2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Elevates Basal B-Cell Intracellular Calcium Concentration and Suppresses Surface Ig- but Not CD40-Induced Antibody Secretion

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INTRODUCTION

Living cells expend a great deal of energy maintaining a large Ca2+ concentration gradient across the plasma membrane, ostensibly to promote its use as an intracellular signaling regulator. In B-lymphocytes, mobilization of calcium has been shown to be an integral event in the stimulation of proliferation via the antigen receptor (Dennis et al., 1987), a response which precedes differentiation to an antibody-secreting cell phenotype. Slg cross-linking initiates the rapid breakdown of phosphoinositides, especially PIP2 (Cambier and Ransom, 1987; DeFranco, 1987). The resulting PLC-mediated liberation of diacylglycerol and IP3 is thought to lead to activation of PKC and release of stored calcium from the endoplasmic reticulum (Chen et al., 1986). Increased PI breakdown and calcium elevation have also been observed in antigen-specific B-cells after administration of the antigen (Grupp et al., 1987; Wilson et al., 1987). PI signaling events in B-cells can be recapitulated by the combination of direct PKC activators (phorbol esters) and calcium ionophores, which elevate intracellular free calcium (Rothstein et al., 1986; Klaus et al., 1986). Exit from the G0 state into the active cell cycle requires the combination of these stimuli.

1 This work was supported by NIH Grant ES 02520 and Training Grant ES 07087 as well as by the Thomas F. Jeffress and Kate Miller Jeffress Memorial Trust.
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B-cells cultured in low extracellular calcium medium do not proliferate normally in response to antigen receptor cross-linking (Dennis et al., 1987), arguing that calcium elevation plays a necessary role in the proliferative response to slg ligation.

TCDD, a halogenated aromatic hydrocarbon (HAH) and an environmentally persistent contaminant known to have been present in Agent Orange, has been shown to be an immunomodulatory agent capable of inducing thymic atrophy (Vos et al., 1980; Thomas and Faith, 1985; Luster et al., 1987) and suppressing humoral immune responses due to an apparent disruption of the differentiative process of B-lymphocytes (Holsapple et al., 1986; Tucker et al., 1986; Dooley and Holsapple, 1988; Luster et al., 1988). TCDD has recently been shown also to inhibit B-cell proliferative responses triggered by either surface Ig cross-linking or the combination of PMA plus calcium ionophore (Karras and Holsapple, 1994). The molecular mechanisms responsible for these activities are poorly understood, although binding to the cytoplasmic Ah receptor (so named because this receptor, in its activated state, induces transcription of genes which segregate with the Ah locus) is believed to mediate at least some of the biological responses to TCDD (Poland and Knutson, 1982; Whitlock, 1987). TCDD-induced alterations in calcium homeostasis have been reported in non-lymphoid tissues, including murine hepatoma cells, guinea pig hepatocytes, and guinea pig papillary muscle (Al-Bayati et al., 1988; Canga et al., 1988; Puga et al., 1992). Previous studies have indicated that TCDD-mediated inhibition of B-cell responses to antigen receptor stimulation (1) requires early addition (i.e., within the first 24 hr of culture with antigen; Tucker et al., 1986), (2) is not the result of aberrant PI metabolism (Luster et al., 1988), and (3) could be modulated by calcium ionophore (Karras and Holsapple, 1994).

Despite the recognized role of calcium in B-cell intracellular signaling events requisite for the proliferative response, the effect of TCDD on B-cell calcium fluxes or compartmentalization has not been studied. The discovery of thapsigargin (Christensen and Schaumburg, 1983; Christensen and Norup, 1985), a sesquiterpene lactone, has provided a tool with which to study calcium flux and compartmentalization in cells. Thapsigargin is believed to liberate stored calcium from the endoplasmic reticulum (ER) by a specific inhibition of the ER calcium–ATPase (Thastrup et al., 1989), resulting in an increase in resting intracellular calcium concentrations in a number of different cell types. Inhibition of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)–ATPases (SERCA) is one mechanism by which the polycyclic aromatic hydrocarbons (PAHs), a class of compounds structurally related to TCDD and the other HAHs, increase intracellular Ca\(^{2+}\) (Krieger et al., 1995) and inhibition of SERCA by PAHs correlates with the ability of many PAHs to maintain a sustained elevation of intracellular calcium in lymphocytes. Thapsigargin, an immunosuppressive agent, produces a similar effect. In this report, we have compared both the effects of TCDD and thapsigargin on B-cell intracellular calcium as well as the actions of TCDD on the IgM secretory responses of B-cells stimulated by either anti-IgM (T-independent stimulus) or activated T\(_h\)-cells expressing CD40 ligand (T-dependent stimulus) plus lymphokines. TCDD was found to selectively suppress anti-IgM- but not CD40 ligand-stimulated IgM secretion. Since surface Ig but not CD40 ligation induced calcium mobilization, we monitored the effect of TCDD on intracellular calcium levels in B-cells. Compared to the ER calcium–ATPase inhibitor thapsigargin, TCDD exposure induced a delayed elevation of basal intracellular calcium concentration of nearly equal magnitude, suggesting that disruption of calcium homeostasis may be a mechanism by which TCDD induces suppression of Ig secretion stimulated by signals initiated through surface Ig.

**MATERIALS AND METHODS**

**Animals.** (C57BL/6 × C3H)F1 (B6C3F1) female mice were obtained from the National Cancer Institute (Frederick, MD). The care and use of these animals conformed to the policies and regulations of the Institutional Animal Care and Use Committee.

**Chemicals and animal dosing regimen.** 2,3,7,8-tetrachlorodibenzo-p-dioxin used for in vivo treatments was obtained through the National Toxicology Program and Bio-Rad Laboratories (Rockville Centre, NY) and was kept as a 100 \(\mu\)g/ml solution in corn oil. TCDD used for in vitro experiments was obtained from Chemsyn Laboratories (Lexena, KS) and stock solutions were dissolved in 100% dimethyl sulfoxide (DMSO). The purity was found to be >98%, as determined by Chemsyn, using gas chromatography/mass spectrometry. No contaminating dibenzofurans were detected. DMSO was purchased from Sigma (St. Louis, MO), and thapsigargin was obtained from LC Laboratories (Waltham, MA). For in vivo studies, mice were administered, by oral gavage, a single dose of 0.2 ml of either corn oil (vehicle) or TCDD (0.3–30.0 \(\mu\)g/kg), in corn oil. Animals were sacrificed 72 hr later to allow for disposition of TCDD and to minimize the effects of gavage handling. B-cells were purified from these animals as described below.

**Reagents.** Goat anti-mouse IgM, F(ab\(^{\prime}\))\(_2\), was purchased from Jackson ImmunoResearch (West Grove, PA). The same antibody was also insolubilized on Sepharose 4B, as described below. Recombinant murine IL-2, -4, and -5 were purchased from Genzyme (Cambridge, MA). Concanavalin A-stimulated rat T-cell supernatant was obtained from Collaborative Biotechnologies (Birmingham, AL). FITC-conjugated anti-mouse Thy 1.2 and goat anti-mouse Ig L3T4 and Lyt-2 were purchased from Becton–Dickinson (San Jose, CA). FITC-conjugated hamster anti-mouse CD3-ε was obtained from Pharmingen (San Diego, CA). HO-13.4 mouse anti-mouse Thy 1.2 culture supernatant was produced in this laboratory from the hybridoma obtained through the American Tissue Culture Bank. F(ab\(^{\prime}\))\(_2\) goat anti-mouse Ig and whole anti-mouse IgM, alkaline phosphatase conjugated, were purchased from Southern Biotechnology (Birmingham, AL).

**Preparation of isolated primary B-cells.** Isolated B-cells were obtained from freshly prepared splenocytes as previously described (Morris and Holsapple, 1991). Splenocyte preparations were depleted of macrophages by overnight adherence in RPMI 1640 containing 5% newborn calf serum. Red blood cells were lysed by Gey’s solution and T-cells removed by...
incubation with anti-Thy 1.2 (HO-13) plus complement. The remaining cells were separated on discontinuous Percoll (Pharmacia) step gradients using previously established methods (DeFranco et al., 1982; Layton et al., 1985; Monroe, 1988). Cells banding at the 1.079/1.070 (56/50% Percoll) interface, previously shown to be susceptible to TCDD-mediated immunosuppression (Morris et al., 1993), were collected and used in all experiments. FACScan analysis of this cell population was carried out as described (Morris and Holsapple, 1991) and found to be \\#98% Ig+ and <1% LYT-2- Lyt-2-. Macrophage and granulocyte contamination was monitored by Wright-Giemsa-stained cyt centrifuge preparations and found to be <1%.

**Cell culture conditions.** All experiments were performed in RPMI 1640 (Gibco) containing 2 mM L-glutamine, 1% (v/v) penicillin-streptomycin (Sigma) solution, and 5 \( \times 10^{-5} \) M 2-mercaptoethanol and supplemented with 5% newborn calf serum (Flow Laboratories).

**Measurement of intracellular calcium.** Intracellular calcium in naive (untreated), vehicle- (0.01% DMSO in vitro or corn oil in vivo), TCDD-(0.3–30.0 nM in vitro or 0.3–30.0 \( \mu \)g/kg in vivo), or thapsigargin (0.3–30.0 nM) treated B-cells cultured with or without anti-IgM (either soluble or insolubilized), pokeweed mitogen, or activated Tc2-cells was measured using a modified version of a previously described method (Yellen et al., 1991). B-cells freshly isolated from spleens of naive, vehicle-, or TCDD-dosed animals or harvested from vehicle-, TCDD-, or thapsigargin-treated cultures were washed and resuspended at 1 \( \times 10^6 \) M CaCl2, 20 mM Hepes buffer, and 5% newborn calf serum. Fura-2 acetoxyxymethyl ester (Calbiochem Corp., La Jolla, CA) was added to the cells at a final concentration of 4 nM and mixed. Under subdued lighting conditions, the cells were rotated at room temperature for 10 min, washed, resuspended as above in HBSS, and incubated with occasional mixing at 37°C for 15 min. The cells were then washed and adjusted to 2.5 \( \times 10^7 \) ml and 200 \( \mu \)l of cell suspension was added to 1.8 ml of prewarmed HBSS containing 1% newborn calf serum in a quartz cuvette. Calcium measurements were made using a SPEX dual-excitation fluorophotometer with excitation wavelengths set at 340 and 380 nm and emission monitored at 505 nm. Maximum and minimum fluorescence ratios were determined using 2.5 \( \mu \)M Fluo-3 and 2.5 \( \mu \)M Fura-2, respectively. Fluorescence ratios were converted to calcium concentrations using SPEX software.

**Analysis of B-cell proliferation.** B-cell proliferative responses were determined using 2.5 \( \times 10^5 \) isolated B-cells/well for anti-IgM or 2 \( \times 10^7 \) well for LPS and PMA plus ionomycin responses in 96-well flat-bottom plates (Costar). Medium (Na+), 0.01% DMSO (VH), or thapsigargin (0.3–30.0 nM in 0.01% DMSO) was preincubated for 18 hr before addition of stimuli for convenience; treatment effects were also observed after shorter incubations (data not shown). Soluble and insolubilized anti-IgM and LPS were added at 10 \( \mu \)g/ml, PMA was added at 100 ng/ml, and ionomycin was added at 400 ng/ml. [\( ^3 \)H]Thymidine (1 \( \mu \)Ci/well) incorporation was assessed for quadruplicate cultures for all experiments for each treatment group following a 16-hr pulse beginning at 32 hr after stimulation. For experiments in which TCDD was administered in vivo, B-cells were isolated, adjusted to the proper concentration, and incubated as described above, except that no in vitro TCDD treatment was performed.

**IgM ELISAs.** Ten thousand B-cells/well were treated with vehicle or TCDD as above and incubated with an equal number of activated or nonactivated Tc2-cells plus IL-2 (10 ng/ml), IL-4 (100 U/ml), and IL-5 (200 U/ml) or 2.0 \( \mu \)g/ml of anti-IgM preparation plus 100 U/ml IL-4 for 48 hr, followed by addition of IL-5 (200 U/ml), replacement of IL-4, and supplementation with a 20% (v/v) Concanavalin A-stimulated T-cell supernatant (Collaborative Biochemicals). In the case of soluble anti-IgM-stimulated cultures, the antibody was removed by washing three times after the 48 hr time point. ELISAs were performed using quadruplicate cultures per treatment group as previously reported (Morris and Holsapple, 1991), except that a F(ab)\(^\gamma\), goat anti-mouse Ig primary antibody and an alkaline phosphatase anti-mouse IgM secondary antibody were used, followed by colorimetric detection using p-nitrophenyl phosphate substrate (Sigma). Culture supernatants were collected on Day 6 and immediately assayed for immunoglobulin content. Spectrophotometric detection of absorbance changes was monitored at 405 nm using a Bio-Rad Model 2550 EIA plate reader. Data were calculated using linear regression analysis of the samples, using a semilog plot of log \( \mu \)g IgM myeloma protein standard/well versus absorbance. Sensitivity was typically <10 ng/ml.

**Insolubilizing IgM on Sepharose.** For experiments with insolubilized F(ab)\(^\gamma\), anti-IgM, the antibody was coupled to cyanogen bromide-activated Sepharose-4B (Sigma). Briefly, the Sepharose beads were swelled with 1 mM HCl and pelleted by centrifugation. An appropriate amount of anti-IgM to give a 1 mg/ml final concentration was added and the slurry was mixed on an automatic vortex at the lowest speed at 4°C for 18 hr. The slurry was washed with borate-buffered saline (BBS), pH 8.0, and the percentage of unbound antibody was determined by spectrophotometry. Unbound active sites were then blocked by mixing on the vortex with an equal volume of 2 M ethanolamine, pH 9.0, for 2 hr at room temperature. The slurry was washed two times with BBS, then once with PBS, pH 7.0, and resuspended in PBS in the appropriate volume to yield a 1 mg/ml concentration of bound anti-IgM.

**Statistical analysis.** Results were analyzed using a one-way analysis of variance. When treatment effects were observed, a Dunnett’s T test was used to specify which treatment groups were significantly different from controls, except for the analysis of intracellular calcium, for which the Duncan multiple range test was used.

**RESULTS**

**TCDD Suppresses IgM Secretion Induced by Soluble or Insolubilized Anti-IgM Plus Lymphokines.**

To determine the basis for the TCDD-induced suppression of the humoral immune response, we set out to examine the effects of TCDD on B-cell differentiation triggered by stimuli that mediate their effects via two separate receptors on B-cells: the antigen receptor and CD40. sIg cross-linking using anti-IgM mimics the B-cell response to a T-independent antigen with repeating antigenic epitopes. Activated Tc1-cells expressing CD40 ligand represent a model with which to study activation signals delivered to B-cells by antigen-stimulated T-cells, as would be provided during contact with a T-dependent antigen.

After B-cells were incubated with either soluble or insolubilized anti-IgM plus IL-4 for 48 hr, followed by washing to remove the antibody, replenishment of IL-4, and addition of IL-5 and a Con A-stimulated rat T cell supernatant, IgM secretion was measured by ELISA. Under these conditions, using either soluble (Fig. 1A) or insolubilized (Fig. 1B) anti-IgM, TCDD preincubation results in significant suppression of IgM production. Similar results using a soluble anti-IgM antibody plus a preparation of B-cell growth factor (BCGF) have previously been reported (Luster et al., 1988).

**TCDD Fails to Suppress IgM Secretion Induced by Activated Tc1-Cells Plus Lymphokines.**

Recent studies have demonstrated that Tc1-cell clones stimulated with either alloantigen or anti-CD3 antibody become activated and, when cultured with resting B-cells, either as
of IL-2, -4, and -5 for secretion of IgM. In contrast to the suppression of IgM secretion by TCDD following stimulation through sIg, TCDD failed to suppress IgM secretion induced by CD40 ligation in the presence of lymphokines (Fig. 2). Since TCDD has been shown to suppress the plaque-forming cell response to the T-dependent antigen SRBC, this result is intriguing and suggests that the T-cell signal may be perceived differently by the B-cell if it has first encountered protein antigen (i.e., antigen specific response versus bystander response).

**sIgM but Not CD40 Ligation Induces Calcium Mobilization in Isolated B-Cells**

Because sIg- but not CD40-stimulated B-cell differentiation was inhibited by TCDD, the calcium mobilization responses of the stimuli acting through these sites were compared. As shown in Fig. 3, both soluble and insolubilized anti-IgM induce immediate calcium mobilization in isolated B-cells, with the response stimulated by anti-IgM-Sepharose being muted in comparison to that of soluble anti-IgM. Recently, a report has shown, by calcium imaging of individual cells, that insolubilized anti-IgD produces rapid and sustained elevation of intracellular calcium of small magnitude compared to soluble anti-IgD (Yamada et al., 1993). In contrast, there have been no reports of increases in intracellular calcium in murine B-cells after stimulation of CD40. In the human system, anti-CD40 monoclonal antibody has been reported not to induce changes in intracellular calcium (Clark et al., 1989). Figure 3 shows that addition of an equal number of activated T<sub>H</sub>2-cells to viable B-cells does not produce

**FIG. 1.** TCDD suppresses IgM secretion induced by soluble or insolubilized anti-IgM plus lymphokines. Isolated B-cells were preincubated with 0.01% DMSO (VH) or TCDD (0.3–30.0 nM in 0.01% DMSO) before addition of 2 μg/ml soluble (A) or insolubilized (B) F(ab)2 GAM IgM and lymphokines (IL-4, 100 U/ml; IL-5, 200 U/ml; Con A-stimulated T-cell supernatant, 20% (v/v); see Materials and Methods for details). Data are presented as the mean μg/ml ± SE for quadruplicate cultures. *Responses significantly different from VH control, p < 0.05; **responses significantly different from VH control, p < 0.01. Results shown are representative of three comparable experiments.

fixed, whole cells, or as membrane-enriched fractions thereof, stimulate contact-dependent B-cell cycle entry (Bartlett et al., 1990; Hodgkin et al., 1990). In the presence of IL-4, strong proliferation is induced and, upon addition of IL-2 and IL-5, secretion of Ig of all isotypes can be measured (Bartlett et al., 1990; Hodgkin et al., 1990). Further work indicated that this inducible T-cell help is transient in nature, independent of antigen or MHC class II restriction and mediated by expression of CD40 ligand (p39) on the T<sub>H</sub>-cell surface (Noelle et al., 1992). It thus represents a B-cell maturation pathway distinct from that driven by sIg.

To explore the effects of TCDD in this model of T-dependent B-cell maturation, we pretreated isolated B-cells with TCDD and 18 hr later stimulated the cultures with anti-CD3-activated and -fixed T<sub>H</sub>2 (act. T<sub>H</sub>2) cells plus the combination of IL-2, -4, and -5 for secretion of IgM. In contrast to the suppression of IgM secretion by TCDD following stimulation through sIg, TCDD failed to suppress IgM secretion induced by CD40 ligation in the presence of lymphokines (Fig. 2). Since TCDD has been shown to suppress the plaque-forming cell response to the T-dependent antigen SRBC, this result is intriguing and suggests that the T-cell signal may be perceived differently by the B-cell if it has first encountered protein antigen (i.e., antigen specific response versus bystander response).

**FIG. 2.** TCDD fails to suppress the B-cell IgM response induced by fixed and activated T<sub>H</sub>2-cells. Isolated B-cells were preincubated as described in the legend to Fig. 1 before stimulation with an equal number (1 x 10<sup>5</sup>/well) of fixed and activated T<sub>H</sub>2-cells and IL-2 (10 ng/ml), IL-4 (100 U/ml), and IL-5 (200 U/ml) for detection of IgM secretion. Non-CD3-activated and -fixed T<sub>H</sub>2-cells (non-act. T) were added as controls for CD40-specific interactions with B-cells. Results are expressed as the mean μg/ml ± SE for quadruplicate cultures. One of two comparable experiments is shown.
noticeable changes in intracellular calcium levels. Isolated B-cells also do not mobilize calcium in response to the T-cell-dependent polyclonal B-cell activator, pokeweed mitogen (PWM). Interestingly, like activated TH-cell-stimulated responses, TCDD also does not suppress B-cell responses stimulated by PWM (Wood et al., 1992).

Elevation of B-Cell Resting Intracellular Calcium Levels after TCDD or Thapsigargin Exposure

Thapsigargin (30 nM), dissolved in DMSO and added directly to isolated B-cells, produces an immediate increase of approximately 1.5-fold in intracellular calcium, followed by a constant rise which reaches micromolar concentrations at about 2.5 min (Fig. 4A). This dynamic increase in intracellular calcium concentration triggers spiking fluctuations of large magnitude shortly thereafter. Direct addition of 30 nM TCDD in DMSO to isolated B-cells produced a similar immediate increase in calcium, but no further elevation up to 5 min after addition (Fig. 4B). Later addition of the DMSO vehicle alone, during the same trial, also produced a similar rise in intracellular calcium, suggesting that this modest increase is the result of addition of the DMSO vehicle (Fig. 4B). In similar experiments, TCDD did not produce increases in intracellular calcium over the first 30 min after treatment (data not shown).

Examination of B-cell intracellular calcium levels 1 hr after either thapsigargin or TCDD exposure (each at 30 nM) shows that intracellular calcium is significantly increased in cells after thapsigargin but not TCDD treatment (Table 1). However, analysis of intracellular calcium content at 18 hr postexposure shows that a dose-dependent elevation is induced by both compounds (Table 1). At identical concentrations (i.e., 0.3–30.0 nM), thapsigargin induces a slightly more potent response than that observed with TCDD. Thus, although both thapsigargin and TCDD treatments of B-cells result in increased resting intracellular calcium concentrations, differences in both the kinetics and the magnitude of these elevations are notable. These results suggest that TCDD and thapsigargin may act through different mechanisms to elevate intracellular calcium in isolated primary B-cells.

Elevation of B-Cell Resting Intracellular Calcium Levels by TCDD Occurs after Either in Vitro or in Vivo Exposure but Does Not Prevent Anti-IgM-Mediated Calcium Mobilization

To study the effects of elevated intracellular calcium levels on early B-cell signaling responses mediated through the antigen receptor, analysis of the effect of TCDD on calcium mobilization induced by soluble F(ab)2 anti-IgM was performed. These experiments were conducted using both 18 hr in vitro (Fig. 5A) and 72 hr in vivo (Fig. 5B) TCDD
Thapsigargin has been shown to induce histamine release and increases in intracellular calcium concentrations in platelets (Thastrup et al., 1987), as well as elevated intracellular calcium in other cell types (Christensen and Schaumburg, 1983; Christensen and Norup, 1985; Thastrup et al., 1989). Because, like TCDD, thapsigargin produces increases in intracellular calcium levels in isolated B-cells, its effects on B-cell responsiveness were studied. Thapsigargin preincubation for 18 hr potently inhibits both LPS- and soluble anti-IgM-stimulated B-cell proliferation (Fig. 6), as well as LPS-stimulated IgM secretion (significant suppression at 3.0 nM; data not shown). Interestingly, low concentrations of thapsigargin (0.3–3.0 nM) superinduce the B-cell proliferative response to PMA plus ionomycin, while higher concentrations (30.0 nM) inhibit it (Fig. 6). This is a different profile of activity than that observed with TCDD, which inhibits PMA

![Graph](image)

**FIG. 4.** Thapsigargin, but not TCDD, induces an immediate rise in intracellular calcium concentration in primary murine B-lymphocytes. Isolated B-cells were prepared for intracellular calcium measurement as described in the legend to Fig. 3 and Materials and Methods. After establishment of baseline calcium levels, either (A) thapsigargin (30.0 nM in 0.01% DMSO; arrow) or (B) TCDD (30.0 nM in 0.01% DMSO; first arrow) followed by vehicle (0.01% DMSO; second arrow) was added and intracellular calcium concentrations were monitored. Results shown are representative of three similar experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Ca(^{2+})] (nM) + SE</th>
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<tr>
<td></td>
<td>1 hr after treatment</td>
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<tr>
<td>VH</td>
<td>89 ± 21</td>
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<tr>
<td>TCDD 30.0 nM</td>
<td>101 ± 25</td>
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<tr>
<td>Thapsigargin 30.0 nM</td>
<td>546 ± 38*</td>
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<tr>
<td></td>
<td>18 hr after treatment</td>
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<tr>
<td>VH</td>
<td>90 ± 6</td>
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<tr>
<td>TCDD 0.3 nM</td>
<td>52 ± 5</td>
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<tr>
<td>TCDD 3.0 nM</td>
<td>164 ± 6</td>
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<tr>
<td>TCDD 30.0 nM</td>
<td>389 ± 58*</td>
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<tr>
<td>Thapsigargin 0.3 nM</td>
<td>187 ± 5</td>
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<tr>
<td>Thapsigargin 3.0 nM</td>
<td>250 ± 12*</td>
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<tr>
<td>Thapsigargin 30.0 nM</td>
<td>482 ± 79*</td>
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* Responses significantly different from VH control, p ≤ 0.05 (Duncan’s multiple range test).
ELEVATION OF B CELL INTRACELLULAR CALCIUM BY TCDD

Lizized anti-IgM was used instead of the soluble form. However, Ig responses triggered by activated T_{H} cells expressing CD40 ligand were refractory to TCDD preexposure. Because sIg cross-linking with anti-IgM antibodies induces calcium mobilization, the effects of TCDD on B-cell calcium levels were assessed. While anti-IgM-stimulated calcium mobilization occurred normally after TCDD exposure, basal intracellular calcium levels were substantially elevated by TCDD. Interestingly, another environmental chemical, dimethylbenz(a)anthracene (DMBA), also appears to disturb lymphocyte calcium management (Archuleta et al., 1993).

Calcium is a primary intracellular signaling messenger which plays a critical role in B-cell activation mediated by sIg. Since cells maintain a large calcium gradient across their plasma membranes, using energy-requiring pumps to either extrude or sequester it from their cytoplasm, it is not surprising that compounds which modulate intracellular calcium would affect B-cell function. However, the results shown here suggest that B-cells responding to stimulation via CD40 alone, in the absence of antigen, are not as sensitive to elevation of intracellular calcium as their sIg-activated counterparts. This result is in keeping with the lack of a detectable calcium signal in purified murine B-cells stimulated through CD40 by fixed and activated T_{H}2-cells (Fig. 3). A similar observation was made earlier using an anti-CD40 monoclonal antibody and human B-cells (Clark et al., 1989).

These results do not eliminate the possibility that a delayed calcium response may occur after CD40 ligation. It is also possible that other monoclonal antibodies to CD40 may act differently or yield different results in the murine system.

While a delayed calcium response may be more likely to occur using anti-CD3-activated, whole T-cells instead of an

**FIG. 5.** Effect of TCDD on calcium concentration in isolated B-cells in response to anti-IgM. Isolated B-cells were isolated from either untreated (A) or in vivo TCDD-treated (B) mice. Untreated B-cells were cultured in medium (naive), DMSO (vehicle), or TCDD (5 nM) for 18 hr before loading with Fura-2 while B-cells from in vivo corn oil- (vehicle) or TCDD (5 μg/kg in corn oil)-treated mice were loaded directly after isolation. Baseline intracellular calcium levels were monitored and the cells were then stimulated with 10 μg/ml soluble F(ab)2 GAM IgM (arrows). One of three comparable experiments is shown.

**FIG. 6.** Preincubation with thapsigargin inhibits B-cell proliferative responses. Isolated B-cells were cultured in quadruplicate at 2 (LPS and PMA plus ionomycin) or 2.5 (soluble anti-IgM) × 10^5 cells/well and preincubated for 18 hr with VH (0.01% DMSO) or thapsigargin (in 0.01% DMSO). Stimuli were added as indicated, and [3H]thymidine incorporation was normalized for responses measured after vehicle preincubation. Results shown are representative of duplicate experiments for each stimuli tested. The control B-cell DNA synthesis values are as follows: LPS, 59,844 ± 1205 cpm; anti-IgM, 33,479 ± 1354 cpm; PMA plus ionomycin, 14,424 ± 1364 cpm; medium, 2528 ± 287 cpm.

**DISCUSSION**

Recently, our understanding of how B-lymphocytes transduce surface receptor-mediated stimulatory signals has greatly increased. It appears that B-cells are induced to proliferate and secrete Ig via several pathways which may be selected by both the type of antigen encountered and the stage of the immune response. In this report, we have compared the consequences of TCDD treatment on different B-cell maturation pathways. We found that, as previously reported (Luster et al., 1988), sIg-mediated antibody responses stimulated by soluble anti-IgM were suppressed by TCDD. Similar suppression was observed when Sepharose-insolubi...
antibody preparation, no calcium transients were observed up to 30 min after addition of the activated T-cells (author’s unpublished observations). More importantly, the data suggest that the signal that is sensitive to TCDD in the T-dependent antibody response is provided by antigen and not by subsequent T-cell help.

Identification of the signaling machinery utilized after CD40 ligation has been elusive; however, it appears that early calcium mobilization is not important for CD40 functional responses in vitro. In contrast, anti-Ig-stimulated antibody response models are affected by elevations in intracellular calcium concentrations of two- to threefold over baseline. The effect of TCDD on proliferative and differentiative responses to anti-Ig is not manifest on Ia expression stimulated by this mitogen (Karras and Holsapple, 1994), indicating that calcium is not involved in all B-cell responses to these stimuli. Recent reports support these observations: Klaus and Parker have shown that B-cell signaling pathways for T-dependent activation of B-cells are qualitatively different from those for activation through slg, with regard to the profile of immediate early genes that are expressed (Klaus and Parker, 1992). In addition, PKC and cAMP agonists, which potently inhibit anti-Ig proliferation of B-cells, do not inhibit activated T-effector-mediated responses (Kawakami and Parker, 1992).

While the magnitude of the immediate calcium mobilization response of Sepharose-coupled anti-IgM is lower than that of soluble anti-IgM (Fig. 3), the duration of this response is protracted. This observation suggests that the intensity of the stimulatory signal delivered through slg is related to the length of time that [Ca$^{2+}$], is elevated rather than the magnitude of the immediate calcium peak. TCDD suppresses the antibody response triggered by each of these stimuli equally, suggesting that a common site of action may be downstream kinases or signaling elements activated by calcium fluxes. TCDD treatment has been reported to activate kinase activity in B-cells (Kramer et al., 1987; Clark et al., 1991). The relationship between increased levels of intracellular calcium and activation of B-cell kinases by TCDD is unknown but has been the focus of a significant amount of speculation (Matsumura, 1994).

Much interest has recently focused on determining the mechanism(s) of the TCDD-mediated interference of B-cell function. The results reported here, together with previous data, suggest that TCDD inhibits B-cell signaling pathways which involve rapid calcium mobilization. Specifically, B-cell responses to anti-IgM, PMA plus ionomycin (Karras and Holsapple, 1994), and LPS (Morris et al., 1993) have been shown to be suppressed by TCDD while stimuli which do not appear to induce rapid B-cell calcium flux, such as activated T-effector cells and PWM, were not affected. TCDD has previously been shown to alter calcium homeostasis in other cell types, including hepatocytes and hepatoma cell lines, as well as papillary muscle cells (Al-Bayati et al., 1988; Canga et al., 1988; Puga et al., 1992), and some of these effects have been shown to be Ah receptor independent (Puga et al., 1992). Although it is as yet unknown if the effects of TCDD on B-cell resting Ca$^{2+}$ levels are Ah receptor dependent, together these results, along with those presented here, support a role for disruption of calcium homeostasis as a common mechanism for TCDD toxicity.

In vitro concentrations of TCDD of 3–30 nM were required in these studies to observe significant suppression of antibody responses and elevation of intracellular Ca$^{2+}$. However, only a small portion of the TCDD added is associated with the cultured cells. Our previous in vitro disposition studies with [3H]TCDD showed that the vast majority of TCDD added to the cultures binds to the plastic culture wells and partitions to the medium rather than to the cells. Calculation of the concentration of TCDD in the cell fraction shows that only 2–3 fm associates with the cells after exposure to a 10 nM dose (Morris et al., 1993). This observation is in good agreement with the amount of TCDD associated with lymphoid tissues following a 5 μg/kg in vivo dose (Neumann et al., 1992).

Thapsigargin has previously been shown to provoke pronounced increases in intracellular calcium levels in various tissues, including human platelets and peripheral blood lymphocytes (Thastrup et al., 1987; Scharff et al., 1988), due to discharge of intracellularly sequestered calcium (Thastrup et al., 1989). The slow kinetics of calcium elevation after TCDD exposure (compared to thapsigargin) may explain the prior observation that immunomodulation by TCDD requires its addition at or near the time of B-cell stimulation (Holsapple et al., 1986; Tucker et al., 1986). These kinetics also suggest that the TCDD-sensitive processes of B-cell activation and differentiation may not manifest until hours after stimulation. TCDD has been reported to induce apoptosis in thymocytes via activation of a calcium-dependent endonuclease (McConkey et al., 1988). However, no changes in viability were evident after TCDD exposure in 6-day antibody response cultures (author’s unpublished observations). The respective effects of TCDD and thapsigargin on B-cells may also be determined by calcium-independent pathways, perhaps some shared and some not, since it is not clear whether these compounds have other activities in addition to calcium mobilization.

The differences in the B-cell response to PMA plus ionomycin after thapsigargin or TCDD exposure suggest that the pattern of calcium release determines how this signal is perceived by the cell. The thapsigargin calcium response in B-cells kinetically resembles that of ionomycin, while that of TCDD is more protracted. With regard to the source of calcium from which flux occurs after TCDD treatment, it appears that calcium influx is not responsible for the accumulation, due to our inability in preliminary studies to document...
increased calcium influx after 18 hr incubation with TCDD (author’s unpublished observations). However, using this technique, we were able to reproduce the calcium influx earlier observed after LPS stimulation (Freedman, 1979) (data not shown). Thus, TCDD appears to elevate intracellular calcium by mobilizing intracellularly sequestered stores. Further experiments will examine this hypothesis. In particular, it will be important to determine if the depletion of extracellular calcium (i.e., by administration of a calcium chelator) has any effect on the ability of TCDD to elevate intracellular calcium.

Although much work remains to be done in order to begin to understand the utilization and management of intracellular calcium by B-lymphocytes, our results suggest that disruption of calcium homeostasis in these cells may result in modulation of function, depending upon the activational stimulus. Inasmuch as slg-stimulated B-cell effector function appears to be inhibited by agents that elevate intracellular calcium levels, drugs and environmentally occurring substances which modulate cellular calcium management may target humoral immunity.

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REFERENCES


