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Halothane Inhibition of Phytohemagglutinin-induced Transformation of Lymphocytes

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Phytohemagglutinin activation of human peripheral lymphocytes to synthesize DNA was inhibited in a dose-dependent manner by the presence of halothane in the cell culture. This occurred at concentrations of halothane approximating those found in arterial blood during clinical anesthesia. Tests of cell viability showed that these levels of halothane did not kill the cells in culture. The studies support, but do not prove, the hypothesis that halothane prevents resting cells from entering the cell cycle when signalled to do so. (Key words: Halothane; Lymphocytes; PHA; Cell cycle; Immune response.)

HALOTHANE has been reported to reduce significantly the killing of normal hematopoietic stem cells by the cytotoxic drug, arabinosylcytosine (ara-C).[1] Halothane also provides a similar but lesser degree of protection against the toxicity of vinblastine. Each of these chemotherapeutic agents exerts its principal effect in one portion of the cycle of cell division, ara-C during the DNA synthesis ("S") phase and vinblastine during mitosis ("M" phase). These drugs also affect processes in other phases of the cell cycle, but the main therapeutic effect of ara-C is inhibition of DNA synthesis, while vinblastine causes disruption of the mitotic spindle and metaphase arrest. These actions are demonstrable only in dividing cells. Halothane could spare normal stem cells by preventing them from entering active cycle where they would be vulnerable. This hypothesis is appealing since it might also explain some of the immunosuppressant effects which have been associated with inhalation anesthetic agents.[2] Confirmation of this postulated action of halothane would require a suitable model wherein cells could be diverted from a non-dividing, resting state into one of active cell division. Once such a model had been developed, halothane could be added to see whether entrance of the cells into cycle could be prevented.

One such cellular system has been the object of intense investigation in the past decade. Normal human peripheral-blood lymphocytes are small cells which do not divide when cultured *in vitro*. In the presence of a variety of chemical compounds known as mitogens, however, the lymphocytes enlarge and, subsequently, undergo mitosis. The addition of the mitogen, the commonest one in use being phytohemaggiutinin (PHA),[3] is the stimulus for this change in cellular activity. Neutrophils do not divide when exposed to this compound. Within two hours after the addition of PHA to a culture of human lymphocytes

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TABLE 1. Halothane's Effect on the Incorporation of Tritiated Thymidine by Human Lymphocytes

Halothane in Medium (mg/100 ml)	DPM/10^6 Mononuclear Cells				Inhibition: $\frac{C-E}{C} \times 100$ (Per Cent)	P
	Control		Experimental			
	Mean	SE	Mean	SE		
3.2	39,049	5,810	31,207	7,422	20	NS
9.2	49,716	2,449	31,428	8,251	37	<0.05
11.4	105,266	9,440	56,630	10,420	47	<0.01
11.6	75,002	11,224	31,926	7,926	57	<0.01
13.0	117,042	5,936	87,040	11,423	25	<0.02
18.1	89,712	4,113	28,104	9,215	69	<0.001
23.0	105,435	2,741	13,520	1,477	87	<0.001

there is active RNA[4,5] and protein[6] synthesis. This is followed in about 22 hours by the onset of DNA synthesis, which reaches a peak at 72 hours.[4] We elected to use this model to test the hypothesis that halothane prevents cells from entering active cycle following a stimulus which would normally cause them to do so.

Materials and Methods

Whole blood, 200 ml, was obtained from each of a number of informed volunteers. It was required that the donors be healthy, have taken no medications recently, and have had nothing to eat for eight hours prior to donation. No donor had recently been exposed to anesthetic agents. All procedures were done aseptically. Blood was withdrawn from an antecubital vein into four 50-ml syringes, then transferred to Erlenmeyer flasks containing acid-washed glass beads. The flasks were swirled gently by hand until the clicking of the glass beads was no longer audible; then this motion was continued for an additional 5 minutes. This resulted in complete defibrination of the blood, which was then transferred to large capped tubes, 30 ml blood per tube. To each tube were then added 3 ml of 4 per cent dextran (mol wt 200,000) in 0.9 N saline solution. The tubes were placed in an almost horizontal position for 50 minutes, during which time the leukocyte-rich serum separated, and then placed upright for an additional 5 minutes. Serum from the top of the tubes was removed with Pasteur pipettes, and a sample examined in a hemocytometer for total leukocyte count and differential. Examining unstained cells by phase microscopy, lymphocytes could be differentiated from monocytes and comprised 60 to 80 per cent of the population of leukocytes. Depending on the cell count obtained, the leukocyte-rich serum was diluted with cell-free autologous serum to produce a resultant suspension containing 10^6 lymphocytes per ml. One ml of this final suspension was added to 2 ml of minimum essential Eagle's medium containing glutamine, penicillin and streptomycin, in a 13 × 100-mm screw-capped glass tube. To each tube was added 0.05 ml PHA-M (Difco), all experiments using PHA from the same lot. Immediately after this, each tube was gassed for 10 seconds with a stream of 5 per cent CO_2–95 per cent O_2, with or without halothane, and sealed by closing its cap. Halothane was delivered by directing the CO_2–O_2 flow through a Fluomatic vaporizer and a Millipore filter to remove all bacteria, then into the tubes via a sterile Pasteur pipette. The tubes were placed in airtight sealed boxes and the boxes were purged with either CO_2–O_2 or CO_2–O_2–halothane in a concentration identical to that placed in each tube. Sequential sampling of the cultures showed equilibration of the liquid phase to occur within 15 minutes. In seven studies, the cells were then incubated at 37 C for 72 hours. Three additional experiments followed the above schedule except that the cells were incubated for 96 hours after PHA.

At the end of the incubation period following the introduction of PHA into the culture tubes, 2 μCi tritiated thymidine (^{3}HT) (5.0 Ci/mM) were added to each culture. The tubes were re-gassed, capped, and reincubated

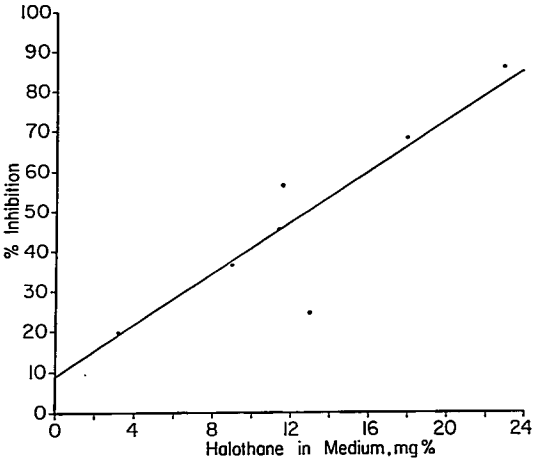

FIG. 1. Plot of halothane inhibition of DNA synthesis 72 hours after addition of PHA to cultures containing 10^6 lymphocytes. Each point represents calculated inhibition of the mean response of 25 cultures with a measured concentration of halothane in their medium *vs.* 25 cultures without halothane, all 50 cultures prepared from blood of same donor on the day of PHA addition. Calculated least-squares regression line is shown.

in the steel box, which was sealed and purged once again with the gas mixture appropriate for the tubes it contained. Three hours later, the cultures were harvested. The contents of each tube were poured through glass-fiber filters held in a multichannel suction device. The filters were then rinsed with two washes of saline solution, 5 ml each, followed by two 5-ml rinses with trichloroacetic acid. Finally, after two 5-ml washes with methanol, the filters were aspirated until nearly dry and removed to dry completely under a heat lamp. Following this, each filter was put in a scintillation vial, a scintillation "cocktail" of toluene (1,000 ml toluene, 4.0 g PPO, 0.25 g POPOP) added, and each vial counted for 10 minutes in a Nuclear Chicago Mark I scintillation counter. Samples were corrected for quench by the channels-ratio method, using a ^{133}Ba external standard. Counting efficiency ranged from 35 to 37 per cent and results were expressed as disintegrations per minute (dpm).

At the time of addition of the ^3HT, three tubes were removed from the halothane-exposed group and liquid samples from each tube were injected into a gas chromatograph. By comparing the areas under the halothane peaks with those produced by liquid standards, we obtained an estimate of the halothane concentration in each sample. Reproducibility of these measurements was ±15 per cent among replicate samples.

Twenty-five tubes were used for radioactivity measurements in both the control and halothane-treated groups in each experiment, utilizing only the blood cells obtained from a single donor. Thus, each experiment was an independent, internally controlled measure of halothane effect on the PHA–lymphocyte response. Results were expressed as the per cent inhibition of this response by halothane and analyzed for statistical significance by an unpaired *t* test between control and halothane values for each experiment. A least-squares regression line was computed and the linearity of regression calculated by analysis of variance.[7] Tests of cell viability followed the method of Stewart and Ingram,[8] in which nuclei of viable cells are counted in a Coulter Counter following digestion of dead cells. Counts of viable nuclei were made from cells maintained in the presence and absence of PHA, both in the presence and in the absence of 31 mg/100 ml halothane in the medium, after 24, 48, and 72 hours of treatment.

Results

Cultures to which no PHA had been added had dpm values of 800–900, while the counts for cells exposed to PHA but not to halothane ranged from 39,000 to 117,000 dpm. The data for seven experiments in which ^{3}HT incorporation was assessed 72 hours following addition of PHA to the cultures are presented in table 1. Since it may be possible that an occasional monocyte was interpreted as being a large lymphocyte, the results are given as dpm/10^6 mononuclear cells, although we feel confident that virtually all these cells were lymphocytes. These data are illustrated in figure 1, in which each experiment is plotted as a single point which represents the per cent inhibition by halothane of the DNA synthesis in the corresponding control culture. The difference between halothane-treated and non-halothane-treated cells was significant at the 5 per cent level or better, except at 3.2 mg/100 ml halothane. The calculated least-squares regression line is shown in the figure. An analysis of variance was done to determine whether the regression was significantly linear, and the result indicated that the probability is greater than 95 per cent that the relationship of halothane concentration in the medium to per cent inhibition of PHA response is linear and not a more complex type of dose–response curve.

Halothane could inhibit the lymphocytic response to PHA by killing the cells. This possibility was investigated in two experiments to assess cell viability. They showed conclusively that halothane did *not* kill cells when present in a concentration of 31 mg/100 ml, a greater concentration than in any of the studies being reported. Halothane did not appear simply to delay the response to PHA, since the three experiments at 96 hours gave results falling very near the regression line established in 72-hour experiments.

Discussion

The bone marrow is regarded as a three-compartment tissue.[9] The first compartment contains stem cells, which have extensive proliferative capacity, but under normal circumstances only a few of these cells are "in cycle." The second compartment is comprised of early differentiated cells which are mostly in cycle, dividing, and giving rise to the third compartment of non-dividing descendants such as erythrocytes and polymorphonuclear neutrophils. Arabinosylcytosine and vinblastine kill cells in the second compartment, causing a depletion of their descendants and signalling the stem cells, somehow, to enter cycle and replenish the early differentiated cell population.

Another example of induction of resting cells into cycle is the antigen-stimulated proliferation of immunologically competent lymphocytes, a fundamental event of the immune response. These cells normally are not in cycle and not dividing, but upon stimulation by an antigen to which they have previously been sensitized they enter the cell cycle and begin the metabolic sequence of events leading to cell division. This subject has been discussed in more detail,[2] in a review of evidence concerning the inhibitory effect of anesthesia on the immune response. It is generally believed that antigenic activation of lymphocytes closely resembles the response of these cells to PHA.

Thus, we have three situations in which the presence of halothane is inhibitory. In the first, toxicity of cycle-specific drugs to marrow stem cells is decreased. This could be the result of a failure of these cells to respond to a signal to enter cycle and divide, thereby providing replacement for early differentiated, dividing cells which had been killed by the drugs. The second situation is the inhibition of the immune response, which also could result if sensitized cells failed to enter cycle upon stimulation by antigen. The third comprises the substance of this study. A known stimulus, PHA, was prevented by halothane from causing a population of non-dividing cells to synthesize DNA preparatory to mitosis. These experiments did not clarify how this happened. DNA synthesis could have been directly inhibited, the transport of tritiated thymidine into the cell nucleus could have been blocked, or the cells could have been prevented from reaching the DNA-synthesizing phase of the cell cycle. This last possibility is, if unproven, at least consistent with other observed cellular effects of anesthetics.

There is no proof yet that halothane prevents the entrance of cells into cycle. Veri-

fication of the hypothesis would require demonstration of halothane inhibition of cycle events earlier than DNA synthesis, such as RNA and protein synthesis. Such studies will be done with the PHA–lymphocyte system in the hope that information about halothane's action which is basic to the understanding of anesthetic effects on other dividing cell populations can be developed.

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Obstetrics

FETAL ACID-BASE STATUS AT HIGH ALTITUDE The arterial oxygen tensions and acid–base statuses of mothers and fetuses were determined in a group residing at an altitude of 150 meters (Lima, Peru) and in another group at an elevation of 4,200 meters (Cerro de Pasco, Peru). Mean Pa_{O_2} in the mothers in labor at 150 meters was 91 mm Hg; at high altitude, 60 mm Hg. Corresponding P_{CO_2}'s were 32 mm Hg and 24 mm Hg. Blood sampled from the fetal scalp during labor had a mean P_{CO_2} of 21 mm Hg at 150 meters and a mean of 19 mm Hg at high altitude (not a significant difference). The fetus at high altitude is born with respiratory alkalosis and metabolic acidosis, in contrast to the fetus at 150 meters, which has both respiratory and metabolic acidosis. The low fetal P_{CO_2} correlates well with the decreased maternal P_{CO_2} at high altitude because of the additive effects of hyperventilation and pregnancy. Despite the low maternal P_{O_2} at high altitude, there was no difference between fetal P_{O_2}'s in highlanders and lowlanders. Apgar scores were good in both groups. Fetal base excess was lower in the high-altitude infants, presumably because of maternal hyperventilation and hypocapnia. (*Sobrevilla, L. A., and others: Human Fetal and Maternal Oxygen Tension and Acid–Base Status during Delivery at High Altitude, Amer. J. Ob. Gyn. 111:1111–1118, 1971.*)