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Halothane Inhibition of RNA and Protein Synthesis of PHA-treated Human Lymphocytes

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Cultured human lymphocytes treated with phytohemagglutinin (PHA) had higher rates of RNA and protein synthesis, as judged by incorporation of the labelled precursors ^{3}H-uridine and ^{14}C-leucine, than did control cultures without PHA. These cultures were prepared from cells of the same donor, as were a third set of cultures which were equilibrated with 2 per cent halothane. The increased rates of RNA and protein synthesis six and 16 hours after PHA addition were inhibited by the halothane, modestly at six hours and strikingly at 16 hours. These experiments provide further evidence that halothane prevents recruitment of resting cells into the active cycle of cell division. (Key words: Anesthetics, volatile: halothane; Blood: lymphocytes; Metabolism: RNA synthesis.)

STUDIES OF THE EFFECT of halothane on the response of human lymphocytes to phytohemagglutinin (PHA) showed that this anesthetic produced a dose-dependent depression of DNA synthesis occurring three days later.[1,2] This inhibition was evidently not due to a decrease in the uptake of tritiated thymidine,[3] so our attention has turned to events earlier in the sequence of changes which follow addition of PHA. During the first 24 hours post-PHA, cultured lymphocytes exhibit an expansion of nuclear volume as part of the process of "blastic" transformation. Compared with controls, cultures equilibrated with 2 per cent halothane showed a statistically significant ($P < .001$) decrease in extent of nuclear enlargement at 24 hours.[4] This suggested that metabolic events such as RNA and protein synthesis, which accelerate soon after addition of PHA, might be inhibited by halothane during this time. The present experiments were done to investigate this possibility.

Methods

Lymphocytes were prepared from venous blood samples obtained from paid donors who were healthy, fasting dental students. They were taking no medication, had not recently been exposed to anesthetic agents, and had signed an informed consent form. All preparative steps were done with sterile supplies. Autologous plasma was prepared by centrifuging samples of heparinized blood (200 USP units of heparin in preservative-free saline solution per 50 ml blood). The remaining blood was used to prepare cells. Tubes of 55 ml capacity had been prepared previously by adding 12 ml of 4 per cent dextran (mol wt 250,000, Sigma) in 5 per cent dextrose in water, 300 mg dry, heat-sterilized carbonyl iron powder (General Aniline and Film Corp., Code 1-1-63763), and heparin as described. After adding 40 ml whole blood to this mixture, the tubes were capped and placed on a rotational mixer in an incubator maintained at 37 C. They were then rotated at 32–35 rpm for one hour to allow phagocytosis of the iron particles. Following this, the preparation was put into siliconized glass tubes and the cells sedimented for 45 to 60 min at 37 C. The leukocyte-rich plasma was pooled in plastic tubes to which a large magnet (75-lb capacity) was applied and the plasma was then decanted slowly into another tube. The latter was then centrifuged at 500 × *g* for 10 min at 2 C; then the supernatant was discarded. The pellet was resuspended in 10 ml of leucine-free minimum Eagle's medium (MEM, with 50,000 units penicillin G and 50 mg streptomycin per 100 ml), mixed thoroughly, the magnet was reapplied and the tube contents decanted. The cell count and differential of this suspension were determined by phase-contrast hemocytometry and the previously prepared autologous plasma used as a diluent to produce a resultant 10^6 lymphocytes per ml.

The cultures were prepared by mixing 17 ml of the cell suspension with 24 ml of leucine-free MEM containing penicillin G and streptomycin as described plus 50 mg glutamine per 100 ml MEM. Three-milliliter amounts of this mixture were placed in small, siliconized glass Petri dishes and covered loosely with larger

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Petri dishes. These dishes, each containing 10^6 cells, were divided randomly between two identical incubators. Each was humidified, kept at 37 C, and contained 5 per cent CO_2 by virtue of a low inflow of 100 per cent CO_2. In addition, one received a very low flow of 95 per cent O_2–5 per cent CO_2 which had passed through a Copper Kettle containing halothane. This additional input was adjusted empirically to maintain a halothane content of 2 per cent ± 0.1 per cent within the incubator. CO_2 and halothane contents were measured by gas chromatography and O_2 by a paramagnetic analyzer. All cultures were allowed to equilibrate overnight in their incubators, 20 in air and 10 in the halothane-containing incubator.

At 8:30 A.M. the next day, PHA was added to each culture in halothane and to 10 of the 20 in air. Reconstituted PHA-P (Difco) was given in a dose of 0.005 ml diluted in saline solution to 0.1 ml. After incubation for either 6 or 16 hours, 2 μCi ^{3}H-uridine (^{3}H-U) and 5 μCi ^{14}C-leucine (^{14}C-L) were added to each culture. These precursors of RNA and protein, respectively, were prepared as follows: uridine-5-^{3}H (5 Ci/mM, Amersham-Searle) and L-leucine-^{14}C(U) (280 mCi/mM, New England Nuclear) were combined and diluted with water to a final solution containing 2 μCi ^{3}H-U and 5 μCi ^{14}C-L per 0.2 ml.

After incubating one hour with the labelled compounds, each culture was harvested onto a glass fiber filter (Schleicher and Schuell, Grade 29) fitted tightly into a suction apparatus, and washed with cold (4 C) solutions. Multiple washes were done with saline solution, 5 per cent trichloroacetic acid (TCA) and methanol. The filters were then dried by gentle heating and placed in counting vials containing 0.5 ml of Soluene-100 (Packard) for one hour at 22 C. A scintillation "cocktail" containing toluene, PPO and POPOP was then added, and after cooling and dark-adapting for at least 3 hours in the scintillation counter, the isotope content of the acid-insoluble precipitate was measured by liquid scintillation counting.

The counter was a Packard Model 3385 Tri-Carb Liquid Scintillation Spectrometer equipped with automatic external standard, Teletype printout, and paper tape perforator. The preset channel selection buttons used were ^{3}H(^{14}C) for the red channel to count tritium in the presence of ^{14}carbon and ^{14}C(^{3}H) for the green channel to perform the reverse count. Ten quenched standards for each isotope were run each time and the paper tape record of each run was later transmitted via telephone coupler to the Vogelback Computing Center of Northwestern University.

There, a computer program allowed calculation and transmission to our laboratory of the following data: quench correction curves for each isotope, channels ratio, counting time, counts per minute (cpm) and disintegrations per minute (dpm) for each isotope in each experimental sample. The equations used to prepare the computer program were:

$$\text{Carbon dpm} = \frac{(\text{cpm green})(^3\text{H eff. red}) - (\text{cpm red})(^3\text{H eff. green})}{(^{14}\text{C eff. green})(^3\text{H eff. red}) - (^{14}\text{C eff. red})(^3\text{H eff. green})}$$

$$\text{Tritium dpm} = \frac{\text{cpm red} - (^{14}\text{C dpm})(^{14}\text{C eff. red})}{^3\text{H eff. red}}$$

Statistical comparisons employed the t-test for non-paired data.

Results

The validity of the experimental methods was tested by several preliminary studies. Cultures prepared in the manner described were incubated in a halothane-containing incubator and gas chromatographic determinations of halothane content of air and culture medium were performed periodically. The time taken for equilibration was between two and three hours, indicating that overnight equilibration was more than enough time. Culture concentrations of 16 to 18 mg/100 ml were achieved, in agreement with findings of a previous study.[3] The flow of 95 per cent O_2–5 per cent CO_2 through the halothane vaporizer was so low that the O_2 content of the halothane incubator air was raised less than 1 per cent. A second question related to whether

TABLE 1. Effects of 2 Per Cent Halothane on RNA and Protein Synthesis by 10^6 Lymphocytes, 6 Hours Post-PHA

	Air, No PHA	Air, PHA	2 Per Cent Halothane, PHA	P, Air vs. Halothane
Experiment 1				
^{3}H-uridine				
Number	10	9	10	
dpm,* mean	1,606	4,703	3,915	
SE	207	483	215	N.S.
^{14}C-leucine				
Number	10	9	10	
dpm, mean	640	1,415	1,176	
SE	42	164	76	N.S.
Experiment 2				
^{3}H-uridine				
Number	10	10	10	
dpm, mean	2,789	9,462	5,970	
SE	291	648	414	<.001
^{14}C-leucine				
Number	10	10	10	
dpm, mean	815	1,660	1,363	
SE	40	46	165	N.S.

* Disintegrations per minute.

the cells would take up labelled precursors from a mixture in the same way they would if presented with them separately. Three groups of 10 cultures containing PHA were incubated six hours in air, then each group was given one of three labelled precursors: 2 μCi ^{3}H-U; 5 μCi ^{14}C-L; a mixture containing these amounts of both isotopes. The harvested cells

TABLE 2. Effects of 2 Per Cent Halothane on RNA and Protein Synthesis by 10^6 Lymphocytes 16 Hours Post-PHA

	Air, No PHA	Air, PHA	2 Per Cent Halothane, PHA	P, Air vs. Halothane
Experiment 3				
^{3}H-uridine				
Number	10	10	10	
dpm,* mean	910	15,156	8,531	
SE	70	347	176	<.001
^{14}C-leucine				
Number	10	10	10	
dpm, mean	1,787	22,001	13,482	
SE	208	1,667	496	<.001
Experiment 4				
^{3}H-uridine				
Number	10	10	10	
dpm, mean	1,733	21,916	11,240	
SE	279	287	224	<.001
^{14}C-leucine				
Number	10	10	10	
dpm, mean	3,193	12,722	9,397	
SE	710	391	336	<.001

* Disintegrations per minute.

gave counts indicating equal uptakes of each isotope, irrespective of whether it was given singly or in combination with the other.

The data from the subsequent studies are given in tables 1 and 2. In table 1, the two experiments done six hours post-PHA are summarized. In every experiment, a group of cultures containing no PHA was run as a control. The distinct increases in both RNA and protein synthesis in the PHA-treated cultures were evidenced by the uptake of appropriate precursors between six and seven hours after PHA was added. Halothane produced a uniform reduction in this uptake in both experiments, although this was statistically significant only in the case of uridine uptake in the second experiment. It should be noted that the rate of RNA synthesis in the absence of PHA was relatively high in cells from that donor, and this may explain the greater response to PHA, and inhibition of this response by halothane.

Table 2 gives the data after 16 hours' incubation following PHA. Here, the results were much more dramatic, and in both experiments there was highly significant inhibition by halothane of the rates of synthesis of RNA and protein. Of the two, the inhibition of RNA synthesis was somewhat greater, amounting to almost 50 per cent reduction in both experiments.

Discussion

Following addition of PHA to cultures, Cooper and Rubin[5] found that changes in lymphocyte RNA metabolism consisted of an initial loss of total cellular RNA, followed by an acceleration of ^{3}H-U uptake into newly synthesized RNA which continued for 24 hours. In their studies, the increase in synthesis of RNA began to be detectable one hour after addition of PHA. This was confirmed by Kay,[6] who also studied ^{14}C-L incorporation into protein and found this also to be increased, beginning about two hours post-PHA. Since publication of those reports, many others have confirmed the early onset of both RNA and protein synthesis of lymphocytes treated with PHA. Therefore, the data from this study are evidence that from the very beginning of the reaction in which these cells enter cycle, halothane is inhibitory. In our previous paper we concluded that "there is no proof yet that halothane prevents the entrance of cells into cycle. Verification of the hypothesis would require demonstration of halothane inhibition of cycle events earlier than DNA synthesis, such as RNA and protein synthesis."[1] Now that these have been demonstrated, an effort is under way to study the influence of halothane on the initial reaction of lymphocytes with PHA.

The clinical significance of these studies relates to the role of anesthesia in producing post-surgical depression of immunity. The PHA effect on lymphocytes is generally conceded to mimic the activation of "T" lymphocytes during the cell-mediated immune response. This form of immunity is active in control of some bacterial and many viral infections, tissue transplant rejection, and immunity to cancer cells. If anesthesia alters the early metabolic events in human lymphocytes stimulated with PHA, it may be that during general anesthesia the patient's immune defenses are decreased. This possible clinical correlation is currently under study.

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