

Apnea and bradycardia due to anaphylaxis to tobacco glycoprotein in the infant rabbit[☆]

William M. Gershan,^{a,*} Carl G. Becker,^b Hubert V. Forster,^c Nathaniel S. Besch,^a
and Timothy F. Lowry^a

^a *Department of Pediatrics, Medical College of Wisconsin, Children's Hospital of Wisconsin, 9000 West Wisconsin Avenue, MS #777A, Milwaukee, WI 53226, USA*

^b *Department of Pathology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*

^c *Department of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*

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Abstract

Prenatal and postnatal exposure to cigarette smoke is associated with an increased incidence of the sudden infant death syndrome, although the cause(s) for this is unknown. Tobacco glycoprotein (TGP), a group of proteins purified from cured tobacco leaves and present in cigarette smoke, have been shown to cause anaphylaxis in excised hearts and lungs of adult rabbits that were neonatally sensitized to TGP and later rechallenged. We sought to determine whether anaphylaxis occurred in live infant rabbits who were neonatally sensitized to TGP. At the age of 1 day, 12 animals were sensitized to TGP (0.1 mg in 0.25 cc alum) via intraperitoneal injection (ipi) followed by a booster ipi at the age of 30 days (TGP-S). Seven animals received ipi of antigen-free alum only (controls). All animals underwent an intravenous TGP challenge at age 42 ± 2 days. Heart rate (HR) and respiratory rate (RR) were recorded for 2 min prior to and 5 min after the challenge. Baseline HR (~ 260) and RR (~ 120) were similar in all animals. Seven TGP-S animals developed apnea (1.9–4.7 s) within 60 s of the challenge while none of the controls did. The TGP-S also became bradycardic (the lowest HR over 50 consecutive beats), with the HR decreasing from 260 to 220 vs the controls, whose HR remained constant (~ 250). We conclude that some rabbits neonatally sensitized to TGP develop apnea and bradycardia upon further intravenous TGP challenge. These studies suggest that cigarette smoke exposure may be associated with a higher rate of SIDS via an anaphylactic mechanism.

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1. Introduction

The sudden infant death syndrome (SIDS)¹ remains the most common cause of death of infants between 1 and 12 months of age in the USA, although the cause

(or causes) of SIDS is not known. Numerous investigations have found a strong association linking both maternal smoking during pregnancy and household exposure to cigarette smoke to an increased risk of SIDS (Aligne and Stoddard, 1997; Bergman and Wiesner, 1976; Blair et al., 1996; Mitchell et al., 1993; Schoendorf and Kiely, 1992; Scragg et al., 1993; Taylor and Sanderson, 1995). In fact, a recent epidemiologic investigation concluded that 2000 SIDS deaths per year in the USA are directly attributable to cigarette smoke (Aligne and Stoddard, 1997). Although no study has specifically found the cause for this association, one hypothesis suggests that nicotine and/or other substances in cigarette smoke impair maturation of brain stem nuclei that regulate respiration (Mactutus, 1989; Moessinger, 1989). Another potential mechanism

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* Corresponding author. Fax: +1-414-266-6742.

E-mail address: wgershan@mcw.edu (W.M. Gershan).

¹ Abbreviations used: ANOVA, analysis of variance; ECG, electrocardiogram; HR, heart rate; PBS, phosphate-buffered saline; RR, respiratory rate; SIDS, sudden infant death syndrome; TGP, tobacco glycoprotein antigens; TGP-S, tobacco glycoprotein sensitized animals; V_T , tidal volume.

relating cigarette smoke to SIDS involves tobacco hypersensitivity or anaphylaxis. Recently published data from autopsies of infants who had died from SIDS suggest that the deaths were anaphylactic, a finding deduced from the elevated levels of mast cell tryptase in serum samples obtained at autopsy (Holgate et al., 1994; Roche, 1992). In these studies, mast cell tryptase was not elevated in serum from the autopsies of infants that died from other causes. These hypotheses are not mutually exclusive, but they may be interactive. For example, an infant with imperfect control of respiration might be less likely to survive an anaphylactic event such as bronchospasm. It has been found, for instance, that mice exposed to tobacco smoke were significantly more susceptible to the lethal effects of histamine than nontobacco exposed mice (Keller and Doyle, 1976).

Specific antigens in cured tobacco and cigarette smoke have been identified to which nearly 40% of adult volunteers, both smokers and nonsmokers, exhibited IgE-dependent immediate hypersensitivity reactions (Becker et al., 1976, 1979). In addition, these antigens, named tobacco glycoproteins or TGP, are present in some commonly eaten foods, including eggplant, green peppers, potatoes, chocolate, coffee, and tomatoes, as well as in ragweed pollen (Becker et al., 1981; Levi et al., 1982). Sensitization of rabbits and guinea pigs by intraperitoneal injection of tobacco antigens on the first day of life rendered the animals exquisitely sensitive to the development of anaphylaxis following further antigenic booster and challenge (Levi et al., 1982). Anaphylaxis was characterized by pulmonary arterial bed vasospasm, causing decreased pulmonary arterial flow, and by sinus tachycardia, decreased cardiac contractility, hypoxic electrocardiographic changes, and various rhythm disturbances. These responses, although found in excised hearts and lungs, suggest that severe allergic reaction, and perhaps even fatal anaphylaxis involving the respiratory and/or cardiac systems, may occur in certain individuals who are exposed to cigarette smoke. The occurrence of an anaphylactic event, even if mild, in an infant with brain stem immaturity might be more likely to result in a SIDS fatality. In the present study, we considered whether similar reactions would occur in live infant rabbits neonatally sensitized to TGP. Our specific hypothesis was that neonatal sensitization of live rabbits with TGP and subsequent rechallenge with TGP results in anaphylaxis, which in some animals is fatal.

2. Methods

2.1. TGP purification

TGP was isolated from flue-cured Virginia Bright tobacco leaves and from cigarette smoke condensate

according to methods described previously (Becker et al., 1976, 1979, 1981; Levi et al., 1982). The tobacco leaves were obtained through the courtesy of Dr. Thomas Osden of the Phillip Morris Company (Richmond, VA, USA). The dry leaves were comminuted in an Oster blender. The powder was then delipidized by sequential extraction with petroleum ether and ethyl ether and dried on a funnel. The dried powder was extracted with Tris (20 mM)–EDTA (1 mM) buffer, pH 8.0, made 3 mM in 2-mercaptoethanol (2ME). During the first hours of extraction the pH was adjusted by drop-wise addition of 1 N NaOH until the pH remained constant at 8.0. The extract was clarified by filtration on extra-fast filter paper and then by centrifugation at 12,000 g at 4°C. The extract was then dialyzed against distilled water using a Spectra/Por 2 dialysis membrane, with a molecular weight cut-off of 12,000–14,000. The extract was then lyophilized to yield a brown powder. Four hundred milligrams of this powder was then redissolved in 40 mL of the Tris–EDTA–2ME buffer described above. This was applied to a 2.6 × 40-cm column of DEAE–Sephacel previously equilibrated with the same buffer. The column was washed with 1 L of the buffer made 1 N in NaCl, resulting in elution of a broad protein peak that retained its brown color because of derivitization by polyphenols. This was again dialyzed against distilled water, lyophilized, and stored for further use in the experiments described herein. Analysis of this material on 12% SDS–polyacrylamide gels revealed the peak to contain approximately seven peptide bands ranging between 7 and 67 kDa, as visualized by staining of the gels with Coomassie brilliant blue and silver staining. Similar peptides were observed when peptides were extracted from cigarette smoke condensate (unpublished observations).

2.2. Animals and TGP sensitization

Newborn New Zealand White rabbits were used in the study, which was approved by the Institutional Review Board of the Medical College of Wisconsin. A total of 19 animals from four litters completed the study (12 TGP-sensitized and seven control rabbits). Animals were randomly selected to be in either the TGP-sensitized (TGP-S) or the control group. However, because one or two animals from each litter typically died from natural causes in the perinatal period, the final groups were not of equal size. Many of the animals that died underwent autopsies by one of the authors. All animals were housed with their mother until they were weaned at approximately 5–6 weeks of age. At 24–48 h of age, TGP-S animals were injected intraperitoneally with 0.1 mg of TGP in 0.25 cc alum, followed by a booster injection of 0.05 mg of TGP in 0.25 cc alum at 30 days of age. Control animals were injected intraperitoneally at 24–48 h of age with 0.25 cc antigen-

free alum, followed by a similar injection at 30 days of age.

At 32–35 days of age, nine TGP-S and all seven control animals had serum collected and analyzed for the presence of IgE antibodies against tobacco antigens by the passive cutaneous anaphylaxis assay. Serum from both TGP-S and control rabbits were diluted in phosphate-buffered saline (PBS) to a final dilution of 1:4. Half of the volume of each sample was heated to 56°C for 1 h. Unheated or heated serum of 0.1 mL or dilutions thereof was injected intracutaneously in the shaved back of normal, adult White rabbits. Seventy-two hours later the prepared rabbits were given intravenous injections of 2 mL of a 1.5% solution of Evans blue in saline. The prepared skin sites were challenged by injection of 0.1 mL of PBS containing 3 µg of TGP or 0.1 mL of PBS as a control. The sites were observed for leakage of Evans blue and development of a wheal within 30 min after injection of antigen.

2.3. Weekly ventilation and behavioral data

To assess differences in ventilation or behavior, respiratory rate (RR) and tidal volume (V_T) were measured once or twice weekly in all animals until 8 weeks of age. Animals were studied unrestrained in a 10-L plexiglass plethysmograph for approximately 5 min. Data were collected, recorded, and analyzed with a computer program (SABB), which calculated respiratory rates and used volume calibrations to calculate breath by breath tidal volumes (Jacky, 1980). Animals were weighed during these collections and were also closely observed during the first month of life, both during these collections and during nonexperimental periods within their litters.

2.4. TGP challenges

All animals underwent an intravenous TGP challenge at 42 ± 2 days of age. Two electrocardiogram (ECG) leads were surgically implanted on both sides of the upper, anterior thorax, and intravenous catheters were inserted into the central ear vein of animals 1 day prior to the challenge. The ECG leads were connected to a general purpose amplifier and recorded on computer software (CODAS) for later playback and analysis. The challenge consisted of a 10-min period of recording of ventilatory and ECG data. The first 2.5 min was the baseline period. At 2.5 min, as a control 0.25 cc normal saline was injected intravenously, and monitoring continued. At 5.5 min, 1 mg of purified TGP in 0.25 cc alum was intravenously injected, and monitoring continued until a total of 10 min had passed, at which time the experiment was stopped. Animals were closely observed during the challenge and unusual behaviors were recorded.

2.5. Statistics

Mean heart rate (HR) and bradycardia in these experiments were calculated using the lowest 50-consecutive-beat HR during a particular period. Because the baseline HR in the animals was typically ~ 250 , bradycardia epochs usually represented a time period of approximately 12–15 s. Data in each experiment were analyzed using analysis of variance (ANOVA) and the paired or unpaired *t* test, when appropriate. The Duncan post hoc test was used to specify differences when ANOVA was significant. Fisher's exact test was used to compare frequencies of apnea in the TGP-sensitized and Control groups. A *P*-value < 0.05 was considered significant. All data are expressed as mean $\pm$ SEM.

3. Results

3.1. Animals and TGP sensitization

Several animals, both from the TGP-sensitized ($n = 5$) and control ($n = 3$) groups, died early in infancy with poor weight gain, suggesting underfeeding as the cause of death. Autopsies performed on some of these animals failed to elucidate the cause of death. Four other animals (two TGP-S and two control) appeared to have died during the first 2 weeks of life from acute trauma, probably caused by the doe.

The passive cutaneous anaphylaxis assay revealed that nine of the nine tested TGP-S rabbits had IgE antibodies that were heat labile and reactive with the TGP antigens (Fig. 1). These antibodies were not

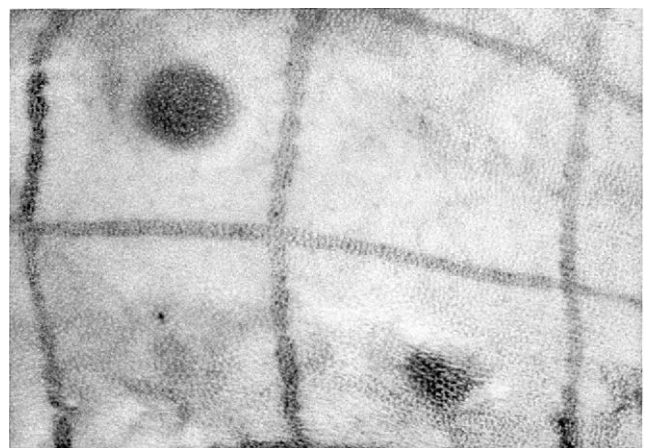


Fig. 1. A passive cutaneous anaphylaxis assay. Note the positive reactions in the upper-left and lower-right quadrants, indicating the presence of IgE antibodies against the TGP antigens (see Methods for details). The negative reaction in the lower-left quadrant is due to heat inactivation of these antibodies; the negative reaction in the upper-right quadrant is due to a lack of antibody activity against the buffered saline control.

reactive with buffered saline control, ovalbumin, or bovine serum albumin. The serum of control rabbits did not contain such antibodies.

3.2. Weekly ventilation and behavioral data

Weight gain patterns were similar in the TGP-S and control animals (data not shown). Weekly breathing frequencies were the same in the TGP-S and control animals (Fig. 2A). Breathing frequency increased in both groups at 6 weeks of age and remained high at 8 weeks of age. Weekly tidal volume measurements were also the same in the two groups (Fig. 2B). Tidal volume per body weight decreased with age in both groups. Additionally, there were no discernible behavioral differences between the two experimental groups.

3.3. TGP challenges

All animals survived the intravenous challenges. No behavioral or physiologic changes occurred during the control saline injections (data not shown). However, all of the TGP-sensitized rabbits, but none of the controls, developed sudden movements consisting of startle

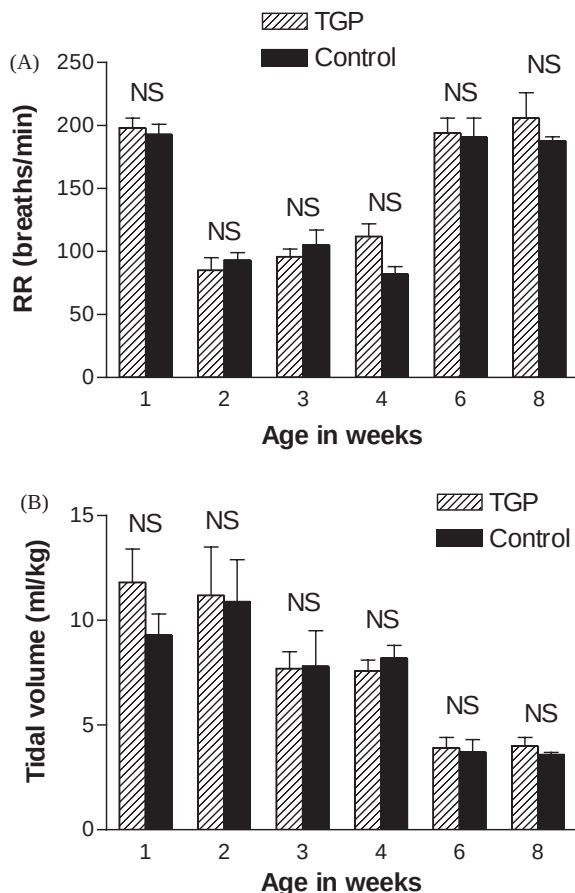


Fig. 2. Weekly breathing frequencies, RR (A), and tidal volumes, in mL/kg (B) in the TGP-sensitized and control animals.

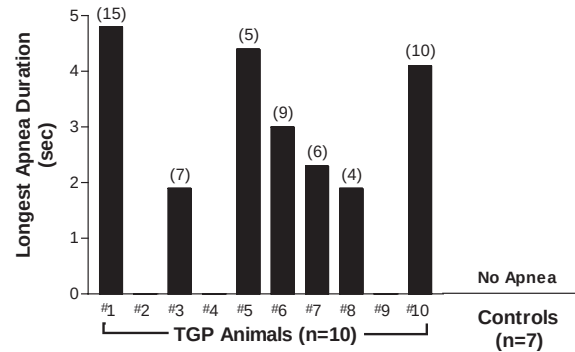


Fig. 3. Apnea occurrence and longest apnea duration during TGP challenges in TGP-sensitized and control animals. The numbers in parentheses above the bar graphs refers to the number of apneas (>1s) during the challenge in that animal. Two TGP-sensitized animals had movement artifacts that precluded the assessment of apnea.

responses within seconds of the TGP injection. In addition, most (seven of 10) of the TGP-S animals developed periods of prolonged apnea as well as irregular breathing approximately 1 min after the TGP injection, while apnea was not seen in the seven control animals ($P < 0.01$, Fig. 3). These apneas and irregular breathing persisted for up to 2 min following the TGP injection. In two of the TGP-S animals, movement artifacts immediately after the TGP injection precluded assessment of apneas. Again, these movements were not seen following TGP injection in the controls nor did they occur following control saline injections in any of the animals. In addition, bradycardia, as defined by the lowest 50-consecutive-beat HR, was seen in the TGP-S animals but not in the controls (Fig. 4A). Both HR (Fig. 4A) and breathing frequency (Fig. 4B) increased in the recovery period, i.e., 2–4 min following the TGP injection, in the TGP-S group but remained constant in the control group. Due to the irregular breathing, movement, and periods of apnea, breathing frequency could not be determined during the response period, i.e., 0–2 min post-TGP injection. Fig. 5 shows a typical TGP challenge response in a TGP-S animal (A) and control animal (B).

4. Discussion

These data have shown that neonatal sensitization of rabbits with TGP and subsequent rechallenge with TGP results in anaphylactic reactions, including bradycardia and irregular breathing characterized by apneas. We believe that these reactions are anaphylactic for several reasons. First, we have shown that the infant rabbits given TGP in the neonatal period and then boosted with TGP at 1 month of age were sensitized to TGP with the development of IgE antibodies against TGP based on the passive cutaneous anaphylaxis assay. None of the

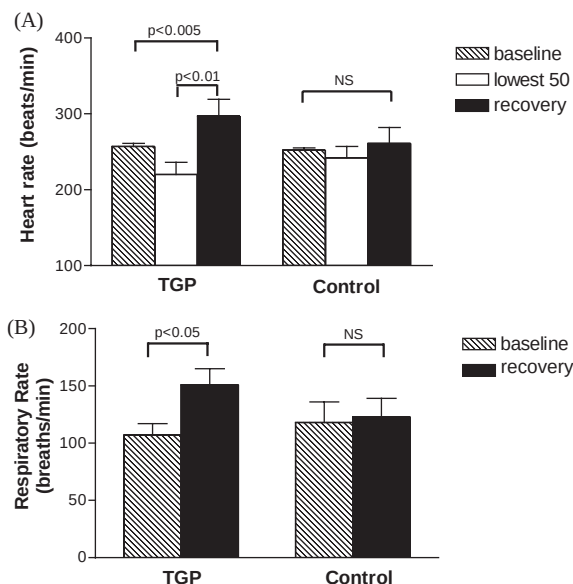


Fig. 4. (A) Heart rates during the TGP challenges in TGP-sensitized and control animals. The HR for each period was calculated using the lowest 50-consecutive-beat HR. The lowest 50 HR during the experiment typically occurred 1–2 min following the TGP injection. The recovery period is defined as 2–4 min post-TGP injection. (B) Breathing frequencies during the TGP challenges in TGP-sensitized and control animals. Due to irregular breathing, movement artifacts, and periods of apnea, breathing frequencies could not be determined during the response period (0–2 min post-TGP injection).

control animals that received antigen-free alum developed these IgE antibodies. Second, only those animals sensitized in the newborn period reacted to the subsequent TGP challenge at 6 weeks of age. Hence, these reactions do not appear to be a direct effect of intravenous TGP, as control animals had no response to the TGP injections. Third, we believe that the time frame of these reactions is consistent with anaphylaxis. Nearly all of the reactions occurred within 30–60 s of completion of the TGP injection, a time frame common to anaphylactic reactions (McGrath, 1997). Last, also consistent with anaphylaxis, is the fact that these reactions were not noted following the intraperitoneal booster injection at 30 days of age but were only seen after the intravenous injection at 6 weeks of age. Anaphylaxis more commonly occurs following intravenous antigen exposure rather than oral or other means of exposure (McGrath, 1997).

Others have suggested that the effects of cigarette smoke are related to a blockade of adrenergic receptors rather than to an immunologic phenomenon (Keller and Doyle, 1976). In that study, mice exposed to tobacco smoke were significantly more susceptible to the lethal effects of histamine compared to non-tobacco-exposed mice, although this effect was diminished by preinjection of the mice with isoproterenol (Keller and Doyle, 1976). However, in our present study, adrenergic blockade would be an unlikely mechanism, since control rabbits had no response to the TGP injection.

Tobacco smoke and its relationship to anaphylaxis and allergy is not a novel concept. As early as 1968, tobacco hypersensitivity was noted based on skin testing and passive transfer techniques (Zussman, 1968). Studies have demonstrated specific precipitins against tobacco antigens in 12% of healthy cigarette smokers, although this has not been found in nonsmokers (Romanski and Broda, 1977). Nearly 40% of adult volunteers, both smokers and nonsmokers, exhibited IgE-dependent immediate hypersensitivity reactions to TGP (Becker et al., 1976, 1979). Additionally, clinically tobacco-sensitive patients have been successfully desensitized using 10% whole-leaf tobacco extract (Levi et al., 1982; Zussman, 1968). Elevated serum IgE levels have been found in cigarette smokers compared to nonsmokers, suggesting that some of the adverse health effects of smoking may be immunologically mediated (Burrows et al., 1981). In addition, total IgE levels are higher in infants whose mothers smoke than in those who live in a nonsmoking environment (Magnusson, 1986).

In support of the cigarette smoke–allergy/anaphylaxis hypothesis are prior studies that have demonstrated the development of IgE antibodies to TGP in rabbits (Becker et al., 1979; Levi et al., 1982) and guinea pigs (Levi et al., 1982). The authors have suggested that “children born of smoking mothers may be extremely hypersensitive to tobacco antigen, because their capacity to produce blocking antibodies has been suppressed” (Becker et al., 1979). These studies found that anaphylaxis developed in isolated hearts and lungs of rabbits and guinea pigs neonatally sensitized to TGP and later exposed to further antigenic boosting and challenge. The anaphylaxis may be related to the systemic absorption and direct toxic effects of the TGP molecule binding to heart and skeletal muscle cells (Santos-Buch et al., 1995). Other potential mechanisms explaining these findings as well as ours include overactivity of a normally protective neural reflex (Houk et al., 1999), alteration of receptor-mediated cell signaling in the heart and/or brain (Slotkin et al., 2001), or a direct effect of histamine on cardiac mast cells (Yeates et al., 2001). However, these possibilities are only speculative and not a focus of this paper.

Unlike isolated rabbit heart studies in which anaphylaxis was characterized by a variety of arrhythmias, including sinus tachycardia, atrioventricular conduction block, atrial and ventricular premature beats, and tachyarrhythmias, including ventricular tachycardia (Levi et al., 1982), we primarily noted bradycardias followed by sinus tachycardia in the intact rabbit. These differences may be explained by the different methodologies used in these experiments, including the use of the intact animal in the current investigations. However, other studies have noted bradycardia during IgE-induced systemic anaphylaxis in the intact rabbit (Halonen et al., 1976, 1980). Bradycardia has also been

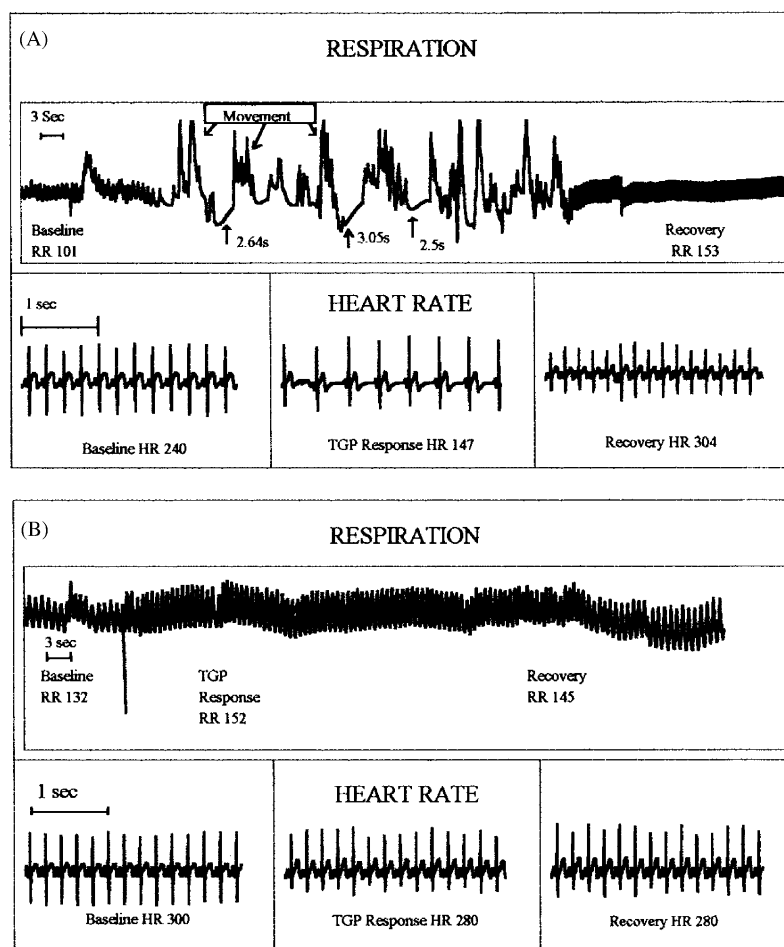


Fig. 5. Typical TGP challenge responses in a TGP-sensitized animal (A) and a control animal (B). Note the irregular breathing and movement, periods of apnea (up arrows), and associated bradycardia that usually occurred approximately 1 min following the TGP injection in the TGP-sensitized animal. Note the relatively constant breathing frequency and heart rate, and lack of movement or apneas, in the control animal.

noted in rabbits during acute upper airway stimulation by cigarette smoke, probably as a result of sympathetic inhibition (Peterson et al., 1983). Although bradycardia was obvious in the present studies, it frequently was difficult to assess the nature of the bradycardia, as *p* waves were often obscured. Additionally, in the previous isolated heart studies, cardiac contractility was frequently decreased, whereas neither contractility nor blood pressure were assessed in the current experiments.

The hypothesis that the anaphylactic reaction would be fatal in some animals was not supported in this study. It is possible that a higher dose of intravenous antigen during the challenge may have resulted in fatalities, but this is only speculative. We also wonder whether a TGP challenge earlier in infancy might prove fatal, though we have not yet completed these studies. Although deaths did not occur, we did note significant apneas in the majority of TGP-sensitized animals. These apneas, longer than 4 s in some animals, should be compared to a usual “breath duration” of 0.5 s. Other studies have also noted abrupt onset of apnea during anaphylaxis in

rabbits (Halonen et al., 1976, 1980). Infantile apnea has been associated with maternal smoking in humans. Studies have found an association between maternal smoking both during and after pregnancy and increased central apnea in infants (Toubas et al., 1986) and between maternal smoking during pregnancy and increased frequency and length of obstructive sleep apneas in newborns and infants (Kahn et al., 1994; Toubas et al., 1986). Although TGP-sensitized animals had obvious and significant reactions during the TGP challenges, their behavior and breathing parameters during weekly measurements were not different from those of the control animals. This suggests that the TGP sensitization by itself had no effect on resting RR, tidal volume, or HR, although it did result in animal vulnerability to subsequent TGP exposure.

This study has its limitations. For instance, the method of both sensitizing and challenging the rabbits was somewhat artificial and nonphysiologic. We hope to duplicate these results via enteral TGP sensitization (and perhaps enteral challenge as well) in the future.

Additionally, since hypotension is such a common manifestation of anaphylaxis, blood pressure monitoring in the rabbits will be essential in further studies. Lastly, as a means of verifying anaphylaxis, both tissue markers and serum alterations, including increases in plasma histamine and tryptase levels and decreases in blood basophil and platelet counts, would be helpful.

The present data are consistent with the concept that cigarette smoke exposure, at least postnatally, may be associated with a higher rate of SIDS via an anaphylactic mechanism. Anaphylaxis, although not a common explanation for SIDS, has been discussed in the literature for nearly 40 years and initially was attributed to a cow's milk sensitivity (Parish et al., 1960). More recently, investigators have found both increased eosinophils in the lungs of infants who have died of SIDS (Baxendine and Moore, 1995; Howat et al., 1994; Roche, 1992), and increased peribronchial mast cells and T and B lymphocytes (Howat et al., 1994). Others have identified elevated concentrations of immunoglobulins in lavage samples in SIDS victims (Forsyth et al., 1989) and increased bound IgG in lung sections of SIDS infants (Ackermann et al., 1979), again suggesting the possible role of hypersensitivity or anaphylaxis. Recent studies have demonstrated increased mast cell activation in SIDS based on elevated serum concentrations of tryptase, a neutral protease produced primarily by mast cells (Holgate et al., 1994; Platt et al., 1994). Others have noted elevated serum concentrations of β -tryptase in SIDS victims (Buckley et al., 2001), again suggestive of anaphylaxis. In one study, 40% of the SIDS infants and none of the control infants had a tryptase level greater than 10 ng/mL, the level that previously has indicated premortem mast cell activation (Platt et al., 1994). The same study found a 20-fold higher chance of having an elevated tryptase level in a SIDS infant compared to a control infant. These studies, along with the present investigation, lend credence to the "anaphylaxis theory" for SIDS. The fact that cigarette exposure is only one of several risk factors for SIDS suggests that SIDS is a multifactorial problem.

Investigators have explained SIDS using the "triple-risk model," in which (1) a vulnerable infant is involved, (2) a critical developmental period in the infant's life is reached, and (3) a certain exogenous trigger(s) or stressor(s) occurs in the infant's environment (Filiano and Kinney, 1994). SIDS only occurs, based on this model, when all three factors are present. Environmental exposure to cigarette smoke may satisfy the triple-risk model by creating a vulnerable infant in utero via tobacco sensitization and by acting as a physiological stressor, perhaps through anaphylaxis in early infancy, the period when SIDS is most prevalent.

In summary, we have shown that most rabbits neonatally sensitized to TGP develop anaphylactic

responses, including apnea and bradycardia, upon further intravenous TGP challenge. This study suggests that some children of smoking mothers have immature control of respiration, are unable to compensate for the consequences of a TGP antigenic challenge and, therefore, are at an increased risk of SIDS.

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