Humoral Immunity to a Metabolite of Halothane, Fluroxene, and Enflurane

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

Trifluoroacetate, a common metabolite of halothane, fluroxene, and enflurane, conjugated to guinea-pig albumin elicits specific serum antibody in guinea pigs. Two classes of antibodies were found: hemolytic, gamma-2, and anaphylactic, gamma-1. Repeated injections of the antigen, trifluoroacetyl-guinea pig albumin, often led to disappearance of circulating antibodies. (Key words: Anesthetics, volatile, halothane; Anesthetics, volatile, fluroxene; Anesthetics, volatile, enflurane; Biotransformation (drug); Allergy; Immune response.)

HEPATIC NECROSIS following anesthesia and operation, although rare (less than 1/35,000), may be fatal. The pathogenesis of the syndrome is unclear, and diagnostic methods to detect susceptible individuals are not available. A diagnostic test for patients with hepatic dysfunction following exposure to halogenated anesthetics could be developed based upon altered immunity in these individuals. The reports of increased incidences of neoplasms in female nurse-anesthetists and abortions in all female operating room personnel could be interpreted as evidence of disturbed immune mechanisms.

Moreover, the increased occurrences of serum hepatitis and hepatic disease in anesthesiologists, compared with the population at large, may reflect an altered immune response. A metabolite of three halogenated anesthetics, trifluoroacetate, conjugated to guinea-pig albumin, has been shown to induce delayed-type hypersensitivity in the guinea pig. The present study demonstrates that humoral antibodies, of both the complement-fixing, 2-mercaptoethanol-resistant gamma-2 type and the anaphylactic gamma-1 type, were detectable following immunization with trifluoroacetyl-guinea-pig albumin (TFA-GPA). However, TFA-GPA appeared to be a weak antigen, since repeated injections resulted at times in ablation of the immune response.

* Assistant Professor of Anesthesiology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114.
† Doctoral Candidate at Tuft University Medical School, Boston.
‡ Assistant Professor of Surgery, Northwestern University Medical School and the Veterans Administration Research Hospital, Chicago. Recipient of a Schweppes Career Development Award.
§ Associate Professor of Medicine, Harvard Medical School, Massachusetts General Hospital.

Received from the Department of Anesthesia at Harvard Medical School at Massachusetts General Hospital, Boston, Massachusetts, and the Laboratory of Surgical Immunology, Northwestern University Medical School and Veterans Administration Research Hospital, Chicago, Illinois. Accepted for publication November 18, 1974. Supported by USPHS grant # GM 15904-06 and # AM 3564 and a grant from Ayerst Laboratories. A portion of this work was presented at the annual meeting of the American Society of Anesthesiologists, San Francisco, October 1973.

Address reprint requests to Dr. Mathieu.

ABBREVIATIONS

CFA = complete Freund's adjuvant
IFC = incomplete Freund's adjuvant
2-ME = 2-mercaptoethanol
PCA = passive cutaneous anaphylaxis
SRBC = sheep erythrocytes
TFA-GPA = trifluoroacetylated guinea-pig albumin
TFA-HA = trifluoroacetylated human albumin
Materials and Methods

ANIMALS

Outbred male albino guinea pigs (English short-hairs, Elm Hill Breeding Farms, Mass.), weighing 450–500 g, were maintained on a standard feed diet supplemented with ascorbic acid.

PREPARATION OF CONJUGATES

The conjugates, trifluoroacylated guinea-pig albumin (TFA–GPA) and trifluoroacylated human albumin (TFA–HA) were prepared as previously described. Human albumin and guinea-pig albumin (Fraction V, crystallized) were used (Sigma Chemical Company, St. Louis, Mo.).

IMMUNIZATION AND BLEEDING

Two immunization schemes were used. In Protocol A, Guinea pigs immunized with TFA–GPA in complete Freund’s adjuvant (CFA) following that after this treatment had cutaneous delayed-type hypersensitivity were used. One week after the skin test, the animals were bled by cardiac puncture. At 2–3-week intervals thereafter, they were rebled, and on the same day received 100 µg of TFA–GPA in saline solution intravenously. In Protocol B, the guinea pigs received 100 µg of TFA–GPA in incomplete Freund’s adjuvant (IFA) intradurally. They were bled by cardiac puncture, and thereafter in alternate weeks were either boosted with 100 µg TFA–GPA in IFA intradermally, or bled.

ANTIBODY ASSAYS

Complement-fixing (hemolyzing) antibodies were detected using sheep erythrocytes [red blood cells] (SRBC) treated with tannic acid for adsorption with TFA–GPA as described by Bloch et al. The antibody titer represented the highest dilution of serum that caused 100 per cent hemolysis of a standard concentration of sheep erythrocytes. Anaphylactic antibodies were sought by the method of Ovary et al. Undiluted, non-absorbed test antisera (0.1 ml) were injected intradermally into shaven skin areas on the backs and flanks of untreated normal albino guinea pigs weighing 300 g. Six hours later, 1 ml of a solution of 1 mg TFA–GPA in 1 per cent Evans blue dye diluted in saline solution was injected intravenously. Positive reactions, as shown by blue spots representing local antigen–antibody reactions resulting in the release of dye from the vessels, appeared 5 to 20 minutes after the injection. Serum samples were scored as either positive or negative.

While the complement-fixing activity of antibody of the IgM class is sensitive to 2-mercaptoethanol (2-ME), that of IgM antibody is resistant to this treatment. Antisera were diluted 1:10 in Veronal buffer, pH 7.4 (stock 5 x contained per liter: NaCl 41.5 g; Na barbital 5.08 g; 17.2 ml of 1.0 N HCl: 0.5 g MgCl2 · 6 H2O; 0.11 g CaCl2 · 2 H2O) and 2-ME was added to a final concentration of 0.1 M. The mixture was incubated at 37 C for 1 hour and dialyzed for 36 hours against the Veronal buffer. pH 7.4, at 4 C. Controls included portions of the serum sample incubated in the absence of 2-ME and dialyzed under the same conditions.

Results

HEMOLYTIC (GAMMA-2) ANTIBODY TO TFA–GPA

Of six guinea pigs immunized with TFA–GPA, according to Protocol A, three showed hemolytic antibody (table 1). Preimmunization sera were negative for all antigens tested. A sample from the first postimmunization bleeding showed positive titers: 1/20,000 against TFA–GPA-coated cells; 1/64 in two animals and 1/20,000 in one animal against TFA–HA-coated cells; 1/64 against GPA-coated cells. These data suggest that antibody activity was directed predominantly toward the trifluoroacetyl moiety combined with GPA. After continued intravenous injections of antigen, there was no detectable hemolytic activity (Bleed 2, Bleed 3). However, when unresponsive animals were subsequently reimmunized by intradermal injection of TFA–GPA in IFA, they again produced hemolytic antibody, although of considerably lower titer than originally.
PASSIVE CUTANEOUS ANAPHYLACTIC PCA
GAMMA-1 ANTIBODY TO TFA–GPA

Sera from animals injected with TFA–GPA were also tested for PCA antibody activity. Four of five sera that no longer possessed detectable complement-fixing gamma-2 antibody (table 1) had positive PCA activity. These results were extended in another experiment. Six guinea pigs were immunized according to Protocol B. The first bleeding, which was negative for hemolytic antibody in six animals, did show PCA antibody in five of these hosts. Four sera were negative, and one contained only gamma-1 activity. Unfortunately, only five guinea pigs survived the second injection of TFA–GPA in IFA. Two animals produced no antibody of either class, one maintained low-grade titers of gamma-1 and gamma-2 antibodies, and two others produced strong antibodies of both classes.

MERCAPTOETHANOL SENSITIVITY OF THE ANTIBODIES

To determine whether a predominately IgM antibody response accounted for the short-lived activity observed, the sera listed in table 2 were treated with 2-ME and retested. This treatment had no effect on the hemolytic titers of the sera, indicating that the activity was due to the 7S, IgG class, rather than the 19S antibody species.

Discussion

Halothane metabolites conjugated to albumin can serve as haptons to induce cell-mediated and humoral immunity. The present study demonstrated that when the hapten is administered in complete Freund’s adjuvant, there is synthesis of hemolytic and anaphylactic antibodies. High-titer hemolytic antibodies appeared seven weeks after the animals had shown cellular immunity by cutaneous reactivity, although a serum sample obtained one week after the skin test was negative.

Studies to evaluate the immunogenicity of TFA–GPA without adjuvant were not attempted. Previous work in our laboratory indicated that this antigen, even in CFA, was weak, necessitating at times two injections in adjuvant before delayed-type hypersensitivity was observed. In order to answer the question whether antibodies could be produced, we used optimal experimental conditions, i.e., use of CFA, and booster injections.

Rosenberg and Wahlstrom claimed that rabbits immunized with a ten-month course of weekly subcutaneous injections of chicken serum globulin and trifluoroethanol, trifluoroaldehyde hydrate, trifluoroacetic acid, or trifluoroacetic anhydride showed specific precipitating antibody. The antibody reacted against all the antigens, independently of the complexes used for immunization. However, the nature of the complexes is unclear, since attempts were not made to quantitate the amount of substitution on the protein molecule. Since antibody activity was heterogeneous serologically and chemically, as evidenced by the multiple precipitin bands, interpretation of the antibody spe-

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antiserum Titers</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-immunization</td>
<td>Bleed 1</td>
<td>Bleed 2</td>
<td>Bleed 3</td>
</tr>
<tr>
<td>TFA–GPA-coated SRBC</td>
<td>0</td>
<td>20,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GPA-coated SRBC</td>
<td>0</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TFA-HA-coated SRBC</td>
<td>0</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HA-coated SRBC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tannned uncoated SRBC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Of six animals immunized, three animals did not respond; the results represent titers obtained in three animals.
† One animal of three had a titer of 20,000; the other two, titers of 64.
specifically is uncertain. The only antibody directed to TFA was shown by a weak precipitin line with trifluoroacetylated rabbit serum albumin. Unfortunately, chicken serum globulin, being a heterologous protein, is a strong antigen in rabbits and would mask any other weak reactions.

Using the immunization protocols outlined above, both complement-fixing and PCA antibodies to TFA-GPA could be detected. The failure to observe either gamma-1 or gamma-2 antibodies following TFA-GPA administration in prior experiments may be explained by the marked fluctuations in antibody titers documented in the present study.

Since the guinea pigs used in this study were an outbred strain, failure to observe uniform responses was not surprising. It is well established that the immune response to both synthetic polypeptides and other antigens is genetically controlled, namely, animals are weak responders or strong responders. Furthermore, there is variation in the intensity of response and in the class of antibody produced; for example, mice and guinea pigs that are weak responders mount only a primary 19S response that does not progress to the predominantly 7S anamnestic response. Therefore, it is not surprising that not all of the guinea pigs responded to TFA-GPA.

Of interest was the disappearance of the hemolytic antibody in response to additional intravenous injections of 100 μg TFA-GPA into animals that had previously responded strongly. This observation may be explained by: 1) the poor immunogenicity of the conjugate which, when repeatedly injected in saline solution, may have acted as a tolerogen. Since the antigen is weak, it may be that very few memory cells were produced, and the response simply faded. Such weak immune responses have been observed with autologous antigens. In this regard, it was rather difficult to induce delayed-type hypersensitivity toward TFA conjugated to autologous carriers. The majority of animals responded only after two injections of antigen in CFA. The disappearance of antibody may also have been due to 2) the production of blocking antibodies, which interfered with complement-fixing activity, or 3) the formation of soluble antigen–antibody complexes.

1) The possibility of a non-anamnestic IgM response seems ruled out by the insensitivity of the antibodies to mercaptoethanol. 5) If immunodeviation, namely a switch in the class of antibody produced from predominantly gamma-2 (hemolytic) to gamma-1 (hemocytotropic) PCA antibody occurred, it was reversible upon reimmunization with antigen in complete Freund’s adjuvant. In some cases, after a course of two injections of antigens in IFA, hemolytic antibodies again appeared, in significantly reduced titers. Therefore, the altered responsiveness was not total, but rather incomplete.

Guinea pigs immunized with TFA-GPA and found positive for gamma-1 anaphylactic and gamma-2 hemolytic antibodies did not have liver biopsies performed, since the technique was used only to investigate the production of immunity and not for study of possible hepatic damage by the hapten–protein complex. However, in a subsequent study, similarly immunized animals ex-

<table>
<thead>
<tr>
<th>Animal</th>
<th>PCA</th>
<th>First Bleeding Hemolytic</th>
<th>Second Bleeding PCA</th>
<th>Hemolytic</th>
<th>Third Bleeding PCA</th>
<th>Hemolytic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>50</td>
<td>+</td>
<td>320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>320</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>320</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The first bleeding was three weeks after intradermal injection of 1 mg TFA-GPA in IFA; the second bleeding, five weeks after injection; the third bleeding, seven weeks after the first injection and one week after the booster.

† ND = not determined.
posed to 1 per cent halothane on five or six occasions for 1 hour at weekly intervals were found to have the same incidences of hepatic necrosis and abnormal liver function tests (bilirubin, SGOT, LDH, alkaline phosphatase) as nonimmunized animals. Moreover, the latter group, exposed to halothane five or six times, with elevated results of liver function tests, did not have circulating antibodies to TFA or manifest delayed-type hypersensitivity to this antigen. These data suggest that delayed hypersensitivity might not play a role in the development of hepatitis associated with halothane in the guinea-pig model, but they might support the concept of hepatic damage occurring in the absence of antibodies.

Indeed, the number of animals studied was small; but our results suggest that antibodies, rather than promoting tissue damage, may protect the animals, perhaps by “mopping up” the antigen. This concept raises two interesting questions. Are patients exposed to halothane repeatedly for repeated surgical interventions (e.g., radium implant) producing antibodies that block subsequent tissue damage? Are individuals chronically and occupationally exposed to halothane also protected by such antibodies? (This would indicate the overall safety rather than the harmful effect of this agent, at least in the experimental situation.) Further investigations to determine the presence of serum antibody to halothane or its metabolites in individuals receiving multiple halothane anesthesia, in individuals chronically exposed to anesthetics, and in patients with unexplained hepatitis after halothane should shed some light on the relevance, if any, of these antibodies.

The present study demonstrates that conjugates of trifluoroacetate, a metabolite of three halogenated anesthetics, and analogous guinea-pig albumin were generally weak immunogens. However, some animals did produce high-titer complement-fixing antibody upon immunization.

The practical application and the relevance of this study, i.e., the production of antibodies specific to trifluoroacetate, are that, after purification by affinity columns, they can be used as markers, thus as a diagnostic handle to identify the presence of TFA in or near the area of necrosis in the liver. For this purpose, we will apply standard immunofluorescent procedures to human and guinea-pig liver biopsies. Such studies can tell us whether TFA is associated with hepatic damage, irrespective of the mechanism involved. The antibody possessed specificity for TFA, thus offering the possibility of a serologic diagnostic test for the presence of this metabolite in sera. We emphasize that our paper describes humoral immunity to trifluoroacetethylated compounds in guinea pigs. It does not, however, describe a definitive, but rather a potential, laboratory test.

The authors gratefully acknowledge the assistance of Professors L.D. Vandam and R.J. Kitz in reviewing the manuscript.

References