Absence of Cellular Hypersensitivity in Patients with Unexplained Hepatitis Following Halothane

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In-vitro 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[1] found *in-vitro* 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 ethanolamine 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 studies[5] 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.

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¶ Winrow M, Topham J: Pharmaceutical Division, I.C.I. Ltd., Cheshire, England, unpublished data.

TABLE 1. Intervals between Jaundice and
In-vitro Tests

| Patients | "Antigens"* | |
	HSA, TFA-HSA	LSP, TFA-LSP
1	3 years	—
2	2½ years	—
3	6 months	—
4	Jaundiced	—
5	2½ years	—
6	Jaundiced	—
7	Jaundiced	—
8	Jaundiced	—
9	5 months	Jaundiced
10	7 months	3 months
11	2 years	21 months
12	—	Jaundiced
13	—	4 weeks
14	—	Jaundiced
15	—	Jaundiced
16	—	6 years

* HSA: human serum albumin.
  TFA-HSA: trifluoroacetylated human serum al-
  bumin.
  LSP: liver specific protein.
  TFA-LSP: trifluoroacetylated liver specific pro-
  tein.

Materials and Methods

ANTIGEN PREPARATION

Human serum albumin (human crystallized
albumin, Dade Division, American Hospital
Supply Company) and human liver specific
protein[15] (kindly prepared and supplied by
Professor K. H. Meyer zum Büschenfelde,
Mainz, Germany) were trifluoroacetylated as
follows: each protein sample was adjusted to
pH 10 with N sodium hydroxide at room
temperature. Redistilled ethyl trifluroacetate,
0.5 ml, was added, and the solution was held
at pH 10 for 90 minutes, during which period
the N sodium 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 al-
bumin (TFA-HSA), liver specific protein (LSP)
and trifluoroacetylated liver specific protein
(TFA-LSP) were assayed by a modified Lowry

technique.[16] 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 clas-
sified by an independent panel of hepatolo-
gists, 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 re-
mainder at intervals following recovery (table
1). Control subjects were normal healthy
adults without previous hepatic disease or his-
tory of exposure to halothane.

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

Samples from patients and controls were
tested for *in-vitro* transformation and migra-
tion-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 CO_2 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/POPOP 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,[17] developed by Federlin et al.,[18] 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 × 10⁷ 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
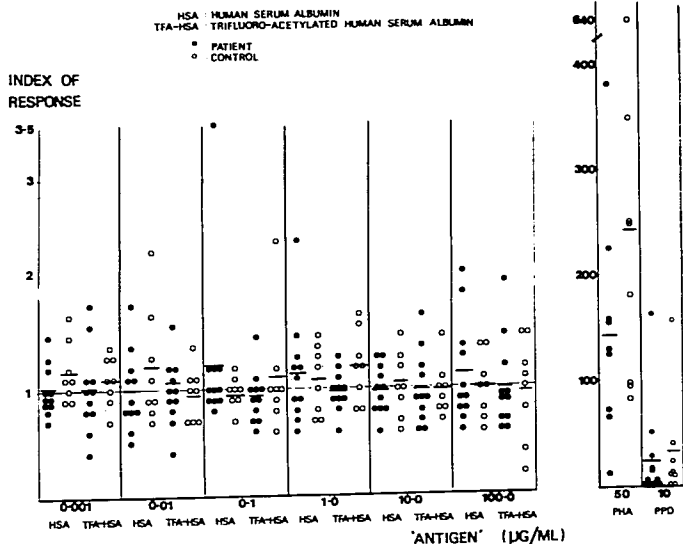
FIG. 1. Transformation *in vitro* of lymphocytes from patients and controls in the presence of human serum albumin and trifluoroacetylated human serum albumin. Responses to phytohemagglutinin (PHA) and purified protein derivative of human tuberculin (PPD) are also shown. The means of responses of patients and controls at each "antigen" concentration are shown thus (—).

halothane, or a metabolite thereof, was capable of forming a complex with protein in the livers of the rabbit[11] and rat.[12-14]

Rosenberg and Wahlstrom[19] immunized rabbits with chicken serum globulin complexed with either trifluoroacetic acid (TFA), trifluoroethanol (TFE), or trifluoroacetaldehyde (TFAld), and demonstrated the production of antibodies to these complexes. Since the authors used heterologous protein and included complexes involving two theoretical halothane metabolites (TFE, TFAld) 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[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 *in vivo* 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.[21] Recent work by Van Dyke and Wood[13] suggested that, although trifluoroacetate binds to some extent to liver proteins, far greater binding, possibly involving free radical formation,[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 transforma-

tion responses are known to persist after clinical hypersensitivity syndromes have subsided,[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.
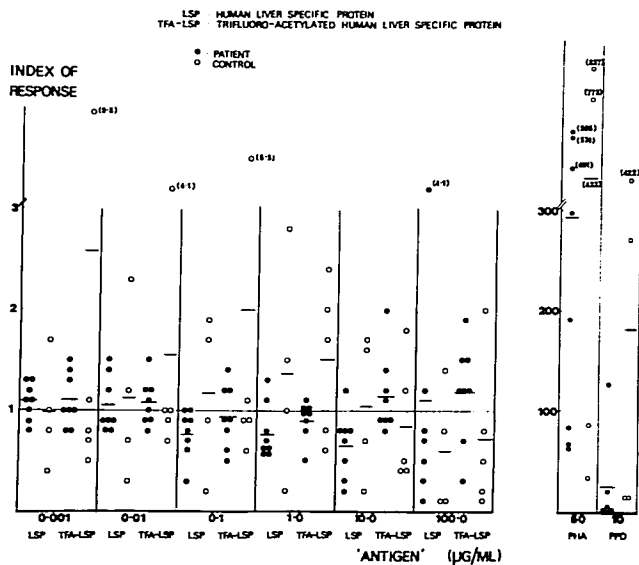
In conclusion, although it is often suggested

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 (—).
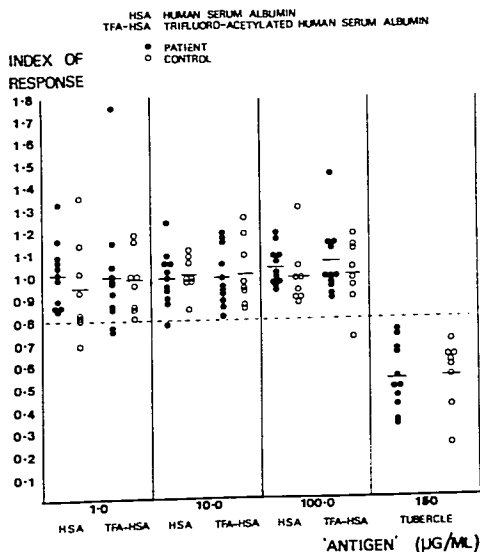
FIG. 3. Migration inhibition *in vitro* of leukocytes from patients and controls in the presence of human serum albumin and trifluoro-acetylated human serum albumin. Responses to heat-killed human tubercle bacilli are also shown. The means of responses of patients and controls at each "antigen" concentration are shown thus (—). Minor inhibition, between 1.0 and 0.8 (dotted line) is usually not considered significant in this system.
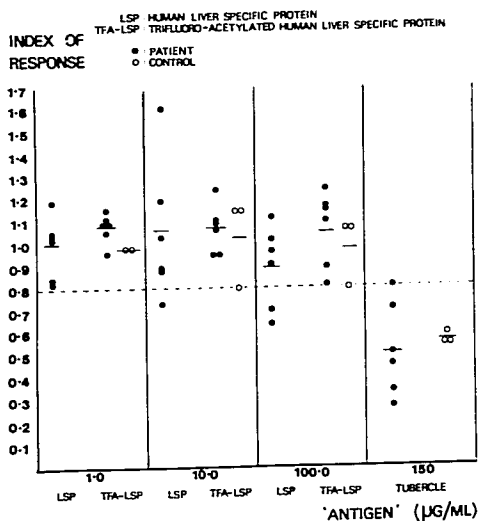

FIG. 4. Migration inhibition *in vitro* of leukocytes from patients and controls in the presence of human liver specific protein and trifluoroacetylated human liver specific protein. Responses to heat-killed human tubercle bacilli are also shown. The means of responses of patients and controls at each "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 *in-vitro* correlates of cell-mediated immunity in these patients provided no evidence in favor of such a hypothesis.

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