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Suppression of Antigen-Specific Th2 Cell-Dependent IgM and IgG1 Production Following Norepinephrine Depletion In Vivo

A. P. Kohm* and V. M. Sanders2*

The mechanism by which the Th2 cell-dependent Ab response is modulated by the sympathetic neurotransmitter norepinephrine (NE) was investigated. Our model system used the severe combined immunodeficient (scid) mouse that was depleted of NE with 6-hydroxydopamine before reconstitution with a clone of β2-adrenergic receptor (β2AR)neg KLH-specific Th2 cells and resting trinitrophenyl (TNP)-specific β2ARpos B cells enriched from the spleens of unimmunized mice. Following challenge with TNP-keyhole limpet hemocyanin (KLH), Ab production in these mice was hapten-, carrier-, and allotype-specific as well as MHC restricted. Depletion of NE resulted in a 50–75% suppression of the primary anti-TNP IgM response compared with that of NE-intact controls, while the secondary IgM response returned to control levels. In contrast, both the primary and secondary anti-TNP IgG1 responses were suppressed by 85 and 40%, respectively. Using NE-intact mice exposed to either a βAR- or αAR-selective antagonist, the effect of NE on the Ab response was shown to be mediated by the βAR. In addition, administration of a β2AR-selective agonist to NE-depleted mice partially reversed the suppressed Ab response that resulted from NE depletion. Expression of the β2AR on TNP-specific B cells was confirmed by radioligand binding, immunofluorescence, and cAMP analysis. Also, while splenic histology was comparable in NE-intact and NE-depleted mice before Ag exposure, follicle expansion and germinal center formation were suppressed in NE-depleted mice after Ag exposure. Taken together, these results suggest that NE stimulation of the β2AR expressed on B cells is necessary for the maintenance of an optimal primary and secondary Th2 cell-dependent Ab response in vivo.


Many groups have described the innervation of both primary and secondary lymphoid organs by sympathetic nerve fibers. These studies demonstrate rich sympathetic innervation penetrating the parenchyma of lymphoid organs via the vasculature that supplies the periarterial lymphoid sheath (PALS), marginal zone, and marginal sinus (8). In addition, electron microscopic studies reveal that sympathetic nerve terminals are in direct apposition to T cells and interdigitating dendritic cells (8), with this junction being ~6 nm wide compared with a typical CNS synapse, which is ~20 nm wide. Following exposure to Ag (9, 10), LPS (11), or IL-1 (11) in vivo, the sympathetic neurotransmitter norepinephrine (NE) is released from sympathetic nerve terminals and is bound by the β-adrenergic receptor (βAR) expressed on resident lymphocytes, which induces an increase in the intracellular concentration of cAMP (7).

A variety of murine immune cells express the βAR, including macrophages, NK cells, B cells, and T cells (reviewed in Ref. 7). Recent data show that the β2AR is differentially expressed on both resting and anti-CD3-activated murine CD4+ effector T cell clones, with detectable expression on Th1 cells, but not on Th2 cells, as determined at the protein level by radioligand binding and immunofluorescence analysis (12, 13), at the mRNA level by RT-PCR (A. P. Kohm et al., manuscript in preparation), and at the functional level by cAMP accumulation following receptor stimulation with a β2AR-selective agonist (12). Furthermore, the functional relevance of differential β2AR expression on Th1 and Th2 cell clones was demonstrated by the finding that β2ARneg Th1 clones pre-exposed to a β2AR-specific agonist before interaction with nonexposed Ag-presenting B cells in vitro produced less IFN-γ than nonexposed Th1 cell controls and resulted in reduced IgG2a production by the B cells (12). As expected, β2ARpos Th2 clones preexposed to a β2AR-specific agonist before interaction with nonexposed B cells produced the same amount of IL-4 as nonexposed Th2 clones and resulted in no change in IgG1 production by the Ag-presenting B cells. In contrast, preliminary findings in our laboratory suggest that stimulation of the β2AR on B cells...
cells during Ag processing, but before interaction with β2AR

Th2 cell clones, results in an enhancement of IgG1, but not IL-4, production. (D. Kasprowicz et al., manuscript in preparation.) Thus, the lack of β2AR expression on clones of Th2 effector cells provides a unique opportunity to study the possible role played by this receptor that is expressed on B cells alone in the NE-induced modulation of the Th2 cell-dependent Ab response both in vitro and in vivo.

In previous studies to determine the role of NE in modulating the Th cell-dependent Ab response in vivo, peripheral sympathetic nerve terminals containing NE were reversibly destroyed in animals by chemical sympathectomy with the neurotoxin 6-hydroxydopamine (6-OHDA) before exposure to Ag. Results from experimental model systems using 6-OHDA-treated animals have been conflicting, showing enhancement (9, 14, 15), suppression (16–18), or no change (19) in the level of Ab production and cell proliferation. One possible source of these conflicting results may be the low frequency of Ag-specific lymphocytes in previous animal model systems that makes it difficult to study the effect of NE on Ag-specific B cell interactions with Ag-specific Th2 effector cells that provide the cytokines necessary for an Ag-specific IgG response. In addition, it has been difficult in previous studies to dissect the effect of NE on CD4+ T cell differentiation into Th1 and Th2 effector cells after the first exposure to Ag from the effect of NE on fully differentiated Th1 and Th2 effector cells themselves.

Although considerable insight into the role of NE in the T cell-dependent Ab response was gained from these previous in vivo studies, we attempted to address some of these concerns by developing an in vivo model system in which an enriched population of resting murine TNP-specific B cells from the spleens of immunized mice and clones of keyhole limpet hemocyanin (KLH)-specific Th2 effector cells were adoptively transferred to scid mice previously depleted of NE by 6-OHDA. This model system enables us to determine the role that NE plays in modulating the Th2 cell-dependent IgM and IgG1 responses and to determine which AR subtype is responsible for mediating the NE-induced effect. Some of the advantages of the model system presented herein, compared with previous model systems, are that there is a high frequency of Th2 cells and B cells responding to a given Ag, the Ab response is limited to a Th2 effector cell-mediated response, the lymphocytes are not present at the time of chemical sympathectomy, and the lymphocytes are not exposed to the burst of NE that is released from nerve terminals immediately after 6-OHDA exposure. Therefore, this model system makes it possible to study the mechanism by which Th2 cells and B cells participate in a Th2 cell-dependent Ab response in the absence of AR stimulation by NE as well as after the administration of pharmacological agents that act as either agonists or antagonists at AR binding sites.

In this report we show that B lymphocytes express the β2AR and that stimulation of this receptor by NE is essential for maintaining an optimal level of IgM and IgG1 production in reconstituted scid mice. The scid mice that were depleted of NE before their reconstitution with clones of KLH-specific Th2 cells and enriched populations of TNP-specific B cells produced less serum anti-TNP IgM and IgG1 following primary immunization with TNP-KLH than NE-intact mice. After secondary immunization these mice produced less IgG1, but control levels of IgM. In addition, although there was no effect of NE depletion on splenic histology and lymphocyte trafficking before immunization, splenic follicle expansion and germinal center formation were less evident in NE-depleted reconstituted scid mice following both primary and secondary immunization compared with those in NE-intact reconstituted scid mice.

Materials and Methods

Animals

Six-week-old female C.B-17/ICR (H-2b, IgG-Δα), C.B-17/ICR scid (H-2b, IgG-Δα), BALB/c (H-2b, IgG-Δα), and B6C3F1 (H-2b) mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All mice were provided autoclaved pellets and water ad libitum. The scid mice received tetracycline HCl (2 mg/ml; Pfizer, New York, NY) in their drinking water 3 days/wk. Mice were permitted 2 wk to acclimate to their environment before being manipulated and were used at 8 wk of age in all experiments. The scid mice were housed under a 12-h light, 12-h dark cycle in microisolator cages contained within a laminar flow system thus maintaining a pathogen-free environment, and all experimental manipulations occurred −4 h into the light cycle.

Reagents and Abs

2,4,6-Trinitrobenzenesulfonic acid was purchased from Fluka (Ronkonkoma, NY), OVA, trinitropheynyl (TPN), and fluorescein (FLU) were purchased from Sigma (St. Louis, MO), and KLH was obtained from Calbiochem (La Jolla, CA). TNP-KLH, TNP-FLU, or FLU-KLH were prepared at a haptenation ratio of 17–24 TNP or FLU molecules/KLH or OVA molecule. Terbutaline, naltol, phenolamine, and metaproterenol were purchased from Sigma. All pharmacologic agents were dissolved in sterile PBS immediately before administration in vivo. Abs used in the ELISA were purchased from PharMingen (San Diego, CA). A rabbit polyclonal IgG directed against an epitope corresponding to aa 399–418 mapping to the carboxyl terminus of the β2AR of mouse origin and control peptide sequences (aa 399–418) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

NE depletion

Chemical sympathectomy was performed at 8 wk of age. Mice received 200 mg/kg injections (i.p.) of 6-OHDA (Sigma) in 0.5 M saline containing 1 × 10−3 M ascorbate as an antioxidant. Mice received three injections of 6-OHDA on alternating days (days −6, −4, and −2 before cell reconstitution), while control mice received ascorbate-only injections on the same injection schedule.

T cell clones

The Th2 cell clone BAC 3.2 was maintained as described previously (12). Viable cells were obtained before use by centrifugation over Lympholyte-M (Accurate, Westbury, NY) 8–14 days after Ag stimulation. Clones were maintained in IL-2-containing medium and were used at least 3 days after exposure to IL-2. BAC 3.2 was tested for the presence of Mycoplasma contamination (Life Technologies, Gaithersburg, MD) and were negative.

TNP-specific B cell preparation

The procedures for enrichment of unprimed TNP-specific B lymphocytes from spleens of nonimmunized mice were adapted from those described by Snow et al. (20) as modified by Myers et al. (21). All procedures were performed at 4°C, except for RBC haptenation and enzyme treatment at 37°C. Briefly, horse RBCs (Colorado Serum, Denver, CO) were haptenated with 20 mg of 2,4,6-trinitrobenzenesulfonic acid per ml of packed RBCs. Spleen cell/haptenated horse RBC suspensions were prepared, and rosette-forming B lymphocytes were separated by velocity and density sedimentation using a discontinuous Percoll gradient. The lymphocyte-bound RBCs were removed by a mild trypsin-pronase treatment, and the lymphocytes were collected over Ficoll. The lymphocytes recovered at the end of the procedure were incubated overnight to allow for reexpression of surface-associated molecules before additional experimentation. Phenotypic and functional characterization of the unprimed TNP-specific B cells have been presented previously, and the resultant cell population contained ~85–90% TNP-specific B cells (22).

Cell transfer and immunization

Two days following the last 6-OHDA injection, all animals received both BAC-3.2 Th2 cells and TNP-specific B cells. Each cell type was prepared for adoptive transfer at 2 × 106 cells in 50 μl of PBS, T and B cell dilutions were prepared separately and were combined only at the time of injection. Cells were injected i.v. into the lateral tail vein in a total volume of 100 μl of PBS. One week after cell reconstitution, sympathectomized animals received primary immunizations i.p. with 100 μg of TNP-OVA, TNP-KLH, or FLU-KLH, delivered in the adjuvant TiterMax Gold (CytRx, Norcross,
GA). In some experiments, mice received an i.p. injection of a β2AR-selective agonist (5 mg/kg terbutaline or metaproterenol; Sigma), a β2AR-selective antagonist (5 mg/kg nadolol; Sigma), or saline at the time of Ag injection.

**Detection of secreted Ag-specific and allototype-specific anti-TNP IgM and IgG1**

Briefly, 96-well, round-bottom Immulon plates (Dynatech, Chantilly, VA) were coated with 100 μg/ml of TNP20-OVA overnight at 4°C, washed with PBS, and blocked with PBS/1% BSA for 1 h at 37°C. Ab-containing serum, diluted between 1/100 to 1/1000, was added to each well. Plates were incubated for 2 h at 37°C in a humidified atmosphere. Wells were washed with PBS/0.05% Tween-20. For Ag-specific assays, a 1/2000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM or IgG1 Ab (Southern Biotechnology Associates, Birmingham, AL) was added to each well for 1 h at 37°C. Non-specific binding was measured in the presence of 5 μg/ml of TNP-KLH (IgG4; Sigma) or MOPC-21 (IgG1 κ; Sigma) and was determined on a plate coated with 2 μg/ml of goat anti-mouse Ig. Color development was determined on a UVmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm.

**Tissue sample preparation for HPLC**

Cell suspensions were prepared from animals following sympathectomy. Cells (spleen and cell fractions) were analyzed separately. All samples were homogenized in 0.1 M HClO4 spun at 11,000 × g, and added to extraction chambers of a centrifuge filter apparatus containing 3.4-hydroxybenzylamine (20 ng/μl) that was used as an internal control. To perform catecholamine extraction, 50 mg of acid-washed alumina was added to each tube, the pH was adjusted to 8.3 with Tris/EDTA, and the tube was vortexed to allow absorption of NE onto alumina. Samples were then washed twice with distilled water, and catecholamines were extracted with 200 μl of 0.1 M HClO4. Samples were stored at −80°C until analysis by HPLC as described previously (10).

**Histology and immunohistochemistry**

Spleen tissue was removed from each animal and stored at −80°C until the time of analysis. For hematoxylin-eosin histology, spleens were fixed in 10% formalin, embedded in paraffin, sectioned by microtome at 8 μm, and stained with hematoxylin and eosin stains. Immunohistochemistry. Immunohistochemical staining was performed using the microwave antigen retrieval method. Sections were heated three times in a microwave oven (900 W) at 350°C for 4 min after denaturation of anti-TNP IgM. The basic solution allows for absorption of NE onto alumina. Tissues were embedded in OCT (Miles, Elkhart, IN) and sectioned at 8 μm. Sections were air-dried for 60 min, O.C.T. was removed by a 5-min incubation in 50% ethanol, and sections were fixed in cold acetone for 5 min. Following fixation, endogenous peroxidase activity was blocked by a 60-min incubation with Universal Block (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After five washes using a cold buffer (0.45 M Tris-HCl and 0.170 M MgCl2·6H2O; pH 7.2) the samples were counted in a Beckman 5500 B gamma counter (Beckman, Palo Alto, CA) with a calculated accuracy of 87%. Both total and nonspecific binding tubes were run for each time point. Results are reported in picomols of IPIN bound. The nonspecific binding was determined in the tubes containing (−)-propranolol at a final concentration of 1 μM. Specific binding of radioligand was determined by subtracting nonspecific binding from the counts occurring in the total binding tubes for each concentration of radioligand used. Densitometry was done using a computer program LIGAND (24).

**cAMP measurement**

Assays were performed using B cells at a density of 1 × 106 cells/ml in serum-free HBSS containing 10 mM HEPES and 1 mM 3-isobutyl-1-methylxanthine (Sigma) to inhibit phosphodiesterase activity. After the addition of terbutaline and incubation at 37°C for 30 min, the reaction was terminated by the addition of 3 parts cold ethanol. The soluble fraction was collected by centrifugation and lyophilized. CAMP measurement was done using a nonisotopic immunoassay system for cAMP (Life Technologies). Results are expressed as picomols of cAMP per 106 cells.

**Immunofluorescence staining**

B cells were resuspended at 5 × 105 cells/ml and were fixed for 20 min at room temperature in 2% paraformaldehyde. Cells were stained using a modification (25) of a previously described procedure (26). All staining Abs and detection reagents were purchased from Santa Cruz Biotechnology. All reagents and washes were performed in the presence of 1% BSA and 0.05% saponin (Sigma), and incubations were conducted at room temperature. Cells were first incubated for 10 min in PBS/BSA/saponin and then stained for 30 min with a polyclonal rabbit anti-mouse β2AR Ab (1 μg/ml final concentration) in the absence or the presence of a mouse β2AR-specific peptide sequence (10 μg/ml final concentration) that corresponds to the epitope of the β2AR recognized by the anti-mouse β2AR Ab. Cells were washed and incubated for 30 min in the tubes containing biotinylated secondary Ab in PBS/BSA/saponin followed by FITC-streptavidin. Cells were washed twice with PBS/BSA/saponin and twice with PBS/BSA without saponin to allow membrane closure. Samples were analyzed on a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) gated on all viable cells. Calibration of the FACStar Plus was manually performed daily using Rainbow Calibration Particles (Sherotech, Libertyville, IL). Results were analyzed using LYSIS II software (Becton Dickinson).

Statistics

Concentration-response data were first analyzed by a one-way ANOVA to determine whether an overall statistically significant change existed previously to using two-tailed unpaired Student’s t test. Statistically significant differences are reported at p < 0.05.

**Results**

**Immunofluorescence staining of the Ab response in reconstituted scid mice**

To determine the Ag specificity of the Ab response in scid mice reconstituted with a clone of KLH-specific Th2 cells and TNP-specific B cells, mice were immunized with TNP-KLH, TNP-OVA, FLU-KLH, or adjuvant alone and evaluated for their ability to produce anti-TNP IgM. As shown in Fig. 1, a significant level of anti-TNP IgM was detected in reconstituted mice immunized with TNP-KLH. In contrast, mice receiving either TNP-OVA or...
Th2 cells (BAC 3.2) and TNP-specific B cells. Reconstituted mice were injected i.p. with 100 
µg of the sibling pairs (H-2d) of the cells of BALB/c mice produce the Ab allotype Igha, while B cells
serum Ab was checked for allotype specificity. It is known that B mice reconstituted with H-2d-expressing B cells produced a sig-
lengthed with TNP-KLH, and serum anti-TNP IgM was measured.
with a KLH-specific Th2 cell clone that was H-2d restricted and
specific B cells of different haplotypes suggests that an MHC-
process it, and present the KLH peptide to the KLH-specific Th2
cell clone.
Next, to determine whether an MHC-restricted cognate interac-
tion occurred between the Th2 cell clone and the B cell to allow for the production of TNP-specific Ab, scid mice were reconstituted with a KLH-specific Th2 cell clone that was H-2d restricted and with TNP-specific B cells enriched from the spleens of unimmu-
B cells of a mismatched hap-
lotypic. Data are presented as the mean micrograms per milliliter of anti-
TNP IgM ± SE from five mice per group. Significant differences from adjuvant-only mice that were reconstituted with B cells of the I-A^d hap-
lotypic are indicated by an asterisk when \( p < 0.05 \).

FIGURE 1.  *Hapten, carrier, and MHC specificity of the Ag-specific Ab response in scid mice reconstituted with a murine clone of KLH-specific Th2 cells (BAC 3.2) and TNP-specific B cells. Reconstituted scid mice were injected i.p. with 100 µg of TNP-KLH, TNP-OVA, FLU-KLH, or adjuvant only. Serum anti-TNP IgM was analyzed by ELISA 2 wk later. In addition, some mice were reconstituted with B cells of a mismatched hap-
lotypic. Data are presented as the mean micrograms per milliliter of anti-
TNP IgM ± SE from five mice per group. Significant differences from adjuvant-only mice that were reconstituted with B cells of the I-A^d hap-
lotypic are indicated by an asterisk when \( p < 0.05 \).

FIGURE 2.  Serum Ab allotype specificity in scid (−/−) mice reconstituted with a clone of KLH-specific Th2 cells (BAC 3.2) and TNP-specific B cells. The scid mice (Igh-C^b) were reconstituted with B cells enriched from the spleens of BALB/c mice (Igh-C^b) and injected i.p. with 100 µg of TNP-KLH. Serum Ab was analyzed 2 wk later for allotype specificity.

Effect of NE depletion on serum IgM and IgG1 levels in scid mice reconstituted with a clone of KLH-specific Th2 cells and TNP-specific B cells
Administration of 6-OHDA to mice induces a long lasting, but not absolute, "Leakiness" of the mutation is defined as the appear-
ance of mature T and B cells in the periphery of CB.17 scid mice. To determine whether leakiness
occurred in reconstituted scid mice at any time after immunization,
splenic NE levels were depleted in the cellular/supernatant fraction 9 days following the last 6-OHDA injection, a time when primary immunization with
TNP-KLH was performed. Approximately 9 wk following chemical sympathectomy (8 wk following primary immunization), a time when secondary immunization was performed, splenic NE levels returned to ~62% of the level in NE-intact control mice, but to 83% of the level in NE-intact mice injected with the vehicle ascorbate. Thus, NE can be depleted almost completely from the spleens of scid mice before cell reconstitution and remain almost completely depleted at the time of cell reconstitution and primary immunization, but NE levels return closer to the levels in ascorbate-injected control mice by the time of secondary immunization.

To determine the effect of NE depletion on the ability of reconstituted scid mice to produce IgM and an Ab isotype characteristic

produced IgM and IgG1 only of the allotype Igh^b. In addition, serum anti-TNP IgM was almost sixfold higher in reconstituted scid mice than in sibling controls, suggesting that the frequency of KLH-
specific T cells and TNP-specific B cells was greater in the recon-
stituted scid mice than in sibling pairs. This result was obtained with all serum samples analyzed regardless of mouse age. Thus, these results suggest that the donor B cells, as opposed to leaky scid B cells, produced the serum anti-TNP Ab in our model system.

To determine the effect of chemical sympathectomy on splenic NE levels in our scid model system, 8-wk-old scid mice were exposed to 6-OHDA dissolved in an ascorbate solution for 3 alternating days 2 days before reconstitution with a clone of KLH-specific Th2 cells and TNP-specific B cells.

While the scid mutation is a homozygous (−/−) mutation, it is not absolute. “Leakiness” of the mutation is defined as the appear-
ance of mature T and B cells in the periphery of CB.17 scid mice in an age- and species-dependent manner (27), but this is less common in CB.17/ICR scid mice. To determine whether leakiness occurred in reconstituted scid mice at any time after immunization, serum Ab was checked for allotype specificity. It is known that B cells of BALB/c mice produce the Ab allotype Igh^b, while B cells of the sibling pairs (+/−) of the scid mouse produce the Ab allotype Igh^b (28). As shown in Fig. 2, scid mice (−/−) reconstituted with TNP-specific B cells isolated from BALB/c mice pro-

Effect of NE depletion on serum IgM and IgG1 levels in scid mice reconstituted with a clone of KLH-specific Th2 cells and TNP-specific B cells
Administration of 6-OHDA to mice induces a long lasting, but not absolute, chemical sympathectomy that results in the depletion of peripheral stores of NE (10, 14, 29). A previous study in rats showed that splenic NE levels decrease ~90–95% immediately following 6-OHDA exposure and return to control levels within 56 days post-6-OHDA exposure, as determined by HPLC (29). To determine the effect of chemical sympathectomy on splenic NE levels in our scid model system, 8-wk-old scid mice were exposed to 6-OHDA dissolved in an ascorbate solution for 3 alternating days 2 days before reconstitution with a clone of KLH-specific Th2 cells and TNP-specific B cells.
2 days following the last 6-OHDA injection. The Ag TNP-KLH (100 μg) was stored at 80°C until the time of analysis. Data are presented as the mean micrograms per milliliter of anti-TNP IgM or IgG1 ± SE from six mice per group. Significant differences from ascorbate-injected controls are indicated by an asterisk when *p < 0.05.

FIGURE 3. Ab production following NE depletion in vivo. A. Splenic norepinephrine concentration of 6-OHDA scid mice. At 8 wk of age, scid mice were depleted of NE with three i.p. injections on alternating days of either 6-OHDA (200 mg/kg) dissolved in 0.01% ascorbate (left panels) or 0.01% ascorbate only (right panels). Two days (2d), 9 days (9 d), and 9 wk (9 w) following the last 6-OHDA injection, spleens were removed, and the NE concentrations of the cell/supernatant fraction (upper panels) and the capsule fraction (lower panels) were determined by HPLC. Data are presented as the mean concentration of NE in picograms per milligram of tissue ± SE from six mice per group. Significant differences from ascorbate-injected controls are indicated by an asterisk when *p < 0.05. B. Kinetics of Ab production by NE-intact and NE-depleted reconstituted scid mice. Equal numbers of the clone of KLH-specific Th2 cells (BAC 3.2) and TNP-specific B cells (2 × 10^6 each) were injected i.v. into either NE-intact scid mice or NE-depleted scid mice 2 days following the last 6-OHDA injection. The Ag TNP-KLH (100 μg) was injected i.p. 1 wk after lymphocyte reconstitution to elicit a primary response and again at 9 wk after reconstitution to elicit a secondary response. Retro-orbital blood samples were collected weekly, RBCs were removed, and the serum was stored at −80°C until the time of analysis. Data are presented as the mean micrograms per milliliter of anti-TNP IgM or IgG1 ± SE from seven mice per group. These data are representative of two separate experiments. Significant differences between the saline control and 6-OHDA groups are indicated by an asterisk at *p < 0.05.

of that produced by B cells provided “help” by a Th2 cell, i.e., IgG1, NE-depleted or NE-intact scid mice were reconstituted with Ag-specific Th2 cells and B cells and immunized with TNP-KLH. As shown in Fig. 3B, the primary IgM response in NE-depleted mice was suppressed 50–75% compared with that of NE-intact controls, while the secondary IgG response returned to control levels. In contrast, both the primary and secondary IgG1 responses were suppressed by ~85% in NE-depleted mice compared with those in NE-intact mice, but by the second week following the secondary immunization, IgG1 production recovered to ~40% of control levels. However, it may be important to note that while NE depletion suppressed the Ab response, it never completely inhibited the response. IgG3 and IgG2a responses were also detected in the sera of reconstituted scid mice, but their levels were ~200- to 300-fold lower than the levels of IgG1. In addition, both IgA and IgE were undetectable in the serum of reconstituted scid mice. Finally, anti-TNP IgM and IgG1 responses were either <6 μg/ml or undetectable in animals that received adjuvant only injections (data not shown). Thus, these results suggest that NE plays a role in maintaining an optimal level of Th2 cell-dependent IgM and IgG1 production in normal mice.

To determine whether NE depletion influenced successful lymphocyte homing in scid mice following cell reconstitution, spleen sections from either NE-depleted or NE-intact reconstituted scid mice before Ag exposure were stained for Th cells and B cells using Abs directed against the markers CD4 and B220, respectively. One week following i.v. injection, transferred B220<sup>+</sup> cells (Fig. 4, A and B) and CD4<sup>+</sup> T cells (Fig. 4, C and D) successfully homed to and populated the splenic white pulp of reconstituted scid mice. B220<sup>+</sup> cells formed tight organized coronas that were adjacent to the less organized CD4<sup>+</sup> T cell containing PALS. In addition, lymphocyte homing and population of both the B cell-containing corona and the T cell-containing PALS were equivalent in both NE-intact (Fig. 4, A and C) and NE-depleted (Fig. 4, B and D) mice, thus suggesting that NE depletion did not affect the ability of the T and B cells in our model system to successfully home to the spleen following i.v. injection into scid mice.

In addition, hematoxylin- and eosin-stained spleen sections were examined before and following Ag exposure. Due to a lack of T and B cells, spleens from nonreconstituted scid mice had limited cellular organization and were devoid of follicles (Fig. 5A) compared with immunocompetent sibling pairs (Fig. 5C). In contrast, spleens from scid mice reconstituted with a clone of Th2 cells and TNP-specific B cells had increased cellular organization into follicles regardless of the NE status of the mice (Fig. 5, B and E), suggesting that successful homing and cellular compartmentalization of the transferred lymphocytes occurred similarly in both NE-intact and NE-depleted mice. Following Ag administration, clonal expansion and germinal center formation were evident in NE-intact mice (Fig. 5C), but were less evident in NE-depleted animals (Fig. 5F). Taken together, these results suggest that NE depletion does not affect the ability of the Th2 cell clones and B cells used in our model to successfully home to the spleen; however, following Ag exposure, less cellular organization into follicles was observed in NE-depleted mice compared with that in NE-intact control mice.

To further investigate the effect of NE depletion on germinal center formation, spleen sections were stained with FITC-labeled PNA 2 wk following secondary Ag exposure. Germinal centers were evident in the NE-intact mice (Fig. 6, A and C), whereas no detectable germinal centers were observed in NE-depleted mice (Fig. 6, B and D). However, it is important to note that PNA<sup>+</sup> germinal center B cells were diffusely scattered throughout the spleen in both groups, suggesting that germinal center formation in NE-depleted mice may be delayed. Thus, it appears that Ag-specific Th2 cells and B cells successfully localized to the spleen in both NE-intact and NE-depleted mice, and following Ag exposure, clonal expansion and germinal center formation were significantly
lower in NE-depleted mice compared with those in NE-intact controls.

The AR subtype responsible for mediating the effects of NE on the Ag-specific immune response

All the above data collected from NE-depleted mice compared with NE-intact mice suggested that NE played a role in maintaining an optimal level of Th2-dependent IgM and IgG1 production in normal mice. To determine the AR subtype responsible for mediating this effect of NE on the in vivo immune response, the αAR-selective antagonist phenolamine and the β2AR-selective antagonist nadolol were injected into reconstituted NE-intact scid mice at the time of Ag exposure to block specific receptor subtypes that could potentially be stimulated by NE. Mice receiving nadolol had lower serum titers of IgM 2 wk following Ag challenge, whereas animals receiving phenolamine had serum titers equivalent to those of saline-injected controls (Fig. 7). Thus, these results suggest that NE modulates the in vivo Ab response via stimulation of the β2AR as opposed to the αAR.

In the present study we used a clone of Th2 cells specific for KLH and a population of murine B cells specific for TNP to reconstitute scid mice. We showed previously that a panel of murine Th2 cell clones do not express a detectable level of the β2AR in either the resting (12) or the activated state (13) compared with either Th1 clones or murine splenic CD4⁺ T cells (30, 31). To determine whether the suppressed Ab response in the absence of NE was due to a lack of β2AR stimulation on the B cell, reconstituted NE-depleted mice were injected with increasing concentrations of either the β2AR-specific agonist terbutaline or metaproterenol at the time of Ag exposure. Results showed that the ligand binding analysis of freshly isolated TNP-specific B cells was conducted using the iodinated β2AR-specific antagonist IPIN. As shown in Fig. 8A, Scatchard transformation of saturation binding data showed that TNP-specific B cells expressed ~620 binding sites/cell with an affinity (Kd) of 0.1 nM. These values are similar to those reported for unFractionated murine splenic B cells, but higher than those reported for murine Th1 cell clones or splenic CD4⁺ T cells. To determine whether the β2AR expressed by TNP-specific B cells was of the β2AR-specific subtype, TNP-specific B cells were exposed to an Ab directed against the β2AR-specific peptide sequence of the cytoplasmic N-terminus of the β2AR protein. As shown in Fig. 8B, TNP-specific B cells expressed a detectable level of the N-terminus of the β2AR, the specificity of which was shown with blocking peptides specific for the immunizing sequence of the β2AR, but not the β1AR. To determine whether the β2AR on TNP-specific B cells was functional, B cells were examined for their ability to accumulate cAMP intracellularly following exposure to the specific β2AR agonist terbutaline. As shown in Fig. 8C, B cells that were exposed to increasing concentrations of terbutaline showed a concentration-dependent increase in the intracellular cAMP concentration. Taken together, these data suggest that an enriched population of splenic TNP-specific B cells expresses a functional β2AR protein.

To determine whether the suppressed Ab response in the absence of NE was due to a lack of β2AR stimulation on the B cell, reconstituted NE-depleted mice were injected with increasing concentrations of either the β2AR-specific agonist terbutaline or metaproterenol at the time of Ag exposure. Results showed that the

FIGURE 4. Detection of CD4⁺ and B220⁺ cells in the spleens of scid mice following i.v. reconstitution. One week following reconstitution, sections of spleen from NE-intact scid mice (A and C) or NE-depleted scid mice (B and D) were stained for either B220⁺ cells (A and B) or CD4⁺ T cells (C and D) as indicated by black staining. Central arteries are marked in all panels with an arrow. A and B are ×325 magnification, and C and D are ×200 magnification. The photomicrographs presented are representative of four mice analyzed per group.

FIGURE 5. Spleen histology of reconstituted scid mice following Ag exposure. Sections of spleen from a nonreconstituted scid mouse (A), an NE-intact reconstituted scid mouse before Ag exposure (B), an NE-intact reconstituted scid mouse 2 wk following Ag exposure (C), a sibling pair mouse (D), an NE-depleted reconstituted scid mouse before Ag exposure (E), and an NE-depleted reconstituted scid mouse 2 wk following Ag exposure (F). Paraffin-embedded spleen sections were stained with hematoxylin and eosin. The photomicrographs presented are representative of six mice analyzed per group.
suppressive effect of NE depletion on Ab production was partially reversed in a dose-dependent manner by either terbutaline or metaproterenol (Fig. 9). In addition, serum anti-TNP IgM was significantly increased in animals receiving chronic injections of metaproterenol each day following NE depletion until the time of Ag exposure. Thus, these results suggested that the Th2 cells and B cells used to reconstitute NE-depleted scid mice remained viable, that the B cells remained responsive to β2AR stimulation, and that the primary consequence of NE depletion may have been to suppress the ability of these cells to function optimally in response to the Th cell-dependent Ag TNP-KLH.

Discussion

In this study we have shown that a population of resting TNP-specific B cells enriched from the spleens of unimmunized mice possess a functional β2AR and that stimulation of this receptor by NE in vivo is necessary to maintain an optimal Th2 cell-dependent IgM and IgG1 response. The ability of NE to maintain optimal Th2 cell-dependent Ab production in NE-intact reconstituted scid mice was suppressed by administration of an antagonist to the βAR, but not by administration of an antagonist to the αAR. In addition, the suppressive effect of NE depletion on Ab production was partially reversed by administration of a β2AR-specific agonist to NE-depleted mice. Although no difference was observed in lymphocyte trafficking or cellular organization within the spleens of either NE-depleted and NE-intact reconstituted scid mice before Ag exposure, the extent of follicle expansion and germinal center formation was suppressed in NE-depleted animals following Ag exposure. Taken together, these data suggest that stimulation of the β2AR on the B cell maintains an optimal level of Th2 cell-dependent Ab production in vivo.

Before Ag exposure, there was no detectable effect of NE depletion on either splenic structure or the ability of lymphocytes to properly home to the spleen in reconstituted scid mice compared with that in NE-intact mice. While it is possible that some of the
cells homed to the peripheral lymph nodes or mucosal lymphoid tissue, it is unlikely, since the T cell clones used in this study are L-selectin negative and are, therefore, unable to cross high endothelial venules to enter the lymph nodes following i.v. reconstitution (32). However, our finding does not support a previous report suggesting that NE depletion in mice prevents lymphocytes from homing properly and that a higher accumulation of adoptively transferred T cells was evident in the spleen and lymph nodes of mice when the T cells were pretreated with a \( \beta \)-AR-selective agonist (33). Thus, the effect of NE depletion on lymphocyte homing remains unclear. Also, the splenic histology of our mice showed that follicular clonal expansion and germinal center formation were less evident following Ag exposure in NE-depleted mice than in NE-intact controls. One possible explanation for this finding is that the lymphocytes present within the spleens of NE-depleted mice may have been in an unresponsive state. However, these cells were able to respond to mitogen stimulation in vitro (data not shown) and appeared to function to some degree following secondary immunization when the level of NE returned to that in controls.

It is possible that the return of IgM production to control levels after secondary immunization was due to a primary response from newly formed B cells migrating from the bone marrow of scid mice following the recovery of NE levels in the spleen. However, this possibility is unlikely, since scid mice rarely generate newly formed B cells (34), and the only Ab allotype produced in the present study was that of the donor cell type. Also, it is likely that the return of secondary IgM production to normal levels was due to the long term survival of the originally transferred donor Th2 cells and B cells within the spleen until a time when NE levels returned to normal, since it has been reported that transferred T and B cells survive indefinitely in a recipient scid mouse (35). This possibility is also supported by the present findings that serum anti-TNP IgM returned to a control level in NE-depleted scid mice following secondary exposure to Ag and that the suppressive effect on IgM production was abolished in these mice.

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**FIGURE 8.** \( \beta \)-AR expression on TNP-specific B cells. A, The binding of \((-\) IPIN to intact, formalin-fixed, TNP-specific B cells measured at 37°C. Specific binding data were fitted by computer-assisted nonlinear regression. The theoretical curves for direct or Scatchard (inset) representations are drawn using the fitted parameters. For Scatchard plots: B/F, bound IPIN/free IPIN; B, bound IPIN (picomolar concentrations). Curves comprise data from one representative experiment from four individual radio-ligand binding experiments. B, An Ab specific for the cytoplasmic domain of the \( \beta \)-AR was used to determine the expression of \( \beta \)-AR on B cells by flow cytometry. To establish the level of specific binding of the anti-\( \beta \)-AR Ab, Ab binding to the receptor was competed with a saturating concentration of the specific peptide sequence used to generate the anti-\( \beta \)-AR Ab or a sequence used to generate the anti-\( \beta \)-AR Ab. Data are presented as histograms showing fluorescence intensity for secondary Ab alone (dotted area), anti-\( \beta \)-AR Ab alone (black area), anti-\( \beta \)-AR Ab and \( \beta \)-AR peptide (shaded area), and anti-\( \beta \)-AR Ab and \( \beta \)-AR peptide (white area). Histograms comprise data of one representative experiment from three individual immunofluorescence experiments. C, Intracellular cAMP concentration of B cells following stimulation by the \( \beta \)-AR-selective agonist terbutaline. Data are presented as mean picomoles of cAMP per 10^6 cells ± SE from data collected in three separate experiments.

**FIGURE 9.** Reversal of the 6-OHDA-induced IgM suppression by a \( \beta \)-AR-specific agonist. Following NE depletion with 6-OHDA (200 mg/kg i.p.) and reconstitution with a clone of KLH-specific Th2 cells and TNP-specific B cells, scid mice were immunized with TNP-KLH (100 \( \mu \)g i.p.) and administered increasing concentrations of either the \( \beta \)-AR-selective agonist terbutaline or metaproterenol (5 mg/kg i.p.). In addition, one group of animals received chronic daily injections of metaproterenol (5 mg/kg i.p.) beginning the day of NE depletion and ending the day of Ag exposure (C5). Two weeks following immunization, serum anti-TNP IgM was determined by ELISA. Data are presented as the mean micrograms per milliliter of serum IgM of six mice per group, and values significantly different from those of NE-depleted mice receiving only Ag are indicated by an asterisk when \( p < 0.05 \).
of NE depletion was partially reversed by the β2AR-selective agonist terbutaline. Thus, the production of secondary IgM and IgG1 in our model system may be due to the long term survival of the donor cells or the expansion of this original population during the primary immune response.

In contrast to the present results showing a suppressive effect of NE depletion on Th2 cell-dependent Ab production, one study by Kruszewska et al. (14) reported a strain-specific enhancement of both cytokine and Ab production in NE-depleted C57Bl/6J (Th1-slated strain) and BALB/c (Th2-slated strain) mice. These studies showed that 6-OHDA treatment of mice enhanced primary serum anti-KLH IgM and IgG1 in KLH-primed C57Bl/6J mice, while NE depletion increased only IgG1 in KLH-primed BALB/c mice. Thus, the effects of NE depletion by 6-OHDA on immune cell function are varied depending on the target organ cells studied, the stimulus used for cell activation, the mouse strain used for study, whether a primary or a secondary response is being measured, and whether the response being measured is generated in vivo or in vitro.

It is also important to note that previous NE depletion studies in vivo used immunocompetent mice with intact mechanisms of precursor development and differentiation to study the role of NE depletion on immune cell function. Thus, since NE and β2AR stimulation have been reported to influence hemopoiesis and modulate the numbers of immune cell progenitors produced in the bone marrow (36), it is possible that NE depletion may remove the ability of this neurotransmitter to influence hemopoiesis in the bone marrow of immunocompetent mice and contribute to the previously discussed conflicting findings. In addition, intracellular cAMP levels have been shown to influence the path of CD4+ T cell differentiation into Th1 and Th2 effector cells (37), suggesting that NE and/or β2AR stimulation may affect the composition of the effector cell population that predominates in NE-depleted immunocompetent mice. Therefore, any conclusions drawn from a study addressing the effects of NE on a resultant immune response in intact animals must also take into consideration the effects of βAR stimulation on both the developmental and effector stages of the response. We have attempted to address some of these possibilities by establishing a model system in which the mouse possesses a high frequency of Ag-specific T and B cell effector populations that cannot be supplemented by newly formed lymphocytes. Thus, our model system eliminates the possibility that the effect of NE depletion on Ab production is due to an effect on lymphocyte development or differentiation and thereby permits study of the role of NE in modulating a Th2-dependent Ab response to a soluble protein Ag when there is a high frequency of both β2AR-αβ Th2 cells and β2AR-αα B cells responding to Ag in a hapten-, carrier-, and MHC-restricted manner.

One concern with all in vivo investigations is the contribution of stress and glucocorticoids to the response. While 6-OHDA does not cross the blood-brain barrier in adult mice to enter the CNS, Callahan et al. (38) showed that 6-OHDA administration to mice increased both Fos protein and corticotropin-releasing factor expression on cells residing within the hypothalamus. This increase in Fos protein within the hypothalamus is believed to have resulted from the peripheral nerve terminal damage induced by 6-OHDA, which, by some mechanism, stimulated the hypothalamic-pituitary-adrenal axis to release glucocorticoids from the adrenal cortex. Therefore, a decreased Ab response in vivo following 6-OHDA-induced depletion of NE may be due to the immunosuppressive effects of glucocorticoids and not a loss of β2AR stimulation by NE. However, another study showed that administration of the glucocorticoid receptor antagonist RU486 in vivo was unable to prevent the effects induced by 6-OHDA (39). Therefore, although 6-OHDA administration may enhance Fos protein and corticotropin-releasing factor expression within the hypothalamus, it appears unlikely that a glucocorticoid effect was responsible for the observed immunosuppressive effects of NE depletion in the present study. However, this possibility will be tested in future studies using our model system.

If stimulation of the β2AR by NE is necessary to maintain optimal Th2 cell-dependent Ab production in vivo, as suggested by the present study and others (12, 13), and if β2AR stimulation leads to an increase in the intracellular concentration of cAMP within the lymphocytes responsible for Th cell-dependent Ab production, then the influence of cAMP accumulation on the ability of lymphocytes to function during a response to Ag needs to be understood. It is possible that the lack of NE stimulation during the primary response to Ag results in a loss of a cAMP-induced effect in the B cell that may remain with the cell until the time of secondary Ag exposure. Such an effect may include the loss of a cAMP-induced effect to either modulate the level of expression of a cell surface molecule on the B cell that is important for optimal B cell function or alter the level of responsiveness of the B cell to cytokines. For example, in vitro stimulation of B cells by either dibutyryl cAMP (40, 41), a β2AR-selective agonist (D. Kasprowicz et al., manuscript in preparation), or NE (D. Kasprowicz et al., manuscript in preparation) enhanced the level of expression of the costimulatory molecule B7-2 on the B cell. Thus, B7-2 expression may be lower on the B cells of mice depleted of NE, since these B cells would not receive a cAMP signal via β2AR stimulation to induce optimal B7-2 expression. As a consequence, it is possible that the lack of enhancement of B7-2 expression on the B cell may hinder optimal B cell function, such as Ab production (D. Kasprowicz et al., manuscript in preparation) and optimal germinal center formation (42). Also, the responsiveness of the B cell to IL-4 may be directly affected by cAMP elevation in the B cell, since there is an apparent role for cAMP in enhancing the level of B cell responsiveness to the Th2 cell-derived cytokine IL-4 (43, 44). Since IL-4 stimulation is necessary for up-regulation of MHC class II on B cells (45, 46), B cell proliferation (47, 48), and B cell switch to IgG Ab production (49–52), it is possible that depletion of NE in vivo will remove a signal that is necessary to generate cAMP in a B cell to increase responsiveness to IL-4. Therefore, the possibility exists that the levels of both B7-2 expression and IL-4 responsiveness by the B cell are affected by NE depletion, and this possibility may partially explain why follicle expansion, Ab production by primary B cells, and germinal center formation were less evident after Ag exposure in NE-depleted mice compared with those in NE-intact controls.

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References


