REVERSIBLE INHIBITION OF NATURAL KILLER CELL ACTIVITY BY VOLATILE ANAESTHETIC AGENTS IN VITRO

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The influence of surgery and general anaesthesia on aspects of the immune system has received considerable attention in recent years. Generally, it has been agreed that surgical trauma produces significant immune impairment but, as yet, there has been no agreement as to the contribution of the anaesthetic agents used (Lee, 1977; Walton, 1979). Investigations of the immunosuppressive effect caused by volatile general anaesthetic agents have taken two main approaches, the most common being to investigate lymphocyte function in patients receiving general anaesthesia. The alternative method has been to perform tests on normal lymphocytes after in vitro exposure to volatile anaesthetic agents. Results from both approaches have been inconclusive and contradictory.

Patients undergoing surgery are at risk of viral and microbial infections, whilst the surgical removal of solid tumours renders the patient susceptible to the appearance of distant metastases. These are clearly unwanted side-effects and are likely to occur in the presence of surgically-induced immunosuppression. To investigate the contribution of general anaesthetic agents to this immunosuppression, a system is required which could quantitate resistance to the formation of metastases, and to viral or microbial infections. This system should be capable of being realistically manipulated in vitro. Natural killer (NK) cells are a subpopulation of lymphocytes known to have key roles in the immune system as they are cytotoxic towards various malignant cells, and to cells infected by viruses and microbes (Herberman and Ortaldo, 1981; Roder and Pross, 1982; Hanna and Burton, 1984; Ortaldo and Herberman, 1984). A decrease in NK activity will render the host more susceptible to infections and, more importantly, to the risk of metastases following tumour surgery. For this reason, and because NK cells can be investigated in vitro, we chose to study the effects of the volatile anaesthetic agents halothane, enflurane and nitrous oxide on NK cell activity in vitro.

MATERIALS AND METHODS

Lymphocyte isolation

Peripheral blood was drawn from normal volunteers into 10-ml heparinized plastic tubes (lithium herapin 125 iu: Johns Products). The heparinized blood was diluted 1:2 with sterile...
phosphate buffered saline (PBS) and the mononuclear cells were isolated by density gradient centrifugation through Histopaque 1077 (Sigma). The mononuclear cells were washed three times and resuspended in the tissue culture medium RPMI-1640 (Flow). Viability, as determined by eosin dye exclusion, was always greater than 95% and contamination by red blood cells and granulocytes was less than 1%.

**NK cells cytotoxicity assay**

NK cell cytotoxicity was detected in a routine chromium release assay utilizing the human erythroleukaemia K562 as the target cells. The K562 target cells were labelled with radioactive chromium by incubating $1 \times 10^6$ cells in the presence of radioactive chromium 100 μCi (CJ.S.4, Amersham) for 60 min at 37 °C in a total volume of 100 μl. After this radioactive labelling the cells were washed three times in RPMI to remove unlabelled chromium and then resuspended at the final concentration of $1 \times 10^6$ ml$^{-1}$ in RPMI + 5% fetal calf serum. One hundred microlitre of the labelled target cells (i.e. $1 \times 10^4$) were added to the wells of V-bottom microtitre trays (Linbro cat. no. 76-222-05) containing either $5 \times 10^5$, $1 \times 10^6$ mononuclear (effector) cells, RPMI or detergent. Therefore, the assays of cytotoxicity were performed at either 50:1 or 10:1 effector cell to target cell ratios. Spontaneous release from the target cells only was determined from the wells containing RPMI and labelled target cells alone, whereas the maximum release was determined from the wells containing detergent and the target cells. All cultures were set up in triplicate and incubated at 37 °C in a carbon dioxide-buffered air atmosphere with or without the volatile anaesthetic agents for 3 h when 100 μl of the supernatant was collected and counted on a gamma-counter (LKB 1260 Multigamma) to determine the amount of chromium released and, therefore, the degree of cytotoxicity. Percent cytotoxicity was determined, according to the following formula, from the mean counts of the triplicate cultures:

$$\text{% cytotoxicity} = \frac{\text{ct min}^{-1} \text{ experimental release} - \text{ct min}^{-1} \text{ spontaneous release}}{\text{ct min}^{-1} \text{ maximum release} - \text{ct min}^{-1} \text{ spontaneous release}} \times 100$$

**Incorporation of volatile anaesthetic agents**

The microtitre trays containing the cytotoxicity cultures were placed inside gas-tight stainless steel autoclave containers and were exposed to the anaesthetic test gases within 15 min of preparation.

The test gases were derived on each occasion from the same Boyle anaesthetic machine and anaesthetic vaporizers. Vaporizers not in use were removed from the Selectatec backbar of the anaesthetic machine. Carbon dioxide 10% was added to all the gas mixtures from a gas cylinder. The consistent output of the MK 3 Fluotec and Enfluratec vaporizers was confirmed by using an Engstrom Emma multi-vapour analyser at the common gas outlet of the Boyle machine. The calibration of the Emma was verified routinely against standardized calibration gases. The autoclave containers were flushed with the anaesthetic or control gas mixture for a minimum of 2 min with a 4-litre min$^{-1}$ gas flow ensuring at least four total volume changes. Adequate flushing was confirmed by ensuring (Datex CO$_2$ monitor) that the cylinder contained the required 10% carbon dioxide. The cylinder was then sealed tightly and placed immediately in a 37 °C incubator for 4 h. The continued presence of the test gases was confirmed before this study by mass spectrometry. Throughout the study there was no evidence of gas loss.

**Reversibility study**

To ascertain the reversibility of the anaesthetic gases, the mononuclear cells were added to the microtitre trays in such a manner as to be already prepared for the addition of the labelled K562 target cells. Before the addition of the target cells the microtitre tray containing the mononuclear (effector) cells was incubated for 3 h in the presence of halothane, enflurane or nitrous oxide. After this exposure the culture trays were allowed to equilibrate with a normal 10% carbon dioxide in air atmosphere. The chromium-labelled target cells were then added for the routine 4 h cytotoxicity assay. Cytotoxicity was determined as described above.

**Statistics**

Student’s paired t tests were used for all the analyses.

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**RESULTS**

The results in figure 1 show clearly that all the anaesthetics produced a dose-dependent reduction
VOLATILE ANAESTHETICS AND NK CELLS

80- Halothane 50:1
40- Enflurane 50:1
20- N2O + Enflurane 50:1
0- CO2 1.5:1
80- 10:1
40- 10:1
20- 10:1
0- 10:1

Fig. 1. Dose dependent reduction of NK cell activity at either 50:1 (upper panel) or 10:1 (lower panel) effector cell to target cell ratios in the presence of halothane (left panel), enflurane (centre panel), or nitrous oxide and a combination of enflurane and nitrous oxide (right panel). The solid lines represent the mean cytotoxicity obtained from triplicate cultures. An overall dose-dependent reduction for all the volatile anaesthetic agents tested is demonstrated and the combination of enflurane and nitrous oxide produced an additive suppressive effect.

in NK cell activity at both the 50:1 and 10:1 effector to target cell ratios. The combination of nitrous oxide and enflurane produced an additive suppressive effect. Although the highest concentration tested exceeded the dose used in clinical anaesthesia, there was not a total abrogation of NK cell activity. To determine in a comparative manner the effects of approximately equipotent and realistic clinical concentrations, the suppressive effects of 60% nitrous oxide, 1% enflurane and 0.5% halothane were tested simultaneously. Table I shows the amount of suppression produced and demonstrates that nitrous oxide had the least suppressive effect whilst enflurane and halothane produced a similar degree of suppression. It should also be noted that the suppression was greater at the 10:1 than the 50:1 effector to target ratios and that at even very high doses (e.g. 3 h at 3% halothane or 12 MAC h) there was still some evidence of NK cell activity. Clearly, the cytotoxic capacity of NK cells was substantially reduced in an atmosphere containing volatile anaesthetic agents; however, it was important to determine the ability of the effector cells to recover from this exposure. Reversibility studies were therefore performed and it was found that the cells did recover and that the reduction of NK activity was evident only during the period of exposure (fig. 2). There was no direct effect of the gases on effector cell viability, nor was there any direct effect on the target cells.

DISCUSSION

It is well established that the combination of surgery and anaesthesia depresses the immune response (Walton, 1978) such that the patient becomes susceptible to microbial and viral infections and, in the case of the patient with cancer, to the development of metastases. The contribution of the anaesthetic to this suppression remains controversial. The evaluation of lymphocyte function from individuals following anaesthesia with or without coincident surgery has led to contradictory findings. Cullen and Van Belle (1975) and Duncan and colleagues (1976) found no evidence for immunosuppression caused by anaesthetics. This view was opposed by Espanol, Todd and Soothill (1974) and Slade and co-workers (1975) who attributed the observed immunosuppression to the inhalation of the anaesthetic. Recent studies comparing the effect of local and general anaesthesia led Whelan and Morris (1982) to conclude that the general anaesthetic agents, and not the surgery, were responsible for immunosuppression. A similar study with a

<table>
<thead>
<tr>
<th>Anaesthetic agent</th>
<th>Effector to target ratio 50:1</th>
<th>P</th>
<th>Effector to target ratio 10:1</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% Nitrous oxide</td>
<td>10</td>
<td>&lt;0.05</td>
<td>18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.5% Halothane</td>
<td>20</td>
<td>&lt;0.001</td>
<td>31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1.0% Enflurane</td>
<td>22</td>
<td>&lt;0.01</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Reversibility

![Graph showing reversibility of anaesthetic-induced suppression of NK cell activity.](image)

Fig. 2. Reversibility of the anaesthetic-induced suppression of NK cell activity. Mononuclear cells were divided into four groups and pre-incubated for 3 h in the presence of either carbon dioxide or nitrous oxide or enfurane or halothane. After this first incubation the cells were then incubated in a 10% carbon dioxide in air atmosphere for 1 h. The cytotoxicity assays were then performed with these cells in the carbon dioxide in air atmosphere. The depicted lines represent the same lymphocyte preparations preincubated with either carbon dioxide or the volatile anaesthetic agents, followed by the 1-h incubation in carbon dioxide before the NK cell assay. The upper panel represents the 50:1 and the lower panel represents 10:1 effector to target ratio. There was no significant difference when comparing cytotoxicity values of the carbon dioxide preincubation cultures with either the nitrous oxide, enfurane or halothane preincubations at the 50:1 (\(P > 0.5\)) or the 10:1 (\(P > 0.4\)) effector to target ratios. Enf = enfurane; Hal = halothane; CO\(_2\) = Control.

contrary finding was reported by Hole (1984), who concluded that general anaesthetics did not interfere with lymphocyte functions. All these studies relied on the investigation of lymphocyte function following the in vivo exposure of individuals to the anaesthetic agents. An alternative method was to expose lymphocytes directly to anaesthetic agents in vitro and this approach has also been inconclusive.

This study was undertaken to investigate the ability of anaesthetics per se to suppress the immune response by directly interfering with lymphocyte function. NK cell cytotoxicity, representing a vital aspect of the immune response, was chosen to serve as a model because of the important role of NK cells in cytotoxicity against virally-infected cells and malignant cells. NK cell activity was assayed in vitro by mixing lymphocytes (from which the NK cell population is derived) with the erythroleukaemia K562 cell line as the target cell population. This is the standard and accepted assay for measuring NK cell activity and it is only the NK cells that are cytotoxic towards the K562 cells (Herberman and Ortaldo, 1981; Roder and Pross, 1982). The results from this study have demonstrated that the inhalation agents nitrous oxide, halothane and enfurane decreased significantly the ability of NK cells to lyse K562 tumour cells in vitro. The inhibition was dose-dependent, with the combined effect of nitrous oxide and enfurane being additive. However, concentrations greater than those used clinically were utilized to overcome the lower solubility of these agents in tissue culture medium than in blood (Hartzell and Johnson, 1985). In a similar study Cullen, Duncan and Ray-Kiel (1976) found that in vitro equilibration of tumour cell-sensitized mouse lymphocytes with halothane or nitrous oxide inhibited the ability of the lymphocytes to kill the tumour cells. In an alternative system Tønnesen, Mickley and Grunnet (1983) found that NK cell activity actually increased after premedication and during the early stages of anaesthesia, but decreased in the postoperative period and remained low for up to 5 days. Superficially, these findings appear contrary to our study. However, Tønnesen, Mickley and Grunnet (1983) tentatively proposed that the augmentation of NK cells was the result, not of an increased activity, but of the mobilization of NK cells from extravasal spaces. Because of a number of in vivo complicating factors it is difficult to attribute any direct effect of the anaesthetic on the NK cell activity from such a study and, therefore, such findings cannot be compared directly with our study. Previous investigations of minor surgery and anaesthesia have demonstrated a reduction in cell-mediated cytotoxicity (Vose and Moudgil, 1976) including NK activity (Møller-Larsen, Møller-Larsen and Haahr, 1983), whilst mitogen-induced blast transformation remained unaffected.

Investigation of approximately equipotent doses demonstrated that nitrous oxide had a lesser inhibitory effect than either enfurane or halothane. It was also shown that the inhibition was greater at the 10:1 than the 50:1 effector to target ratios for the anaesthetic agents tested. The conclusion that can be drawn from this observation is that, at optimal conditions (i.e. 50:1), NK cell cytotoxicity is more resistant to the anaesthetic-mediated inhibition than at suboptimal conditions (i.e. 10:1). It is tempting to extrapolate to the in vivo
situation and propose that perhaps those surgical patients who, before surgery, have a reduced immune function as a consequence of their disease (e.g. cancer) will experience a greater reduction in NK cell function than other “healthier” surgical patients.

The most significant finding of this study was that the cells soon recovered from the effects produced by the anaesthetic. The inhibition of NK cell cytotoxicity would, therefore, only occur during the presence of the anaesthetic agents. Consequently, the anaesthetic per se can only be directly responsible for intra- and immediate post-anaesthetic suppression. The important question unanswered from this and other in vitro studies is the combined effect of surgery and anaesthesia. Although the direct effect of anaesthesia alone appears to be minimal, it is important to determine its effect when combined with surgery to determine if a greater immunosuppression results that can be attributed directly to the surgery alone.

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REFERENCES


