

Thiopental Inhibition of Tumor Immunity

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The ability of leukocytes to kill tumor cells appears central to the defense against neoplastic growth. The authors determined the effect of thiopental on this phenomenon *in vitro* by incubating ⁵¹Cr-labelled YAAC-1 tumor cells obtained from the peritoneal cavities of syngeneic A/JAX white mice with immune leukocytes from the peritoneal cavities of allogeneic C57/black mice. Tumor-cell death was quantitated by the amount of ⁵¹Cr released into the medium following tumor-cell lysis. Thiopental, in concentrations used during routine anesthesia, inhibited tumor-cell killing in a dose-related manner. Inhibition of cytotoxicity ranged from 8.6 per cent at 2.8×10^{-5} M thiopental to 38.1 per cent at 8.5×10^{-5} M thiopental. Moreover, this inhibitory effect was additive to that previously demonstrated with halothane, and was related to the duration of exposure to the anesthetic. It is postulated that thiopental and other anesthetics contribute to the inhibition of leukocytic responsiveness observed in patients with malignancies who have undergone surgical procedures. (Key words: Immune response, thiopental; Cancer, thiopental; Anesthetics, intravenous, thiopental.)

THE DEVELOPMENT and spread of malignant tumors in experimental animals and man depend upon a balance between the frequency of occurrence of neoplastic cells and the ability of normal immune defense mechanisms to effect their destruction. Studies of experimental cancer in animals suggest that anesthetics may enhance development of metastases by reducing immunologic rejection of carcinogens and/or neoplastic cells.¹ Such immunologic potentiation of cancer by anesthesia need not be dependent only upon the direct effects of anesthetic drugs, but may also be related to the neurohumoral and physiologic responses to the state of anesthesia.

One of the defense mechanisms limiting neoplastic growth depends upon tumor-cell killing by sensitized leukocytes. This mechanism can be assessed by *in-vitro* assays wherein tumor cells are incubated with sensitized leukocytes from patients or animals bearing that tumor.² The amount of tumor-cell lysis can be quantitated by the use of vital dyes, by measuring the adherence of tumor cells to culture plates, or by release of radioactive labels from tumor cells during lysis.

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When suitable allowance is made for various serum factors, these *in-vitro* assays of tumor-cell killing correlate with the *in-vivo* clinical status and prognosis of patients with cancer.³ Such assays, generally referred to as "cell-mediated cytotoxicity assays," require direct leukocyte-tumor-cell interaction, do not require complement or circulating antibody, and are thought to be mediated by proteins secreted into the tumor from sensitized leukocytes.⁴

Tumor cells are widely disseminated at the time of surgical resection in spite of acceptable surgical techniques.^{5,6} Hence, the ultimate survival of the patient who has cancer must depend on his or her ability to destroy these cells before they become established metastases. However, postoperative patients have reduced immunologic defenses,^{7,8} including responses to membrane antigens associated with tumor immunity.^{9,12} It is not yet known what role anesthetic agents play in this immunosuppression, although we previously reported *in-vitro* inhibition of cell-mediated cytotoxicity by clinical concentrations of halothane and nitrous oxide.¹³ Since anesthesia is often induced by a barbiturate, we have examined the *in-vitro* effect of thiopental on tumor-cell killing by sensitized mouse leukocytes. We found that thiopental, in concentrations achieved systemically in clinical practice, significantly impairs cell-mediated cytotoxicity.

Methods and Materials

To assess cytotoxicity, we utilized the YAAC-1 mouse tumor and a method modified from that described by Berke.¹⁴ The details of the assay are reported elsewhere.¹³ In brief, YAAC-1 ascitic tumor (target) cells were carried in syngeneic A/JAX white mice and obtained for use by peritoneal lavage. Because these mice have no resistance to this tumor, being killed in 10-14 days after its intraperitoneal injection, they serve as a source of pure tumor cells. These target cells were washed, counted, suspended in essential medium (RPMI-1640 supplemented with penicillin and streptomycin), and incubated with chromium-51 for an hour. After labelling, the target cells were washed, examined for viability by trypan blue exclusion, and resuspended in essential medium.

Sensitized leukocytes were obtained by peritoneal lavage from allogeneic C57/black-6 mice ten days after intraperitoneal injection of the YAAC-1 tumor. These mice normally reject the tumor, and by ten days only macrophages and lymphocytes specifically sensitized to the tumor remain in the peritoneal cavity. These immune leukocytes function as effector or killer cells (PEC) in the assay.

TABLE 1. Effect of Thiopental on Cell-mediated Cytotoxicity (Means \pm SE)

Thiopental Concentration	Number of Studies	Per Cent Cytotoxicity		Per Cent Inhibition	P
		Air	Thiopental		
2.8×10^{-5} M	11	36.2 ± 4.8	34.5 ± 5.1	8.6 ± 5.1	N.S.
5.7×10^{-5} M	14	36.8 ± 4.0	29.4 ± 4.7	25.5 ± 6.8	≤ 0.01
8.5×10^{-5} M	9	34.6 ± 5.8	23.8 ± 6.5	38.1 ± 9.1	≤ 0.01

TABLE 2. Inhibition of Cell-mediated Cytotoxicity Related to Duration of Exposure to Thiopental, 5.7×10^{-5} M, Mean Values (\pm SE)

Duration of Exposure	Number of Studies	Per Cent Cytotoxicity		Per Cent Inhibition	P
		Air	Anesthetic		
1 hour	6	47.1 (± 9.2)	45.4 (± 9.8)	5.7 (± 7.1)	< 0.5
2 hours	6	47.1 (± 9.2)	42.3 (± 9.3)	11.6 (± 4.2)	< 0.05
3 hours	6	47.1 (± 9.2)	43.3 (± 10.3)	11.2 (± 5.1)	< 0.10
4 hours	6	47.1 (± 9.2)	40.7 (± 9.0)	13.3 (± 7.5)	< 0.05

The cells were washed, counted, and resuspended in essential medium. PEC and labelled target cells were then mixed in 35×10 -mm petri dishes at a ratio of 100:1. Heat-inactivated fetal calf serum (1 per cent) was added to sustain cell viability. Control dishes of labelled target cells in medium alone (without PEC) were also prepared to determine spontaneous release of ^{51}Cr , while maximum possible release of chromium was measured by osmotic lysis of tumor cells in distilled water.

All dishes were incubated for four hours in humidified 5 per cent CO_2 and air at 37 C. Following incubation, the medium was aspirated, the dishes washed with phosphate-buffered saline solution, and the cells removed by centrifugation. Target-cell lysis was determined by counting the amount of ^{51}Cr that had been released into the supernatant. Results were expressed as percentages of tumor cells killed, or percentage cytotoxicity, and were calculated as follows:

Per cent cytotoxicity

$$= \left[\frac{\text{CPM}_{\text{PEC+YAAC}} - \text{CPM}_{\text{YAAC}}}{\text{CPM}_{\text{Max}} - \text{CPM}_{\text{YAAC}}} \right] \times 100$$

where: CPM = counts per minute; YAAC = cultures containing target cells alone; PEC + YAAC = cultures containing both PEC and target cells; CPM_{max} = maximum ^{51}Cr released when target cells were lysed in distilled water.

To examine the effect of thiopental, a stock solution of 2.5 per cent sodium thiopental \S was diluted in RPMI-1640 and added to the cultures of PEC before mixture with target cells. Control cultures for determining spontaneous and maximum ^{51}Cr release were treated similarly. Cultures containing thiopental were compared with similar cultures containing cells from the same animal but treated only with an equal volume of RPMI. All experiments were paired and performed in triplicate. The effect of thiopental was measured at 2.8×10^{-5} M, 5.7×10^{-5} M, and 8.5×10^{-5} M.

Percentage inhibition of cytotoxicity resulting from exposure to thiopental was calculated as:

Per cent inhibition in anesthetic

$$= \left[\frac{\text{per cent cytotoxicity in air} - \text{per cent cytotoxicity in anesthetic}}{\text{per cent cytotoxicity in air}} \right] \times 100$$

To determine whether the effect of thiopental varied with duration of exposure, cultures were prepared as described, but the thiopental was added after 0, 1, 2 or 3 hours of incubation. The total incubation period remained constant at four hours. Reversibility of the phenomenon was assessed by preincubating PEC in thiopental for two hours, after which the cells were washed twice, mixed with labelled target cells, and incubated for four hours in air as described above.

The combined effect of thiopental and halothane was measured in a separate set of experiments by incubating paired dishes for four hours in air, halothane, 1 per cent, thiopental 5.7×10^{-5} M, or a combination of halothane and thiopental. Halothane was delivered to the chambers from a calibrated vaporizer and the concentration in the incubator continuously monitored by a Beckman LB II infrared gas analyzer. PEC were pre-equilibrated in the anesthetic under study for 30 minutes before addition of the target cells in order to assure the presence of halothane at the time of cellular interaction.

Because stock solutions of sodium thiopental contain 6 per cent sodium carbonate buffer, an additional set of experiments was performed to compare cytotoxicity in a sodium carbonate-buffered thiopental solution with that in an equal concentration of sodium carbonate alone. No change in pH resulted from addition of either test solution to the essential medium. Viability of effector cells in thiopental was examined by their ability to exclude trypan blue dye.

All data were analyzed by Student's t test for paired data.

\S Pentothal, Abbott Laboratories.

Results

Thiopental, in concentrations found in blood after induction of anesthesia, reversibly inhibited cell-mediated cytotoxicity in a dose- and time-related manner. The inhibition was additive to that produced by halothane.

The lytic activity of PEC in air varied considerably from day to day due to differences in macrophage content of the ascitic fluid and to differences in immunologic responsiveness among mice. The quantitative influence of the anesthetic also varied with the adequacy of the animal's tumor immunity. Thiopental inhibition of tumor-cell killing was greatest when the lytic activity of PEC in control cultures was less than maximal. Hence, analysis of the effect of thiopental in each experiment is valid only when the result is compared with the paired control without anesthetic, and quantitative comparisons among different groups of experiments cannot be made.

Inhibitions of cytotoxicity following four-hour exposure to thiopental ranged from 8.6 per cent at 2.8×10^{-5} M to 38.1 per cent at 8.5×10^{-5} M and were statistically significant at all concentrations above 2.8×10^{-5} M (table 1). Thiopental inhibition was also related to duration of exposure (table 2), although the extent of inhibition was of only marginal significance following two and three hours of exposure. Neither the spontaneous release nor the maximum release of ^{51}Cr from tumor cells was affected by thiopental. There was no alteration in culture pH or PEC viability with the concentrations of thiopental used, and the effectiveness of tumor-cell killing was not modified by sodium carbonate alone. Hence, it would appear that thiopental inhibits target-cell killing by an effect on immune PEC rather than by modification of tumor-cell integrity or by production of artificial changes in the culture medium. The effect of thiopental was reversible, as a two-hour exposure of PEC to thiopental prior to mixture with target cells was not associated with significant inhibition of tumor-cell killing (table 3).

Halothane and thiopental had additive inhibitory effects on cell-mediated cytotoxicity (table 4). Thiopental, 5.7×10^{-5} M, inhibited cytotoxicity to an ex-

TABLE 4. Effects of Halothane and Thiopental on Cell-Mediated Cytotoxicity (Means \pm SE)

	Number of Studies	Per Cent Cytotoxicity		Per Cent Inhibition	P
		Air	Anesthetic		
Thiopental, 5.7×10^{-5} M	10	41.7 \pm 3.1	33.9 \pm 4.3	20.4 \pm 7.6	≤ 0.01
Halothane, 1 per cent	10	41.7 \pm 3.1	32.8 \pm 3.6	20.5 \pm 7.1	≤ 0.05
Thiopental, 5.7×10^{-5} M, plus halothane, 1 per cent	10	41.7 \pm 3.1	23.3 \pm 3.7	44.5* \pm 7.6	≤ 0.001

* Also significantly different from response with each anesthetic alone ($P \leq 0.001$).

tent equivalent to that caused by 1 per cent halothane, and, when used in combination, their effects were summated.

Discussion

Inhibition of cell-mediated cytotoxicity by anesthetics has been demonstrated *in vitro* using local anesthetics,¹⁵ volatile and gaseous agents,¹³ and now thiopental. Drug effects alone may not, however, account for all the impairment of cytotoxicity seen postoperatively in patients,^{9,16} as the inhibitory effects of the neurohumoral stress response to anesthesia and surgery may also be contributing factors. Furthermore, while inhibition of tumor-directed responses by single anesthetics may not be clinically important, the summated effects of the multiple anesthetics so often used in modern anesthesia may be very significant. While extrapolation of animal data to the human situation must be done with caution, the recent demonstration of reduced cytotoxicity during anesthesia for minor surgery¹² suggests that anesthesia may in fact affect human resistance to cancer.

The phenomenon of cell-mediated cytotoxicity begins with the intimate contact of tumor cells with sensitized leukocytes and terminates with an alteration of the target-cell membrane predisposing to osmotic lysis.³ The interaction of target and effector cells occurs within the first few minutes of culture and destines target cells to ultimate death. The fatal blow is dealt by means of proteins synthesized in the effector leukocyte and secreted into the target cell by way of microtubules within the effector cell and microfilaments between the cells. Hence, to lyse a tumor cell, the effector cell must be viable, be capable of achieving intimate contact with the tumor cell, and possess an active mechanism for protein synthesis and secretion.

It is possible that anesthetic drugs may interfere with several steps in the reaction sequence. Since effector-cell viability and target-cell suscepti-

TABLE 3. Reversibility of Thiopental Effect on Cell-mediated Cytotoxicity (Means \pm SE)

Thiopental Concentration	Number of Studies	Per Cent Cytotoxicity		
		Air	Pre-exposure to Thiopental for Two Hours	Thiopental Added for Two Hours
5.7×10^{-5} M	4	44.5 (± 10.2)	45.4 (± 9.7)	34.1 (± 15.1)
8.5×10^{-5} M	4	36.5 (± 10.9)	37.8 (± 10.7)	33.4 (± 9.6)

bility to osmotic lysis were not affected in our model, the inhibition of cell-mediated cytotoxicity must be explained by reduced cellular interaction or by impaired protein synthesis and secretion. While barbiturates¹⁹ and halothane²⁰ are capable of impairing cellular motility *in vitro*, it is doubtful that inhibition results from reduced cellular movement and membrane contact. Inhibition occurred despite the fact that thiopental was added to some cultures after one or two hours of incubation, after the time when direct cell interaction is thought to occur. For similar reasons, we do not feel that physical alterations in the membranes, such as are known to be produced by halothane,²¹ are "masking" antigenic sites, for inhibition by thiopental was not confined to the critical time during the initial few minutes of culture when recognition and interaction occur.

More likely, the anesthetics affect the lytic stage of the reaction sequence, when synthesis and secretion of mediators lead to tumor destruction. Volatile anesthetics are capable of dose-related inhibition of microtubule and microfilament organization,²² both critical to protein production. Barbiturates are similarly capable of reducing protein synthesis and secretion,²³ although the mechanism involved is less clear. Alternatively, elevated levels of cyclic AMP can be induced by halothane,²⁴ and are capable of inhibiting cytotoxicity.³ The effect of thiopental on cyclic nucleotides has not been investigated.

The inhibition of cytotoxicity by thiopental, 5.7×10^{-5} M (equivalent to peak blood levels after induction doses of 4 mg/kg²⁵), and halothane, 1 per cent, is parallel to that shown previously by equianesthetic doses of halothane and nitrous oxide.¹³ We have also demonstrated that the effects of thiopental and halothane on the reaction are additive, although complete dose-response curves are necessary to establish such a relationship with certainty.

While a four-hour exposure to thiopental *in vitro* is of little relevance to clinical anesthesia, it is a useful example of an intravenous anesthetic maintaining a fixed level of "anesthesia" in culture. Since equal reductions in immune resistance to tumor cells can be demonstrated *in vitro* with approximately equianesthetic concentrations of anesthetic vapors (halothane), gases (nitrous oxide) and intravenous agents (thiopental), it is possible that enhanced tumor growth resulting from exposure to anesthetics could occur as a consequence of the state of anesthesia rather than as a function of a particular agent. Reduced anti-tumor activity of mouse leukocytes in the presence of agents with such varied physical and chemical properties but with similar anesthetic potentials suggests that exposure of immune leukocytes to anesthesia may be as important to enhanced tumor development as the properties of any individual anesthetic drug.

While many aspects of tumor immunity are still open to question,²⁶ there is little doubt that the immune system is intimately involved in the control of cancer. Failure of normal immune mechanisms has been associated with increased incidences of malignancy in animals and man, particularly with tumors of reticuloendothelial origin.²⁷ Barbiturates have been reported to enhance the development of spontaneous and induced tumors in experimental animals without possessing tumorigenic potential themselves, presumably by reducing host defenses.^{28,29} In view of the reduced tumor immunity reported to occur after clinical anesthesia in human subjects,^{9,12} and in view of our data showing anesthetic inhibition of cytotoxicity *in vitro*, we should seriously consider whether all anesthetics are potentially capable of enhancing the development and spread of malignancy in animals and man.

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