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RESEARCH ARTICLE

Establishment and comparison of delayed-type hypersensitivity models in the $B_6C_3F_1$ mouse

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Abstract

The objective of these studies was to establish and compare delayed-type hypersensitivity (DTH) models, using keyhole limpet hemocyanin (KLH), sheep red blood cells (SRBC), and *Candida albicans* as sensitizing antigens, for their capability to assess a DTH response (utilizing footpad swelling as the endpoint) with minimal confounding factors resulting from antigen-specific antibody (Ab) production. The key elements of the DTH are the sensitization dose, time interval between sensitization and challenge [i.e. the challenge interval (CI)], and the challenge dose. Models were established by first determining the challenge dose, or the amount of antigen that produced no greater footpad swelling 24-h post-injection than the trauma induced by injection of physiological saline. Time-course studies determined the CI that produced a peak response for each antigen. Dose-response sensitization studies were conducted to determine the optimum sensitization concentration (i.e. maximum footpad swelling with minimal impact by antigen-specific Ab production). Footpad swelling decreased dose-responsively with increasing KLH sensitization concentration and corresponded to a dose-responsive increase in KLH-specific Ab levels. In the SRBC model, footpad swelling decreased at the high dose (1×10^9 SRBC/mouse), and a corresponding increase in SRBC-specific Ab was observed at this dose level. A dose-responsive increase in footpad swelling was observed in the *C. albicans* model up to 3×10^7 organisms/mouse, while antigen-specific antibody levels were not different from background (unsensitized) levels following sensitization with any concentration of *C. albicans* (up to 1.2×10^8 organisms/mouse, the highest concentration tested). Finally, each model was evaluated for its ability to detect immunosuppression following exposure to benzo[a]pyrene (B[a]P), with the *C. albicans* model demonstrating greater sensitivity than the other models. These results indicate that, of the three models examined here, the *C. albicans* DTH model may be the most appropriate model for evaluating effects on cell-mediated immunity when conducting immunotoxicological investigations.

Keywords: *Delayed-type hypersensitivity models; Candida albicans; sheep red blood cells (SRBC); keyhole limpet hemocyanin (KLH)*

Abbreviations: Ab, antibody; ABTS, 2,2′-azino-bis[3-ethyl-benzthiazoline-6-sulfonic acid]; ANOVA, analysis of variance; BCG, Bacillus Calmette-Guérin; BSA, bovine serum albumin; C. albicans, Candida albicans; CHT, chitosan; CI, challenge interval; CMI, cell-mediated immunity; CO, challenge only; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; HI, humoral immunity; KLH, keyhole limpet hemocyanin; O.D, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; sc, subcutaneous; SRBC, sheep red blood cells; T_H1, T-helper type 1; T_H2, T-helper type 2; TI, T-independent antigen; TI-1, T-independent antigen, type 1; TI-2, T-independent antigen, type 2.

Introduction

The search for holistic models that provide an accurate assessment of effects on cell-mediated immunity (CMI), such as the delayed-type hypersensitivity (DTH) response, is currently receiving much attention within the field of immunotoxicology. The DTH response has been well characterized since being discovered by Robert Koch in the late 1800s (Kaufmann and Schaible, 2005). The response is cell-mediated and is antigen-specific for a wide variety of antigens, including proteins, grafted tissue, and mycobacteria (Black, 1999). A number of DTH models have since been developed and are currently employed, utilizing a

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variety of sensitizing antigens, including sheep red blood cells (SRBC), *Candida albicans*, tetanus toxoid, attenuated viable *Mycobacterium bovis* [Bacillus Calmette-Guérin (BCG)], keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and ovalbumin (OVA). BSA and OVA (and occasionally KLH) have historically been administered with an adjuvant at the time of sensitization, producing a Jones–Mote hypersensitivity reaction *in lieu* of a 'classic, tuberculin-type' DTH (Black, 1999).

Because the DTH response is antigen-specific, the choice of sensitizing antigen and sensitizing dose are particularly important when developing a model to assess the potential effects of a xenobiotic on the DTH response. Specifically, antigens such as SRBC and KLH are T-dependent antigens that are frequently used in immunotoxicology for evaluating humoral immunity (HI) by inducing antibody (Ab) responses, and it has been documented that increased Ab production correlates with a decreased DTH response to the sensitizing antigen (Mackaness et al., 1974; Lagrange et al., 1980; Morikawa et al., 1991). Thus, for each DTH model, it is imperative that an appropriate sensitizing dose of the antigen be utilized such that antigen-specific Ab production is minimal.

The purpose of these studies was to establish and compare DTH models, using KLH, SRBC, and *C. albicans* as sensitizing antigens without adjuvant, for their ability to assess a DTH response (utilizing footpad swelling as the endpoint) with minimal confounding factors resulting from antigen-specific Ab production. The hypotheses were that antigen-specific antibody, produced in response to sensitization with KLH and SRBC, but not *C. albicans*, would diminish the footpad swelling response, and that the formation of antigen-Ab complexes would contribute to the footpad swelling observed in models demonstrating a significant Ab component.

Materials and methods

Animal husbandry

Female $B_6C_3F_1$ mice (8–15 weeks of age) from Taconic Farms (Germantown, NY) were utilized in these studies. Animals were quarantined for at least 1 week prior to use and were determined to be free of hepatitis and Sendai virus by serology testing. Mice were maintained on NTP 2000 Laboratory Diet and tap water *ad libitum*. Ambient temperatures were maintained at 21–24°C, relative humidity was maintained between 40% and 70%, and a 12-h light/dark cycle was utilized. Mice were randomly assigned to respective treatment groups (typically eight animals per group). All animal procedures were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility under an animal protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Antigens

SRBC in Alsever's (Lampire Biological Laboratories, Pipersville, PA) were washed three times, diluted in 0.9% sodium chloride (NaCl) solution, and administered at doses of 1×10^6–1×10^9 cells/mouse [0.1 mL/mouse subcutaneously (*sc*) into the right flank] for sensitization and at 1×10^8–8×10^8 cells/mouse (0.04 mL/mouse *sc* into right footpad) for challenge. KLH (Pierce Biotechnology, Rockford, IL) stock was prepared at 10 mg/mL, diluted in 0.9% NaCl, and administered at 50–400 μg/mouse (0.1 mL/mouse; *sc* into the right flank) for sensitization and at 6.25–400 μg/mouse (0.04 mL/mouse; *sc* into right footpad) for challenge. The *C. albicans* DTH model utilized here was modified from the procedure established in the laboratory of Dr. Steve Ullrich (Nghiem et al., 2002; Ramos et al., 2002). Formalin-fixed *C. albicans* (AlerChek Inc., Portland, ME) was diluted in 0.9% NaCl and administered for sensitization at 1×10^5–1.2×10^8 organisms/mouse (0.1 mL/mouse *sc* into the right flank; larger volumes were used to achieve sensitization concentrations greater than 3×10^7 organisms/mouse). The *C. albicans* antigen, chitosan [(CHT; (AlerChek Inc.)], was administered at 27.5–55.0 μg/mouse (0.04 mL; *sc* into the right footpad) for challenge in the *C. albicans* DTH model.

Experimental design

Mice were sensitized by *sc* injection of the appropriate antigen in a volume of 0.1 mL into the right flank. On the day of challenge, duplicate measurements of the right footpad of each mouse were made (pre-measurement) with a digital micrometer (Mitutoyo Corp., Tokyo, Japan), followed by *sc* injection of the appropriate challenge antigen into the right footpad in a volume of 0.04 mL. Twenty-four (± 2) hours post-challenge, the thickness of the right footpad was measured in duplicate, and the footpad swelling for each mouse was calculated (average post-challenge thickness—average pre-challenge thickness). Data are reported in terms of footpad swelling in units of mm × 100.

For each model, the challenge level, i.e. the amount of antigen that would produce no greater swelling than an injection of 0.9% NaCl, was first determined. This was accomplished by evaluating the footpad swelling in naïve (unsensitized) mice following *sc* administration (into the right footpad) of either 0.9% NaCl solution or the antigen of interest. Footpad thickness was measured before injection and 24 and 48 h after injection. From these results, appropriate challenge levels were determined for use in all subsequent studies.

Time course studies were completed for each antigen in order to determine the challenge interval (CI), i.e. the time interval between sensitization and challenge that produced a peak response. Animals were sensitized to the antigen of interest and subsequently challenged at one of several timepoints ranging from 2 to 14 days after sensitization. Each study also contained a group of mice that were challenged but not sensitized [challenge only (CO)]. Finally, sensitization dose-response studies, evaluating both footpad swelling and antigen-specific Ab production, were conducted according to the peak CI determined for each model. Following post-measurement of the right footpad 24 h after challenge, animals were humanely euthanized by CO_2 inhalation, and blood was obtained via cardiac puncture for collecting serum. Each study also contained a CO group.

Evaluation of antigen-specific antibody production by ELISA

Enzyme-linked immunosorbent assay (ELISA) was utilized to determine the production of antigen-specific total Ig^+ Ab in each DTH model. A modification of the ELISA procedure established in this laboratory and described by Temple et al. (1993) was used to determine the serum titers of the antigen-specific total Ig^+ Ab for each antigen. Briefly, Immulon-2 microtiter plates (Thermo Electron Corporation, Milford, MA) were coated with 100 µL of the appropriate antigen diluted in sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO) to an appropriate concentration (SRBC membrane antigen: 10 µg/mL; KLH and CHT: 5 µg/mL) and incubated at 4°C overnight. Prior to each subsequent step, plates were washed three times with assay buffer [PBS with 0.05% Tween 20 (Sigma) for SRBC and CHT ELISAs; PBS (Sigma) with 0.1% milk (Carnation Instant Dry Milk Powder obtained from a local grocery) for KLH ELISAs] and allowed to sit for 1 h, followed by three additional washes. Serum samples were diluted in assay buffer and added to microtiter plates. Each serum sample was assayed as 10 serial, two-fold dilutions, starting at a 1:2 dilution. After incubation at room temperature for 60 min, the plates were washed, and the secondary Ab (i.e. human-adsorbed horseradish peroxidase-conjugated goat anti-mouse Ig (H + L) Ab; Southern Biotech, Birmingham, AL) was then added at a 1:500 dilution (1:1000 for KLH ELISAs), and the plates were allowed to incubate for ≈ 60 min. The plates were subsequently washed and the peroxidase substrate ABTS [2,2′-azino-*bis*(3-ethyl-benzthiazoline-6-sulfonic acid); Sigma] was added to each well. After 45 min of incubation at room temperature, the resultant color in each well was read at 405 nm on a $Thermo_{MAX}$ microplate reader (Molecular Devices Corp., Sunnyvale, CA), and results were obtained using Softmax (v.2.32; Molecular Devices Corp). Using the procedure of Kawabata et al. (1995), the linear portion of a log–log plot of optical density (OD) versus dilution was identified, interpolation at 0.5 OD was completed (using Softmax), and the value recorded. The titer was defined as the reciprocal of the serum dilution for which the OD was 0.5; results were reported as $\log_2$ (titer). Samples for which the OD was less than 0.5 at the 1:2 starting dilution were assigned a value of 0, indicating the absence of detectable antigen-specific Ab.

Histology and pathology

After assessing the DTH response to *C. albicans* utilizing the previously determined optimum sensitization conditions, mice from each of three groups (naïve, challenge only, or sensitized and challenged) were anesthetized by CO_2 inhalation and euthanized by cervical dislocation. The right foot of each animal was excised and placed into 10% buffered formalin. Histological and pathological evaluation was conducted by EPL, Inc. (Sterling, VA). Cross-sections were prepared from each sample and stained with hematoxylin and eosin (H&E) prior to pathology evaluation. Images were obtained using a Zeiss Axioplan microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) and captured with a Spot Insight CCD camera and Spot Basic software (SPOT Imaging Solutions, Sterling Heights, MI).

Serum transfer

Donor mice were sensitized with KLH (400 µg/mouse) *sc* in the right flank 5 days prior to euthanization. An additional group of naïve mice were utilized as donors of naïve serum. On the day of serum transfer, donor mice were euthanized by CO_2 inhalation and then subjected to cardiac puncture to obtain blood for collecting serum. Harvested blood was allowed to clot at room temperature for ≈ 2 h, after which it was centrifuged ($1000 \times g$) for 10 min. The resultant serum of several mice was pooled (four mice per pool), thereby generating individual pools for each recipient mouse, and 0.5 mL was administered by intraperitoneal (*ip*) injection to each recipient mouse. One day following serum transfer, all recipient groups and a CO group were challenged in the right footpad with KLH following pre-measurement of the footpad thickness. Post-measurements were obtained 24 h after challenge. Serum was also evaluated by ELISA for KLH-specific Ab titers.

Model sensitivity to the effects of benzo[a]pyrene (B[a]P) on the DTH response

Benzo[a]pyrene (B[a]P; Sigma), a well-documented immunosuppressant, was administered *sc* in corn oil vehicle (VH) for 14 days at 5, 20, and 40 mg B[a]P/kg. Mice were sensitized with the appropriate antigen (50 µg KLH, 1×10^8 SRBC, or 1×10^7 *C. albicans* organisms, per mouse) according to the previously established CIs so that pre-measurement and challenge were conducted on day 15 (i.e. 1 day after the last dose of B[a]P). Positive control mice were sensitized according to the previously determined antigen-specific CI and received 50 mg cyclophosphamide/kg by daily *ip* injection for the last 4 days prior to challenge.

Statistical analysis

Results represent the mean ± standard error obtained from eight animals per group. Statistical analysis of all data was performed using JMP® 5.0 (SAS Institute, Inc., Cary, NC) by first using Bartlett's test for homogeneity of variances, followed by an analysis of variance (parametric or non-parametric as necessary). Ad hoc pair-wise comparisons were made using Dunnett's test and/or the Tukey–Kramer test for parametric data and the Wilcoxon Rank Test for non-parametric data. In all evaluations, $P < 0.05$ indicated a statistically significant difference.

Results

Determination of challenge level

Figure 1 depicts results of the challenge-only studies for SRBC (Figure 1A), KLH (Figure 1B), and CHT (Figure 1C). Significant increases in footpad swelling as compared to

saline control were observed at challenge levels greater than 4×10^8 SRBC/mouse (≥18.4 vs. 9.06 mm × 100) and 50 μg KLH/mouse (23.0 vs. 5.62 mm × 100). Challenge with CHT at both 27.5 and 55 μg/mouse resulted in swelling no different than that following injection of 0.9% NaCl (Figure 1C). From the results of each study, a challenge level was selected for use in the remainder of these experiments, such that swelling following injection of the antigen was not significantly greater than the swelling imparted by injection of 0.9% NaCl. The challenge levels selected for use in all remaining experiments were: 1×10^8 SRBC/mouse for the SRBC model (Figure 1A), 12.5 μg KLH/mouse for the KLH model (Figure 1B), and 55 μg CHT/mouse for the *C. albicans* model (Figure 1C).

Sensitization time-course results

Footpad swelling in the SRBC model was statistically significantly increased over the CO control group at all timepoints except day 2 (Figure 2A). The peak response for this model (67.8 mm × 100) occurred when animals were sensitized 4 days prior to challenge (CI = 4 days). Similarly, in the KLH model, all sensitized groups demonstrated statistically significantly increased footpad swelling as compared to CO controls, with the exception of the day 2 group, with a CI of 4 days providing the peak response (43.2 mm × 100; Figure 2B).

Figure 2C depicts time-course results for the *C. albicans* model. Again, footpad swelling was statistically significantly increased over CO controls at all timepoints, with the exception of day 2. However, in contrast to the SRBC and

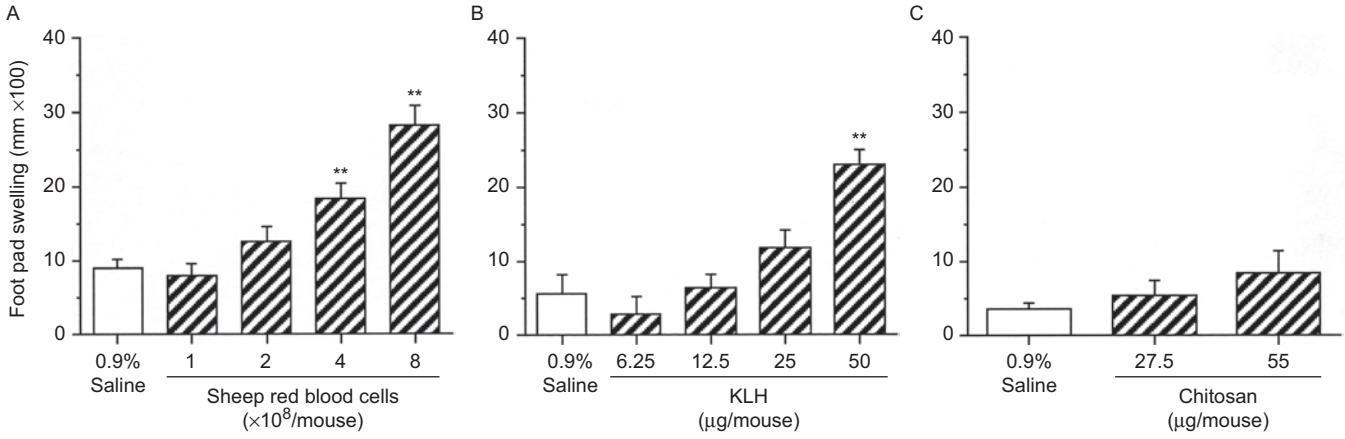

Figure 1. Footpad swelling in naïve mice following challenge with sheep red blood cells (SRBC), keyhole limpet hemocyanin (KLH), or *Candida albicans*. Pre-measurements of the right footpad of naïve mice were obtained with a digital micrometer prior to challenge with either 0.9% NaCl (vehicle) or antigen in a volume of 40 μL. (A) SRBC (1×10^8–8×10^8 cells/mouse); (B) KLH (6.25–50 μg/mouse); and (C) chitosan (27.5 or 55 μg/mouse). The thickness of the right footpad was measured 24 h later, and the change in footpad thickness for each mouse was calculated (post-challenge–pre-challenge thickness). The data are expressed as footpad swelling (mm × 100). Values represent the mean (± SE) derived from eight animals per group; $^{**}P < 0.01$ versus control.

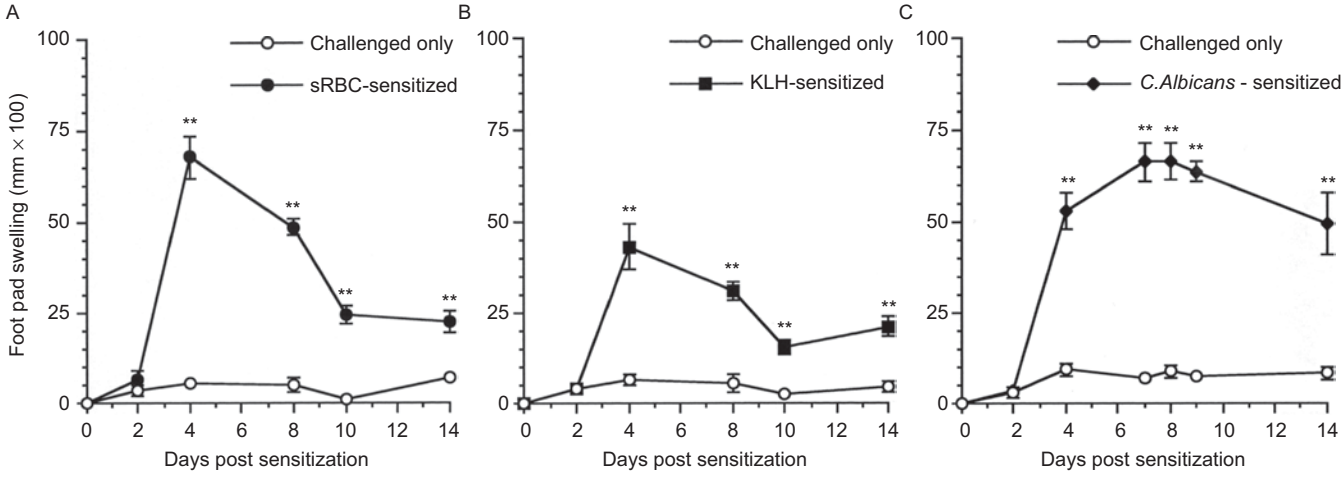

Figure 2. Sensitization time-course studies for sheep red blood cells (SRBC), keyhole limpet hemocyanin (KLH), and *Candida albicans* delayed-type hypersensitivity models. On day 0, mice were sensitized with either 1×10^8 SRBC/mouse, 50 μg KLH/mouse, or 1×10^7 *C. albicans* organisms/mouse in a volume of 0.1 mL *sc* in the right flank. On days 2, 4, 8, 10, and 14 (SRBC and KLH models) or days 2, 4, 7, 8, 9, and 14 (*C. albicans* model), pre-measurements of the right footpad were obtained with a digital micrometer, and mice were subsequently challenged in the right footpad with either 0.9% NaCl (vehicle) or the appropriate challenge dose of antigen in a volume of 40 μL. The thickness of the right footpad was measured 24 h later, and the change in footpad thickness for each mouse was calculated (post-challenge–pre-challenge thickness). The background footpad swelling was determined in a group of mice that were challenged but not sensitized (challenge only). The data are expressed as footpad swelling (mm × 100). Values represent the mean (± SE) derived from eight animals per group; $^{**}P < 0.01$ versus control.

KLH models, the peak response for the *C. albicans* model (66.4 mm × 100) occurred at a CI of 7–8 days. From the results of these time-course studies, the peak CIs selected for use in all subsequent experiments were: 4 days for the SRBC and KLH models and 8 days for the *C. albicans* model.

Sensitization dose-response results

The SRBC model demonstrated increasing footpad swelling (up to 69.4 mm × 100) with no detectable antigen-specific Ab up to 1×10^8 SRBC/mouse (Figure 3). At the high dose (1×10^9 SRBC/mouse), there was a decrease in footpad swelling (49.0 mm × 100; Figure 3A) and a corresponding statistically significant increase in antigen-specific Ab levels (mean $\log_2$ titer = 4.7; Figure 3B).

In the KLH model, a dose-responsive decrease in footpad swelling was observed (Figure 4A), which corresponded to a dose-responsive increase in KLH-specific Ab production (mean $\log_2$ titer = 7.6 for the 400 µg/mouse group; Figure 4B). Footpad swelling was greatest at the 50 µg/mouse dose

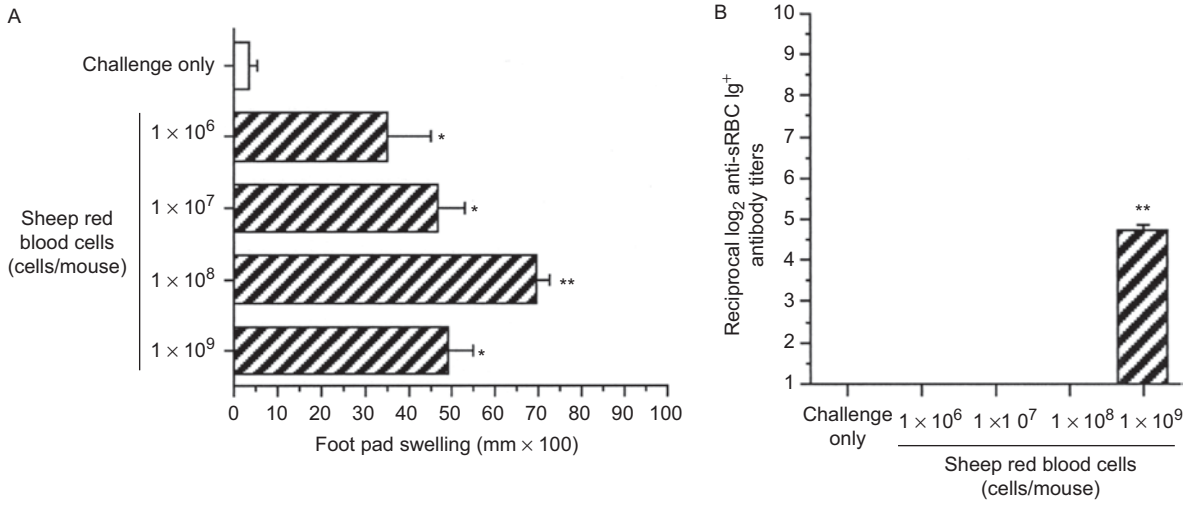

Figure 3. Dose-response of footpad swelling and antigen-specific antibody production using the sheep red blood cells (SRBC) delayed-type hypersensitivity (DTH) model. Mice were sensitized with 1×10^6–1×10^9 SRBC/mouse in a volume of 0.1 mL *sc* in the right flank 4 days prior to challenge. On the day of challenge, pre-measurements of the right footpad were obtained with a digital micrometer, and mice were subsequently challenged in the right footpad with 1×10^8 SRBC/mouse in a volume of 40 µL. The thickness of the right footpad was measured 24 h later, and the change in footpad thickness for each mouse was calculated (post-challenge–pre-challenge thickness). The background footpad swelling was determined in a group of mice that were challenged but not sensitized (challenge only). The data are expressed as (A) footpad swelling [mm x 100], and (B) $\log_2$(titer). Values represent the mean (± SE) derived from eight animals per group; $^*P < 0.05$ or $^{**}P < 0.01$ versus control.

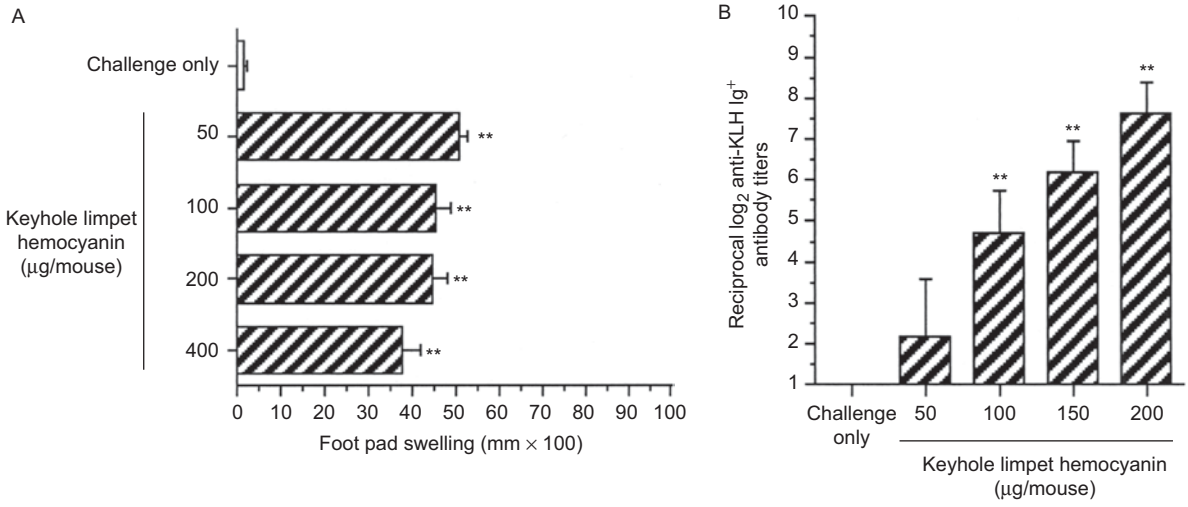

Figure 4. Dose-response of footpad swelling and antigen-specific antibody production using the keyhole limpet hemocyanin (KLH) delayed-type hypersensitivity model. Mice were sensitized with 50–400 µg KLH/mouse in a volume of 0.1 mL *sc* in the right flank 4 days prior to challenge. On the day of challenge, pre-measurements of the right footpad were obtained with a digital micrometer, and mice were subsequently challenged in the right footpad with 12.5 µg KLH/mouse in a volume of 40 µL. The thickness of the right footpad was measured 24 h later, and the change in footpad thickness for each mouse was calculated (post-challenge–pre-challenge thickness). The background footpad swelling was determined in a group of mice that were challenged but not sensitized (challenge only). The data are expressed as (A) footpad swelling [mm x 100], and (B) $\log_2$(titer). Values represent the mean (± SE) derived from seven or eight animals per group; $^{**}P < 0.01$ versus control.

(50.9 mm × 100; Figure 4A), and low levels of anti-KLH Ab were detected at this sensitization level (mean $\log_2$ titer = 2.2; no Ab was detected in three of the seven animals; Figure 4B). However, the mean Ab titer for the 50 μg/mouse group was not significantly different from the CO (unsensitized) control group.

In the *C. albicans* model, a dose-responsive increase in footpad swelling was observed, up to a sensitization concentration of 3×10^7 organisms/mouse (82.3 mm × 100; Figure 5A), with no additional increase in footpad swelling up to 1.2×10^8 organisms/mouse (data not shown). Antigen-specific Ab was not detected at any sensitization level up to 3×10^7 organisms/mouse (Figure 5B). Increasing the sensitization concentration to 1.2×10^8 organisms/mouse failed to promote antigen-specific antibody production (data not shown).

Serum transfer

Due to the high Ab titers observed in the KLH DTH studies and because the CI for the KLH DTH was consistent with the timing resulting in a peak Ab response to KLH (Shea, 2003), serum transfer studies were conducted in order to determine whether the footpad swelling observed was an Arthus reaction (i.e. swelling resulting from the formation of antigen–antibody complexes) or a combination of an Arthus reaction and a DTH response. To determine what contribution, if any, the formation of antigen–antibody complexes made to the footpad swelling observed in the KLH DTH model, serum was transferred from mice sensitized with KLH (400 μg/mouse) to naïve recipients. Another group of mice received serum from unsensitized naïve mice. A third group—receiving no serum transfer—was utilized as the challenge only group. Results indicated no differences in footpad swelling between any of the groups (data not shown). ELISA results confirmed that the titers of KLH-specific total antibody in serum from both KLH-sensitized and naïve mice (data not shown) were similar to titers observed in sensitization dose-response studies shown in Figure 4B.

Histology and pathology analysis in the C. Albicans DTH model

Images of H&E-stained histology samples (Figure 6A, 6B, and 6C at 40×; Figure 6D and 6E at 100×) from the right feet of naïve (Figure 6A), challenged only (Figure 6B), and sensitized and challenged mice (Figure 6C) demonstrate distinct differences in the number of immune cells present. Challenge with CHT resulted in a moderate neutrophilic inflammation with minimal macrophage and lymphocyte infiltration at 1 day post-challenge (Figure 6D). In contrast, samples from mice that had been sensitized with *C. albicans* and subsequently challenged with CHT exhibited more severe neutrophilic inflammation, edema, and greater numbers of accompanying macrophages and lymphocytes than the CO group (Figure 6E).

Effects of benzo[a]pyrene on the DTH response using each model

B[a]P exposure for 14 days resulted in statistically significant immunosuppressive effects in each of the DTH models employed. In the KLH model (Figure 7A), footpad swelling was significantly decreased by 48% at the high B[a]P dose (40 mg/kg), with no effect observed at 20 mg B[a]P/kg. Similarly, no effect was observed at the 20 mg B[a]P/kg dose level in the SRBC model (Figure 7B), while the high dose produced a significant decrease (40%). In contrast, statistically significant decreases in footpad swelling were observed at both the 20 and 40 mg B[a]P/kg exposure groups when the DTH response was evaluated utilizing the *C. albicans* model (Figure 7C). Footpad swelling was significantly decreased 45% at the 20 mg B[a]P/kg exposure level and 61% at the

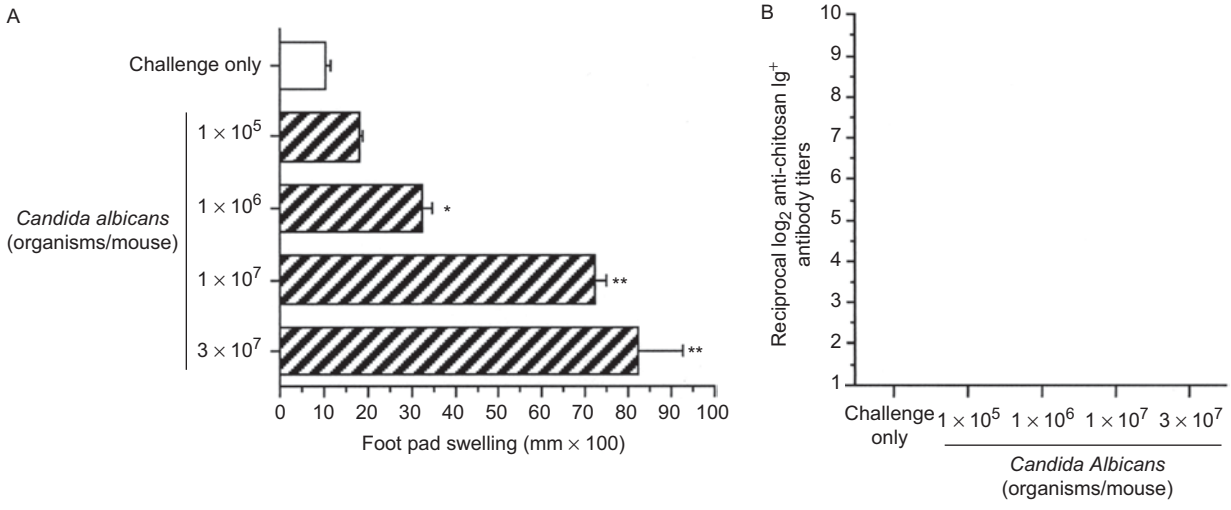

Figure 5. Dose-response of footpad swelling and antigen-specific antibody production using the *Candida albicans* delayed-type hypersensitivity model. Mice were sensitized with 1×10^5–3×10^7 *C. albicans* organisms/mouse in a volume of 0.1 mL *sc* in the right flank 8 days prior to challenge. On the day of challenge, pre-measurements of the right footpad were obtained with a digital micrometer, and mice were subsequently challenged in the right footpad with 55 μg chitosan/mouse in a volume of 40 μL. The thickness of the right footpad was measured 24 h later, and the change in footpad thickness for each mouse was calculated (post-challenge–pre-challenge thickness). The background footpad swelling was determined in a group of mice that were challenged but not sensitized (challenge only). The data are expressed as (A) footpad swelling [mm x 100], and (B) $\log_2$(titer). Values represent the mean (± SE) derived from eight animals per group; $^*P < 0.05$ or $^{**}P < 0.01$ versus control.

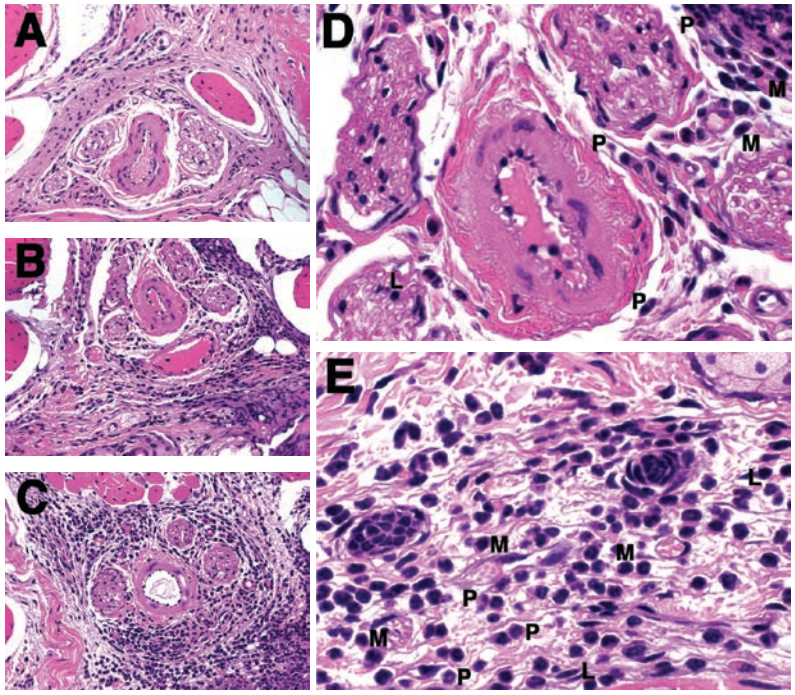

Figure 6. Histology images of feet from mice following evaluation for delayed-type hypersensitivity using the *Candida albicans* model. Mice were sensitized with 1×10^7 *C. albicans* organisms and challenged 8 days later with chitosan in the right footpad. Twenty-four hours after challenge, the right footpad was excised, stored in 10% buffered formalin, and processed for histopathology. Naïve mice and mice that were challenged but not sensitized (challenge only) were also evaluated. Samples were stained with hematoxylin and eosin (H&E). (A) Naïve; (B) and (D), challenge only; (C) and (E), Sensitized and Challenged. Magnifications for (A), (B), and (C) are 40×; for (D) and (E), the magnifications are 100×. L, lymphocyte; P, PMN; M, macrophage.

40 mg B[a]P/kg exposure level. Studies from this laboratory (unpublished data) and by Dean et al. (1983) have demonstrated—using the SRBC antigen—that the humoral immune response is affected to a greater extent than CMI following B[a]P exposure. Similar data using the KLH antigen is not available from either our laboratory or the literature.

Discussion

Based upon the results of these studies, the optimum conditions for evaluating the DTH response in $B_6C_3F_1$ mice using SRBC, KLH, and *C. albicans* as sensitizing antigens are given in Table 1. A CI of 4 days produced a peak response in both the KLH and SRBC models, consistent with previous reports of the peak time course for the SRBC DTH (Hurtrel et al., 1984). Both SRBC and KLH, being T-dependent antigens capable of initiating a humoral immune response, also produce peak Ab responses at a CI of 4–5 days in the mouse (Temple et al., 1993; Shea, 2003). In contrast, the *C. albicans* DTH demonstrated a peak response at 7–8 days post-sensitization, which is closer to the minimum CI of 15 days reported for BCG (Hurtrel et al., 1984) than are the CIs for either SRBC or KLH. Histopathological analyses of feet from mice in the *C. albicans* DTH model indicated that there were greater numbers of macrophages and lymphocytes in samples from mice that were both sensitized and challenged as compared to those from the CO group. The infiltration of these cell types is consistent with a classical DTH response (Hurtrel et al., 1984; Black, 1999).

Each of the three sensitizing antigens examined here can be used to conduct an evaluation of the DTH response using the recommended optimal conditions. Of the three, the KLH DTH model demonstrated greater inter-assay variability and consistently gave results that were less robust than the other two models. In addition, this model demonstrated significant anti-KLH Ab titers at sensitization concentrations only twice the optimal concentration with corresponding decreases in footpad swelling. Because of the potential for antibody production to interfere with the ability to assess a true cell-mediated response in the DTH assay, this model may not be an appropriate choice.

The SRBC DTH model demonstrated no detectable serum anti-SRBC Ab up to a sensitization concentration of 1×10^8 SRBC/mouse. However, significant levels of anti-SRBC Ab were detected following sensitization at the high dose (1×10^9 SRBC/mouse) that corresponded to a significant decrease in footpad swelling following challenge. Even though there was no appreciable Ab response at the optimum sensitization level (1×10^8 SRBC/mouse), the use of this model may be contraindicated. If, for example, the immunomodulatory properties of a drug are unknown and the drug stimulates humoral immune responses, drug-dependent increases in Ab production may correlate with a decreased DTH response such that the compound might be incorrectly deemed an immunosuppressant of CMI. It is therefore important that a model be employed that is unlikely to be subject to confounding by the production of antigen-specific Ab.

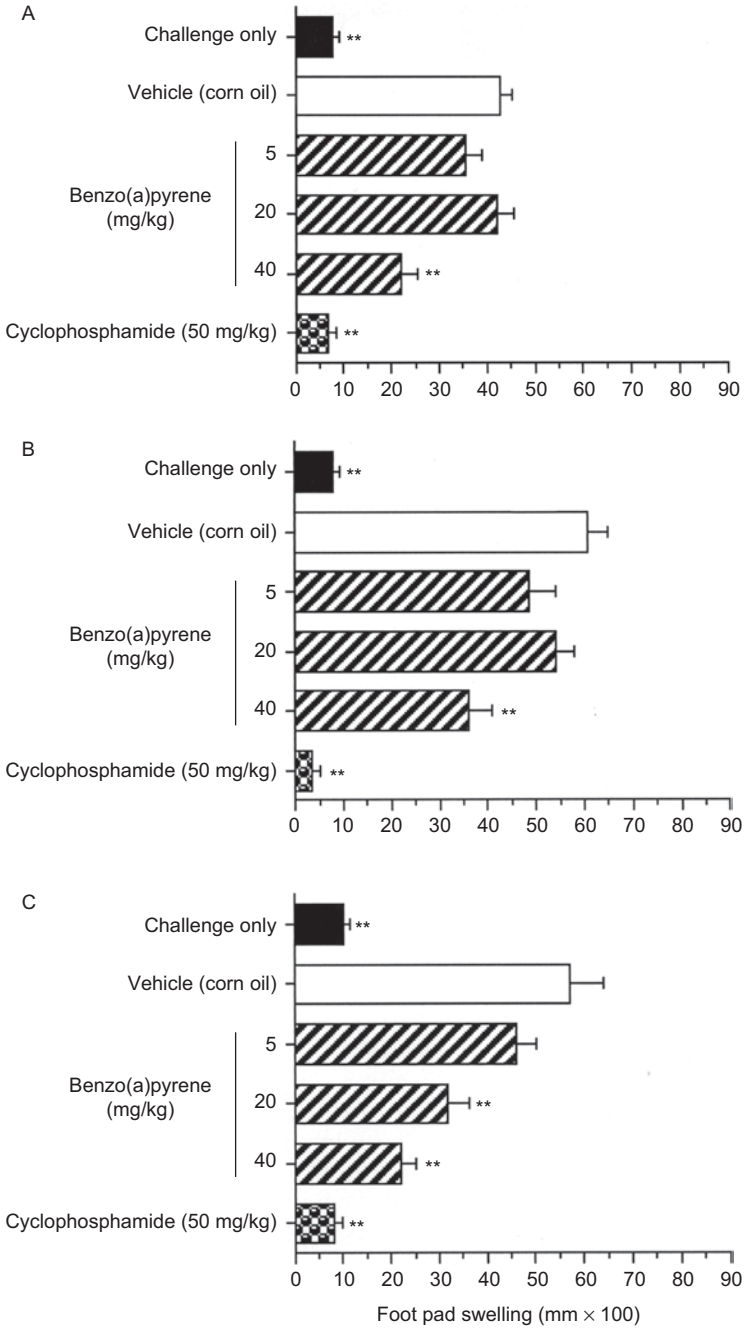

Figure 7. Effects of B[a]P on the delayed-type hypersensitivity (DTH) response using the keyhole limpet hemocyanin (KLH), sheep red blood cells (SRBC), and *Candida albicans* models. Mice were administered either vehicle [corn oil; VH] or B[a]P *sc* daily for 14 days. Positive control mice were administered 50 mg/kg cyclophosphamide (CPS) for the last 4 days prior to challenge. Sensitization with the appropriate antigen was conducted according to the antigen-specific challenge interval (CI) so that pre-measurement and challenge was conducted 1 day after the last exposure to B[a]P. (A) KLH model; (B) SRBC model; and (C) *C. albicans* model. On the day of challenge, pre-measurements of the right footpad were obtained with a digital micrometer, and mice were subsequently challenged in the right footpad with the appropriate concentration of antigen in a volume of 40 μL. The thickness of the right footpad was measured 24 h later, and the change in footpad thickness for each mouse was calculated (post-challenge–pre-challenge thickness). The background footpad swelling was determined in a group of mice that were challenged but not sensitized (challenge only). The data are expressed as footpad swelling (mm × 100). Values represent the mean (± SE) derived from eight animals per group; $^{**}P < 0.01$ versus control.

The primary importance of CMI and the minor role of HI in the host response to *Candida* infection have been well documented. Sinha et al. (1987) reported that there were no differences in either host resistance or the DTH response to *C. albicans* between B-cell deficient and control (wild-type) mice, and they concluded that B-lymphocytes have no appreciable role in protecting against infection with this organism. Indeed, it has been reported that T-helper type 1 (T_H1) responses are necessary in order to resist and clear *C. albicans* infection (Mencacci et al., 1999). Herzyk et al. (1997) reported low serum titers of anti-*C. albicans* Ab following multiple inoculations with the organism, furthering the

Table 1. Summary of the optimum conditions for the SRBC, KLH, and *C. albicans* DTH models in the $B_6C_3F_1$ mouse.

DTH model	Sensitization concentration (per mouse)	Sensitization interval (days)	Challenge concentration (per mouse)
SRBC	1×10^8 cells	4	1×10^8 cells
KLH	50 μg	4	12.5 μg
C. albicans	1×10^7 organisms	8	55 μg CHT

C. albicans, *Candida albicans*; CHT, chitosan; DTH, delayed-type hypersensitivity; KLH, keyhole limpet hemocyanin; SRBC, sheep red blood cells.

consensus that CMI, and not HI, is the primary host-defense mechanism against *C. albicans*.

In the present studies, the challenge antigen used in the *C. albicans* model was CHT, a polysaccharide formed by the deacetylation of chitin, which is the primary structural element of the cell wall of fungi. Polysaccharides are classified as T-independent (TI) antigens for their ability to activate B-lymphocytes without T-lymphocyte help. TI antigens are divided into two groups: Type 1 (TI-1), which are mitogens that elicit polyclonal B-lymphocyte activation via Toll-like receptors, and Type 2 (TI-2), which are polysaccharides that initiate antigen-specific activation by interacting directly with the B-lymphocyte receptor (Mond et al., 1995a, 1995b). Historically, B-lymphocytes have been thought to be incapable of forming a memory response to TI-2 antigens, although recent work has identified a distinct phenotype of memory B-lymphocytes that are specific for TI-2 antigens (Obukhanych and Nussenzweig, 2006).

Current research has indicated that both chitin and CHT are capable of activating macrophages and promoting T_H1 cytokine production while down-regulating T_H2 cytokines (Shibata et al., 1997, 2001; Chen et al., 2008; Lee et al., 2008; Lee, 2009; Muzzarelli, 2010). This is consistent with the robust DTH (i.e. T_H1) response and the absence of a notable antibody response in the *C. albicans* model. Although there is also some evidence of chitin up-regulating T_H2 allergic responses (Elias et al., 2005; Lee et al., 2008; Lee, 2009), the evidence in favor of the T_H1 polarization theory is more abundant at the present time.

At the time of challenge in the *C. albicans* DTH model (day 8), nodules/lesions were observed at the site of sensitization following injections of 3×10^7 organisms and greater, consistent with previous reports (Pearsall and Lagunoff, 1974; Herzyk et al., 1997). However, at the optimum dose (1×10^7 organisms/mouse), no nodules were observed. With no significant increase in footpad swelling at sensitization concentrations $> 1\times10^7$ organisms/mouse, there is no apparent benefit to increasing the sensitization concentration beyond this level.

These studies also indicate the absence of detectable anti-CHT Ab in serum from mice sensitized with up to 1.2×10^8 *C. albicans* organisms. The antigenicity of CHT is weak (Felse and Panda, 1999), and the generation of antibodies to this polysaccharide is quite difficult (El-Gueddari et al., 2002). Indeed, we were unable to generate anti-CHT serum Ab titers in mice after five administrations of CHT with alum over 4 weeks (data not shown). It has been reported that several injections of chitin over an extended period of time, using Freund's complete and incomplete adjuvants, was necessary in order to obtain sufficient antibody titers to this polysaccharide (Spindler-Barth and Buss, 1997). Given this weight of evidence, it is likely that exposure to CHT in the absence of an adjuvant is not sufficient to generate a measurable antibody response, suggesting little, if any, concern for the generation of significant anti-CHT Ab levels in this DTH model.

Utilizing each of the three DTH models, results indicated that B[a]P, a well-documented immunosuppressant of HI, is also suppressive of CMI, in contrast to reports otherwise (Dean et al., 1983; White, 1992). A previous report from our laboratory demonstrated conflicting results when the DTH response was evaluated following B[a]P exposure (White, 1992). Specifically, no effect was seen in the KLH DTH (utilizing [^{125}I]-5-iododeoxyuridine [IUdR] incorporation in an ear challenge model), while footpad swelling in the SRBC DTH was significantly decreased at 40 mg/kg, in agreement with the results presented here. Because B[a]P had previously been indicated as having dramatic effects on HI (in the plaque assay) with no effect on CMI, that previous report hypothesized that the SRBC DTH may have had a significant antibody component contributing to the swelling observed, i.e. an Arthus reaction, and that the KLH DTH was perhaps a better model. However, the KLH serum transfer study presented here and a previous study by Hurtrel et al. (1984) utilizing SRBC suggest this is not the case.

Although each of the three models was able to detect the cell-mediated immunosuppressive effects of B[a]P, the *C. albicans* model was more sensitive to these effects than were the SRBC and KLH DTH models, lending greater evidence to its advantage over these other models. The *C. albicans* model is therefore a robust, sensitive model for assessing effects on the DTH response without significant potential for interference by the production of antigen-specific Ab. The weight of evidence here suggests that, of the three models evaluated, the *C. albicans* model is the most appropriate choice for evaluating the effects of any given xenobiotic on the DTH response.

Conclusion

We have successfully established and optimized DTH models using SRBC, KLH, and *C. albicans* as sensitizing antigens. Results demonstrated the potential for antigen-specific Ab production to decrease footpad swelling in the SRBC and KLH models, which were each insensitive to the immunosuppressive effects of B[a]P at 20 mg/kg, in contrast to the *C. albicans* DTH model. Due to both the absence of an appreciable humoral immune response in the *C. albicans* DTH model and a greater sensitivity at detecting cell-mediated immunotoxic effects when utilizing this model, we conclude that the *C. albicans* DTH model is a more appropriate choice than the SRBC and KLH models for assessing the cell-mediated immunotoxic effects of a drug or compound.

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Declaration of interest

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