the clinical consequences of the *in vitro* effect. Firstly, the studied PMN suspension, containing 10^6 PMN/ml, cannot be compared with normal human blood, containing usually less than 10^4 PMN/ml. Secondly, only 10% of the PMN total body content, representing intravascular PMN, are in contact with the drugs at their plasma concentrations. The essential defense against invading microorganism is provided by the other 90%, located in the tissues where they remain functional for 1–2 days.[21] Thus, *in vivo*, the kinetics of the drug in the infected tissues should be the major determinant of its effect on PMN oxygen consumption rather than its plasma kinetics.

During their metabolic activation, PMN generate highly reactive oxygen species such as superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$).[22] This generation is essential for bacterial killing. Thus inhibition of the oxidative activity of the stimulated PMN is a predisposing factor for infection. *In vivo*, inherited abnormalities of the oxidative activity of PMN, such as chronic granulomatous disease, result in an increased susceptibility to pyogenic infections. The inhibitory effect on PMN function of some anesthetic agents

could be involved in the occurrence of postoperative infections. However, the clinical relevance of the inhibitory effect of benzodiazepine compounds observed in our study remains to be determined.

References

1. Hakim J, Cramer E, Boivin P, Troube H, Boucherot J: Quantitative iodination of human blood polymorphonuclear leukocytes. Eur J Clin Invest 5:215–219, 1975
2. Kvarstein B: Oxygen consumption during the initial stage of human leucocyte phagocytosis of polystyrene latex particles. Scand J Clin Lab Invest 25:337–348, 1970
3. Baehner RL, Nathan DG: Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N Engl J Med 278:971–976, 1968
4. Graham EA: The influence of ether and ether anesthesia on bacteriolysis, agglutination and phagocytosis. J Infect Dis 8:147–175, 1911
5. Mathieu D, Mathieu A: Effect of intravenous anesthetics on leukocyte migration. ANESTHESIOLOGY 53:S30, 1980
6. Mathieu A, Mathieu D, Hyslop N: Effects of induction agents and nonvolatile anesthetics on chemotaxis of polymorphonuclear leucocytes. ANESTHESIOLOGY 51:S56, 1979
7. Mougdil GC, Allan RB, Russell RJ, Wilkinson PC: Inhibition, by anesthetic agents on human leukocyte locomotion towards chemical attractants. Br J Anaesth 49:97–105, 1977
8. Nunn JF, O'Morain C: Nitrous oxide decreases motility of human neutrophils *in vitro*. ANESTHESIOLOGY 56:45–48, 1982
9. Nunn JF, Sturrock JE, Jones AJ, O'Morain C, Segal AW, Coade SB, Dorling J, Walker D: Halothane does not inhibit human neutrophil function *in vitro*. Br J Anaesth 51:1101–1108, 1979
10. Cullen BF: The effect of halothane and nitrous oxyde on phagocytosis and human leukocyte metabolism. Anesth Analg 53:531–536, 1974
11. Welch WD, Zaccari J: Effect of halothane and N_2O on the oxidative activity of human neutrophils. ANESTHESIOLOGY 57:172–176, 1982
12. Welch WD, Zaccari J: Enflurane, but not isoflurane, inhibits neutrophils. ANESTHESIOLOGY 55:A293, 1981
13. Auclair C, Gautero H, Boivin P: Effect of salicylate–copper complex on the metabolic activation in phagocytizing granulocytes. Biochem Pharmacol 29:3105–3109, 1980
14. Kanto J: Benzodiazepines as oral premedicants. Br J Anaesth 53:1179–1188, 1981
15. Mandelli M, Tognoni G, Garattini S: Clinical pharmacokinetics of diazepam. Clin Pharmacokinet 3:72–91, 1978
16. Rey E, d'Athis P, Giraux P, de Lauture D, Turquais JM, Chavinie J, Olive G: Pharmacokinetics of clorazepate in pregnant and nonpregnant women. Eur J Clin Pharmacol 15:175–180, 1979
17. Clarke RSJ, Dundee JW, McGowan Waw, Howard PJ: Comparison of the subjective effects and plasma concentrations following oral and IM administration of flunitrazepam in volunteers. Br J Anaesth 52:437–445, 1980
18. Hillestad L, Hansen T, Melsom H, Drivenes A: Diazepam metabolism in normal man. I. Serum concentrations and clinical effects after intravenous, intramuscular and oral administration. Clin Pharmacol Ther 16:479–484, 1974
19. Ochs HR, Greenblatt DJ, Lauven PM, Stoeckel H, Rommelsheim K: Kinetics of high dose IV diazepam. Br J Anaesth 54:849–852, 1982
20. Van der Kleijn E, Van Rossum JM, Muskens ET, Rijntjes NV: Pharmacokinetics of diazepam in dogs, mice and humans. Acta Pharmacol Toxicol (Copenh) 29(suppl 3):109–127, 1971
21. Wade BH, Mandell GL: Polymorphonuclear leukocytes: Dedicated professional phagocytes. Am J Med 74:686–693, 1983
22. Babior BM: Oxygen-dependent killing by phagocytes. N Engl J Med 298:659–668, 1978