Experimental Immunity to a Metabolite of Halothane and Fluoxene:

Cutaneous Delayed-type Hypersensitivity

Alix Mathieu, M.D.,* Diana DiPadua, M.A.,† John Mills, M.D.,‡ Barry Kahan, M.D., Ph.D.§

Trifluoroacetate (TFA), a common metabolite of halothane and fluoxene, conjugated to human and guinea pig albumin induces specific delayed-type hypersensitivity in guinea pigs. Histologic examination of cutaneous hypersensitivity reactions characterized by erythema and induration 24–48 hours after intradermal challenge with antigen revealed perivascular mononuclear infiltration. These findings support the hypothesis that trifluoroacetate, a metabolite of halothane and fluoxene, can serve as a hapten to elicit specific cellular immune responses. (Key words: Anesthetics, volatile: halothane; Anesthetics, volatile: fluoxene; Biotransformation: halothane; Biotransformation: fluoxene; Allergy, hypersensitivity: trifluoroacetate.)

Among patients with postoperative hepatitis, halothane, fluoxene and methoxyflurane may be the precipitating cause in a small number of predisposed individuals. Two possible mechanisms of injury have been generally suggested: 1) a genetically determined metabolic error, or 2) an autoimmune reaction wherein the host develops sensitivity toward self-components through conjugation of these substances to halothane metabolites. The nature of the immune response which might mediate hepatic damage remains unclear. Evidence favoring the role of humoral antibody or that of cellular mechanisms has not been definitive. In parallel with the current interest in cell-mediated autoimmune diseases, e.g., experimental allergic encephalomyelitis, thyroiditis, orchitis, and arthritis, Paronetto's suggested that the lymphocytes of afflicted patients were exquisitely sensitive to halothane–plasma sonicates in vitro. These results have not been generally accepted, although a number of investigators have proposed that antigenic complexes of halothane metabolites could activate specific thymus-derived T lymphocytes, which then result in hypersensitivity reactions in the liver, and possibly other sites.

The purpose of the present investigation was to determine whether trifluoroacetate, a metabolite of halothane and fluoxene, could function as a hapten in the induction of cellular immunity. While the capacity to act as a hapten is essential for the generation of an immune response, this potential does not suggest that the mode of hepatic damage is necessarily the result of such a mechanism. In accord with previous observations on the induction of autoimmune diseases employing tissue extracts emulsified in complete Freund's adjuvant, the test materials conjugated to albumin were administered in this form.

Materials and Methods

Animals

Since Hughes (1972)' reported hepatic lesions in the Hartley strain of guinea pigs following several exposures to 1 per cent
halothane, and inasmuch as this species offers an excellent animal model for investigations in cell-mediated immunity, outbred male albino guinea pigs (English short hair) weighing 450–500 g were obtained from Elm Hill Breeding Farms, Massachusetts. Animals were maintained on a standard feed diet supplemented with ascorbic acid. Prior to inception of the experiments, the animals were divided into the various groups at random.

**PREPARATION OF THE CONJUGATES**

Several investigators (E.N. Cohen and J.R. Trudell, The urinary metabolites of halothane, abstract, ASA meeting, 1971;²⁵) have found that trifluoroacetate is the main urinary and hepatic metabolite of halothane and fluoroxyne in guinea pigs and mice. This compound was used in the immunologic studies. The trifluoroacetylated proteins were prepared using the procedure described by Goldberger and Anfinsen.² Protein in the substituted trifluoroacetylated albumins was determined by the Lowry procedure using recrystallized bovine serum albumin as a standard. Samples of the lyophilized substituted protein were sent for fluorine analyses to Schwartzhoff Microanalytical Laboratories, Woodside, New York.

The lyophilized trifluoroacetylated human albumin contained 1.47 per cent fluorine. When corrected for actual protein content, the conjugate was composed of 60 moles fluorine/mole human albumin. Similarly, the trifluoroacetylated guinea-pig albumin contained 60 moles fluorine/mole guinea-pig albumin and fluorine was reported to be 1.54 per cent.

**IMMUNIZATION**

Each guinea pig received intradermal injections into the four footpads of a total of 50 μg trifluoroacetate conjugated to human albumin in complete Freund’s adjuvant (Difco) (table 1). [Complete Freund’s adjuvant (CFA) is a mixture of Mycobacterium tuberculosis and oils such as Arlacel and Bayol.] Three weeks later, each animal was challenged with 10 μg each of the hapten-protein complexes, including trifluoroacetyl-human albumin (TFA–HA), trifluoroacetyl-guinea-pig albumin (TFA–GPA), guinea pig albumin (GPA), human albumin (HA), and tuberculin as purified protein derivative (PPD) (intermediate strength). Animals were also skin tested with halothane and methoxyflurane alone, or with sonicated serum proteins. Two additional groups of guinea pigs were immunized with trifluoroacetyl-guinea pig albumin conjugates (table 2). An intradermal booster injection into the nape of the neck of 50 μg in complete Freund’s adjuvant was given three weeks later, when the first set of cutaneous reactions was negative.

**SKIN TESTS**

Skin tests were performed on the closely clipped, but not depilated, flanks of guinea pigs. These tests consisted of injecting 10 μg of each soluble antigen in saline solution intradermally. Skin reactions were read at 1/2, 6, 12, 18, 24, 48, and 72 hours for 1) the maximum diameter of erythema, 2) the extent of induration at the test site compared with a fold of normal skin, and 3) the presence of hemorrhage or necrosis. The extent of erythema was tabulated as the maximum diameter (in mm); induration was graded on a scale of + to ++. Before the final result was recorded, animals were depilated with Nair (a commercial depilatory cream).

**HISTOLOGY**

Skin reactions were excised and fixed in 10 per cent buffered formalin. Histologic sections were stained with hematoxylin–eosin, periodic acid–Schiff, and methyl-green pyronin.

**Results**

**NON-IMMUNIZED ANIMALS**

Six control, nonimmunized guinea pigs were challenged intradermally with 10 μg each of HA, TFA–HA, GPA, TFA–GPA. They failed to manifest cutaneous reactions to the conjugates. When normal animals were challenged
TABLE 1. Cutaneous Reactions of Guinea Pigs Preimmunized with 50 μg of Trifluoroacetyl-Human Albumin in Complete Freund’s Adjuvant

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>Number of Responders/Number Tested</th>
<th>Average Diameter of Erythema (mm)</th>
<th>Induration*</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA–HA</td>
<td>3/3</td>
<td>13</td>
<td>2+</td>
<td>Present</td>
</tr>
<tr>
<td>HA</td>
<td>3/3</td>
<td>20</td>
<td>2+</td>
<td>Present</td>
</tr>
<tr>
<td>TFA–GPA</td>
<td>3/3</td>
<td>11</td>
<td>1+</td>
<td>Absent</td>
</tr>
<tr>
<td>GPA</td>
<td>0/3</td>
<td>0</td>
<td>—</td>
<td>Absent</td>
</tr>
<tr>
<td>Tuberculin</td>
<td>3/3</td>
<td>5</td>
<td>—</td>
<td>Absent</td>
</tr>
<tr>
<td>Halothane</td>
<td>0/3</td>
<td>0</td>
<td>—</td>
<td>Present</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>0/3</td>
<td>0</td>
<td>—</td>
<td>Present</td>
</tr>
</tbody>
</table>

* Expressed on a scale 1+ to 4+.

TABLE 2. Cutaneous Reactions of Guinea Pigs Preimmunized with 50 μg of Trifluoroacetyl–Guinea-pig Albumin in Complete Freund’s Adjuvant

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>Number of Responders/Number Tested</th>
<th>Average Diameter of Erythema (mm)</th>
<th>Induration*</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA–GPA</td>
<td>17/17†</td>
<td>11</td>
<td>2+</td>
<td>Present</td>
</tr>
<tr>
<td>HA</td>
<td>0/51</td>
<td>0</td>
<td>—</td>
<td>Absent</td>
</tr>
<tr>
<td>TFA–HA</td>
<td>4/51</td>
<td>4</td>
<td>1+</td>
<td>Absent</td>
</tr>
<tr>
<td>Tuberculin</td>
<td>17/17</td>
<td>12</td>
<td>2+</td>
<td>Present</td>
</tr>
<tr>
<td>GPA</td>
<td>0/17</td>
<td>0</td>
<td>—</td>
<td>Absent</td>
</tr>
</tbody>
</table>

* Expressed on a scale 1+ to 4+.
† Three animals responded after one injection; the remaining 14 responded only after a booster injection of the same antigenic preparation of TFA–GPA.
‡ This table represents two experiments. Only the five animals in the first experiment were tested for reactivity to HA and TFA–HA.

with either 100 per cent halothane or 1 per cent halothane diluted with physiologic saline solution (0.9 per cent), there was severe local toxicity with immediate necrosis of all layers of the epidermis. Thus, while the conjugates were nontoxic, the parent compounds were extremely irritating.

ANIMALS IMMUNIZED WITH CONJUGATES OF HUMAN SERUM ALBUMIN

Animals immunized with 50 μg of TFA–HA manifested cutaneous hypersensitivity to TFA conjugated to human serum albumin and to HA alone. By 24 hours following challenge erythema and induration had appeared at the test sites. The reactions were most intense at 48 hours, at which time central necrosis was evident in some animals. The positive reactions observed at sites inoculated with TFA–GPA indicated cross reactivity to other hapten conjugates and to the carrier by itself (table 1).

Neither halothane nor methoxyflurane injected alone or administered in the form of sonicates elicited delayed-type hypersensitivity reactions. On the contrary, the response to these materials was an immediate local reaction with prominent cutaneous necrosis. At a dilution of 1/1000, neither an immediate nor a delayed reaction could be elicited.

As would be expected from immunization of hosts with complete Freund’s adjuvant, the sensitized hosts manifested strong reactions to tuberculin. However, mycobacterial sensitivity was less than that which each host mounted against heterologous hapten–protein conjugate.
no carrier-specific component could be identified. Skin-test challenge with guinea-pig albumin alone had negative results. The reactivity manifested by these animals was, therefore, unequivocally directed toward the hapten, trifluoroacetate (fig. 1).

HISTOLOGY

Histologic examination of 24–48-hour skin-test sites on sensitized guinea pigs supported the hypothesis of delayed-type hypersensitivity reactions which had been formulated from the gross appearance and time course of the reactions. In agreement with Turk's description of the prototype tuberculin delayed-type reaction, we observed erythema and induration at the skin test site after 6 hours, with maximum intensity after 24–48 hours. The erythema and induration usually persisted 48–72 hours. Histologic study of the TFA-GPA reaction in an animal sensitized to TFA-GPA (see fig. 2) with hematoxylin–eosin stains disclosed a distinct, predominant perivascular infiltration of mononuclear cells in the deep dermis. Also of interest were the increased vascularization of this tissue, evidence of hypertrophy of endothelial cells, and disruption of the connective tissue, the last probably resulting from edema. A methyl-green pyronin stain showed pyroninophilic transformed lymphocytes in the skin lesion, further suggesting a cellular immune reaction. Blastic transformation of lymphocytes is rarely if ever seen in normal skin.

Discussion

Delayed hypersensitivity, more commonly referred to as "cellular immunity," is one of the immune responses expressed by a host to a foreign substance usually called an "antigen." This type of defense mechanism is usually beneficial, affording protection against bacteria, viruses, and fungi, but in some situations it may cause potentially harmful effects, as seen in contact dermatitis, the rejection of transplanted tissue, and certain immune disorders.

The work presented herein demonstrates the induction of specific delayed-type hypersensitivity to a metabolite of halothane and
fluroxene, trifluoroacetate, conjugated to a protein, serum albumin. When trifluoroacetate–guinea-pig albumin conjugates were administered to guinea pigs, a large measure of the immunologic specificity appeared to be conferred by the trifluoroacetate group alone, in a fashion similar to that found by Leskowitz and Jones, using azobenzencarsonate as a hapten. A contribution of the determinants adjacent to the site of attachment of the hapten was suggested by the reactions of animals immunized with trifluoroacetyl–human albumin. In this case, not only were challenge reactions to the TFA–HA hapten–carrier complex significantly stronger than those to the TFA–GPA complex as observed in the reverse approach with immunization with guinea-pig complexes, but also the host was responsive to inoculation of human albumin alone. While the exact molecular nature of the effective immunizing hapten–peptide moiety could be elucidated by immunologic techniques, such as induction of specific unresponsiveness or desensitization with cross-reacting conjugates, the specificity of the observed reactions suffices to prove that the reactivity was developed in response to the trifluoroacetyl group per se.

The demonstration that trifluoroacetate compound has the capacity to stimulate a response in an animal model system suggests that human beings could be sensitized in a similar fashion. However, the relationship between experimental sensitization with adjuvant and possible clinical responses to
Inhalant agents in the absence of adjuvant is uncertain. Since the present work suggests that at least one of the metabolites of fluoroxene and halothane can function as a hapten in the immune response, an observation never before established, further investigations in this area are justified. We** have recently observed the production of humoral antibodies in animals immunized with the same hapten conjugates. We are presently investigating whether hapten—carrier conjugates could be used as tools to detect evidence of immune responses in patients afflicted with noninfectious postoperative hepatitis. Finally, studies are in progress to determine whether experimental hepatic damage can be engendered by appropriate treatment of normal animals with combinations of conjugates, hepatotoxins, and immunoreactive drugs.


The authors gratefully acknowledge the help of Drs. Richard J. Kitz and Edward Lowenstein in reviewing the manuscript. They appreciate the suggestions of Dr. Myron B. Lauer, and the unlimited use of the laboratory facilities of Dr. Ronald Malt and Dr. Paul Russell. The photograph of the guinea pig was taken by Dr. Neville Davis.

References