INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of “sectioning” the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.
Siadat Pajouh, Majid

GENERATION OF HALOTHANE INDUCED ANTIBODY IN GUINEA PIGS AND ITS POSSIBLE ROLE IN THE PATHOGENESIS OF HALOTHANE INDUCED LIVER INJURY

The University of Arizona

University Microfilms International  300 N. Zeeb Road, Ann Arbor, MI  48106
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages ______
2. Colored illustrations, paper or print ______
3. Photographs with dark background ______
4. Illustrations are poor copy ______
5. Pages with black marks, not original copy ______
6. Print shows through as there is text on both sides of page ______
7. Indistinct, broken or small print on several pages ______
8. Print exceeds margin requirements ______
9. Tightly bound copy with print lost in spine ______
10. Computer printout pages with indistinct print ______
11. Page(s) _________ lacking when material received, and not available from school or author.
12. Page(s) _________ seem to be missing in numbering only as text follows.
13. Two pages numbered ______. Text follows.
14. Curling and wrinkled pages ______
15. Dissertation contains pages with print at a slant, filmed as received ______
16. Other______________________________________________________________

University
Microfilms
International
GENERATION OF HALOTHANE INDUCED ANTIBODY IN GUINEA PIGS AND ITS POSSIBLE ROLE IN THE PATHOGENESIS OF HALOTHANE INDUCED LIVER INJURY

by

Majid Siadat Pajouh

A Thesis submitted to the Faculty of the DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY In Partial Fulfillment of the Requirements For the Degree of MASTER OF SCIENCE In the Graduate College THE UNIVERSITY OF ARIZONA 1986
STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or by the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: M. Siadat Pajouh

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

ANDREA K. HUBBARD
Research Assistant Professor, Microbiology/Immunology

Date 12/15/86
ACKNOWLEDGMENTS

My special thanks and appreciation to Dr. Andrea K. Hubbard for all her guidance and assistance during my Graduate Program, research training and preparation of this thesis; Dr. David Lucas for his guidance and helpful comments during this study.

The author wishes to express his sincere gratitude to Dr. A.J. Gandolfi for his intellectual guidance and financial support throughout this study.

Finally, I wish to express my gratitude to Tim Roth, Richard Lind, and John Levy for the technical assistance they graciously offered and Patricia Kime for her professional assistance in the typing of this thesis.

This work was supported in part by NIH Grant GM34788.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Physical, Chemical and Physiological Properties of halothane</td>
<td>2</td>
</tr>
<tr>
<td>Biotransformation of Halothane</td>
<td>3</td>
</tr>
<tr>
<td>Animal Models</td>
<td>5</td>
</tr>
<tr>
<td>Clinical Features</td>
<td>8</td>
</tr>
<tr>
<td>Etiology</td>
<td>8</td>
</tr>
<tr>
<td>A. A Role for Mediated Immunity in Halothane Hepatitis</td>
<td>9</td>
</tr>
<tr>
<td>B. A Role for Humoral Immune Response in Halothane Hepatitis</td>
<td>10</td>
</tr>
<tr>
<td>STATEMENT OF PURPOSE</td>
<td>12</td>
</tr>
<tr>
<td>MATERIALS</td>
<td>13</td>
</tr>
<tr>
<td>Chemicals</td>
<td>13</td>
</tr>
<tr>
<td>Animals</td>
<td>15</td>
</tr>
<tr>
<td>METHODS</td>
<td>16</td>
</tr>
<tr>
<td>Synthesis of Trifluoroacetylated-Guinea Pig Serum Albumin (TFA-GPA)</td>
<td>16</td>
</tr>
<tr>
<td>Studies on the Generation of Halothane Induced Antibodies in Strain 2 Guinea Pigs</td>
<td>16</td>
</tr>
<tr>
<td>Study of the Effect of Immunization with TFA-GPA on the Potentiation of Liver Injury in Halothane Exposed Guinea Pigs</td>
<td>17</td>
</tr>
<tr>
<td>Study of the Correlation between Halothane Induced Liver Injury and SGPT in Guinea Pigs and the Possible Role of Halothane Induced Antibody in the Liver Injury</td>
<td>18</td>
</tr>
</tbody>
</table>
## TABLE OF CONTENTS - Continued

**METHODS - Continued**

- Characterization of Halothane Induced Antigen with Halothane Induced Antibodies from Guinea Pigs and Rabbits... 19

**ANALYSIS**

- Assessment of Antibody Response and SGPT Levels........ 20
- Immunization of Guinea Pigs with TFA-GPA............ 21
- Detection of Halothane Induced Antigens............... 21
  - Ammonium Sulfate Precipitation....................... 21
  - DEAE Sepharose Chromatography...................... 22
  - Coupling of the antibody to CNBr-Sepharose........ 22
  - SDS-PAGE........................................ 23
  - Western Blot Technique............................. 23

**ANALYTICAL ASSAYS**

- 25

**RESULTS**

- Halothane Induced Antibodies in Strain 2 Guinea Pigs...... 26
- Halothane Induced Antibodies in Strain Amana Guinea Pigs.................................................. 29
- Halothane Induced Antibodies in Hartley Guinea Pigs...... 29
- The Effect of Pre-Existing Anti-TFA Antibodies on Halothane Toxicity................................. 33
- Chronology of Halothane Induced Liver Injury in Guinea Pigs.............................................. 33
- Characterization of Halothane Induced Antigens with Halothane Induced Antibodies from Guinea Pigs and Rabbits... 37

**DISCUSSION**

- 49

**CONCLUSION**

- 55

**REFERENCES**

- 56
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Association between halothane induced antibody titers and SGPT levels in a Strain 2 guinea pig following multiple halothane exposures</td>
<td>27</td>
</tr>
<tr>
<td>2.</td>
<td>Lack of association between halothane induced antibody titers and SGPT levels in a Strain 2 guinea pig</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>Halothane induced antibody titers and SGPT levels in an Amana guinea pig following halothane exposures</td>
<td>30</td>
</tr>
<tr>
<td>4.</td>
<td>Halothane induced antibody titers and SGPT levels in an Amana guinea pig following halothane exposures</td>
<td>31</td>
</tr>
<tr>
<td>5.</td>
<td>Halothane induced antibody titers in Hartley guinea pigs following multiple halothane exposures</td>
<td>32</td>
</tr>
<tr>
<td>6.</td>
<td>SGPT values in Hartley guinea pigs following multiple halothane exposures</td>
<td>34</td>
</tr>
<tr>
<td>7.</td>
<td>The induction of a secondary antibody response in an immunized (TFA-GPA) Amana guinea pig following halothane exposures</td>
<td>35</td>
</tr>
<tr>
<td>8.</td>
<td>The induction of a secondary antibody response in an immunized (TFA-GPA) Amana guinea pig following halothane exposures</td>
<td>36</td>
</tr>
<tr>
<td>9.</td>
<td>Photomicrograph of the liver section from a Hartley pig on day one following the first halothane exposure</td>
<td>38</td>
</tr>
<tr>
<td>10.</td>
<td>Same photomicrograph as Figure 9 with a higher magnification</td>
<td>39</td>
</tr>
<tr>
<td>11.</td>
<td>Photomicrograph of the liver section from a Hartley guinea pig on day three following the first exposure</td>
<td>40</td>
</tr>
<tr>
<td>12.</td>
<td>Same photomicrograph as Figure 11 with a higher magnification</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Illustration Description</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>13.</td>
<td>Photomicrograph of the liver section from a Hartley guinea pig on day seven following the first exposure</td>
<td>42</td>
</tr>
<tr>
<td>14.</td>
<td>Same photomicrograph as Figure 13 with a higher magnification</td>
<td>43</td>
</tr>
<tr>
<td>15.</td>
<td>Photomicrograph of the normal liver section from a Hartley guinea pig</td>
<td>44</td>
</tr>
<tr>
<td>16.</td>
<td>Identification of halothane induced proteins in the livers of exposed guinea pigs and rabbits</td>
<td>47</td>
</tr>
<tr>
<td>17.</td>
<td>Identification of halothane induced proteins in the livers of exposed guinea pigs and rabbits</td>
<td>48</td>
</tr>
</tbody>
</table>
ABSTRACT

This study has focused on investigating a role for the immune system in mediating halothane induced hepatitis. A guinea pig model of halothane hepatitis explored the humoral immune response induced by multiple halothane exposures and the potential role this response might play in contributing to liver damage. Three different strains (strain 2, Amana, and Hartley) of guinea pigs were exposed to 1% halothane (under 21% O\textsubscript{2} or 80% O\textsubscript{2}) for 4 hours at two week intervals. Blood samples were taken at various days post each exposure and evaluated for antibodies cross-reactive with trifluoroacetylated-guinea pig serum albumin (TFA-GPA) in an enzyme linked immunosorbent assay (ELISA). Additionally, SGPT (serum glutamate pyruvate transaminase) as well as liver histology were evaluated as markers of hepatic damage. Each strain demonstrated a halothane induced antibody that was cross-reactive with TFA-GPA. The concentration of this antibody in Hartley guinea pigs was approximately six fold higher than in the other two strains. Moreover, the peak of liver necrosis occurred on day 3 post each exposure as evidenced by degenerated hepatocytes and fat deposition. In addition, guinea pigs from strain Amana were immunized with TFA-GPA and then exposed to halothane to determine if pre-existing antibodies would exacerbate halothane induced liver damage. Immunization of guinea pigs with TFA-GPA generated a specific anti-TFA-GPA antibody. However,
presence of this antibody did not potentiate the liver damage induced by halothane exposure. In experiments to identify any halothane induced antigens, both halothane induced antibodies and specific anti-TFA-GPA antisera detected five unique antigens in liver homogenates from halothane exposed guinea pigs. Thus multiple halothane exposures to guinea pigs do induce an antibody response which not only cross-reacts with TFA-GPA but also potentially can recognize halothane induced antigens in the guinea pig liver.
INTRODUCTION

Halothane, or 2-bromo-2-chloro-1,1,1 trifluoroethane(CF₃CCLBrH), a volatile anesthetic, was introduced into clinical practice in 1956 (Bunker et al., 1969). Because of its superior properties, halothane soon became the most commonly used anesthetic in general surgery. Since some halogenated compounds have a history of inducing liver necrosis, prolonged testing in animals preceded the introduction of halothane into the market. Imperial Chemical Industries (ICI) stated that, "The introduction of the halogen atoms into ethane has produced a desired, non explosive anesthetic with high potency". They also claimed that low toxicity could be expected because of the strength of fluorine-carbon bond, suggesting no substantial chemical alteration of the molecule in the body (Aach et al., 1968). It has been proven that halothane has many advantages over other anesthetics; However, the first cases of liver dysfunction following halothane administration were reported in 1958 (Virtue et., 1958) and were soon followed by similar reports (Brit et al., 1965). As a result of increasing clinical and public concern of severe liver necrosis following halothane anesthesia, the National Halothane Study was instituted in 1963 (Bunker et al., 1969). This retrospective study was designed to assess the real risk to the patient. At the completion of the study it was found that postoperative severe liver injury could be explained by circulatory shock, sepsis or chronic hepatic disease. However, the
study could not rule out the possibility of halothane induced hepatocellular damage following single or multiple administration of halothane.

Since the publication of the National Halothane Study, an enormous amount of research has been done in an attempt to confirm the existence, incidence and, more recently, the cause of halothane induced hepatitis. The rationale in these efforts is in part due to the fact that, halothane is still a widely used anesthetic in many countries. Furthermore, insight into the mechanism of halothane hepatitis may predict the likelihood of toxicity with new anesthetic agents.

**Physical, Chemical and Physiological Properties of Halothane**

Halothane is a colorless and volatile liquid with a molecular weight of 197, a boiling point of 50.2 and vapor pressure at 20°C of 243 mm Hg. When stored in amber bottles or in clinical vaporizers and protected from light, halothane is stable and degradation products are not found even after long periods of storage. Its stability is further enhanced by the addition of 0.01% thymol (Goodman, The Pharmacological Basis of Therapeutics, 1967).

In general, halothane has a great many of the qualities that have been listed for an ideal anesthetic agent. It is non-irritating and does not cause nausea. Halothane is nonexplosive which is critical considering the variety of electrical equipment in operating rooms. Since halothane is a very potent anesthetic, it can be administered at 1% concentration in the presence of 99% oxygen. Furthermore, it is
capable of producing anesthesia to any depth. Concentration of halothane vapor required to induce and maintain anesthesia depends on several factors, including the physical status of the patient, the degree of sedation from preanesthetic medication and the adjunctive use of other anesthetics. Halothane is considerably cheaper than enflurane and particularly isoflurane, two relatively new anesthetics that are used as halothane substitutes. However, halothane has some disadvantages. It causes only moderate muscle relaxation, often requiring adjunctive use of a specific relaxant. It is also a respiratory depressant, therefore it reduces the respiratory amplitude and slightly increases the rate of respiration. Additionally, halothane depresses cardiovascular functions with a moderate depth of anesthesia, hypotension and reduced cardiac output (Bunker et al., 1969).

**Biotransformation**

Halothane (CF₃CHBrCl), was initially considered to be refractory to metabolism. However, further investigation revealed that it is metabolized in man and animals (Van Dyke et al., 1969). Since then, the metabolism of halothane has been extensively studied in order to determine the possible role of its metabolites or reactive intermediates in the pathogenesis of halothane induced hepatitis. It is clearly shown that liver microsomal cytochrome P-450 metabolizes halothane through two different pathways (Maiorino et al., 1979; Sipes et al., 1980). Under aerobic conditions, halothane is metabolized by the oxidative pathway, whereas, under anaerobic conditions, the
metabolism is through the reductive pathway. During halothane oxidation, trifluoroacetyl halide (CF$_3$COCl) intermediate is produced and can either potentially acetylate liver macromolecules to form trifluoroacetylated adducts or be hydrolysed to form trifluoroacetic acid (CF$_3$COOH) (Jeremy et al., 1985). The result of halothane reduction in the reductive pathway is 1-chloro-2,2,2-trifluoroethyl radical (CF$_3$CHClBr$^-$). This reactive intermediate may also react with liver microsomal proteins, lipids, or other unidentified targets, to form adducts or can abstract a hydrogen atom to form 1-chloro-2,2,2 trifluoroethane (CF$_3$CH$_2$Cl). This radical can be further reduced to form 1-chloro-2,2,2 trifluoroethyl carbanion (CF$_3$CHCl:), which with the elimination of a fluoride ion results in 1-chloro-2,2-difluoro ethylene (CF$_2$CHCl) (Jeremy et al., 1985). Bromide is released as a result of both oxidative and reductive pathways. However, the release of fluoride is only representative of halothane metabolism through the reductive pathway. It has been shown that halothane can be metabolized by liver microsomal enzymes to its metabolites (Van Dyke et al., 1965).

In addition, Servin et al demonstrated that hyperthyroid patients metabolize halothane to a greater degree than do euthyroid patients (Servin et al., 1986); hyperthyroid patients have higher plasma bromide and fluoride levels after halothane exposure. It has also been shown, in rats pretreated with phenobarbital and exposed to halothane, that bioactivated metabolites of halothane can covalently bind to liver subcellular macromolecules (Gandolfi et al., 1980). In addition, it has been demonstrated that liver sections from rats four hours after an
intraperitoneal injection of halothane can be stained, preferentially in centrilobular region, with anti-TFA antibodies. These data suggest that halothane metabolites or reactive intermediates may form antigens which could induce an immune response to mediate liver injury.

Animal Models

Three models have been developed to assess halothane associated liver injury. The first model describes phenobarbital pretreated rat exposed to halothane in an hypoxic environment (McLain et al., 1979). Phenobarbital treatment and hypoxia, which are necessary for liver damage in this animal model, favors the metabolism of halothane through the reductive pathway (Cousins et al., 1979). Recently the validity of the phenobarbital/hypoxic (PB/hypoxic) rat as a model of halothane hepatotoxicity, has come into question (Van Dyke et al., 1982). The ease of reproducibility in this model follows a seasonal variation (Strunin et al., 1983). In addition, a second model of liver injury demonstrated that hypoxia alone could produce hepatic necrosis in phenobarbital pretreated rats (Shingu et al., 1982). Furthermore, enflurane and isoflurane are both capable of producing hepatic necrosis in PB/hypoxic, starved rats (Van Dyke et al., 1982). Since metabolism of enflurane and isoflurane is less than halothane and reductive metabolism is minimal, an alternative mechanism (indirect toxicity) for hepatotoxicity in this rat model is more likely (Shingu et al., 1983). In addition, the incidence of hepatic necrosis was higher in PB/hypoxic rats exposed to high concentrations of halothane for shorter periods
than to low concentrations for longer periods (Shingu et al., 1982). Since it has been shown that the metabolism of halothane is proportionally greater at low (subanesthetic) concentrations (Sawyer et al., 1971), this is the converse of what one would expect if metabolites were directly responsible for toxicity in this model. Rats pretreated with triiodothyronine ($T_3$) prior to halothane exposure is the third model of halothane toxicity. It has been demonstrated that centrilobular necrosis occurs in this $T_3$ model following halothane exposure for two hours (Uetrecht et al., 1983). In addition, $T_3$ pretreated fasted animals have lower SGPT levels than fed animals.

As an alternate mechanism to chemical induced hepatotoxicity the immune system may also mediate damage. A rabbit model was developed in order to study the humoral immune response to a reactive intermediate of halothane and the possible role of this response in the pathogenesis of halothane induced liver injury (Callis et al., 1985). Although anti-TFA antibodies were generated, no significant halothane induced liver necrosis or elevation in SGPT levels, a marker of liver necrosis, has been evidenced in this animal model. Consequently, a guinea pig model of halothane induced hepatotoxicity was developed. This model appears to mimic more closely the human situation, since neither enzyme induction nor hypoxia is required for the production of liver necrosis in guinea pigs (Lunam et al., 1985). Although hypotension occurs in this model with both halothane and isoflurane (Lunam et al., 1983), hepatic necrosis is only seen with halothane, suggesting that hypotension is unlikely to be responsible for the liver
damage observed. Recent studies with this model have also revealed that liver necrosis can be produced under both oxidative and reductive conditions (Lunam et al., 1985).

Lunam et al also demonstrated that exposing of the guinea pigs to 1% halothane in 21% oxygen resulted in liver damage in these animals evidenced by SGPT elevation and observation of scattered foci of necrosis throughout the liver lobules. They also showed that damage was present on the second and third days after anesthesia and by day seven the livers had recovered. In addition, they demonstrated that administration of halothane in 14% or 80% oxygen did not alter the extent or incidence of liver damage. Hughes et al also demonstrated the induction of hepatic lesions in guinea pigs after repeated administration of halothane. Studies by Lind et al revealed that there are sex and strain differences in the response of guinea pigs to halothane anesthesia (Lind et al., 1985). They demonstrated that females of inbred Strain 2 and male and females of Strain 13 were the most susceptible to halothane exposure as evidenced by extensive centrilobular necrosis as well as elevations in SGPT levels. In addition, inbred Hartley guinea pigs were totally refractory to necrosis, whereas, outbred Amana and male inbred Strain 2 guinea pigs showed an intermediate hepatotoxic response. Although the guinea pig has several advantages over other models, none of the models can completely mimic the fulminant hepatic necrosis seen in humans. Liver necrosis in guinea pigs is similar to the mild hepatic necrosis observed in humans after halothane administration.
Clinical Features

Two forms of halothane associated hepatitis have been reported in patients (Pohl et al., 1982). A mild form, characterized by minor elevations in SGPT levels is reported in about 20% of the patients (Wright et al., 1975). A more severe form of halothane hepatitis, however, is characterized by high SGPT levels and massive hepatic necrosis. This form is much rarer (1 out of 30,000) and is often fatal (Mushin et al., 1971).

Etiology

The etiology of halothane induced hepatitis has always been a controversial issue among investigators. A variety of mechanisms have been suggested over the years on the basis of studies in both humans and animals. These include liver hypoxia (Shingu et al., 1982), peroxidation (Brown et al., 1977), recrudescence of viral hepatitis (Bergani et al., 1980), bioactivation (Sipes et al., 1976) and hypersensitivity (Dienstag et al., 1980). Bioactivation and hypersensitivity are the more popular mechanisms as a cause than the others cited. In bioactivation, damage is chemotoxic and caused directly by reactive metabolite(s) of halothane (Jee et al., 1980). Alternatively, liver damage may result from an immunologic hypersensitivity reaction (Walton et al., 1976). In this case, covalent binding of metabolic intermediate(s) to hepatic proteins or lipoproteins results in a hapten carrier antigen which could evoke an autoimmune response leading to hepatic necrosis in susceptible individuals.
A number of clinical features suggests that drug hypersensitivity or allergy has a role in the pathogenesis of halothane hepatitis (Dienstag et al., 1980). These include rash, arthralgia, peripheral blood eosinophilia, hepatic eosinophilia, granuloma, serum autoantibodies, and a history of atopy. Additionally, epidemiologic data indicate that the disease occurs more commonly after repeated exposures and with each exposure the latency period shortens and severity increases.

**Cell Mediated Immunity in Halothane Hepatitis**

In support of a hypersensitivity mechanism, investigators have attempted to demonstrate a cell mediated immune response to halothane or to one of its metabolite(s) in patients with halothane induced hepatitis. Vergani et al demonstrated the inhibition of leukocyte migration in eight of the twelve patients with halothane hepatitis in response to liver homogenates obtained from rabbits pretreated with halothane (Vergani et al., 1978). Such sensitization appeared transient during early acute illness and was not observed in normal controls, persons exposed to halothane in whom hepatitis did not develop, or in patients with liver diseases unrelated to halothane. In addition, Paronetto and Popper demonstrated a positive lymphocyte transformation test in patients with suspected halothane hepatitis (Paronetto et al., 1970). However, other investigators were unable to confirm this observation (Walton et al., 1976). The demonstration by Uehleke et al that halothane metabolite(s) could bind to liver
macromolecules suggested that patients could become sensitized to these metabolite(s) during halothane exposure (Uehleke et al., 1973). In support of this, Reves et al showed the induction of delayed hypersensitivity response in guinea pigs to a halothane metabolite (TFA) (Reves et al., 1976). This sensitization did not, however, exacerbate the liver injury on subsequent halothane exposures.

A Role for Humoral Immune Response in Halothane Hepatitis

Humoral immune responses in mediating liver injury has also been extensively studied in patients with halothane induced hepatitis. Anti mitochondrial antibody has been shown to exist in patients with jaundice after halothane anesthesia (Rodriguez et al., 1969), but not in jaundiced patients treated with other drugs (methyldopa, perphenazine, amino cyclic acid). In addition, Vergani et al detected circulating antibodies that bound to the surface membrane of halothane-altered rabbit hepatocytes in nine of fourteen patients with unexplained hepatitis following halothane administration (Vergani et al., 1980). Vergani et al also showed that normal lymphocytes could be cytotoxic to halothane altered (but not diethylether altered) rabbit hepatocytes after incubating them with serum from patients with halothane induced hepatitis. In addition, they claimed specificity for this test since serum from patients with acetaminophen-induced liver failure did not render lymphocytes cytotoxic to acetaminophen-pretreated hepatocytes. They also mentioned that the presumed halothane altered membrane antigen was only produced when the oxidative
pathway was activated (Neuberger et al., 1981). Furthermore, Satoh et al demonstrated that liver sections from rats four hours after an intra-peritoneal injection of halothane could be stained, preferentially in the centrilobular region, with anti-TFA serum (Satoh et al., 1985). Mathieu et al reported that trifluoroacetate, a common metabolite of halothane and a fluoroxene, conjugated to guinea pig albumin (GPA) could elicit specific serum antibody in guinea pigs (Mathieu et al., 1975). In this case, two classes of antibodies, hemolytic gamma 2 and anaphylactic gamma 1 were identified.

Callis et al demonstrated the induction of halothane induced antibodies, cross reactive with trifluoroacetylated rabbit serum albumin (TFA-RSA) in rabbits exposed multiple times to halothane under oxidative conditions (Callis et al., 1985, 1986). A greater titer of halothane induced antibody was generated under 75% O₂ than under 14% O₂ in rabbits, suggesting a possible role for oxidative metabolite(s) of halothane in the production of a nonself antigen in the liver. In order to assess halothane induced antibody, Kenna et al developed an enzyme linked immunosorbent assay (ELISA), using as antigen liver microsomal fractions from rabbits exposed to halothane. Using this antigen, they detected halothane induced antibodies in sera from 16/24 patients with halothane associated liver failure (Kenna et al., 1984). Furthermore, they demonstrated that this antibody did not exist in sera from 26 normal blood donors, five healthy anesthesists, nor in 12 patients who had received multiple halothane anesthesia without complication.
Statement of Purpose

The objective of this research project is to investigate, in a guinea pig model, a role for the immune system in mediating halothane induced hepatitis.

The Specific Aims of this project are as follows:

1) To assess the humoral immune response of guinea pigs (three different strains) to a halothane metabolite (TFA) following multiple halothane exposures.

2) To correlate the appearance and level of halothane induced antibody with markers of liver damage such as SGPT and tissue injury (histology).

3) To determine if immunization of guinea pigs with TFA-GPA prior to halothane exposure may potentiate the immune response or liver necrosis.

4) To determine if halothane exposure in guinea pigs can induce liver proteins detected by anti-TFA antibodies from halothane exposed guinea pigs or rabbits.
# MATERIALS

## I. Chemicals

Chemicals and biochemicals used in this study are as follows:

<table>
<thead>
<tr>
<th>Name</th>
<th>Quality</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane (fluothane)</td>
<td>U.S.P.</td>
<td>Ayerst Lab., New York, NY</td>
</tr>
<tr>
<td>Ketamine</td>
<td>U.S.P.</td>
<td>Parke-Davis, Morris Plains, NJ</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>G.C.</td>
<td>EM Science, Cherry Hill, NJ</td>
</tr>
<tr>
<td>JB-4 embedding kit, component B</td>
<td>T.M.</td>
<td>Polyscience, Inc., Warrington, PA</td>
</tr>
<tr>
<td>(methacrylate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JB-4 embedding kit, component A</td>
<td>T.M.</td>
<td>Polyscience, Inc., Warrington, PA</td>
</tr>
<tr>
<td>(methacrylate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematoxylin</td>
<td>Cert. for Histology</td>
<td>Polyscience, Inc., Warrington, PA</td>
</tr>
<tr>
<td>Gelatin powder</td>
<td>N.F.</td>
<td>G.T. Baker, Phillipsburg, PA</td>
</tr>
<tr>
<td>Horse radish peroxidase conjugated goat</td>
<td>DEAE</td>
<td>Cooper Biomedical Lab., Malver, PA</td>
</tr>
<tr>
<td>anti guinea pig immunoglobulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABTS</td>
<td>PFS</td>
<td>Sigma Chemical Company, St. Louis, MO</td>
</tr>
<tr>
<td>Sodium dodecyl</td>
<td>E.G.</td>
<td>Sigma Chemical Company, St. Louis, MO</td>
</tr>
<tr>
<td>Name</td>
<td>Quality</td>
<td>Vendor</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>Serum glutamate transaminase reagent</td>
<td>SGPT</td>
<td>Sigma Chemical Company, St. Louis, MO</td>
</tr>
<tr>
<td>Ethylthioltrifluoroacetate</td>
<td>E.G.</td>
<td>Pierce Chemical Co., Packford, IL</td>
</tr>
<tr>
<td>Guinea pig serum albumin</td>
<td>Fractions pure (99%)</td>
<td>Sigma Chemical Co., St. Louis, MO</td>
</tr>
<tr>
<td>BioRad protein assay</td>
<td>R.G.</td>
<td>BioRad Lab., Richmond, CA</td>
</tr>
<tr>
<td>Freund complete and incomplete adjuvant</td>
<td>R.G.</td>
<td>Sigma Chemical Co., St. Louis, MO</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>Grade 1 purified</td>
<td>Sigma Chemical Co., St. Louis, MO</td>
</tr>
<tr>
<td>Cyanogen bromide activated sepharose-4B</td>
<td>Pure</td>
<td>Pharmacia Fine Chem., Switzerland</td>
</tr>
<tr>
<td>Glycine</td>
<td>E.G.</td>
<td>U.S. Biochem. Corp., Cleveland, OH</td>
</tr>
<tr>
<td>Ethyl glycol</td>
<td>R.G.</td>
<td>Em Science, Cherry Hill, NJ</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>E.G.</td>
<td>BioRad Laboratory, Richmond, CA</td>
</tr>
<tr>
<td>Coomassie blue</td>
<td>E.G.</td>
<td>BioRad Laboratory, Richmond, CA</td>
</tr>
<tr>
<td>Goat anti guinea pig or rabbit Ig conjugated horse radish peroxidase</td>
<td>Affinity Chromatography pure</td>
<td>American Qualex, LaMirada, CA</td>
</tr>
<tr>
<td>1-chloro-4-nephtol</td>
<td>E.I.A.</td>
<td>BioRad Laboratory, Richmond, CA</td>
</tr>
</tbody>
</table>
II. Animals

1. Six inbred male Strain 2 guinea pigs, weighing 450-600 g, (University of Arizona, Tucson, AZ)

2. Nine outbred female Amana guinea pigs, weighing 450-500 g, (University of Arizona, Tucson, AZ)

3. Twenty outbred female Hartley guinea pigs, weighing 400-500 g, (Harlan Sprague Dawley Inc., Indianapolis, IN)
METHODS

I. Synthesis of Trifluoroacetylated-Guinea Pig Serum Albumin (TFA-GPA)

The synthesis of TFA-GPA was initiated by the addition of ethyl thioltrifluoroacetate (Pierce Chemical Co., Packford, IL) to guinea pig serum albumin (Sigma Chemical Co., St. Louis, MO) as described by Goldberger and Anfinsen (Goldberger et al., 1962). This reaction occurs between ethyl thioltrifluoroacetate and the lysine residue on guinea pig serum albumin. The synthesized TFA-GPA was dialized against water for two days at 4°C and subsequently lyophilized. The concentration of protein in TFA-GPA was determined by the BioRad protein assay (BioRad Lab., Richmond, CA) and the ratio of free lysine to TFA-bound lysine was also assessed (Kokode et al., 1969). The final concentration of protein in TFA-GPA was 80% and 70% of the lysine residue were conjugated with TFA.

II. Studies on the Generation of Halothane Induced Antibodies in Strain 2 Guinea Pigs

Six inbred male Strain 2 guinea pigs (University of Arizona, Tucson, AZ), weighing 450-600 g, were housed in stainless steel cages and maintained at room temperature (25°C). A standard feed diet fed libitum was supplemented with fresh cabbage. The animals were exposed,
four times, to 1% halothane (Ayerst Laboratories, New York, NY) 21% oxygen and 88% nitrogen for four hours. The interval between each exposure was two weeks. Exposures were performed in an exposure chamber equipped with vaporizer (Ohio Medical Products, Madison, OH), circulating fan, heater and thermometer. Halothane concentration was monitored by gas chromatography and oxygen concentration was monitored by an oxygen electrode.

Blood samples were taken, on days three and seven after each exposure, by cutting the vein in the toenail; blood flow was enhanced by placing the hind leg into an apparatus to which a vacuum pump had been connected (Hochman et al., 1983). Before each bleeding, guinea pigs were anesthetized with ketamine (Park-Davis, Morris Plains, NJ) by injecting 0.1 mg/0.1 ml ketamine, into the leg. Seven days after the fourth exposure animals were sacrificed by cervical dislocation while under deep ether anesthesia. Samples of liver tissue were fixed in 10% phosphate buffered formalin. Tissue was embedded in methacrylate and thin sections (2 micron) prepared. Tissue sections were stained with hemotoxylin and eosin and evaluated for hepatic necrosis under the light microscope.

III. Study of the Effect of Immunization with TFA-GPA on the Potentiation of Liver Injury in Halothane Exposed Guinea Pigs.

Nine inbred female Amana guinea pigs (University of Arizona, Tucson, AZ) were housed and fed as described above. Animals were divided into two groups. The first group of four guinea pigs were immunized with TFA-GPA and then exposed two times to 1% halothane, 80%
O₂, 19% N₂ for four hours. The interval between exposures was two weeks. The second group of four guinea pigs were only exposed to halothane under the same conditions as the first group. Animals in both groups were bled prior to halothane exposure and on days three, five, and seven after each exposure. Plasma samples were assessed for any antibody specific or cross reactive with TFA-GPA in an ELISA. SGPT levels and liver necrosis were also assessed as is described in Analyses Section. One guinea pig served as the ketamine control; receiving 0.1 ml ketamine prior to blood sampling and its plasma was assayed for SGPT.

IV. Study of the Correlation between Halothane Induced Liver Injury and SGPT in Guinea Pigs and the Possible Role of Halothane Induced Antibody in the Liver Injury

Twenty outbred female Hartley guinea pigs (Harlan Sprague Dawley Inc., Indianapolis, IN), weighing 400-500 gr, were housed and fed as described previously. Two guinea pigs served as controls (did not receive halothane) and the other eighteen were exposed to 1% halothane, 80% oxygen and 19% N₂ for four hours. Nine guinea pigs were selected randomly and divided into three groups of three animals per group. The three animals in each group were sacrificed on days one, three, seven post exposure. Two weeks after the first exposure, the nine remaining guinea pigs were anesthetized with halothane and sacrificed in a similar manner as the other guinea pigs. Blood samples were taken from the inferior vena cava and the resulting plasma was assessed for halothane induced antibody and SGPT. Liver sections were
also prepared and evaluated for liver necrosis at each sacrificing time.

V. Characterization of Halothane Induced Antigens with Halothane Induced Antibodies from Guinea Pigs and Rabbits

The source of antigen in this experiment was liver from either New Zealand white rabbit or Hartley guinea pig exposed to 1% halothane under the oxidative conditions. Plasma from the Hartley guinea pigs or rabbits, exposed to 1% halothane under oxidative conditions, were separately pooled and used as a source of halothane induced antibody. Additionally, pooled sera from Amana guinea pigs, immunized with TFA-GPA and exposed to halothane, were also pooled and used as a source of antibody in this experiment. A normal guinea pig liver and plasma served as a control in this study. Plasma and liver proteins were precipitated with ammonium sulfate. Plasma precipitated proteins were separated on a DEAE ion exchange column in order to isolate the IgG fractions from other plasma proteins. The IgG antibodies were then immobilized by coupling to activated sepharose 4B beads and subsequently were mixed with liver proteins. Antigen-antibody complexes removed from sepharose matrix were electrophoresed through a discontinuous slab gel (SDS-PAGE). Fractionated liver proteins on the gel were then transferred to nitrocellulose paper and were detected by Western blot technique.
ANALYSES

I. Assessment of Antibody Response and SGPT Levels

The presence and titer of halothane induced antibodies, cross-reactive with TFA-GPA, was assessed using an enzyme linked immunosorbent assay (ELISA). Each well in microtiter plates (Costar, Cambridge, MA) was coated with TFA-GPA (5.0 ug/well) in 0.5 M carbonate buffer (pH=9.6) overnight at 4 C. The next day, the plates were washed three times with 0.01 M phosphate buffered saline with 0.5% Tween 20 (PBS-Tween 20) pH=8.2, followed by the addition of 3% gelatin (G.T. Baker, Phillipsburg, PA) and incubation at 37 C for one hour. Plates were again washed three times with PBS-Tween 20. Each plasma sample was serially diluted and each dilution tested in triplicate. After a one hour incubation at room temperature and another wash with PBS-Tween 20, horse radish peroxidase (HRP) conjugated goat anti guinea pig immunoglobulin in 0.05% gel (Cooper Biomedical Laboratories, Malvern, PA) was added and incubated for exactly one hour at room temperature. Plates were then washed three times and the substrate for the peroxidase, ABTS (Sigma Chem. Co., St. Louis, MO) was added. After a ten minute incubation at room temperature the reaction was terminated by the addition of 50 ul of 5% sodium dodecyl sulfate (SDS) (Sigma Chemical Corp., St. Louis, MO). The degree of color development was determined at a wavelength of 410/490 nm by a Titertek Microelisa Reader (Flow Laboratories, McLean, VA). This procedure represents a
modification of the ELISA method described by Satoh et al (Satoh et al., 1985). The reciprocal of the antibody dilution which yields an optical density (O.D) of 0.3 was used for quantitation of antibody in the ELISA.

Serum glutamate pyruvate transaminase (SGPT) levels were determined as markers of liver damage in each plasma sample (Sigma Chemical Co., St. Louis, MO). The results were expressed in Wroblewski-LaDue units.

II. Immunization of guinea pigs with TFA-GPA

Two mg TFA-GPA were emulsified in 0.5 ml water and 1.5 ml of Freunds complete adjuvant (Difco Lab., Detroit, MI) by sonication. Five 0.1 ml of injections were injected into each leg (I.M.) and along the back (S.C.) of the four guinea pigs. Two weeks after immunization, guinea pigs were bled and plasma samples assessed for specific anti TFA-GPA antibody by ELISA. A booster immunization of 1 mg TFA-GPA in 0.5 ml water and 1.5 ml of incomplete Freunds' adjuvant was given to these guinea pigs three weeks after primary immunization. One week after the booster immunization, blood samples were drawn from the animals and plasma samples were assayed for specific antibody against TFA-GPA.

III. Detection of Halothane Induced Antigens

(A) Ammonium sulfate precipitation (Garvey et al., Methods in
Immunology): Proteins of less than 200,000 daltons in the plasma were precipitated by ammonium sulfate (50% saturation) (Sigma Chemical Co., St. Louis, MO). Proteins in the guinea pig liver homogenate were also precipitated by ammonium sulfate (80% saturation). The precipitated proteins in the plasma and liver homogenates were then centrifuged and resuspended in 20 mM phosphate buffer containing 0.5 M NaCl pH=7.4.

(B) DEAE sepharose chromatography: The precipitated proteins in the plasma were applied to a DEAE sepharose column in order to purify the IgG fraction. Three milliliter fractions were collected and assayed for halothane induced antibody (IgG class only) in an ELISA. Tubes containing the IgG fractions were collected and pooled.

(C) Coupling of the antibody to CNBr activated sepharose: These IgG fractions were coupled to CNBr-activated sepharose (Pharmacia Fine Chemicals, Switzerland). Any remaining active sites in the matrix were blocked with 0.2 M glycine (U.S. Biochemical Corp., Cleveland, OH. In addition, any unabsorbed antibody or nonspecific binding was removed by successive washing with 0.1 M carbonate buffer with 0.5 M NaCl (pH=8.3) and 0.1 M acetate buffer pH=4. Next, 200 ml of liver antigen (ammonium sulfate precipitated) at 1 mg protein per ml, was mixed with 150 ul of the CNBr-sepharose-Ab complex and incubated
overnight at 4 C. The next day, the solution was centrifuged and the pellet containing CNBr-sepharose-antigen-antibody complex was washed three times with 20 mM PBS (pH=8.3) to remove any unbound antigen. The pellet was then transferred to 1 ml microfuge tube and 300 ul of the sampling buffer containing 2% SDS, 10% glycerol, 0.001% bromothimol blue in 62.5 mM tris buffer (pH=6.8) was added to the pellet. These microfuge tubes were rocked for 10 minutes at room temperature and centrifuged at 2838 g for ten minutes. The antigen was fractioned by SDS-PAGE electrophoresis (Bio-Rad Lab., Richmond, CA) and was identified through western blot technique (Hoffer Scientific Instruments, San Francisco, CA).

(D) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Antigen removed from sepharose matrix was electrophoresed through gradient SDS-PAGE gel (Bio-Rad Lab., Richmond, CA). The protein bands on the gel were subsequently transferred to nitrocellulose paper by a technique described by Hoefer Scientific Instruments, San Francisco, CA. The gel was subsequently stained with coomassie blue (Bio-Rad Lab., Richmond, CA).

(E) Western Blot Technique: The nitrocellulose paper with the transferred proteins was first pre-incubated with 3% gelatin in 20 mM tris buffer pH=7.4, for two hours at room temperature. Next, the purified guinea pig or rabbit
antibody at the appropriate dilution was incubated with
the nitrocellulose paper overnight at room temperature.
The following day, the nitrocellulose paper was washed
with 20 mM tris buffer with 0.25% gelatin. Goat anti
guinea pig or rabbit conjugated with horse radish
peroxidase (American Qualex, LaMirada, CA) was added for a
two hour incubation with agitation at room temperature.
Any excess second antibody was washed away with 20 mM tris
buffer with 1% gelatin and 0.05% Tween 20. The guinea pig
or rabbit liver protein bands were evidenced by the
addition of color developing reagent containing HRP, 20 mm
tris buffer pH=7.4, methanol, 1-chloro-4-naphtol (Bio-Rad
Lab., Richmond, CA).
ANALYTICAL ASSAYS

Data are expressed in values from individual animals. Values could not be pooled for groups of animals due to the large variation between high and low responders. Therefore, analytical assays were not performed.
RESULTS

I. Halothane Induced Antibodies in Strain 2 Guinea Pigs

Multiple halothane exposures in Strain 2 guinea pigs induced an antibody cross reactive with TFA-GPA. The titer of this antibody response varied between animals as evidenced by the disparity in antibody titers between different guinea pigs. Elevation in levels of SGPT were observed in all animals, indicating hepatocellular damage by halothane exposure in guinea pigs. All six animals exposed showed an increase in the level of SGPT on day three after the first exposure. Four out of six guinea pigs also showed an elevation in the levels of SGPT on either days three or seven following the second, third or fourth exposure. A representative chronology of SGPT and antibody levels which appear related is shown in Figure 1. The peak of antibody response in this animal and two other guinea pigs (data not shown) was on day three after each exposure and subsequently decreased by day seven. In these three guinea pigs, an association was observed between the titer of antibody and the SGPT levels. The decrease in the titer of antibody in these three guinea pigs might be due to the formation of immune complexes or binding of antibodies to antigens on the surface of hepatocytes. In the other three guinea pigs, no consistent association was observed between antibody titer and SGPT level (Figure 2). No significant liver necrosis was observed histologically in these animals. However, a moderate to high degree of fat deposition was
Figure 1. Association between halothane induced antibody titers and SGPT levels in a Strain 2 guinea pig following multiple halothane exposures.

Strain 2 guinea pigs were exposed to 1% halothane and 21% oxygen four times at two week intervals. The reciprocal of the antibody dilution which yields an optical density of 0.3 is used for quantitation of antibody in ELISA. SGPT values are expressed in Wroblewski Ladue units. Arrows show the time of each exposure.
Figure 2. Lack of association between halothane induced antibody titers and SGPT levels in a Strain 2 guinea pig.

Strain 2 guinea pigs were exposed to 1% halothane and 21% O₂ four times at two week intervals. The quantitation of the antibody titer and SGPT units are the same as described in Figure 1. Arrows show the time of each exposure.
observed in all guinea pigs.

II. Halothane Induced Antibodies in Amana Guinea Pigs

In Amana guinea pigs, after halothane exposure, an antibody was induced that was also cross-reactive with TFA-GPA. In addition, SGPT levels were also increased in these animals and a correlation was observed between the antibody titers and SGPT levels. One exposed guinea pig showed a peak of halothane induced antibody as well as SGPT on day three following the first exposure (Figure 3). In contrast, another exposed guinea pig had the peak of antibody on day seven after the second exposure (Figure 4).

III. Halothane Induced Antibodies in Hartley Guinea Pigs

Multiple halothane exposures in Hartley guinea pigs resulted in the generation of an antibody that was cross-reactive with TFA-GPA (Figure 5). The magnitude of the immune response in Hartley guinea pigs was higher than in Strain 2 and Amana animals perhaps because of increased oxygen concentration during the exposure (80%) or because of differences in susceptibility to halothane exposure between these strains of guinea pigs. The same increase in the concentration of halothane induced antibody, due to an increase in oxygen tension during the exposure, has already been shown in the rabbit model. The concentration of halothane induced antibody after the second exposure was higher as compared to the first exposure (Figure 5), indicating the
Figure 3. Halothane induced antibody titers and SGPT levels in an Amana guinea pig following halothane exposures.

Amana guinea pigs were exposed to 1% halothane and 80% oxygen two times at a two week interval. The reciprocal of the antibody dilution which yields an optical density of 0.3 is used for quantitation of antibody in ELISA. SGPT values are expressed in Wroblewski Ladue units. Arrows represent the time of each exposure.
Figure 4. Halothane induced antibody titers and SGPT levels in an Amana guinea pig following the second halothane exposure.

Amana guinea pigs were exposed to 1% halothane and 80% oxygen two times at a two week interval. Arrows represent the time of each exposure. The quantitation of the antibody titers and SGPT units are the same as described in Figure 3.
Figure 5. Halothane induced antibody titers in Hartley guinea pigs following multiple halothane exposures.

Hartley guinea pigs were exposed to 1% halothane and 80% oxygen two times at a two week interval. Eighteen Hartley guinea pigs were sacrificed at these sacrificed times following halothane exposures. Each point represents the value from one guinea pig. Antibody levels at 350 represent background values of controls. The reciprocal of the antibody dilution which yields an optical density of 0.5 is used for quantitation of antibody in ELISA. Arrows represent the time of each exposure.
probable induction of a secondary humoral immune response to halothane. Furthermore, the elevation of SGPT values was observed on days one and three after the first exposure followed by a decline on day seven (Figure 6). In contrast, the SGPT values were only elevated on day three after the second halothane exposure. As seen with the other strains, there is a variation among guinea pigs in SGPT and halothane induced antibody levels.

IV. The Effect of Pre-existing Anti-TFA Antibodies on Halothane Hepatotoxicity

Immunization of four Amana guinea pigs with TFA-GPA resulted in the induction of a specific anti-TFA-GPA antibody response indicating that TFA-GPA was immunogenic. Antibody levels one month post immunization evidenced titers from 60,000 to 120,000 when reacted with TFA-GPA in an ELISA. The subsequent challenge of these immunized Amana guinea pigs with halothane (80% \( O_2 \)) generated a secondary antibody response in two out of three guinea pigs (Fig. 7, 8). Although the two guinea pigs showed an elevation in SGPT titer following halothane anesthesia, no correlation was observed between the SGPT values and antibody titers in these animals. Evaluation of the liver sections from immunized-exposed guinea pigs showed no difference between the immunized-exposed and exposed only groups, indicating that immunization of guinea pigs with TFA-GPA did not potentiate the liver necrosis in these animals.

V. Chronology of Halothane Induced Liver Injury in the Guinea Pigs
Figure 6. SGPT values in Hartley guinea pigs following halothane exposures.

Hartley guinea pigs were exposed to 1% halothane and 80% oxygen two times at a two week interval. These SGPT values are from the same 18 Hartley guinea pigs as described in Figure 5. An SGPT score of 18 represents the background values of controls. Each point represents an SGPT value from one guinea pig. SGPT values are expressed in Wroblewski Ladue units. Arrows show the time of each exposure.
Figure 7. The induction of a secondary antibody response in an immunized (TFA-GPA) Amana guinea pig following halothane exposures.

An Amana guinea pig was immunized with TFA-GPA and then exposed two times to 1% halothane and 80% oxygen at a two week interval. The reciprocal of the antibody dilution which yields an optical density of 0.5 is used for quantitation of antibody in ELISA. SGPT values are expressed in Wroblewski Ladue units. Arrows show the time of each exposure.
Figure 8. The induction of a secondary antibody response in an immunized (TFA-GPA) Amana guinea pig following halothane exposure.

An Amana guinea pig was immunized with TFA-GPA and then exposed two times to 1% halothane and 80% oxygen at a two week interval. The quantitation of the antibody titers and SGPT units are the same as described in Figure 7. Arrows represent the time of each exposure.
Evaluation of the chronology in the development of the hepatic lesion in Hartley guinea pigs revealed that 1% halothane exposure under oxidative conditions (80% O₂) generates a transient yet moderate to severe hepatic injury in these animals. On day one following the first exposure, there are foci of hepatic necrosis (Fig. 9, 10) characterized by hepatocytes with darker and more dense nuclei as well as elevations in SGPT levels. The peak of necrosis was observed on day three following the first exposure, evidenced by extensive foci of necrosis around central veins, characterized by degenerated hepatocytes, hepatocytes filled with fat droplets, infiltration of inflammatory cells and occasionally hepatocytes filled with calcium deposits (Fig. 11, 12). On day seven following the first exposure, hepatocyte recovery was apparent. Non specific vacuolization, a non pathogenic phenomena with unknown etiology, was observed in all six guinea pigs on day seven following the first and second exposures. This non-specific vacuolization may be due to impaired food and water consumption in exposed animals. Thus, they do not have a normal hepatic architecture (Figure 13, 14). In addition, the exact pattern of liver injury and recovery was observed on day one, three and seven following the second exposure.

VI. Characterization of Halothane-Induced Antigens with Halothane Induced Antibodies from Guinea Pigs and Rabbits

Halothane induced antigens from exposed guinea pigs or rabbits were identified with two types of antibodies: a pool of antibody from halothane exposed Hartley guinea pigs and TFA-GPA immunized Amana
Figure 9. Photomicrograph of the liver section from a Hartley guinea pig on day one following the first halothane exposure.

Liver section from a Hartley guinea pig one day following the first halothane exposure was examined under light microscopy. A developing foci of necrosis around central vein is evidenced by hepatocytes with dense nuclei and darker cytoplasm. Hematoxylin-eosin stain 40x.
Figure 10. The same photomicrograph as Figure 9 with a higher magnification: 400x.

Upon closer examination of Figure 9, a moderate deposition of fat droplets can be seen. The hepatocytes with darker nucleus and cytoplasm appear to be in the process of degeneration forming a foci of necrosis. Hematoxylin-eosin stain. 400x.
Figure 11. Photomicrograph of the liver section from a Hartley guinea pig on day three following the first exposure.

A liver section from a Hartley guinea pig on day three following the first exposure showed a massive centrilobular necrosis. Infiltration of inflammatory cells as well as fat deposition are also seen. The SGPT value was 275 units (control value 22). Hematoxylin-eosin: 40x.
Figure 12. Same photomicrograph as Figure 11 with a higher magnification: 400x.

Closer examination of Figure 11 shows an area of necrosis evidenced by degenerated hepatocytes (pink cytoplasm without nucleus), inflammatory cells and fat deposition. A demarcation between the degenerated area (left) and normal hepatocytes (right) is also shown. Hematoxylin-eosin. 400x.
Figure 13. Photomicrograph of the liver section from a Hartley guinea pig on day seven following the first exposure: 40x.

The liver section obtained from a Hartley guinea pig on day seven following the first exposure demonstrates a non-specific vacuolization, a nonpathogenic phenomena with an unknown etiology. Hematoxylin-eosin. 40x.
Figure 14. The same photomicrograph as Figure 13 with a higher magnification. Hematoxylin-eosin: 400x.
Figure 15. Photomicrograph of the normal liver section from a Hartley guinea pig. Hematoxylin-eosin: 40x.
guinea pigs (Figure 16) or a pool of TFA-RSA immunized and halothane exposed white New Zealand rabbits (Figure 17). Antisera from exposed and immunized animals were pooled to increase the detection of any cross-reactive antigens. Using antisera from a different species confirmed the immunogenic potential of TFA in more than one species. The heavy chain of IgG (approximately 41.2 Kd) as well as the light chain of IgG (approximately 20.7 Kd) can be seen in proteins from control guinea pigs (lane 3), exposed guinea pigs (lane 4) and exposed rabbits (lane 5) (Figure 16). This immunoglobulin is undoubtedly from the affinity column used to purify the liver proteins (see Methods). In addition control guinea pigs, exposed guinea pigs, and exposed rabbits share a protein with a molecular weight of 10.9 Kd. The detection of this protein may be due to nonspecific binding of guinea pig halothane induced antibody to normal guinea pig and rabbit proteins and perhaps could be eliminated by using a more dilute primary antibody (guinea pig halothane induced antibody). Five unique liver proteins with the molecular weights of 77.7 Kd, 69.8 Kd, 53.8 Kd, 28.4 Kd, 25.6 were shared between exposed guinea pigs and exposed rabbits indicating that anti-TFA antibody in the guinea pigs detected not only halothane induced antigen in guinea pigs, but also cross-reacted with halothane induced antigens in rabbits. The absence of these five liver proteins in control guinea pig livers suggests that these protein antigens are generated as a result of halothane exposure.

Figure 17 demonstrates the detection of halothane induced antigens by antibody from rabbits immunized with TFA-RSA and subse-
quentely exposed to halothane. This antibody was reacted with control guinea pig proteins, halothane exposed guinea pig liver proteins, and halothane exposed rabbit liver proteins. Five protein bands, with the molecular weights of 91.6 Kd, 76.3 Kd, 61.6 Kd, 53.9 Kd and 46.0 Kd were shared between exposed guinea pigs and rabbits. In addition, three bands with the molecular weights of 34.4 Kd, 26.4 Kd, and 24.2 Kd were seen only in exposed rabbits. Although halothane induced antibody in the rabbits will cross-react with halothane induced proteins in the guinea pigs, this antibody is more specific for halothane induced antigens in rabbits. Furthermore, the absence of protein bands in control guinea pig liver homogenates suggests that these proteins are generated only following halothane exposure.
Figure 16. Guinea pig and rabbit halothane induced antigens were separated in a western immunoblot and identified by both guinea pig halothane induced antibody and guinea pig specific anti-TFA-GPA. Lanes 1 and 6 (M) represent the following molecular weight markers: phosphorylase b (97,400 Kd), bovine serum albumin (68,000 Kd), ovalbumin (43,000 Kd), d-chromotrypsinogen (25,700 Kd), B-lactoglobulin (18,400 Kd), and lysozyme (14,300 Kd). Lane 2 (IG) represent the heavy and light chains of IgG. Lane 3 (C) represent unexposed guinea pig liver proteins. Lane 4 (GP) represent liver protein bands from an exposed guinea pig. Lane 5 (K) represent liver proteins from an exposed rabbit. Five unique halothane induced proteins with the molecular weights of 77.7, 69.8, 53.8, 28.4, 25.6 Kd were detected by this pool of antibody. Heavy and light chains of immunoglobulins are evidenced by two protein bands with the molecular weights of 41.2 and 20.7, respectively.
Figure 16. Identification of halothane induced proteins in the livers of exposed guinea pigs and rabbits.
Figure 17. Guinea pig and rabbit halothane induced antigens were identified by a pooled plasma from rabbit halothane induced antibody and rabbit specific anti-TFA-RSA. Lane 1 unexposed guinea pig liver proteins. Lane 2 exposed guinea pig liver proteins. Lane 3 exposed rabbit liver proteins. Five halothane induced antigens with the molecular weights of 91.6, 76.3, 61.6, 53.9 and 46.0 Kd are detectable by this antibody. In addition this antibody is able to detect 3 extra proteins in rabbit liver homogenates with the molecular weights of 34.4, 26.4 and 24.2 Kd.
Figure 17. Identification of halothane induced proteins in the livers of exposed guinea pigs and rabbits.
DISCUSSION

This study has been focused on the induction of a humoral immune response which could potentially recognize halothane induced antigens in guinea pigs. Speculation still remains as to the possible role of this antibody in the pathogenesis of halothane hepatitis. The information obtained by exposing three different strains of guinea pigs (Strain 2, Amana, Hartley) to halothane confirms the presence of antibodies cross-reactive with a trifluoroacetyl halide moiety, a reactive intermediate formed during halothane metabolism. The antigen used for detecting halothane induced antibodies (TFA-GPA), is probably similar, but not identical to the true antigen. Therefore it is possible that other humoral immune responses with a different specificities could have been evoked in these animals.

The peak of antibody in Strain 2 guinea pigs is on day three following the first exposure and is followed by a decline on day seven (Figure 1). This rapid decline in the titer of halothane induced antibody could be due to the binding of this antibody to free halothane induced antigens in the circulation which leads to the formation of immune complexes. In addition, this decline might also have resulted from the binding of this antibody to halothane induced antigen on the surface of hepatocytes in the liver. The rationale behind the above assumptions is due to the detection of immune complexes both in halothane hepatitis patients (Roth et al., 1986) and animal models
Furthermore, it has been shown that halothane induced antibody in halothane hepatitis patients is capable of binding to the surface membrane of halothane altered rabbit hepatocytes (Vergani et al., 1986). All Strain 2 guinea pigs showed an increase in SGPT values on day three after the first exposure, indicating the occurrence of liver injury in these animals following halothane administration. The susceptibility of all Strain 2 guinea pigs to halothane exposure is due to the fact that they are inbred and as a result have a very similar genetic background. This is in agreement with a report by Lunam et al who described a genetic basis for susceptibility of guinea pigs to halothane hepatotoxicity (Lunam et al., 1986). Although in three out of six guinea pigs a relationship was observed between antibody titer and SGPT levels, the possible cause and effect relationship between these two factors requires more investigation. The lack of observing liver necrosis in these animals may be due to the fact that the peak of liver injury in guinea pigs proved to be on day three after each exposure in susceptible animals. These liver samples were from animals sacrificed on day seven following the fourth exposure. Thus, only fat deposition was observed in these liver sections, indicating that hepatocytes were in the stage of recovery.

The increase in halothane induced antibody titer in non-immunized strain Amana guinea pigs as compared to Strain 2 is most likely due to an increase in oxygen concentration during halothane exposure from 21% to 80%. Increases in the levels of halothane induced antibody in this case may be due to increases in the total quantity of
oxidative metabolites of halothane. The infiltration of inflammatory cells observed in the liver section obtained from an exposed Amana guinea pig, sacrificed on day three post first exposure may be due to the presence of nonself antigens generated as a result of halothane exposure.

The concentration of antibody, induced by halothane administration, in Hartley guinea pigs was higher compared to the two other strains (approximately 6 fold). Since it is known that the magnitude of the immune response to antigens is genetically determined (Dixon et al., The Biology of Immunologic Disease, 1983), the higher titer of halothane induced antibody in Hartley guinea pigs could be due to the genetic background of these animals. The fact that the titer of this antibody is higher following the second exposure than the first exposure whereas SGPT levels are increased after both exposures indicates that liver injury following first exposure might have been mediated by chemotoxic factors instead of the immune response. However, the injury following the second exposure could be mediated by a humoral immune response as well as chemotoxic factors. In this case, the argument that this antibody might be the result of liver necrosis can not be ruled out. The report of liver necrosis, in outbred Hartley guinea pigs, in this study is in contrast to the report by Lind et al who described inbred Hartley guinea pigs as non-responders to halothane anesthesia (Lind et al., 1986). This descrepancy might be explained by the fact that each group of animals were obtained from different sources. In addition, in this study, outbred animals have been used
whereas Lind et al used inbred Hartley guinea pigs. Thus, genetic predisposition may be important in susceptibility to halothane induced liver damage both in humans and animals.

The result of immunization of Amana guinea pigs with TFA-GPA prior to halothane administration indicates the generation of specific anti TFA-GPA antibody response as well as a halothane induced antibody in these animals. Since guinea pig albumin was a self antigen for these guinea pigs, specific anti-TFA-GPA antibody would have been generated primarily against TFA moiety indicating that TFA is immunogenic. However, the failure of pre-immunization to exacerbate liver damage suggests that specific anti TFA-GPA antibody may not have a role in the pathogenesis of halothane induced liver injury in guinea pigs. In all likelihood, TFA-GPA is similar but not identical to a halothane induced antigen in the liver.

In most Hartley guinea pigs, the degree of liver necrosis after halothane administration was associated with an increase in SGPT levels. One guinea pig on day three following the first exposure showed the highest degree of liver injury and evidenced the highest SGPT value of 275 units as well as extensive centrilobular necrosis (Figure 11). In addition infiltration of inflammatory cells, composed of polymorphonuclear leukocytes as well as monocytes, were also observed in the necrotic areas of the liver of this animal. It has been demonstrated that inhibition of halothane metabolism during exposure by prior administration of SKF-525A prevented subsequent development of severe necrosis (Lunam et al., 1985). However,
administration of liver enzyme inducers such as phenobarbital prior to halothane exposure did not exacerbate the liver necrosis in guinea pigs (Lind et al., 1986). Although halothane metabolism is important in the initiation of halothane hepatitis, other factors may be important in its perpetuation.

The initiation of liver necrosis in Hartley guinea pigs one day after the first halothane anesthesia is most likely due to the direct toxicity of halothane metabolites which rupture the hepatocytes and release halothane induced antigens as well as other liver proteins. This process continues until day three following the first exposure when the necrosis reaches to its highest level. Between days three to seven following the first exposure halothane metabolites are gradually decreased and by day seven hepatocytes have recovered. This mechanism might also occur in hepatocytes following the second exposure to halothane. However, this time halothane induced antibody along with halothane metabolites may mediate liver injury in these animals.

A similarity between halothane induced antigens in guinea pigs and rabbits, whether detected by anti-TFA antibodies from guinea pigs (Figure 16) or rabbits (Figure 17) can be found in two protein bands with comparable molecular weights: 77.7 Kd and 53.8 Kd proteins in guinea pigs versus a 76.3 Kd and 53.9 Kd proteins in rabbits. Although guinea pigs demonstrate signs of liver injury following halothane exposure, rabbits do not show any elevation in SGPT values or change in liver histology. The detection of similar halothane induced proteins in these animals indicates that a common mechanism of hepatotoxicity,
perhaps with different magnitudes, may be involved in both animal models. In addition, the signs of hepatotoxicity in guinea pigs are detectable only when the animals are exposed to 1% halothane for four hours not two hours as in rabbit exposures. Perhaps exposing rabbits to halothane for four hours may result in a pattern of hepatotoxicity similar to that seen in guinea pigs.
CONCLUSION

Detection of halothane induced antibody in guinea pigs that appears to have the potential for recognizing halothane induced proteins in the guinea pig liver suggest a possible role for the humoral immune response in the pathogenesis of halothane hepatitis. The advantage of the guinea pig model appears to be the similarity in the route of halothane metabolism and histological distribution of liver damage between this animal model and that reported in man (Lunam et al., 1985). Therefore, it is possible that comparable mechanisms of hepatotoxicity exist in humans and guinea pigs. The development of other animal models that could mimic more completely fulminant halothane hepatitis as seen in humans, would reveal additional facts about the pathogenesis of this syndrome. Although an association was observed between the titers of halothane induced antibody and SGPT levels, more work on the potential of this antibody in mediating liver damage by complement fixation or perhaps antibody dependent cellular cytotoxicity should be performed. Perhaps then, one would be able to speculate on the cause and effect relationship between these parameters. Furthermore, explaining the role of cell mediated immunity in the pathogenesis of this disease could contribute to more understanding in the initiation and perpetuation of halothane hepatitis.
REFERENCES


