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The Effect of Halothane on Phytohemagglutinin-induced Transformation of Human Lymphocytes in Vitro

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The authors determined the effect of halothane on phytohemagglutinin-induced transformation of human lymphocytes *in vitro*, measured in terms of incorporation of tritiated thymidine. After 57 hours of exposure, 0.5 per cent halothane had not inhibited transformation, 1 per cent halothane had caused 16 per cent inhibition, and 2 per cent halothane had caused 43 per cent inhibition. When lymphocytes were incubated in 2 per cent halothane for 24, 48, 72, 96, and 120 hours, inhibition was evident within 48 hours and increased progressively with time. These data suggest, but do not prove, that halothane interferes with DNA synthesis. Cell viability studies confirmed that these effects of halothane were not the result of cell death. Inhibition of PHA-induced lymphocyte transformation *in vitro* is correlated with depressed cell-mediated immunity *in vivo*. Immunosuppressant effects of halothane could play a role in postoperative infections, in the metastatic spread of cancer during operation, and in organ transplant immunology. (Key words: Halothane; Lymphocyte transformation; Phytohemagglutinin; Tritiated thymidine; DNA synthesis; Immune response.)

A RECENT REVIEW [1] summarized evidence that anesthetics can interfere with several phases of the immune response. They may cause bone marrow depression, inhibit phagocytosis, inhibit macrophage mobility, decrease antibody production, and decrease host resistance to infection and malignancy. There have been no reports of the effects of anesthetic agents on transformation of lymphocytes.

Lymphocyte transformation [2] is an intermediate step in cell-mediated immunity and results from the interaction of resting sensitized lymphocytes with specific antigen *in vivo*. Transformation may also be produced *in vitro* when lymphocytes are stimulated by specific antigen or a nonspecific mitogen such as phytohemagglutinin (PHA).[3] Resting lymphocytes stimulated with antigen or PHA enlarge, actively synthesize DNA, RNA, and protein, and subsequently divide. Diminished lymphocyte transformation *in vitro* has been correlated with depressed immunity *in vivo* and has been observed after operation,[4, 5] in certain disease state,[2] in the elderly,[6] and in patients taking corticosteroids,[7] barbiturates,[8] salicylates,[9] and immunosuppressants.[10] This study was undertaken to determine the effects of halothane on lymphocyte transformation *in vitro* so that inhibitory factors *in vivo*, such as elevated cortisol levels, could be excluded.

Methods

Whole blood from seven healthy volunteers was collected in preservative-free heparin. Carbonyl iron (300 mg) and 15 ml of 1 per cent aqueous methylcellulose were added to each 35 ml of blood and phagocytosis of iron by granulocytes was allowed to proceed at 37 C for 45 minutes. The mixture was then transferred to siliconized glass tubes and the erythrocytes and iron-laden polymorphonuclear cells were allowed to settle at 37 C for an additional 45 minutes. Lymphocytes in the methylcellulose–plasma layer were then aspirated, pooled, collected by centrifugation, and washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium [11] for removal of residual iron particles. A final cell suspension of 0.5×10^6 lymphocytes per ml in 10 per cent pooled normal human AB serum and RPMI 1640 medium (including 100 units

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TABLE 1. Effect of Halothane on Lymphocyte Incorporation of ^{3}H-thymidine after 57 Hours of Exposure*

	Halothane Concentration	PHA-P (μg Protein/ml)					
		0	3.125	6.25	12.5	25	50
Donor 1	Control	5.8 ± 0.7	166.5 ± 3.7	199.4 ± 3.4	202.3 ± 3.0	195.5 ± 4.3	144.0 ± 4.6
	0.5 per cent	6.2 ± 0.4	157.4 ± 1.8	185.7 ± 5.2	198.3 ± 2.9	219.2 ± 11.2	172.0 ± 4.9†
Donor 2	Control	3.3 ± 0.5	156.8 ± 9.9	206.8 ± 2.5	205.2 ± 4.6	175.2 ± 11.6	142.2 ± 3.8
	0.5 per cent	2.7 ± 0.2	133.4 ± 2.9	218.1 ± 4.2	207.8 ± 2.6	198.3 ± 3.3	162.3 ± 4.5†
Donor 3	Control	4.6 ± 0.3	134.9 ± 3.1	160.5 ± 3.6	157.5 ± 6.2	144.7 ± 5.5	119.2 ± 3.6
	1.0 per cent	3.9 ± 0.6	126.4 ± 6.0	138.4 ± 3.7†	135.0 ± 2.1†	124.6 ± 3.8	93.9 ± 8.0
Donor 4	Control	5.3 ± 0.6	198.1 ± 1.9	223.5 ± 3.6	206.1 ± 2.6	191.4 ± 3.8	179.8 ± 2.7
	1.0 per cent	5.7 ± 0.6	162.4 ± 2.9†	179.0 ± 3.5†	172.5 ± 3.0†	161.5 ± 4.1†	145.5 ± 3.7†
Donor 5	Control	5.6 ± 0.7	136.1 ± 4.1	157.3 ± 2.4	159.6 ± 6.5	150.2 ± 7.4	141.5 ± 2.9
	2.0 per cent	3.8 ± 0.4	69.4 ± 1.6†	93.9 ± 2.8†	94.7 ± 1.2†	88.7 ± 1.3†	76.9 ± 1.8†
Donor 6	Control	3.7 ± 0.4	165.1 ± 4.0	211.3 ± 2.3	218.7 ± 1.7	215.8 ± 2.3	180.5 ± 2.4
	2.0 per cent	4.2 ± 0.3	85.7 ± 2.2†	152.7 ± 2.4†	160.7 ± 2.2†	151.1 ± 2.3†	126.0 ± 2.6†

* Data expressed as dpm (disintegrations per minute) × 10^3 ± SE.
† Significantly different from control ($P < 0.01$).

TABLE 2. Effect of Halothane on Lymphocyte Incorporation of ^{3}H-thymidine after 96 Hours of Exposure*

	Halothane Concentration	PHA-P (μg Protein/ml)					
		0	3.125	6.25	12.5	25	50
Donor 1	Control	3.9 ± 0.2	123.2 ± 1.8	147.5 ± 8.0	154.2 ± 6.4	156.5 ± 7.3	152.8 ± 3.6
	0.5 per cent	4.5 ± 0.6	113.1 ± 5.6	135.1 ± 7.4	148.5 ± 3.6	161.8 ± 13.2	148.9 ± 4.6
Donor 2	Control	5.1 ± 1.0	96.9 ± 3.9	146.1 ± 3.7	159.2 ± 6.2	173.6 ± 5.7	158.7 ± 5.1
	0.5 per cent	3.2 ± 0.6	103.6 ± 3.9	144.9 ± 6.6	142.0 ± 7.2	144.8 ± 6.0†	142.5 ± 5.4
Donor 3	Control	4.6 ± 0.8	191.0 ± 8.9	186.6 ± 11.3	190.3 ± 12.3	167.6 ± 9.4	165.4 ± 4.5
	1.0 per cent	5.4 ± 0.8	166.1 ± 6.4	144.7 ± 7.5	139.3 ± 3.0†	130.9 ± 5.5	106.3 ± 2.1†
Donor 4	Control	7.1 ± 0.6	195.9 ± 4.9	193.0 ± 8.0	191.5 ± 0.5	183.1 ± 2.9	168.5 ± 6.0
	1.0 per cent	3.6 ± 0.4†	165.5 ± 4.6†	164.1 ± 2.6	152.2 ± 2.6†	135.4 ± 2.4†	110.8 ± 4.6†
Donor 5	Control	3.7 ± 0.3	179.4 ± 2.3	185.1 ± 3.0	177.9 ± 2.0	163.9 ± 3.0	140.1 ± 3.3
	2.0 per cent	3.4 ± 0.6	112.8 ± 2.7†	151.6 ± 1.8†	133.8 ± 3.6†	110.6 ± 4.9†	94.3 ± 2.5†
Donor 6	Control	2.7 ± 0.5	96.9 ± 5.5	123.8 ± 4.5	149.6 ± 4.3	147.8 ± 3.3	139.0 ± 6.9
	2.0 per cent	2.7 ± 0.3	77.3 ± 2.2†	112.2 ± 5.0	142.5 ± 4.6	132.5 ± 4.6	97.6 ± 5.2†

* Data expressed as dpm (disintegrations per minute) × 10^3 ± SE/ml culture.
† Significantly different from control ($P < 0.01$).

penicillin, 100 μg streptomycin, and 300 mg glutamine/ml) was prepared.

One-milliliter samples of the final cell suspension were placed in 5-ml loosely-capped polypropylene tubes. Phytohemagglutinin-P (Difco Laboratories, Detroit, Michigan), reconstituted in 0.14 M NaCl, was added to the cultures in doses of 3.125, 6.25, 12.5, 25, and

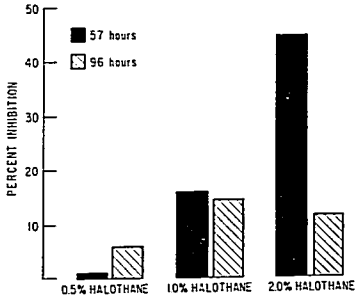

FIG. 1. Concentration-dependent inhibition of lymphocyte transformation by halothane. Per cent inhibition was determined at the dose of PHA inducing maximum incorporation of ^{3}H-thymidine into control cells, and was calculated by dividing the difference between dpm values in corresponding control and halothane-treated cells by the dpm for controls.

50 μg/ml (dose expressed as weight PHA protein). Six tubes were prepared for each concentration of PHA, as well as for controls without PHA. Lymphocyte counts were performed in cetrimide diluent with a Model B Coulter counter. Cell viability studies were performed using the pronase-cetrimide system described by Stewart and Ingram.[12]

Lymphocytes were cultured in two separate identical incubators at 37 C in an atmosphere of humidified 5 per cent carbon dioxide (confirmed by infrared analysis) in air. Halothane vapor was delivered to one incubator and the concentrations within the chamber were measured by infrared analysis and gas chromatography. Cells from Donors 1 through 6 were exposed to 0.5, 1.0, or 2.0 per cent halothane and cultures were terminated after 57 or 96 hours of incubation by quick freezing in liquid nitrogen. Cells from Donor 7 were incubated in 2 per cent halothane and cultures were terminated after 24, 48, 72, 96, and 120 hours.

Transformation was quantitated in terms of DNA synthesis, as determined by the incorporation of tritiated (^{3}H) thymidine in a three-hour labelling period. ^{3}H-thymidine (300 mCi/mM), 30 μCi, was added to each culture. Nuclear protein was precipitated with cold 5 per cent trichloroacetic acid (TCA). The precipitate was washed once in 5 per cent TCA, again in 0.14 M NaCl, and 0.5 ml of Soluene (Packard) was then added to dissolve the precipitate. After 12 hours the Soluene was neutralized by addition of 20 μl of glacial acetic acid and the mixture was transferred to scintillation-counting vials by three washes with 5-ml volumes of toluene–Liquiflor scintillation fluid. Radioactivity was measured in a model 3003 Packard Tricarb scintillation counter, with results corrected for quench (using an external standard) and expressed as disintegrations per minute (dpm).

The time needed for equilibration of halothane in the ambient atmosphere and in the culture suspension was also determined. Tubes containing RPMI 1640 medium and 10 per cent serum were exposed to 1 per cent halothane for 72 hours. At hourly intervals for four hours, and at various times thereafter, tubes were removed and the medium analyzed for halothane content by gas chromatography.

Data were analyzed by the unpaired t test. Values were considered significant if $P < 0.01$.

Results

Lymphocytes exposed to 0.5 per cent halothane (Donors 1 and 2) responded to PHA stimulation in the same way as cells not exposed to halothane. In general, there was no significant difference between incorporation of ^{3}H-thymidine into treated cells and controls at any dose of PHA during either incubation period. Significant differences between the control and halothane-treated cultures occurred only with 50 μg PHA after 57 hours (table 1) and with 25 μg PHA after 96 hours (table 2).

One per cent halothane significantly inhibited transformation of lymphocytes (Donors

TABLE 3. Time-related Effects of 2 Per Cent Halothane on Lymphocyte Incorporation of ^{3}H-thymidine*

Time (Hours)	Control (dpm × 10^2)	2 Per Cent Halothane (dpm × 10^2)	Per Cent Inhibition
24	9.7 ± 0.6	11.8 ± 0.8	—
48	127.5 ± 1.9	106.3 ± 2.9†	16.6
72	194.2 ± 4.2	126.9 ± 2.9†	34.7
96	166.7 ± 12.5	112.0 ± 4.5†	32.7
120	131.4 ± 0.6	56.9 ± 7.2†	56.8

* Determined at that dose of PHA inducing a maximum response in control cells.

† Significantly different from control ($P < 0.01$).

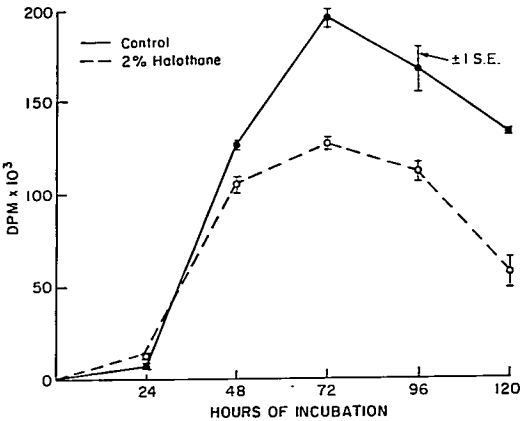

FIG. 2. Time-related effects of 2 per cent halothane on lymphocyte incorporation of ^{3}H-thymidine at the dose of PHA inducing a maximum response. Data refer to the response of lymphocytes from Donor 7 only.

3 and 4) after both 57 and 96 hours of exposure. After 57 hours inhibition averaged 16 per cent, and the differences from control were uniform regardless of dose of PHA. After 96 hours the decreases in responses to all doses of PHA averaged 23 per cent, with inhibition tending to be greater with the higher doses.

Inhibition of transformation was greatest with 2 per cent halothane (Donors 5 and 6). After 57 hours the mean decrease in response to PHA was 38 per cent, and after 96 hours it was 21 per cent. This inhibition did not appear to be a function of PHA concentration. After 57 hours the responses of 2 per cent halothane-treated cells were significantly different from control values with all doses of PHA.

When only the peak response to PHA (regardless of dose) was considered, inhibition of lymphocyte transformation was linearly related to halothane concentration (fig. 1). After 57 hours of exposure, 0.5 per cent halothane caused no significant inhibition of ^{3}H-thymidine incorporation, 1 per cent halothane caused 16 per cent inhibition, and 2 per cent halothane caused 43 per cent inhibition. After 96 hours, however, inhibition of transformation was less a function of halothane concentration. Inhibition increased from 6 per cent with 0.5 per cent halothane to 14 per cent with 1 per cent halothane, but no further increase resulted from exposure to 2 per cent halothane.

Inhibition of lymphocyte transformation was not secondary to cytocidal effects of halothane *in vitro*. There was no significant difference between cell viability counts when non-PHA-stimulated lymphocytes exposed to 2 per cent halothane were compared with controls. Lymphocytes given no PHA also had the same low level of thymidine incorporation whether exposed to halothane or not.

Results of the experiment with lymphocytes from Donor 7, examining the time-related effects of halothane, are shown in table 3 and figure 2. After 24 hours of exposure to 2 per cent halothane there was minimal incorporation of ^{3}H-thymidine by either halothane-exposed cells or controls. During the ensuing two, three, four, and five days, however, this halothane concentration caused a progressive decrease in transformation. At the peak stimulating dose of PHA, inhibition ranged from 17 per cent after 48 hours of exposure to 56 per cent after 120 hours. This increase in inhibition with time was not apparent, however, when lymphocytes from Donors 5 and 6 were exposed to 2 per cent halothane for 57 and 96 hours.

Any time-dependent inhibition by halothane could not result from progressive uptake of anesthetic by the cells and medium. Halo-

thane concentrations in medium exposed for 72 hours to 1 per cent ambient halothane rose to 4.5 mg/100 ml within an hour and stabilized at 6.09 mg/100 ml (±0.06 SE) after nine hours. Thus, equilibrium of halothane in the atmosphere and the liquid culture medium was attained early in the incubation period.

Discussion

Transformation of lymphocytes has been advocated as an *in-vitro* index of immunologic competence.[13, 14] Although transformation of lymphocytes in response to PHA stimulation resembles the response to specific antigens, these reactions differ. PHA stimulates 60–80 per cent of all lymphocytes to transform, whereas antigenic stimulation transforms fewer than 50 per cent of only those lymphocytes previously sensitized to the antigen. In addition, peak DNA synthesis occurs two to three days (57 hours in our laboratory) after PHA stimulation, as opposed to five to seven days after antigenic stimulation. Nevertheless, transformation in response to PHA is characteristic of normal lymphocytes, and inhibition of the response is correlated with certain types of deficiency in immune responses.[10, 15, 16]

In recent years various techniques for determining the effects of disease, drugs, etc., on PHA-induced lymphocyte transformation have been reported. All aspects of the technique used in this study, including cell concentration, serum source, serum concentration, incubation period, PHA dose range, label concentration, and labelling period, have been thoroughly studied, and the final method was selected for optimal sensitivity and reproducibility. By using a carbonyl iron–methylcellulose cell-purification technique we were able to obtain a final cell suspension containing at least 98 per cent mononuclear cells. We were thus able to minimize possible concomitant effects of granulocytes on PHA response and ^{3}H-thymidine incorporation.[17] Multiple doses of PHA were used for two reasons. First, PHA dose was quantitated in terms of protein content. The mitogenic activity relative to protein content is not known, and the ratio may vary from one batch of PHA to the next.[18, 19] Second, the doses of PHA capable of inducing maximum responses are not always the same for cells from different donors.[19–21] In order to compare the peak response of one group of cells with that of the next it was necessary to stimulate with a wide range of doses.

We conclude that halothane, at clinically useful concentrations, is an inhibitor of PHA-induced human lymphocyte transformation *in vitro*. After 57 hours of exposure this inhibition is linearly related to halothane concentration. At the maximum stimulating dose of PHA, 0.5 per cent halothane caused no significant inhibition of ^{3}H-thymidine incorporation, 1 per cent halothane caused 16 per cent inhibition, and 2 per cent halothane caused 43 per cent inhibition.

In general, the differences between responses of halothane-treated cells and controls did not vary as a function of PHA dose. With 0.5 per cent halothane, however, low doses of PHA stimulated halothane-treated cells and controls to respond similarly, but high doses (50 μg) of PHA caused halothane-treated cells to transform better than controls. The reason for this enhanced response to the high dose of PHA is not clear. PHA is thought to act by binding to specific receptor sites on the cell membrane,[22–25] and we may speculate that halothane interferes with this binding.

PHA-stimulated lymphocytes transform in a characteristic temporal fashion,[2, 26, 27] although they do not represent a synchronous cell population. In the first 72 hours following PHA stimulation, lymphocytes change from their normal resting state to enter a synthetic phase of activity. There is morphologic enlargement of small lymphocytes to large lymphoblasts, with a simultaneous considerable increase in synthesis of cellular protein, RNA, and DNA. In our laboratory a maximum wave of DNA synthesis is observed at 57 hours, but a high level of DNA synthesis may be maintained for as long as 72 hours.[28] After 72 hours the cells enter a proliferative phase. Large numbers of mitotic figures appear, the large lymphoblasts divide to form a new population of small lymphocytes, and a second wave of DNA synthesis may ensue.

By harvesting cultures after both 57 and 96 hours, we tried to separate halothane's effects

on the synthetic phase of the lymphocytic response to PHA from its effects on the proliferative phase. If the effects of halothane on cell division were limited solely to a colchicine-like inhibition of mitosis, as suggested by several reports,[29-32] we could have expected normal incorporation of ^{3}H-thymidine during the first 48–72 hours after PHA stimulation, but inhibition thereafter. Halothane inhibited incorporation of radioactive thymidine following both incubation periods, however, and therefore it must affect both phases of the response to PHA. This conclusion was reinforced when we incubated cells from Donor 7 in 2 per cent halothane for 24, 48, 72, 96, and 120 hours. Inhibition was observed at all times after 24 hours and, in this particular instance, increased progressively with time.

These data suggest, but do not prove, that halothane inhibits DNA synthesis. Although decreased incorporation of exogenous tritiated thymidine into nuclear protein may reflect inhibition of DNA synthesis, a similar decreased incorporation of label could result from halothane's interference with cell membrane and cytoplasmic thymidine-transport mechanisms.[31]

These *in-vitro* data provide evidence that clinical concentrations of halothane may have significant depressant effects on cell-mediated immunity. If this effect is also apparent *in vivo*, in the clinical setting, anesthetic-induced immunosuppression could play an important role in postoperative infections, in the metastatic spread of cancer due to "seeding" during operation, in organ transplant immunology, and in the development of allergic reactions during anesthesia. In addition, it is known that patients who receive immunosuppressants or have immune deficiency disease are more prone to develop lymphoid cancer.[13, 33] It may be that chronic exposure to the immunosuppressant effects of anesthetics accounts for the reported above-average incidence of such lymphoid malignancies in anesthesiologists.[34] Further investigation along these lines is warranted.

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Paraphernalia

POSITION OF CVP CATHETERS The position of a central venous catheter introduced percutaneously through an arm vein in each of 300 patients was determined radiologically. Sixty-two per cent of the catheter tips were in the subclavian vein, innominate vein or superior vena cava; 16 per cent were in the internal jugular vein, and 15 per cent were in the right atrium or ventricle. Two were found coiled in the subclavian vein. In no case was the incorrect position evident clinically.

There were no complications in this group of patients since the aberrant catheters were repositioned immediately. Potential complications of improper positioning include misleading pressure measurements, thrombophlebitis, perforation of the vein or cardiac chambers (atrium or ventricle), knotting or breaking of the catheter, and inadvertent rapid transfusion of cold blood or fluids with the possible occurrence of arrhythmias. (*Langston, C. S.: The Aberrant Central Venous Catheter and Its Complications, Radiology 100:55–59, 1971.*)