Absence of Cellular Hypersensitivity in Patients with Unexplained Hepatitis Following Halothane


In-citro tests of cell-mediated immunity were performed using blood obtained from subjects with unexplained hepatitis following halothane anesthesia to determine whether sensitization to potentially antigenic products of halothane metabolism might exist. Both lymphocyte transformation and leukocyte migration-inhibition tests were undertaken in the presence of trifluoroacetylated human liver specific protein and trifluoroacetylated human serum albumin. All tests in the presence of these potential antigenic complexes were negative. The results support the view that cell-mediated hypersensitivity to trifluoroacetylated proteins does not contribute to the pathogenesis of hepatic dysfunction following halothane anesthesia. (Key words: Anesthetics, volatile, halothane; Liver, hepatitis; Allergy, hypersensitivity, halothane.)

The direct association between halothane and postoperative hepatic dysfunction (“halothane hepatitis”) remains controversial. Of the hypotheses put forward to explain such an association, that of hypersensitivity to halothane itself, or to a metabolite thereof, has achieved wide support.

Paronetto and Popper found in-citro transformation, in the presence of halothane, of lymphocytes of patients with alleged halothane hepatitis. They interpreted their results as evidence that the hepatic damage in these patients was a manifestation of cell-mediated hypersensitivity to halothane. This work has been extensively criticized,2-4 and subsequent investigators have been unable to confirm the findings.5-7 In 1972, Jones Williams et al.8 published a preliminary report of positive migration inhibition in the presence of halothane of leukocytes of two patients who also had hepatic dysfunction following exposure to halothane. Appropriate control experiments were not undertaken, and this work has not since been confirmed. Paronetto and Popper, and Jones Williams et al., used halothane itself as the potential antigen, although halothane is a small molecule (mol wt 197.4) that is unlikely to be immunogenic in its own right. Although it was originally suggested that halothane was an inert molecule,9 it has subsequently been shown that it is extensively metabolized in the body, with trifluoroacetate the major, and trifluoroacetyl ethanamine the minor, metabolite.10 There is no evidence that halothane itself is capable of combining with macromolecules, but recent work has shown that metabolites of halothane may combine with proteins in the liver.11-14 and it seems possible that the complexes so formed may be immunogenic. Therefore, we have extended our earlier studies5 of cell-mediated immunity in patients with possible “halothane hepatitis” to include representative conjugates.

In this study we exposed lymphocyte or leukocyte populations from patients with histories of post-halothane jaundice to artificially produced conjugates of trifluoroacetate with both human liver specific protein and human serum albumin. We report the results of both lymphocyte transformation and leukocyte migration-inhibition tests undertaken to seek evidence of cellular hypersensitivity to these potential antigens.

* Senior Lecturer.
† Lecturer, Division of Immunology, Kennedy Institute of Rheumatology.
‡ Director, Division of Immunology, Kennedy Institute of Rheumatology.
§ Professor. Present address: Chief, Department of Anesthesia, Baylor University Medical Center, 3500 Gaston Avenue, Dallas, Texas 75246.

Received from the Anesthetics Unit, The London Hospital, London E.1, England, and the Division of Immunology, Kennedy Institute of Rheumatology, Bute Gardens, London, W.6, England. Accepted for publication January 13, 1976.

Address reprint requests to: Dr. B. Walton, Anesthetics Unit, The London Hospital, London, E1 1BB, England.

TABLE 1. Intervals between Jaundice and *In-vitro* Tests

<table>
<thead>
<tr>
<th>Patients</th>
<th>HSA, TFA-HSA</th>
<th>LSP, TFA-LSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 years</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>2½ years</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>6 months</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Jaundiced</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>2½ years</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>Jaundiced</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>Jaundiced</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>Jaundiced</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>5 months</td>
<td>Jaundiced</td>
</tr>
<tr>
<td>10</td>
<td>7 months</td>
<td>3 months</td>
</tr>
<tr>
<td>11</td>
<td>2 years</td>
<td>21 months</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>Jaundiced</td>
</tr>
<tr>
<td>13</td>
<td>—</td>
<td>4 weeks</td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>Jaundiced</td>
</tr>
<tr>
<td>15</td>
<td>—</td>
<td>Jaundiced</td>
</tr>
<tr>
<td>16</td>
<td>—</td>
<td>6 years</td>
</tr>
</tbody>
</table>

* HSA: human serum albumin.
TFA-HSA: trifluoroacetylated human serum albumin.
LSP: liver specific protein.
TFA-LSP: trifluoroacetylated liver specific protein.

**Materials and Methods**

**Antigen Preparation**

Human serum albumin (human crystallized albumin, Dade Division, American Hospital Supply Company) and human liver specific protein (kindly prepared and supplied by Professor K. H. Meyer zum Bärenfelde, Mainz, Germany) were trifluoroacetylated as follows: each protein sample was adjusted to pH 10 with NaOH at room temperature. Redistilled ethyl trifluoroacetate, 0.5 ml, was added, and the solution was held at pH 10 for 90 minutes, during which period the NaOH hydroxide uptake was 3.8 ml. Each sample was dialyzed against 0.01 M phosphate buffer (pH 7.2) at 4°C, with five changes of buffer in 48 hours. Subsequent analysis showed extensive trifluoroacetylation of the proteins.

The concentrations of human serum albumin (HSA), trifluoroacetylated human serum albumin (TFA-HSA), liver specific protein (LSP) and trifluoroacetylated liver specific protein (TFA-LSP) were assayed by a modified Lowry technique. Dilutions of these antigens, which initial toxicity studies had shown did not suppress normal lymphocyte transformation and leukocyte migration, were prepared in Eagle's M.E.M. (Flow Laboratories).

**Patients and Controls**

During a three-year nationwide survey of postoperative jaundice, 203 patients were visited (by B.W.) during their acute illness. The case histories of all patients were classified by an independent panel of hepatologists, without knowledge of the anesthetic agents involved. The panel identified a group of 78 patients with biochemical evidence of hepatocellular damage, in whose cases no etiologic factor (such as cirrhosis, septicemia, cardiovascular collapse) could be identified. Of the 78 patients, 76 had received halothane as part of their final anesthesia. Sixteen of the 76 patients who had unexplained hepatitis following halothane were investigated in this study. Blood was taken from eight patients during their acute illness, and from the remainder at intervals following recovery (table 1). Control subjects were normal healthy adults without previous hepatic disease or history of exposure to halothane.

Blood samples were collected into sterile glass bottles containing 20 units of preservative-free heparin (Evan Medical)/ml blood and delivered to the laboratory at ambient temperature, within two hours of collection.

Samples from patients and controls were tested for *in-vitro* transformation and migration-inhibition responses to the potential “antigens” (TFA-HSA and TFA-LSP) and “antigen controls” (HSA and LSP).

**Lymphocyte Transformation Tests**

A micro-whole blood culture method was used. Whole blood, 0.1 ml, was placed in culture tubes to which 0.1 ml of “antigen” had been added. The “antigens” were used in final concentrations ranging from 100 µg/ml, in one-tenth dilutions, to 0.001 µg/ml. In addition, cells were exposed to purified protein derivative of human tuberculin (P.P.D. 292, Central Veterinary Laboratory, Weybridge) at a final concentration of 10 µg/ml, and to phytohemagglutinin (PHA, reagent grade MR10, Wellcome Reagents Ltd.) at a final...
concentration of 50 μg/mL. The volume of each culture was then made up to 1.0 ml with Eagle’s M.E.M., buffered with sodium bicarbonate, and supplemented with penicillin G sodium and streptomycin. Cells were exposed to each “antigen” concentration in triplicate cultures, and control cultures were incubated without “antigen.” Cultures were incubated for six days at 37°C in an atmosphere of 5 per cent CO₂ in air, and 1 μCi tritiated thymidine (Radiochemicals, Amersham; specific activity 5 Ci/mmol) was added 24 hours before harvesting. The cultures were treated sequentially with 3 per cent acetic acid (to lyse erythrocytes), and buffered saline solution and trichloroacetic acid (to precipitate proteins and nucleic acids) and dehydrated with methanol. The cells were solubilized in hyamine hydroxide (Koch Light Laboratory), counted in PPO/POPPOP in toluene, and the quench-corrected disintegrations per minute (dpm) were determined for each culture. The results represent the mean for each set of triplicate cultures and are expressed as an index of response (IR): dpm in the presence of “antigen” divided by dpm without “antigen.”

LEUKOCYTE MIGRATION-INHIBITION TESTS

The micromethod modification of the original technique of Bendixen and Soborg, developed by Federlin et al., was employed. Leukocytes were separated from the samples of whole blood by gravity sedimentation. The cells were centrifuged at 150 × g for 10 minutes, washed three times in Eagle’s M.E.M. containing 10 per cent horse serum (Wellcome Laboratories) and resuspended in the medium at approximately 6 × 10⁶ leukocytes/ml. Microcapillaries (Drummond Instrument Company) were filled with the leukocyte suspension, plugged at one end with wax, centrifuged at 600 × g for 5 minutes, cut at the cell-fluid interface and secured within migration chambers (Sterilin). The chambers were immediately filled with Eagle’s M.E.M. (containing 10 per cent horse serum), to which “antigen” had been added in concentrations of 100, 10, and 1.0 μg/mL. In addition, the migrating cells were exposed to heat-killed human tubercle bacilli (Central Veterinary Laboratory, Weybridge) at a concentration of 150 μg/mL. The chambers were covered with glass slips and incubated at 37°C for 18 hours. The migration area of the leukocytes was mapped by projection microscopy and measured by planimetry. The mean of the migration areas from six capillaries at each “antigen” concentration was taken and compared with the mean of 12 control migrations in medium alone. The results were expressed as a migration index: mean migration area in presence of “antigen” divided by mean migration area in medium alone.

Results

Lymphocyte transformation responses to the “antigen” TFA-HSA and “antigen control” HSA are shown in figure 1 and responses to TFA-LSP and LSP in figure 2. The mean indices of response for “antigen” and “antigen control” concentrations between 0.001 and 100 μg/mL demonstrate that there was no significant stimulation in whole-blood cultures from either patients or control subjects, compared with the positive responses to 50 μg/mL PHA and 10 μg/mL PPD. Indices greater than 2 were observed in a few experiments. However, these occurred in samples from both patients and controls, in response to “antigen” and “antigen controls” and since the results did not follow a dose–response curve they were not regarded as significant. It was concluded that TFA-HSA and TFA-LSP were not antigenic at the concentrations used.

Leukocyte migration-inhibition results are shown in figures 3 and 4. The mean indices of response for “antigen” and “antigen control” concentrations of 1.0, 10.0, and 100 μg/mL and heat-killed tubercle bacilli at 150 μg/mL are shown. Significant inhibition (migration indices less than 0.8) was obtained in the presence of heat-killed tubercle. However, the mean indices of inhibition in samples from both patients and controls in the presence of “antigen” and “antigen controls” provided no evidence for cellular hypersensitivity to either TFA-HSA or TFA-LSP.

Discussion

The possibility that a halothane metabolite might achieve immunogenic status by combining with protein became evident when several groups of investigators demonstrated that
halothane, or a metabolite thereof, was capable of forming a complex with protein in the livers of the rabbit\textsuperscript{11} and rat\textsuperscript{12-14}.

Rosenberg and Wahlström\textsuperscript{19} immunized rabbits with chicken serum globulin complexed with either trifluoroacetic acid (TFA), trifluoroethanol (TFE), or trifluoroacetaldehyde (TFAl), and demonstrated the production of antibodies to these complexes. Since the authors used heterologous protein and included complexes involving two theoretical halothane metabolites (TFE, TFAl) for which no evidence had appeared, the possible relevance of their work to unexplained hepatitis following exposure to halothane was unclear. More recently, Mathieu and colleagues\textsuperscript{20} demonstrated that, in guinea pigs, trifluoroacetate, when conjugated with homologous protein, was capable of inducing specific delayed-type hypersensitivity. However, these investigators advised caution in extrapolating their results (involving sensitization in the presence of adjuvant) to possible rare clinical responses in man.

Against this background, our negative results may be discussed in four ways. First, it is possible that the technique of chemical trifluoroacetylation did not produce the appropriate immunogenic complex, for whereas this method trifluoroacetylates many sites on protein molecules, it is unlikely that chemical substitution \textit{in vitro} would occur at more than one or two such intramolecular sites. However, it might be argued that excessive trifluoroacetylation might render recognition of the antigenic determinants of TFA–protein complexes more likely when patient's lymphocytes were subsequently exposed to these
complexes in vitro. Second, as halothane is metabolized in the liver, it was felt that liver specific proteins were the carrier molecules most likely to be recognized, for it appears that the elicitation of cell-mediated immune responses to drug metabolite–protein complexes may require both the correct hapten and carrier molecules.\textsuperscript{24} Recent work by Van Dyke and Wood\textsuperscript{10} suggested that, although trifluoroacetate binds to some extent to liver proteins, far greater binding, possibly involving free radical formation,\textsuperscript{14} may be occurring with as yet unidentified alternative metabolites. Third, in addition to the possibility that the "wrong" hapten–protein conjugates were being used, a number of patients were no longer jaundiced at the time their blood samples were obtained for in-vitro testing (table 1). However, lymphocyte transformation responses are known to persist after clinical hypersensitivity syndromes have subsided,\textsuperscript{22,23} and it was, therefore, felt that had these patients had cellular hypersensitivity to the hapten–protein complexes concerned, we should have been able to detect it. Fourth, it is possible that our patients, classified as having unexplained hepatitis following halothane, did not have "halothane hepatitis." The diagnosis of "halothane hepatitis" remains predominantly one of exclusion, and observer bias often influences that diagnosis. It was felt, therefore, that independent classification, without knowledge of the anesthetic agents involved, would be most likely to identify patients whose postoperative hepatitis might relate to the anesthetic agents used.

In conclusion, although it is often suggested

![Diagram](image)

**Fig. 2.** Transformation in vitro of lymphocytes from patients and controls in the presence of human liver specific protein and trifluoroacetylated human liver specific protein. Responses to phytohemagglutinin (PHA) and purified protein derivative of human tuberculin (PPD) are also shown. The means of responses of patients and controls at each "antigen" concentration are shown thus (—).
Fig. 3. Migration inhibition in vitro of leukocytes from patients and controls in the presence of human serum albumin and trifluoroacetylated human serum albumin. Responses to heat-killed human tubercle bacilli are also shown. The means of responses of patients and controls at each “antigen” concentration are shown thus (---). Minor inhibition, between 1.0 and 0.8 (dotted line) is usually not considered significant in this system.

Fig. 4. Migration inhibition in vitro of leukocytes from patients and controls in the presence of human liver specific protein and trifluoroacetylated human liver specific protein. Responses to heat-killed human tubercle bacilli are also shown. The means of responses of patients and controls at each “antigen” concentration are shown thus (---). Minor inhibition, between 1.0 and 0.8 (dotted line) is usually not considered significant in this system.
that the rare occurrence of hepatic dysfunction following exposure to halothane is a manifestation of cellular hypersensitivity, the \textit{in-vitro} correlates of cell-mediated immunity in these patients provided no evidence in favor of such a hypothesis.

The authors thank Professor R. Wright (Professor of Medicine, University of Southampton), Dr. R. Williams (Consultant Physician, Kings College Hospital, London), and Dr. N. McIntyre (Consultant Physician, Royal Free Hospital, London) for their classification of the patients with postoperative jaundice. Protein assays were performed by Mr. J. Biffen, Biochemistry Department, The London Hospital Medical College. Analysis of the triluoroacetylated proteins were performed by Dr. H. Gregory, Chemistry Department, Imperial Chemical Industries Ltd. Liver specific protein was kindly supplied by Professor Meyer zum Büschenfelde, Mainz, Germany. The skilled assistance of Mrs. J. Walters and Miss E. Wilson is gratefully acknowledged.

References