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# 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Stimulation of Tyrosine Phosphorylation in B Lymphocytes: Potential Role in Immunosuppression

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**SUMMARY**

The prototype halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is carcinogenic and toxic in experimental animals. At the cellular level, TCDD toxicity is often expressed as an inhibition or alteration in normal cell maturation. In this respect, we and others have demonstrated that exposure of experimental animals to TCDD causes immunosuppression, including inhibition of B lymphocyte maturation and antibody synthesis. Although the immunological effects of TCDD are well described, little is known about its mechanism of action. In the present studies, it was found that TCDD increases membrane protein phosphorylation, which is, in part, associated with tyrosine-specific phosphorylation in B lymphocytes. This increase in phosphorylation occurred within minutes following TCDD treatment and was not associated with protein kinase C. The increase in tyrosine kinase by TCDD appears to be primarily due to *de novo* synthesis of new protein, because the protein synthesis inhibitors puromycin and cycloheximide, as well as the transcriptional inhibitor actinomycin D, partially inhibited the effect, although increased activity of preexisting protein cannot be fully dismissed. The dose response for increased phosphorylation by TCDD was identical to that we previously reported for inhibition of antibody synthesis, suggesting that immunosuppression by TCDD may be expressed through alterations in regulatory processes controlled by tyrosine kinases. These studies are discussed in terms of the potential role of TCDD-induced tyrosine phosphorylation in immunosuppression.

Halogenated aromatic hydrocarbons are persistent environmental pollutants with potential adverse effects on human health. TCDD has served as the prototype of these compounds in experimental studies, due to its potent toxicity in laboratory animals and potential widespread human exposure through industrial accidents, landfills, and TCDD-containing herbicides such as the defoliant Agent Orange (reviewed in Ref. 1). TCDD also acts as a potent liver carcinogen in rodents (2), possibly through its tumor-promoting properties (3). At the cellular level, TCDD toxicity is characterized by abnormal cell proliferation and/or differentiation.

The immune system is one of the more sensitive targets for TCDD, with toxicity affecting both B and T lymphocytes (for review, see Ref. 4). In mice, suppression of humoral immune responses occurs when splenic lymphocytes are incubated with nanomolar concentrations of TCDD or when mice are exposed to similar concentrations *in vivo*. Results using various mouse strains that differ at the *Ah* locus and receptor antagonists have suggested that immunosuppression is mediated via the Ah receptor (5, 6), although non-Ah receptor-mediated events have also been reported (7). Recently, we have used lymphocyte maturation as an *in vitro* model system to analyze the stages of B lymphocyte development that are most sensitive to TCDD immunosuppression (8). Using a variety of functional, biochemical, and phenotypic markers, these studies demonstrated that TCDD selectively inhibits terminal cell differentiation, without altering earlier events, including cell activation or proliferation. Analysis of the kinetics of exposure, however, demonstrated that TCDD must be present before or at the time of antigen addition, i.e., when B lymphocytes are at the resting ($G_0$) stage of the cell cycle, for maximum suppression to occur, suggesting that TCDD may alter an early event in lymphocyte activation, which leads to abnormal cellular development.

The events associated with B lymphocyte maturation have

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**ABBREVIATIONS:** TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PMA, phorbol 12-myristate-13-acetate; FBS, fetal bovine serum; anti-Ig, F(ab')$_2$ fragment of goat anti-mouse IgM; BCGF, mixture of B cell growth factors; PFB, phosphate-free buffer; PKC, protein kinase C; EGTA, ethylene glycol-(aminoethylether)-tetraacetic acid; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; EGF, epidermal growth factor; IL-4, interleukin 4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; BCA, bicinchoninic acid.

been intensely investigated, and it is apparent that receptor-regulated activity plays an integral role. For example, among normal tissues, the spleen is especially rich in tyrosine protein kinase activity, and many of the antigen recognition events and interleukin-dependent processes in T and B lymphocyte maturation have been associated with protein phosphorylation. Protein phosphorylation, particularly tyrosine phosphorylation, first identified as the product of viral oncogenes, has been associated with the regulation of many systems critical to cell function, including receptors for growth factors and other mitogenic compounds (9). Recent observations that TCDD modulates EGF receptor expression in epithelial cells (10) and tyrosine kinase activity in hepatocytes (11) suggested that a similar event may occur in B cells, which might be related to antibody suppression. In the present studies, we demonstrate that *in vitro* exposure of B lymphocytes to TCDD increases membrane protein phosphorylation and, in particular, stimulates tyrosine-specific protein phosphorylation. This effect may be associated with a gene product, because increased tyrosine kinase activity was, in part, inhibited by protein and RNA synthesis inhibitors. Evidence is also presented suggesting that the inhibitory effects of TCDD on antibody production are directly associated with alterations in regulatory processes controlled by tyrosine phosphorylation.

## Experimental Procedures

**Animals and chemicals**. Six- to 8-week-old C57BL/6 and B6C3F$_1$ (C57BL/6 × C3H/HeN) mice were purchased, through a National Cancer Institute contract, from Charles River Laboratories. TCDD, with greater than 99% purity (lot 851-144-II) (12), was a gift from Dow Chemical Co. (Midland, MI) and was suspended in FBS (Hyclone, Ogden, UT) to a known concentration, as previously described (5). PMA, cyclohexamide, puromycin, and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO). Concentrated stock solutions of the protein and RNA synthesis inhibitors were prepared in phosphate-buffered saline. Anti-Ig was obtained from Cooper Biomedical (Malvern, PA) and dialyzed extensively against saline. B cells were activated using anti-Ig and a T cell supernatant containing B cell-priming (IL-4) and growth factors (interleukin 5), which we designate BCGF and which was obtained from Cytokine Technology International, Inc. (Buffalo, NY). This is a mixture of $M_r$ 20,000 factors prepared according to the method of Metha *et al.* (13), which does not contain interleukin 2 but which supports B cell proliferation in T cell-depleted cultures (8). Recombinant IL-4 was purchased from Genzyme (Boston, MA). Isotopes used included carrier-free [$^{32}$P]orthophosphoric acid and [$\gamma$-$^{32}$P]ATP (12.5 Ci/mmol) obtained from Dupont-NEN Products and a $^{14}$C-methylated standard protein mixture purchased from Amersham Corporation. Other reagents used include poly(Glu,Ala,Tyr) (6:3:1) from Sigma, diisopropylfluorophosphate from Aldrich Chemical Company, Bio-Rad protein assay reagent from Bio-Rad Laboratories, and BCA protein assay reagent from Pierce Chemical Co. All chemicals were reagent grade or better.

**Experimental design**. For *in vitro* studies, vehicle-control FBS or TCDD-containing FBS was added directly to B lymphocytes and incubated at 37° in 5% $CO_2$/air, for 4 hr. PMA was dissolved in ethanol before dilution in culture medium and added to cells so that the final concentration of ethanol did not exceed 0.01%. All manipulations involving TCDD or PMA were carried out in either a chemical fume hood or a class II biological safety cabinet. In some experiments, mice were exposed by oral gavage to 10 μg/kg TCDD in corn oil or to corn oil alone and were sacrificed after 72 hr. Ethoxyresorufin-*O*-deethylase activity, an indicator of mixed function oxidase activity, was assayed as previously described (6) to establish the effectiveness of the *in vivo* administration.

**Isolation of B lymphocytes**. Small dense B lymphocytes were isolated as previously described (8), using two successive treatments with anti-Thy-1.2 monoclonal antibody plus complement (Accurate Chemicals, Westbury, NY) to remove T cells and centrifugation over a Ficoll gradient (1.0875 g/ml). Greater than 90% of these cells were in $G_0$, as determined by flow cytometric analysis of DNA and RNA using acridine orange and a two-step staining protocol (8). T cell depletion was confirmed periodically by the inability of the processed cells to respond to phytohemagglutinin mitogen and by the presence of less than 2% Thy-1.2$^+$ cells.

**Analysis of changes in protein phosphorylation**. The procedure for labeling cells with $^{32}PO_4$ was previously described by Chaplin *et al.* (14). B cells were washed in PFB (0.14 M NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 20 mM Tris·HCl, pH 7.35), resuspended to a concentration of 2.5 × 10$^7$ cells/ml in PFB containing 10% phosphate-free FBS or in phosphate-free serum that contained 10 nM TCDD, and incubated at 37° for 60 min. After phosphate depletion, [$^{32}$P]orthophosphate (0.25 mCi/ml) was added, and the cells were incubated for an additional 1 hr at 37°. Cells were activated by treatment with either 10 nM PMA or 50 μg/ml anti-Ig plus 5% BCGF. After a 20-min incubation at 37°, the cells were washed and adjusted to 5.0 × 10$^7$ cells/ml in cold sonication buffer (50 mM Tris·HCl, pH 7.3, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 2 mM EDTA, 10 mM diisopropylfluorophosphate). The cells were disrupted by sonication, and the lysate was centrifuged at 40,000 × *g* for 90 min. The pellets containing plasma membrane proteins were analyzed for phosphorylated protein by SDS-polyacrylamide electrophoresis on 10% polyacrylamide slab gels and autoradiography, as previously described (15).

**Assay of PKC**. B lymphocytes were resuspended, at a concentration of 5 × 10$^7$ cells/ml, in RPMI 1640 culture medium containing 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamycin, 1 mM sodium pyruvate, 0.1 μM nonessential amino acids, and 5 × 10$^{-5}$ M 2-mercaptoethanol, supplemented with 10% FBS. The cells were divided into 2.5-ml aliquots, stimulated with either 10 nM PMA, 40 μg/ml anti-Ig plus 5% BCGF, or medium alone, and incubated for 10 min at 37°. Incubation was terminated by addition of 8.5 ml of cold sonication buffer (20 mM Tris-acetate, pH 7.0, 0.33 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 1 mM PMSF, 10 μg/ml leupeptin). The cells (1.25 × 10$^8$ cells/treatment) were pelleted, resuspended in 4 ml of the buffer described above, and sonicated. Cell lysates were centrifuged at 100,000 × *g* for 1 hr. The pelleted membranes were resuspended in 1 ml of sonication buffer containing 1% Nonidet P-40, resuspended with a glass rod, and stirred on ice with a microbar for 1 hr. Both cytosol and membrane fractions were applied to a 1-ml DEAE-Sephacel column equilibrated in 20 mM Tris-acetate, pH 7.0, 10 mM 2-mercaptoethanol, 1 mM PMSF. Protein was eluted with a step-wise gradient, in 2-ml fractions, of equilibration buffer containing 50 mM, 150 mM, and 300 mM NaCl. The majority (90–95%) of the PKC activity was eluted in the fraction containing 150 mM NaCl, with the residual activity residing in the 300 mM NaCl fraction. Fractions were assayed for protein (Bio-Rad) and PKC activity within 1–2 days of isolation. PKC activity was determined using the method of Kraft *et al.* (16). Experiments were replicated a minimum of three times, with representative data being reported. Net PKC activity was obtained by subtracting the cpm incorporated in the presence of EGTA from cpm incorporated in the presence of calcium, phosphatidylserine, and diolein, and it is expressed as pmol of $PO_4$ incorporated/min × mg of protein.

**B lymphocyte tyrosine-specific protein kinase assay**. Twenty-milliliter volumes containing 5 × 10$^6$ cells/ml were incubated for 3 hr at 37°, in RPMI 1640 medium containing 10% FBS, with or without TCDD. The cells were pelleted by centrifugation at 500 × *g* for 10 min and resuspended in 2 ml of PIPES buffer (50 mM PIPES, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin). Cells were disrupted by sonication, diluted with 2 ml of PIPES buffer containing 500 mM sucrose, and centrifuged at 400 × *g* for 4 min to remove

undisrupted cells and nuclei. The lysates were then centrifuged at 100,000 × *g* for 60 min, and the supernatants were discarded. Pellets were resuspended in 0.5 ml of PIPES buffer containing 10% glycerol, dispersed with a glass rod, and further suspended by stirring for 1 hr on ice. Protein was assayed by the BCA method, and the samples were adjusted to contain equivalent amounts of protein and frozen at −70° until assayed.

The membrane-associated tyrosine-specific kinase activity of B lymphocytes was assayed using a modification of the procedure described by Earp *et al.* (17, 18). The activity of these kinases is affected by the concentrations of $Mg^{2+}$, $Mn^{2+}$, $VO_4^{3-}$, and Triton X-100. Preliminary experiments established optimal conditions for tyrosine kinase activity and further demonstrated that 50 mM PIPES, pH 7.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 100 μM $Na_3VO_4$, and 0.1% Triton X-100 minimized basal enzyme activity but allowed the demonstration of increases in enzyme activity (data not shown). Kinase activity was measured by addition of 10 μl of assay buffer to 50-μl aliquots of membrane protein and incubation at room temperature for 15 min. Activity was initiated by addition of 10 μl of 1 μCi/μmol [$^{32}$P]ATP (5 μCi/reaction) and incubation on ice for 3 min. The reaction was terminated by addition of 70 μl of 2× SDS sample buffer and heating at 100° for 3 min (15). Sixty-microliter aliquots of the reaction mixture were added to 10% SDS-polyacrylamide gels with 4% discontinuous stacking gels, which were electrophoresed until the bromophenol blue marker ran off the gels. The gels were fixed in a mixture of water/methanol/acetic acid (7:5:1) for 1 hr, and one gel was stained for protein by the method of Reisner *et al.* (19). A replicate gel was hydrolyzed in 1 N potassium hydroxide for 1 hr at 55° under constant shaking, to help establish whether these were tyrosine-specific phosphorylations. To confirm that this represented tyrosine-specific phosphorylation, an exogenous substrate, poly(Glu,Ala,Tyr) (6:3:1) of molecular weight 25,000, was included in the assay buffer at a concentration of 0.7 mg/ml in most of the studies. The addition of tyrosine-containing synthetic peptides as phosphorylatable substrates is routinely used to support tyrosine-specific activity (20).

Gels were destained in fixing solution for 2 hr and dried with a Bio-Rad model 443 slab dryer. The dried gels were autoradiographed on Kodak X-OMAT XAR film, with Dupont Cronex Lightning Plus intensifier screens. Molecular weights of proteins were estimated from $^{14}$C-methylated protein standards. All autoradiographs were scanned using a LKB Ultrascan XL linked to an IBM PC, and peak heights and integrations were determined using the common baseline and signal option.

## Results and Discussion

Preliminary studies from this laboratory (21) demonstrated that *in vitro* exposure of B lymphocytes to TCDD altered the phosphorylation patterns of cytoplasmic proteins at concentrations similar to those that inhibit antibody synthesis (5, 8). These findings were extended to investigate the effects of TCDD on protein phosphorylation in membrane preparations from B lymphocytes. An autoradiograph depicting the protein phosphorylation profiles of membranes from control and TCDD-treated B lymphocytes stimulated with either PMA or anti-Ig plus BCGF is shown in Fig. 1. Exposure of B lymphocytes to TCDD induced a moderate quantitative enhancement of phosphorylation, which was best observed by comparing membranes from unstimulated control cells with membranes from TCDD-treated cells (Fig. 1, *lane A* versus *lane B*). TCDD caused an increase in phosphorylation of proteins with estimated molecular weights of 105,000, 64,000, 61,000, 54,000, 50,000, 42,000, 38,000, and 17,000. In general, the increases in protein phosphorylation in B cells pretreated with TCDD still occurred even after the cells were treated with PMA or anti-Ig plus BCGF (Fig. 1, *lanes B, D,* and *F*). However, PMA treatment had a slight inhibitory effect on TCDD-mediated increases in protein phosphorylation, whereas anti-Ig treatment slightly enhanced the effects seen with TCDD. The effects of PMA treatment on protein phosphorylation were distinct from those seen with TCDD. Stimulation of B lymphocytes with PMA resulted in the phosphorylation of a $M_r$ 33,000 protein (Fig. 1, *lane C*) that was not apparent in unstimulated control cells (Fig. 1, *lane A*). However, TCDD treatment followed by PMA stimulation did not affect the increased phosphorylation of the $M_r$ 33,000 protein observed in PMA-stimulated cells (Fig. 1, *lane C* versus *lane D*). Anti-Ig plus BCGF stimulation of B lymphocytes resulted in enhanced phosphorylation of three proteins ($M_r$ 64,000, 61,000, and 50,000) and phosphorylation of a $M_r$ 24,000 protein that was not present in unstimulated control cells (Fig. 1, *lane A* versus *lane E*). TCDD pretreatment followed by anti-Ig plus BCGF stimulation did not affect the phosphorylation of the $M_r$ 24,000 protein but enhanced the phosphorylation of the $M_r$ 64,000, 61,000, and 50,000 proteins (Fig. 1, *lane E* versus *lane F*). These results demonstrate that an early response to TCDD in B lymphocytes is an increase in membrane protein phosphorylation, which is distinct from responses to PMA and anti-Ig. Increased phosphorylation of membrane proteins also occurs in hepatocytes after *in vivo* exposure to TCDD in rats and has been suggested to contribute to the altered function and expression of the EGF receptor (22). This increase in hepatic membrane protein phosphorylation is associated with increased activity of a number of protein kinases, including PKC, in rats and guinea pigs (23).

Initiation of events leading to antibody synthesis in B lym-

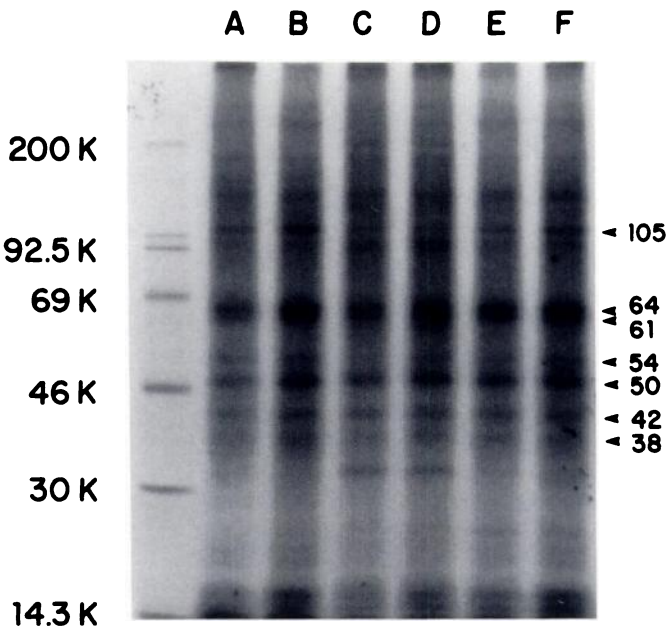


**Fig. 1.** Effect of TCDD on phosphorylation of membrane proteins. B lymphocytes were isolated as described in Experimental Procedures, and 5 × $10^7$ cells were suspended in 2 ml of PFB, with 10% control FBS or serum containing 10 nM TCDD, and incubated for 1 hr at 37°; 0.5 mCi of [$^{32}$P]orthophosphate was added and incubations were continued for 1 hr. Cells were then treated with 10 nM PMA or 50 μg/ml anti-Ig plus 5% BCGF, and incubation was continued for an additional 20 min. Membrane proteins were isolated and electrophoresis and autoradiography were performed as described in Experimental Procedures. Autoradiographs were scanned using a LKB Ultrascan XL linked to an IBM PC. *Lane A*, control; *lane B*, 10 nM TCDD; *lane C*, 10 nM PMA; *lane D*, 10 nM TCDD and 10 nM PMA; *lane E*, 50 μg/ml anti-Ig and 5% BCGF; *lane F*, 10 nM TCDD, 50 μg/ml anti-Ig, and 5% BCGF.

phocytes requires the cross-linking of membrane immunoglobulin by antigen, resulting in increased phosphatidylinositol-4,5-bisphosphate hydrolysis and the release of inositol trisphosphate and diacylglycerol (reviewed in Ref. 24). These products act as second messengers in mobilizing ionized intracellular calcium and stimulating PKC activity. The increase in PKC activity is thought to mediate a number of secondary responses in B cells, including depolarization of the cell membrane, increased expression of the class II major histocompatibility molecule (Ia), and entry into the cell cycle. In view of the importance of PKC activity in the stimulation of B lymphocytes, we investigated whether TCDD-mediated increases in phosphorylation were due to an increase in PKC activity. As shown in Table 1, TCDD had no effect on membrane-associated PKC activity. As expected, PMA, a known activator of PKC, caused PKC to translocate from the cytoplasmic to the membrane fraction, a response characteristic of enzyme activation. Stimulation of cells with anti-Ig/BCGF activated PKC, but to a lesser extent than did PMA. TCDD treatment did not cause either stimulator to activate PKC. Similarly, PKC was not activated after *in vitro* exposure of B lymphocytes to TCDD, when examined over a 4-hr period, a time sufficient for PMA-induced activation (data not shown). These data are consistent with earlier studies that demonstrated that TCDD does not inhibit phosphatidlyinositol hydrolysis (8), an event that precedes PKC activation. In contrast to the findings in lymphocytes, studies by Bombick *et al.* (23) reported that TCDD caused an increase in PKC activity in rat hepatocytes, suggesting that different signaling events may exist in lymphocytes and hepatocytes.

Studies of cellular transformation by oncogenic viruses and of the activities of protooncogenes have suggested a role for tyrosine-specific protein kinases in controlling normal and aberrant cellular proliferation and differentiation, in part through regulation of the expression of cell surface receptors for growth factors (9). Because TCDD affects B lymphocyte maturation (8), which is regulated by a complex series of growth and differentiation factors, many of which involve tyrosine kinases, the effect of TCDD exposure on tyrosine-specific kinase activity was investigated. Two major tyrosine-specific

TABLE 1

**Effect of TCDD exposure on PKC activity**

PKC activity is expressed as pmol of $PO_4$ incorporated/min × mg of protein and was determined for both cytoplasmic and membrane fractions, as described in Experimental Procedures. Data are expressed as mean ± standard error for triplicate observations. Percentage of membrane-associated activity was calculated from the formula: $[cpm_{membrane}/(cpm_{cytoplasm} + cpm_{membrane})] \times 100$.

| Treatment[a] | Cytoplasmic PKC activity | Membrane PKC activity | Membrane-associated |
|---|---|---|---|
| | *pmol/min × mg of protein* | | % |
| Control | 1751 ± 81 | 478 ± 27 | 21 |
| 10 μg/kg TCDD | 1322 ± 46 | 374 ± 41 | 22 |
| 100 nM PMA | 418 ± 77 | 2058 ± 74 | 83[b] |
| 10 μg/kg TCDD plus 100 nM PMA | 141 ± 41 | 1435 ± 79 | 91[b] |
| Anti-Ig/BCGF | 905 ± 96 | 836 ± 52 | 48[b] |
| 10 μg/kg TCDD plus anti-Ig/BCGF | 674 ± 43 | 793 ± 120 | 48[b] |

[a] Groups of five mice were exposed to 10 μg/kg TCDD or vehicle and sacrificed 48 hr later. B lymphocytes were prepared as described in Experimental Procedures and incubated with either PMA or anti-Ig/BCGF for 30 min.

[b] Significantly different from control at $p < 0.05$.

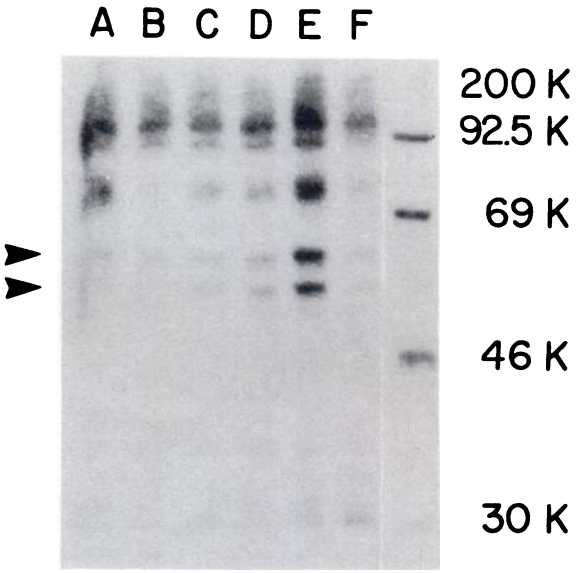


**Fig. 2.** Phosphorylation of endogenous membrane proteins by tyrosine kinase activity in B lymphocytes treated with TCDD. Cells were exposed to various concentrations of TCDD for 3 hr, after which membranes were isolated and assayed for tyrosine-specific kinase activity, as described in Experimental Procedures. Phosphorylation at tyrosine residues was determined by resistance to alkaline hydrolysis at 55° for 1 hr. *Arrowheads*, proteins that have previously been characterized to be major tyrosine kinases in B lymphocytes. *Lane A*, control; *lane B*, 1.25 nM TCDD; *lane C*, 2.5 nM TCDD; *lane D*, 5.0 nM TCDD; *lane E*, 10 nM TCDD; *lane F*, 10 nM PMA.

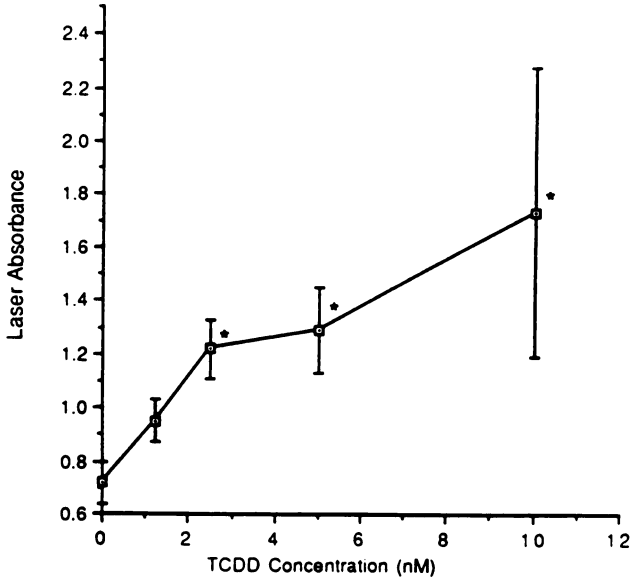


**Fig. 3.** Stimulation by TCDD of phosphorylation of a $M_r$ 61,000 protein. Tyrosine phosphorylation was assayed as described in Experimental Procedures. The phosphorylation of the $M_r$ 61,000 protein was quantitated by laser densitometry from four independent experiments, and the absorbance was normalized between experiments to the response of an internal control of 10 nM PMA. Graphed are the means ± standard deviations of the absorbance of the $M_r$ 61,000 protein. *, Statistically different from control by analysis of variance and Dunnett's multicomparison test, at $p \leq 0.05$.

kinases of murine B lymphocytes have been previously identified, consisting of $M_r$ 54,000 and 61,000 proteins (17, 18). As shown in Fig. 2, TCDD caused a dose-dependent increase in the autophosphorylation of the $M_r$ 54,000 and 61,000 kinases

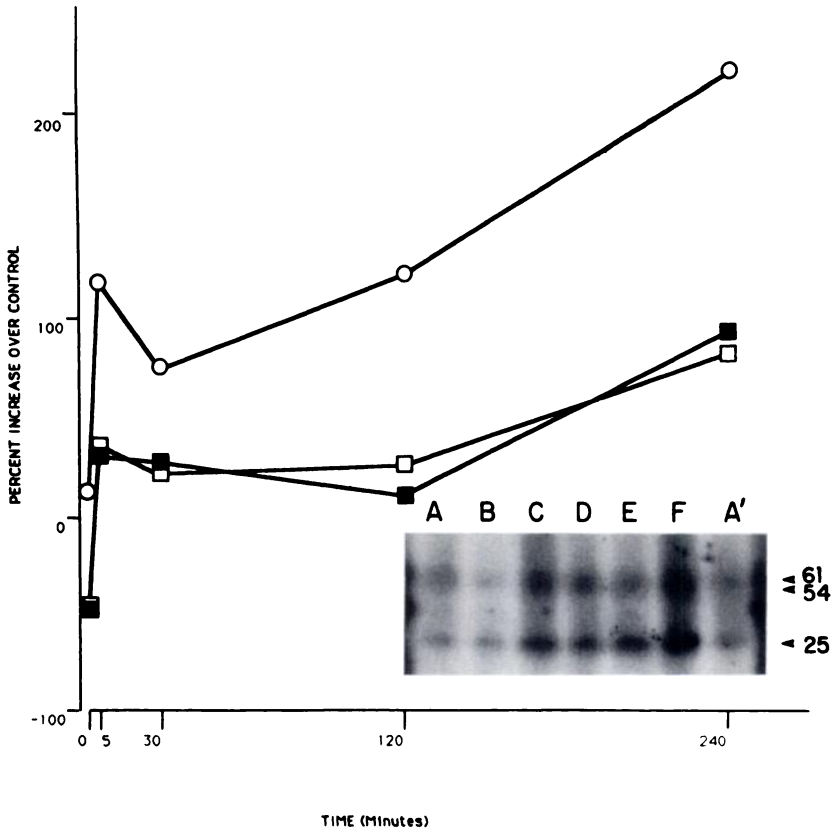


**Fig. 4.** Time course for increase in tyrosine phosphorylation in B lymphocytes exposed to 10 nM TCDD. Cells were exposed to TCDD for the specified time periods, diluted in medium, pelleted by centrifugation, and frozen. Tyrosine-specific activity was determined as described in Experimental Procedures, except that poly(Glu,Ala,Tyr) ($M_r$ 25,000 random polymer) was added to the reaction mixture as an exogenous substrate to confirm the tyrosine nature of the phosphoproteins. Autoradiograms were scanned with a LKB Ultrascan XL linked to an IBM PC, and peak absorbance was determined using a common baseline. Data were standardized as percentage increase in absorbance over the respective control response and graphed versus time following TCDD exposure for the $M_r$ 54,000 (□) and 61,000 (■) substrate, as well as for the exogenous substrate poly(Glu,Ala,Tyr) (○). *Inset*, an autoradiogram: *lane A*, control; *lane B*, 10 nM TCDD, 0 min; *lane C*, 10 nM TCDD, 5 min; *lane D*, 10 nM TCDD, 30 min; *lane E*, 10 nM TCDD, 120 min; *lane F*, 10 nM TCDD, 240 min; *lane A'*, control at 240 min.

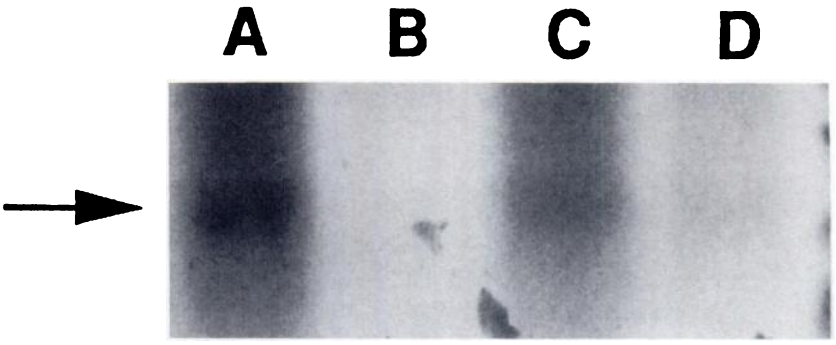


**Fig. 5.** Inhibition of TCDD-stimulated tyrosine phosphorylation in the presence of protein and RNA synthesis inhibitors. Membrane proteins from B cells treated with TCDD in the presence of 100 μM puromycin, 10 μM cycloheximide, or 0.1 μM actinomycin D were assayed for tyrosine-specific kinase in the presence of the exogenous substrate poly(Glu,Ala,Tyr), as described in Experimental Procedures. *Lane A*, control; *lane B*, 0.1 μM actinomycin D; *lane C*, 10 μM cyclohexamide; *lane D*, 100 μM puromycin. Autoradiograms were scanned with an LKB Ultrascan XL. The absorbance over background was control, 0.81; actinomycin D, 0.02; cyclohexamide, 0.55; puromycin, 0.08.

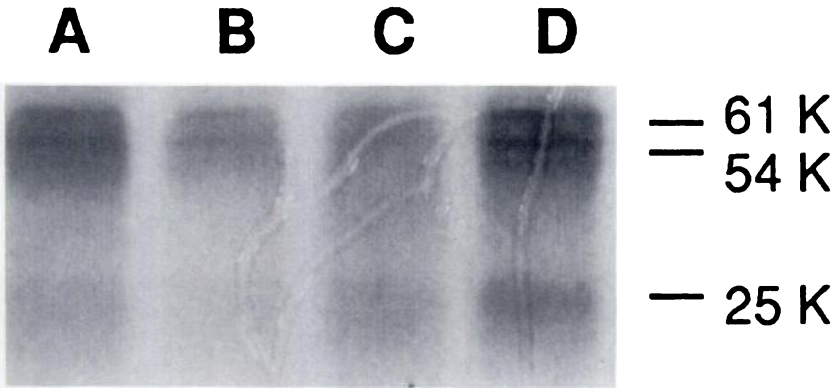


**Fig. 6.** TCDD-stimulated tyrosine phosphorylation in activated B cells. B cells were incubated for 36 hr in the presence of 5 μg/ml anti-Ig plus IL-4 (200 units/ml). Stimulated or freshly prepared B cells were treated with 10 nM TCDD for 3 hr. Membrane proteins were prepared and assayed for tyrosine-specific phosphoproteins in the presence of the exogenous substrate poly(Glu,Ala,Tyr), as described in Experimental Procedures. *Lane A*, anti-Ig and IL-4; *lane B*, anti-Ig, IL-4, and 10 mM TCDD; *lane C*, control; *lane D*, 10 mM TCDD.

at tyrosine residues, as preliminarily suggested by resistance to alkaline hydrolysis. TCDD also increased the tyrosine-specific phosphorylation of $M_r$ 78,000 and 90,000 proteins. These latter two proteins have not been previously described and may be substrates for tyrosine-specific kinases or unidentified kinases; however, increases in the phosphorylation of these proteins were not uniformly demonstrated in all experiments. In contrast, PMA did not affect the major tyrosine kinases of B lymphocytes. Shown in Fig. 3 is the increase in phosphorylation of the $M_r$ 61,000 protein as a function of TCDD concentration.

A modest but statistically significant increase over the control response was observed at concentrations of 2.5 nM TCDD and greater. A similar response was observed for increased phosphorylation of the $M_r$ 54,000 protein (data not shown).

Fig. 4 depicts the results of a time course study on the expression of tyrosine activity in B lymphocytes exposed to 10 nM TCDD. A synthetic exogenous substrate, poly(Glu,Ala,Tyr) ($M_r$ 25,000), was included in the reaction mixture to help confirm that phosphorylation occurred on tyrosine residues after TCDD treatment. The increase in substrate activity could be detected as early as 5 min after exposure to TCDD and appeared to continue to increase for at least 4 hr. Increased phosphorylation of the $M_r$ 54,000 and 61,000 proteins paralleled the increased phosphorylation of the exogenous peptide and, coupled with the base-hydrolyzed gels (e.g., Fig. 2), provided additional evidence that these phosphoproteins are associated with tyrosine-specific kinase activity.

As previously indicated, TCDD is believed to exert many of its responses via binding to the Ah receptor, nuclear translocation of the receptor-TCDD complex, and increased transcription rate of specific genes (for review, see Refs. 25 and 26). This can occur rapidly, as evidenced by the increase in the transcription rate of a gene(s) responsible for arylhydrocarbon hydroxylase activity that occurs within minutes after addition of TCDD to mouse hepatoma cells (25). In order to help establish whether the observed effects on tyrosine phosphorylation involved a transcriptional event, specific protein and RNA inhibitors were added during the 4-hr incubation period in which B cells were treated with TCDD. The transcriptional and translational inhibitors employed were 100 μM puromycin, 10 μM cycloheximide, and 0.1 μM actinomycin D. At these concentrations, protein synthesis, or RNA synthesis in the case of actinomycin D, was inhibited in lipopolysaccharide-stimulated lymphocytes by at least 90%, without any additional loss in cell viability as determined by trypan blue exclusion (data not shown). TCDD-mediated increases in tyrosine phosphorylation were inhibited 32% by cycloheximide treatment, whereas puromycin and actinomycin D treatment inhibited the response by 90 and 98%, respectively, indicating that TCDD-induced tyrosine phosphorylation is at least partially mediated by transcription events (Fig. 5). However, considering the rapid onset of the effects of TCDD on tyrosine kinase activity demonstrated in the time course experiments and the incomplete block by the metabolic inhibitors, it is possible that TCDD may also be affecting preexisting proteins. In this respect, the binding activity of the estrogen receptor can be regulated by its degree of phosphorylation, independent of gene products (27), presumably via a secondary messenger system. We have compared the relative differences in TCDD-inducible tyrosine-specific phosphorylation in B cells from *Ah* congenic B6D2 ($Ah^d/Ah^d$)$NE_9$ and C57BL/6 ($Ah^b/Ah^b$) mice.[3] Basal tyrosine kinase activity was markedly higher in the congenic mice, compared with the $Ah^b/Ah^b$ strain, and no dose-dependent increase could be obtained with increasing concentrations of TCDD. This is in contrast to other TCDD responses, in which, given a sufficient dose, the same maximum response in both $Ah^b/Ah^b$ and $Ah^d/Ah^d$ mice occurs (for review, see Refs. 25 and 26). Further studies will be needed to clarify the difference in these responses.

[3] Clark, G. C., Luster, M. I., unpublished observations.

Similar to our results in B lymphocytes, others (11, 28) have recently reported that an early response to TCDD in the liver is an increase in tyrosine-specific protein kinase activity. This response precedes other biochemical changes associated with TCDD exposure, including down-regulation of the EGF receptor, and suggests a role for TCDD/tyrosine kinase interactions in altering receptor expression or receptor-mediated responses to growth factors. In fact, TCDD alters the binding affinity of a number of hormone receptors including estrogen and glucocorticoid receptors (29). In human keratinocytes, the binding of TCDD to the Ah receptor precedes altered expression of at least two receptor systems, the EGF and the glucocorticoid receptors (10, 30), which are involved in the control of epidermal cell proliferation (30). The ability of TCDD to alter tyrosine-specific protein kinase activity in B lymphocytes may similarly lead to altered receptor-mediated responses, affecting B lymphocyte maturation. In this respect, we have previously demonstrated that TCDD prevents terminal differentiation of B cells (5, 8), which occurs at concentrations found to increase tyrosine-specific protein kinase activity (~2 nM). This relationship was explored in the present studies by comparison of the influence of TCDD on tyrosine phosphorylation in preactivated ($G_1$/S) B cells, which are resistant to immunosuppression by TCDD (5, 8), and resting ($G_0$) cells. Activation of B cells, i.e., transition from $G_0$ to the $G_1$/S phases, was accomplished by incubation of lymphocytes with anti-Ig plus IL-4 for 36 hr at 37°, which typically results in greater than 70% of the B cells entering into the cell cycle (31). As shown in Fig. 6, anti-Ig/IL-4-activated B cells ($G_1$/S) were resistant to TCDD-mediated increases in tyrosine kinase activity (Fig. 6, *lane A* versus *lane B*) and actually produced a 45% decrease in total activity, as measured by laser densitometry, whereas tyrosine-specific phosphorylation in freshly prepared ($G_0$) cells treated with TCDD (Fig. 6, *lane C* versus *lane D*) was increased by 80%. As previously reported (32), B cell activation, alone, was associated with specific tyrosine phosphorylation. These were not, however, associated with TCDD.

Taken together these results demonstrate that TCDD stimulates tyrosine kinase-specific phosphorylation in B lymphocytes and this response coincides with inhibition of antibody synthesis. However, determination of a direct cause and effect relationship between these phenomena awaits further studies. There is, nonetheless, ample evidence showing that many of the events in lymphocyte maturation are associated with tyrosine kinase activity. For example, Gold *et al.* (32) demonstrated that activation of tyrosine kinase activity occurs concurrently with phospholipase C-stimulated activation of B cells. Furthermore, many of the interleukin-dependent events associated with lymphocyte regulation are associated with tyrosine kinase activity (reviewed in Ref. 24). Additionally, a number of reports have suggested that membrane-associated tyrosine protein kinases play a role in controlling differentiation of lymphoid/myeloid cell lines. For example, differentiation of the monomyelocytic cell lines HL-60 and U-937 correlates with the induction and increased tyrosine protein kinase activity of the c-*src* gene product, $pp60^{c\text{-}src}$ (33, 34). A correlation between increased levels of tyrosine protein kinase activity and lymphoid differentiation has also been observed in leukemic B cells (35). The recent cloning of the *hck* gene, which appears to code for a tyrosine protein kinase that regulates aspects of differentiation in cells of hematopoietic origin, also supports a role

for tyrosine-specific protein kinases in controlling lymphocyte differentiation (36, 37).

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