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Investigations into the Immunotoxicity and Allergic Potential Induced by Topical Application of *N*-Butylbenzenesulfonamide (NBBS) in a Murine Model

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Abstract

N-Butylbenzene sulfonamide (NBBS) is a commonly used plasticizer found in numerous products. Due to its extensive use, lack of adequate toxicological data, and suspicion of toxicity based on the presence of structural alerts, it was nominated to the National Toxicology Program for comprehensive toxicological testing. The purpose of this study was to evaluate the potential for hypersensitivity and immune suppression following dermal exposure to NBBS using a murine model. NBBS tested negative in a combined irritancy/local lymph node assay (LLNA), classifying it as nonirritating and nonsensitizing. To estimate the immunosuppressive potential of NBBS, assays that assessed immunotoxicity were performed, including the immumnoglobulin (Ig) M response to T-cell-dependent antigen sheep red blood cells (SRBC), using the plaque-forming cell (PFC) assay and immune cell phenotyping. After a 28-d treatment with NBBS, mice exposed to the lowest concentration (25% NBBS) showed a significant increase in IgM-producing B cells in the spleen. No marked changes were identified in immune cell markers in the lymph node. In contrast to body weight, a significant elevation in kidney and liver weight was observed following dermal exposure to all concentrations of NBBS. These results demonstrate that dermal exposure to NBBS, other than liver and kidney toxicity, did not apparently induce immunotoxicity in a murine model.

N-Butylbenzenesulfonamide (NBBS) is a common plasticizer used in polyacetals, polycarbonates, polysulfones, and polyamides, in flexible tubing, and in the production of films, transparent coating, and plastic resins (Kumar et al. 2007; Strong et al. 1991). NBBS (1) enables easier machining and removal of plastics from molds, (2) produces a better finish, and (3) imparts heat stability (Proviron Fine Chemicals 2003). In addition to its plasticizer properties, NBBS possesses antifungal properties (Kim et al. 2000), and was described as a starting reagent for synthesis of a proposed sulfonyl carbamate herbicide as well as other industrial chemicals and drugs (Stephens 1976). In the United States alone, production volumes of NBBS in 2006 ranged from 1,000,000 to <10,000,000 lb, according

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to the 2006 Inventory Reporting database. Environmental studies showed that NBBS is a groundwater contaminant and not readily biodegradable. NBBS was detected in pre- and posttertiary treatment water samples taken at a water treatment plant in southern California and ranged from approximately 1 to 16.3 μg/L (Soliman et al. 2007). Its presence was also demonstrated in different locations within the San Francisco Estuary (1999 and 2000) and in the Santa Ana River (2002) from variou sources including atmospheric deposition, sewage treatment plant and industrial wastewater effluent discharge, boating activities, and agricultural and urban runoff, ranging from 111 to 454 ng/L (Oros et al. 2003). In addition, NBBS was also identified in the effluents of doue major treatment plants that discharge into the Santa Ana River (concentration from 0 to 94 ng/L, <0.5 mg/L) (Gross et al. 2004). In Europe in 2001, NBBS was detected in eight locations in the Rhine River (92 to 190 ng/L), which flows through highly industrialized areas in Switzerland, Germany and the Netherlands and is an important reservoir of drinking water (Schwarzbauer and Heim 2005).

Due to its widespread production and use, there may be significant potential for human exposure to NBBS, through ingestion, inhalation, and cutaneous routes. Oral exposure is possible due to its presence in contaminated water sources or due to potential leaching from consumer products (Skjevrak et al. 2005). However, inhalation and dermal exposure are the most common forms of occupational exposure that occur in rubber and plastic industries and all other chemical product and preparation manufacturing (U.S. EPA 2010b). The National Institute for Occupational Safety and Health (NIOSH) National Occupational Hazards Survey (1981–1983) estimated that 1534 employees (1169 female and 365 male) were potentially exposed to NBBS (NIOSH n.d.).

In spite of the large production volume and high exposure potential, there are insufficient toxicological data to adequately characterize potential human health risks to NBBS. As a result, NBBS was nominated to the National Toxicology Program (NTP) for comprehensive toxicological testing and evaluation. Results from limited animal studies suggest that this chemical may induce developmental, reproductive, and/or neurotoxic effects. In studies evaluating reproductive effects, NBBS was administered by oral gavage at 100, 200, or 400 mg/kg to male and female Wistar rats 2 wk prior to mating. Males were dosed for a minimum of 28 d, while females were administered until postpartum day 3. At the high dose, adverse effects noted in parental animals included abnormal gait, tremors, hunched posture, piloerection, lethargy, and significant decreases in body weight, while low-dose females showed significant increases in body weight during the premating period. Reproductive performance was impaired in 9 of 12 high-dose mating pairs. A significant reduction in absolute testis and epididymides weight was observed in high-dose males. Histopathological evaluation of the testes and epididymides noted several effects, including Sertoli cell vacuolation, spermatid retention, multinucleated giant cells, desquamation of germ cells, absence of spermatids, atrophy of testis, and oligospermia. An elevated rate of offspring loss (pre- and postimplantation, and postpartum) was found for high-dose female rats, and mean pup weight was decreased (IUCLID 2007). In another study, pregnant mice were administered NBBS (500 or 750 mg/kg/d) via intraperitoneal (ip) injection and examined on gestation day 13. The number of live fetuses, average crown–rump lengths, body weight, and placental weight in both treatment groups were significantly reduced compared to control dams (Hashimoto et al. 1991).

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In vitro studies support a role for NBBS cytotoxicity in Neuro-2a and C6 glioma cells. Following a 72-h incubation using conventional assays for cell viability and function, micromolar concentrations of NBBS inhibited cell growth, produced morphological changes, and altered cell function in these cell lines. A lower concentration of NBBS was required to inhibit DNA synthesis in Neuro-2a cells (10 p*M*) than in C6 glioma cells (100 p*M*), suggesting that neuronal cells may be more sensitive than glial cells to NBBS-mediated toxicity. The effects of NBBS on DNA synthesis in C6 glioma cells occurred as early as 24 h after exposure to a concentration of 10 p*M* as shown by an 18% inhibition of thymidine incorporation, with 30% inhibition at 72 h. At an NBBS concentration of 250 p*M*, DNA synthesis was markedly inhibited (70%) at 72 h. C6 glioma cells exposed to 100 or 250 p*M* NBBS exhibited markedly reduced or absence of immunoreactivity with antibodies against glial fibrillary acidic protein and S-100 protein, while Neuro-2a cells incubated with 1 or 10 p*M* NBBS demonstrated significantly less staining for the 160-kD neurofilament subunit protein (Nerurkar et al. 1993). NBBS (35–2000 μg/ml) also was found to be cytotoxic to human lymphocytes following exposure to 1000 or 2000 μg/mL (IUCLID 2007).

Due to the high production volume of NBBS, high potential for human exposure, and lack of dermal toxicological data, this study was performed to evaluate the immunotoxicity following subchronic dermal exposure to NBBS in a murine model.

Materials and Methods

Animals

Female BALB/*c* and B6C3F1 mice were used in these studies. BALB/*c* mice have a T-helper (Th2) bias and are commonly used to evaluate potential immunoglobulin (Ig) E-mediated sensitization and therefore used in the hypersensitivity studies (Klink and Meade 2003; Woolhiser et al. 2000). B6C3F1 mice are the strain of choice for immunotoxicity studies and were used to evaluate the IgM response to sheep red blood cells (SRBC) (Luster et al. 1992). All mice were purchased from Taconic Farms (Germantown, NY) at 5–9 wk of age weighing 18–20 g and allowed to acclimate for a minimum of 5 d before they were randomly assigned to treatment groups. Animals were weighed and individually identified via tail markings using a permanent marker. A preliminary analysis of variance on body weights was performed to ensure homogeneous distribution of animals across treatment groups. Animals were housed 5 per cage (except for concentration range-finding studies, which required mice to be housed 3 per cage) in ventilated plastic shoebox cages with hardwood chip bedding, fed NIH-31 modified 6% irradiated rodent diet (Harlan Teklad), and provided tap water from water bottles, ad libitum. The temperature in the animal facility was maintained between 18 and 73°C with relative humidity between 30 and 70%. The light/dark cycle was maintained on 12-h intervals. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited National Institute for Occupational Safety and Health animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

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Chemicals

N-Butylbenzene sulfonamide (CAS number 3622-84-2; Figure 1), acetone vehicle (CAS number 67-64-1), USP grade phosphate-buffered saline (PBS), the positive control for the LLNA alpha-hexylcinnamaldehyde (HCA, CAS number 101-86-0), the irritancy control 2,4-dinitrofluorobenzene (DNFB, CAS number 70-34-8), and cyclophosphamide (CP) control for plaque-forming cell assay (PFC assay) (CP, CAS number 50-18-0) were purchased from Sigma Aldrich (St. Louis, MO).

Concentration Range-Finding Study

An initial range-finding study was conducted to determine the test article concentrations to be used in the subsequent studies. Mice (3 per group) were exposed topically on each ear to 25 μl vehicle (acetone) or 100% NBBS for 3 consecutive days. Animals were allowed to rest for 2 d following the last exposure and then euthanized via CO_2 inhalation. Mice were observed daily for signs of distress or extreme irritation, for any signs of chemical-induced toxicity (ruffled fur, abnormal posture, isolation in the cage, abnormal exudates from the eyes, nose, or anus), and for local signs of irritation, including ear erythema and swelling. Animals were weighed the day prior to the first exposure and on d 4 prior to euthanasia.

Combined Irritancy and Local Lymph Node Assay

To determine the irritancy and sensitization potential of NBBS, a combined irritancy/LLNA was conducted and performed according to the method described in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Peer Review Panel report (National Institute of Environmental Health Sciences [NIEHS] 1999) and as previously described (Anderson et al. 2013).

In Vivo IGM Response to the T-Cell-Dependent Antigen SRBC

The primary IgM response to sheep red blood cells (SRBC) was enumerated using a modified hemolytic plaque-forming cell (PFC) assay of Jerne and Nordin (1963). B6C3F1 mice were exposed over a 28-d period dermally (25 μl per dorsal surface of each ear). This exposure schedule is based on immune-toxicity guidelines set forth by the NTP. Groups of mice (5/group) were exposed to one of three concentrations of test agents or acetone vehicle over a 28-d period for each mouse. Systemic toxicity was evaluated weekly by clinical observations and change in body weight. The PFC assay was conducted as previously described (Anderson et al. 2013). Results were expressed as specific activity (IgM PFC per 10^6 spleen cells) and total activity (IgM PFC per spleen).

Immune Cell Phenotyping

Animals were euthanized by CO_2 inhalation 24 h after the final exposure, weighed, and examined for gross pathology. Blood was collected in ethylenediamine tetraacetic acid (EDTA)-coated vacutainer tubes following transection of the abdominal aorta, and hematological analysis was conducted (see later description). The liver, spleen, kidneys, and thymus were removed, cleaned of connective tissue, and weighed. Spleen and DLN cell suspensions were prepared by mechanical disruption of tissues between frosted microscope slides in phosphate-buffered saline (PBS) and counted on a cellometer (Nexcelom). Cells

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($1–2 \times 10^6$) were aliquoted into a 96-well U-bottom plate and washed in staining buffer (PBS + 1% bovine serum albumin + 0.1% sodium azide). Cells were resuspened in staining buffer containing anti-mouse CD16/32 antibody (clone 2.4G2) for blocking of Fc receptors (BD Biosciences). Cells were next resuspended in staining buffer containing a cocktail of fluorochrome-conjugated antibodies specific for cell surface antigens: CD45-Allophycocyanin (clone 30-F11), CD3e-V500 (500A2), CD4-Allophycocyanin-H7 (GK1.5), CD8a-PE-CF594 (53-6.7), CD45R/B220-Alexa Fluor 700 (RA3-6B2), NK1.1-FITC (PK136) (BD Biosciences), CD11c-eFluor 450 (N418), and CD11b-PerCP-Cyanine5.5 (M1/70) (eBioscience). Cells were washed in staining buffer and fixed in Cytofix buffer (BD Biosciences). Within 24 h, cells were resuspended in staining buffer and analyzed on an LSR II flow cytometer (BD Biosciences). Data analysis was performed with FlowJo 7.6.5 software (TreeStar, Inc.). Leukocytes were first identified by their expression of CD45, and the cells were further identified as follows: CD4 T cells (CD4+ CD3+), CD8 T cells (CD8+ CD3+), B cells (B220+ CD3-), NK cells (NK1.1+, CD3-), and dendritic cells (CD11b+ CD11c+).

Hematology

Selected hematological parameters were evaluated using a Hemavet 950 automatic hematology analyzer (Drew Scientific, Waterbury, CT). Endpoints analyzed included peripheral erythrocyte and leukocyte counts, leukocyte differentials (lymphocytes, neutrophils, monocytes, basophils, and eosinophils), platelet counts, hematocrit, hemoglobin levels, mean corpuscular hemoglobin (MCH) and hemoglobin concentration (MCHC), mean corpuscular volume (MCP), mean platelet volume (MCV), and platelet distribution width (PDW).

Statistical Analysis

Data were first tested for homogeneity using the Bartlett's chi-squared test. If data were homogeneous, a one-way analysis of variance (ANOVA) was conducted. If ANOVA showed significance at $p < .05$ or less, Dunnett's multiple range *t*-test was used to compare treatment groups with the control group. For dose-response studies, linear trend analysis (linear trend test) was performed to determine whether NBBS had exposure concentration-related effects for specific endpoints. Differences were considered to be significant if $p < .05$ compared to vehicle controls. Statistical analysis was performed using Graph Pad Prism version 5.0 (San Diego, CA).

Results

In Vivo Studies

Dermal exposure of female BALB/*c* mice to the high concentration of NBBS (100%) produced no marked changes in body weight and there were no signs of overt toxicity or visual signs of inflammation at the exposure sites (data not shown). For this reason, 100% was selected as the highest test dose of NBBS, along with 50% and 25% (in acetone), to be used in subsequent investigations. A numerical increase in ear swelling was observed following exposure to NBBS (8.4, 7.2, and 5.6% for 25, 50, and 100% respectively) (Figure 2A). DNFB (0.3%), used as a positive control for irritancy studies, showed a mean

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significant rise of 241% ear swelling after exposure. While a dose-dependent elevation in lymphocyte proliferation was observed, it did not reach statistical significance. Exposure to 25, 50, or 100% NBBS produced SI (stimulation index) values of 0.85, 1.9, and 2, respectively; therefore, an EC3 (threefold increase compared to vehicle control) value could not be calculated and NBBS was not considered to be sensitizing (Figure 2B). HCA (30%) was used as a positive control for the LLNA and resulted in an average SI value of 10.3 (data not shown).

Dermal Exposure to NBBS for 28 d Results in Increased Liver and Kidney Weights

In contrast with body weight data, a significant increase in kidneys and liver weight was observed following exposure of female B6C3F1 mice to all concentrations of NBBS (Table 1). The liver weight rose 28, 50, and 59%, and kidney weight increased 17, 19, and 23%,following exposure to 25, 50, and 100% NBBS, respectively. No marked changes were noted for the other organs evaluated. Dermal exposure to NBBS altered blood lymphocyte count with an elevation of 18, 17, and 19%, respectively, for increasing concentrations of NBBS compared with control, resulting in a dose-dependent rise, although statistical significance was not obtained. Exposure to NBBS did not markedly alter any other of the analyzed hematological parameters (Table 2). Flow cytometric analysis of the DLN did not result in significant alterations in numbers and frequency for any of the lymphocyte markers and subpopulations examined (Table 3). Due to a technical issue, evaluation of the splenic immune markers could not be conducted. There was no difference in spleen cellularity following exposure (data not shown).

Dermal Exposure to NBBS Did Not Suppress the IgM Response to SRBC

To evaluate immune suppressive potential, the murine splenic IgM response to SRBC was examined in female B6C3F1 mice following a 28-d exposure to NBBS. While suppression of IgM response to SRBC was not observed, mice exposed to the lowest concentration of NBBS (25%) demonstrated a significant increase of PFC/spleen, (87% compared with vehicle-treated mice), suggesting an elevation in IgM antibody response to SRBC (Figure 3B). In addition, although it did not reach significance, there was a numerical rise in PFC/10^6 cells following exposure to the lowest concentration of NBBS. Mice exposed to the positive control, cyclophosphamide (CP), showed a significantly reduced specific spleen IgM response and total IgM response compared to vehicle control. No marked changes in body weight were observed for these animals.

Discussion

According to the CDC, more than 13 million workers in the United States are exposed to chemicals that can be introduced through the skin. Occupations and applications with the highest risks for dermal chemical exposure include chemical, pharmaceutical, and plastic/rubber industries, paint and adhesive applications, and some synthesis intermediates products. Occupational skin exposures may result in numerous diseases, which adversely affect an individual's health and capacity to perform at work, resulting in significant economic losses, including decreased productivity, medical expenses, and loss of work because of illness, with associated costs estimated to exceed $1 billion annually in the

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United States alone (Cashman et al. 2012; Mancini et al. 2008). Dermal exposure to hazardous agents may lead to a variety of occupational diseases, including skin diseases such as irritant and allergic dermatitis, as well as systemic toxic effects on the body. In certain instances dermal exposure is the principal route of exposure, especially for chemicals that are relatively nonvolatile. For example, studies of workers in the rubber industry suggested that exposure to genotoxic chemicals present in the workplace is greater via skin than via lung. Vermeulen at al. (2003) evaluated the potential influence of skin aberrations, such as hand dermatitis and traumata of the skin, and acetylation status on urinary mutagenicity levels, in relation to inhalation and dermal exposure. It was noted (based on the levels of genotoxic compounds found in urine of rubber workers) that skin might be the primary route of exposure. It is important to note that while the mice in these investigations were exposed to relatively high (25–100%) concentrations of NBBS, these studies were conducted for the purpose of hazard identification. However, while actual human exposure assessment has not been conducted, it is expected that exposure to the neat form of this chemical may occur occupationally.

Despite the high potential for occupational exposure to NBBS (2010b), there are many gaps in the characterization of the human risk resulting from exposure to this substance, starting with exposure and absorption assessment. Occupational Safety and Health Administration Permissible Exposure Limit (PEL) and American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) values are not available for this chemical. A common indicator of dermal absorption potential is the relative solubility of a material in octanol and water (octanol– water partition coefficient, Kow), expressed in the logarithmic form as log Kow. NBBS has a log Kow of 2.1 (Proviron Fine Chemicals 2003), which suggests significant skin penetration; however, investigations addressing the toxicity and immunotoxicity of this chemical following dermal exposures are lacking.

In an attempt to fill some of the data gaps associated with NBBS-related health effects, this study was performed to investigate potential immunomodulatory effects of NBBS after a 28-d dermal exposure in a murine model. Irritancy and sensitization potential were evaluated using a combined LLNA. The murine LLNA is considered the gold standard for assessing human skin sensitization potential by various regulatory authorities (Cockshott et al 2006). It is based upon the concept that chemical sensitizers induce a primary proliferation (induction phase) of lymphocytes, through previous stimulation of keratinocytes and Langerhans cells, in the lymph nodes draining the site of chemical application, which may be quantified using measurement of radiolabeled thymidine incorporation into lymphnode cellular DNA. However, based on the findings from our study, NBBS did not induce lymphocyte proliferation and is therefore not considered to be a dermal irritant or sensitizer.

NBBS was also evaluated for immunosuppression in a murine model by examining the antibody plaque-forming cell (PFC) response to sheep red blood cells (SRBC). This assay was reported to be one of the best predictors of immunotoxicity in mice, and one of the most sensitive endpoints available to assess chemical induced alteration to the immune system (Luster et al. 1988). The PFC assay quantifies production of specific antibody through enumeration of antibody-producing cells following immunization with SRBC. It is based upon the antibody response to SRBC, a T-dependent antigen that requires functional

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integration of several cell populations including macrophages, T-helper cells, and B cells (Luster et al. 1988). Alterations in function or numbers of any of these populations may result in a suppressed immune response to pathogens. Interestingly, while suppression of the immune response was not observed in the present study, data demonstrated that the lower concentration of NBBS (25% in acetone) induced a significant increase in the IgM response in the spleen. However the biological significance of this finding is questionable due to the lack of change in spleen cellularity and absence of dose response.

In vitro metabolism studies demonstrated that NBBS was metabolized to 2-hydroxy-NBBS through a proposed cytochrome P-450 mechanism (U.S. EPA 2010a). Distribution investigations conducted in rats showed that NBBS (1 mg/kg) rapidly enters the brain, cerebrospinal fluid, and blood within 1 min post intravenous (iv) injection and is present in all tissues and organs examined, including liver, skeletal muscle, kidneys, and fat, by 24 h postexposure (Kumar et al. 2007). Toxicokinetic studies on female rats dosed with stable isotope labeled NBBS by iv injection estimated that 1 min after administration, liver and kidney together received 15% of the dose, blood carried 9%, and muscle accounted for 52% of the dose (Kumar et al. 2007). In the present investigation, while no marked changes were observed in the immune assays, significant increases in liver and kidney weights were observed following exposure in the absence of alterations in spleen, thymus. and body weight. In support of our findings, previous studies reported abnormal histology following NBBS (U.S. EPA 2010a). Rats dosed via gavage for 28 d at 0, 50, 150, or 1000 mg/kg of NBBS displayed enlarged liver and kidneys in animals in the high-exposure group. In addition, the histopathology confirmed for all concentrations centrilobular hypertrophy of hepatocytes, multifocal necrosis in the liver, and hyaline droplet formation in duct epithelium of renal papilla in most animals (U.S. EPA 2010a). Amacher et al. (1998) reported that a 10–50% rise in liver weight (similar to the increases observed in our study) is a typical response to xenobiotic exposure due to hepatic enzyme induction as a result of elevation in workload demand to initiate metabolic clearance. While increased metabolic activity supporting a potential hepatotoxic and nephrotoxic role for NBBS is suggested by these studies, specific pathways and functional capacity were not evaluated; therefore, additional studies are necessary to fully understand this finding.

While dermal exposure to NBBS did not induce marked hematological alterations in the present study, other investigations reported such changes. For example, rats orally gavaged with NBBS (150 mg/kg) for 28 d showed a decrease in erythrocyte count and hemoglobin (U.S. EPA 2010a). In a single-dose threshold study, NBBS produced a dose- and time-dependent suppression of erythropoiesis when administered by a variety of routes to adult guinea pigs and rats when exposed to NBBS by aerosol inhalation, topical application to skin and mucous membranes, and gavage (U.S. EPA 2010a). Acute toxicity occurred at NBBS exposures of 3 mg/m^2 body surface area, and consisted of decreased reticulocyte counts, histological changes suggestive of altered vascular permeability, and death. With a single dose of 1.5 mg/m^2, leukopenia (in guinea pigs), transient increased capillary permeability, and reduced hemoglobin (after 1 mo) developed (U.S. EPA 2010a). A potential explanation for the differences in these findings may be different duration and route of exposure, different models and species, and different exposure concentration. While

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our study did not show marked alterations in red blood cells, a dose-dependent increase in lymphocytes was observed following hematological analysis.

These are the first studies addressing the immunotoxicity and allergic potential induced by topical application of NBBS in a murine model. While these findings do not suggest that dermal exposure to NBBS produces immunotoxic effects, these observations raise concern regarding potential liver and kidney toxicity following exposure to this chemical.

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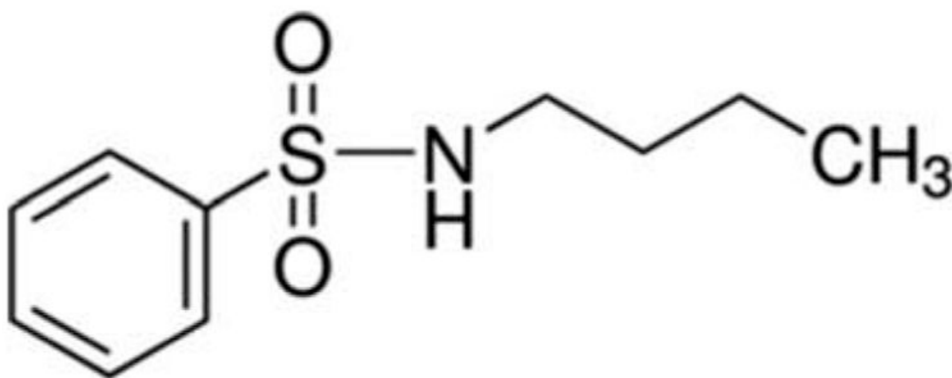

Figure 1.
Chemical structure of NBBS.

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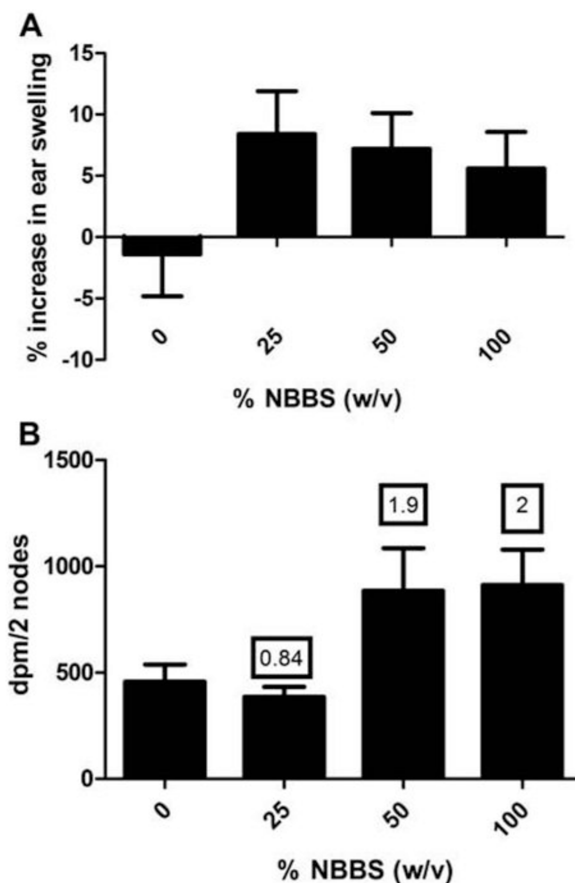

Figure 2.

Irritancy and allergic sensitization potential after dermal exposure to NBBS. Analysis of irritancy (A) and the allergic sensitization potential (B) of NBBS using the LLNA. DPM represents [^{3}H]thymidine incorporation into draining lymph node cells of BALB/*c* mice following exposure to vehicle or concentration of NBBS (25–100%). SI value is the stimulation index (fold change over vehicle control). Bars represent mean (± SE) of five mice per group.

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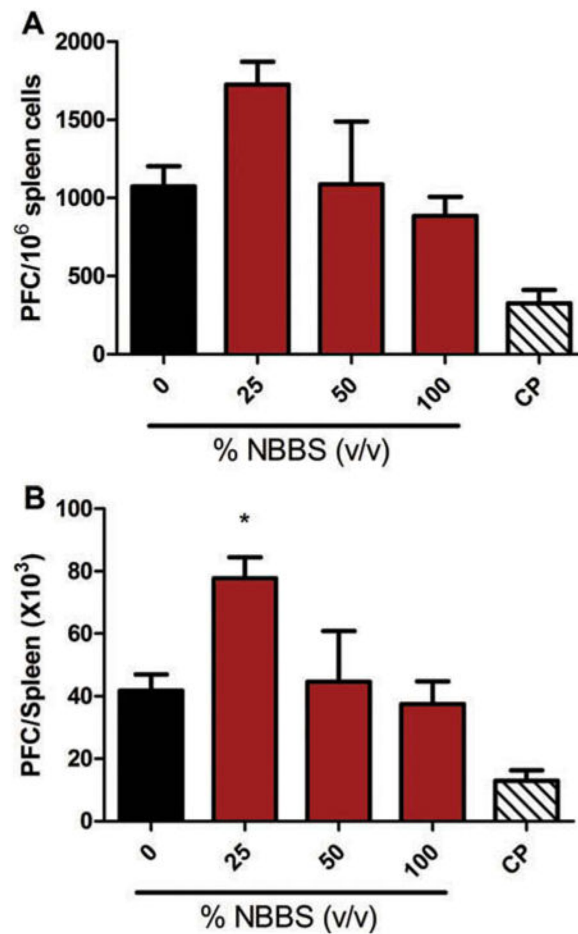

Figure 3.

NBBS does not suppress the spleen IgM response to SRBC. Analysis of antibody producing cells following a 28-d dermal exposure to NBBS (25-100%). The (A) specific and (B) total activity IgM response to SRBC was evaluated in the spleen. Bars represent mean fold change (± SE) of six mice per group. Cyclophosphamide (CP) was included as the positive control. Significance is indicated as $*p < .05$.

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Table 1

Body/Organ Weights of Female $B_6C_3F_1$ Mice Dermally Exposed to NBBS for 28 d

	NBBS (w/v)			
Parameter	**0%**	**25%**	**50%**	**100%**
Body weight (g)	19.31 ± 0.67	20.43 ± 0.83	20.56 ± 0.74	19.91 ± 0.30
Kidney weight				
(mg)	243 ± 6	302 ± 14**	312 ± 15**	312 ± 12$^{**\#\#}$
% bw	1.27 ± 0.02	1.48 ± 0.03**	1.51 ± 0.03**	1.57 ± 0.05$^{**\#}$
Spleen weight				
(mg)	69 ± 2	98 ± 22	74 ± 3	69 ± 4
% bw	0.36 ± 0.01	0.48 ± 0.11	0.36 ± 0.02	0.35 ± 0.01
Thymus weight				
(mg)	43 ± 4	37 ± 6	46 ± 3	49 ± 4
% bw	0.23 ± 0.02	0.21 ± 0.01	0.22 ± 0.01	0.24 ± 0.01
Liver weight				
(mg)	901 ± 30**	1226 ± 57***	1439 ± 41**	1479 ± 44$^{**\#\#}$
% bw	4.67 ± 0.10	6.01 ± 0.17***	7.01 ± 0.11**	7.42 ± 0.16$^{**\#}$

Note. Values are expressed as means (± SE) for each group; bw, body weight. Significantly different from acetone controls at

$^{**}p < .05$ or

$^{***}p < .01$. Linear trend:

$^{\#}p < .001$,

$^{\#\#}p < .05$.

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Table 2

Hematology Parameters of Female $B_6C_3F_1$ Mice Dermally Exposed to NBBS for 28 d

	NBBS			
Parameter	**0%**	**25%**	**50%**	**100%**
Hemoglobin (g/dl)	12.42 ± 2.04	14.06 ± 0.56	12.80 ± 1.72	14.30 ± 0.29
Erythrocytes (M/μl)	7.460 ± 1.27	8.454 ± 0.33	7.712 ± 1.04	8.520 ± 0.19
Platelets (K/μl)	312 ± 130.5	383 ± 131.2	458 ± 150.2	409.2 ± 119.7
Hematocrit (%)	40.50 ± 7.03	46.74 ± 1.85	42.12 ± 5.75	47.66 ± 1.36
MCV (fl)	53.96 ± 0.54	55.30 ± 0.15	54.54 ± 0.25	55.90 ± 0.72
MCH (pg)	16.84 ± 0.38	16.64 ± 0.28	16.60 ± 0.17	16.78 ± 0.31
MCHC (g/dl)	31.28 ± 0.93	30.10 ± 0.46	30.48 ± 0.27	30.04 ± 0.44
PDW (%)	24.64 ± 1.75	21.80 ± 0.64	22.26 ± 0.74	23.82 ± 1.43
Leukocytes (K/μl)	4.256 ± 1.07	3.704 ± 0.84	3.552 ± 0.83	3.792 ± 0.77
Percent lymphocytes	63.16 ± 4.61	74.38 ± 2.36	74.21 ± 1.43	74.85 ± 0.90[#]
Percent neutrophils	24.28 ± 2.61	18.77 ± 2.28	18.39 ± 1.30	18.00 ± 0.69
Percent monocytes	6.43 ± 1.01	4.29 ± 0.48	4.118 ± 0.78	4.36 ± 0.88
Percent eosinophils	4.806 ± 1.15	2.064 ± 1.21	2.504 ± 1.17	2.228 ± 0.78
Percent basophils	1.314 ± 0.39	0.494 ± 0.24	0.784 ± 0.32	0.548 ± 0.17

Note. MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; PDW, platelet distribution width; MCHC, mean corpuscular hemoglobin concentration. Values are expressed as the means (± SE) for each group. Linear trend:

[#] $p < .05$.

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Table 3

Effects of Dermal Exposure to NBBS for 28 d on Draining Lymph Nodes Cell Number and of Lymphocyte Subpopulations in Female $B_6C_3F_1$ Mice

	NBBS			
Parameter	**0%**	**25%**	**50%**	**100%**
DLN, number (× 10^6)	2.176 ± 0.29	3.404 ± 0.45	3.120 ± 0.34	3.411 ± 0.69
B cells, number				
(× 10^5)	4.6 ± 0.34	6.3 ± 0.74	5.7 ± 0.84	6.4 ± 1.3
Percent of dLN	22.10 ± 2.4	18.62 ± 0.92	17.92 ± 1.04	18.66 ± 0.33
Neutrophils				
(number)	566 ± 201	843 ± 180	656 ± 307	581 ± 169
Percent of dLN	0.02 ± 0.007	0.03 ± 0.005	0.02 ± 0.008	0.02 ± 0.06
$CD4^+$ T cells, number				
(× 10^6)	1.4 ± 0.37	1.7 ± 0.23	1.6 ± 0.15	1.7 ± 0.37
Percent of dLN	46.76 ± 2.19	48.54 ± 1.07	51.54 ± 1.31	49.24 ± 0.97
$CD8^+$ T cells				
(× 10^6)	0.67 ± 0.10	1.084 ± 0.14	0.91 ± 0.09	1.025 ± 0.18
Percent of dLN	29.78 ± 0.77	31.84 ± 0.45	29.50 ± 0.57	30.54 ± 0.73
Dendritic cells				
(number)	8456 ± 3810	7760 ± 702	8390 ± 1609	10573 ± 3023
Percent of dLN	0.23 ± 0.03	0.24 ± 0.03	0.26 ± 0.03	0.29 ± 0.04
NK cells				
(× 10^4)	2.8 ± 0.1	2.8 ± 0.3	2.6 ± 0.3	2.7 ± 0.4
Percent of dLN	0.81 ± 0.06	0.85 ± 0.06	0.85 ± 0.10	0.83 ± 0.07

Note. Mice were dermally exposed to vehicle (acetone) or different concentrations of NBBS for 28 d. The mice were euthanized 24 h after the final exposure, DLN were removed, and total cells counted. Numbers and frequency of B and T cells, and subsets of T-cells ($CD4^+$ and $CD8^+$), natural killer (NK), and dendritic cells were enumerated. Values represent the means (± SE) for each group. Significantly different from acetone controls at

$^*p < .05$.