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2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ENHANCES ANTIBODY PRODUCTION AND PROTEIN KINASE ACTIVITY IN MURINE B CELLS

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SUMMARY: Treatment of murine spleen cells with 30 nM TCDD resulted in an approximately 3 fold increase in unstimulated antibody production after 3 days in culture. This response was not accompanied by increased cellular proliferation and may represent an effect of TCDD on B cell activation or differentiation. Since PMA is capable of activating B cells, presumably via PKC, we have compared the effects of PMA and TCDD on protein kinase activation and phosphorylation of endogenous proteins in a highly purified preparation of B cells. In contrast to a reduction of cytosolic PKC activity, the expected effect of PMA, TCDD caused an increase in basal kinase activity with no effect on PKC activity. Addition of either PMA or TCDD resulted in enhanced phosphorylation of a similar profile of proteins, including proteins of Mr 12.2, 14.6, 29.2, 52.3 and 62.7 KDa. Addition of TCDD also resulted in the increased phosphorylation of a protein of Mr 45.2, which was unaffected by PMA. Combined treatment with PMA and TCDD resulted in additive responses. The additive effects of PMA and TCDD suggest an interaction at the level of protein phosphorylation which is mediated by different kinases. Therefore, TCDD may be stimulating B cells via an early effect on an unidentified protein kinase.

The effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) vary widely depending on the target cell as well as species of the organism, but are frequently manifested as effects on gene expression and differentiation (1-3). While the TCDD receptor, a cytosolic protein, has been investigated in detail (4,5), the biochemical mechanism(s) which mediate the effects of TCDD have

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The following abbreviations were used: LPS, lipopolysaccharide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PKC, protein kinase C; PMA, phorbol 12-myristate, 13-acetate; PS, phosphatidylserine; DO, diolein.

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not been well characterized. Previous reports by Bombick, et al. (6), have implicated an effect of TCDD on PKC activity as having a role in TCDD effects on the liver. However, we recently observed in EL4 cells that TCDD has no direct effect on PKC activity or on specific binding by phorbol esters to their receptor, which is putatively PKC (7).

Murine B lymphocytes are activated by biologically active phorbol esters such as PMA, with a concomitant stimulation of PKC (8) and phosphorylation of several endogenous proteins (9,10). Treatment of B cells with PMA provides signals which then allow the cell to respond mitogenically to immunoglobulin or calcium ionophore (9). Treatment of murine spleen cells with TCDD results in suppression of stimulated (e.g. by LPS, DNP-ficoll or sheep erythrocytes) antibody production, which is not accompanied by an effect on proliferation (11). This suppression by TCDD is not dependent on T cells, and was therefore interpreted to represent an effect of TCDD on B cell differentiation (12). In the present studies we have examined the effects of TCDD on basal (nonstimulated) antibody production by murine spleen cells. In addition, we have compared the effects of PMA and TCDD on protein kinase activation and phosphorylation of endogenous proteins of B cells in order to identify a potential pathway for this effect of TCDD. In these latter studies we have used a rigorously purified population of splenic B cells in order to completely eliminate any contribution by contaminating T cells or macrophages.

METHODS

Purified B cells were obtained from female B6C3F1 mice (Litton Bionetics, Frederick, MD) by depletion of macrophages and T cells from spleen cell suspensions as previously described (13). Briefly, spleens were aseptically removed and a single cell suspension was prepared. Macrophages were depleted first by adherence to plastic petri dishes (Falcon, Oxnard, CA) for 1 hour, then by passage of nonadherent cells over Sephadex G-10 columns by a modification of

the method of Jerrels, et al. (14). Macrophage depleted cells (greater than 99.4% macrophage-free according to nonspecific esterase stain, kit 90-A1, Sigma) were depleted of T cells by incubation with monoclonal antibody anti-thy 1.2 (Accurate Chemical, Westbury, NY) for 40 min. at 4°C followed by washing and suspension in 1:10 Low-Tox rabbit complement (Accurate) and incubation for 50 min. at 37°C. Viable B cells were separated from dead cells and red blood cells by centrifugation through Lympholyte-M (Accurate) for 18 min. at 500g. Recovered B cells were washed twice in phosphate-free buffer. B cells were 91-93% pure as determined by fluorescein-conjugated anti-mouse thy 1.2 (Becton-Dickinson, Mountain View, CA) and enumeration via fluorescence microscopy (Olympus model BH-2) and have been extensively characterized elsewhere (13). Measurement of antibody producing cells was performed using suspensions of spleen cells from B6C3F1 mice in 24-well cluster plates (Falcon), at 5 x 10^6 cells/ml/well, which were incubated with rocking at 37°C in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2 (11). The response was measured against sheep erythrocytes (Colorado Serum), which were densely coupled with trinitrophenyl by the method of Rittenberg and Pratt (15), and antibody forming cells were measured by a modification of the Jerne plaque assay (11,12).

Protein kinase activity was measured by a modification of the method of Witt and Roskowski (16) as described previously (7). Briefly, B cells were treated with TCDD +/or PMA for 30 min., then centrifuged at 500g for 10 min. and resuspended in homogenization buffer containing 300 mM sucrose, 5 mM EDTA, 5 mM EGTA, 150 mM tris pH 7.4, 5 mM leupeptin and 20 mM benzamidine. Cells were homogenized by 30 strokes of a Dounce homogenizer then centrifuged at 100,000g for 1 hour. The supernatant or cytosol was then used in kinase assays. Phosphorylation experiments were performed after 1 hour preincubation with 50 uCi/ml $^{32}PO_4$ (ICN Pharmaceuticals, Irvine CA). Labeled cells (10^6/tube) were treated for the times indicated at 37°C, then reactions were stopped by addition of an equal volume of 20% SDS, 10% glycerol containing bromophenol blue and 5 mM 2-mercaptoethanol. Samples were boiled and equal volumes were loaded onto lanes of 10% acrylamide slab gels. Dried gels were exposed to Kodak XAR-5 film and bands of interest were excised and counted in 5 ml scintillation fluor.

2,3,7,8-TCDD was obtained through the National Toxicology Program, and stored in 1% DMSO; PMA was obtained from P-L Biochemicals(Worthington, MA) and stored in 100% DMSO. The vehicle had no effect on these assays at the final concentrations used, with the exception of the kinase assay as described in Results.

RESULTS AND DISCUSSION

The effect of TCDD on antibody production by B6C3F1 mouse splenocytes using haptenated sheep erythrocytes as the target is illustrated in Figure 1. On days 3 and 4, cultures treated with 30 nM TCDD contained approximately three times the number of

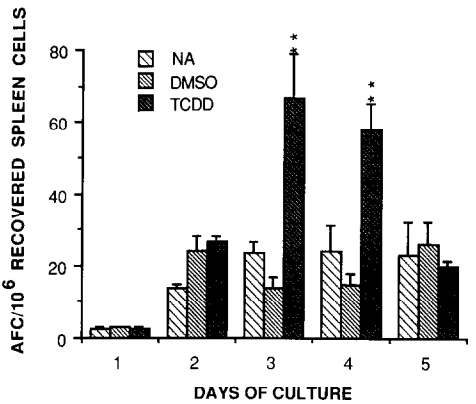


FIGURE 1. EFFECT OF TCDD ON THE BACKGROUND ANTIBODY RESPONSE OF MURINE SPLENOCYTES. Suspensions of spleen cells were incubated with medium (naive, NA), DMSO or 30 nM TCDD at 37°C, rocking, in 10% CO_2, 7% O_2 and 83% N_2 for the number of days indicated, then antibody forming cells (AFC) were determined as described in Methods. Data are from one of four similar experiments performed in triplicate. **$p<0.01$ vs. NA or DMSO.

antibody forming cells as compared to naive or vehicle (DMSO) treated cultures. The time course of antibody production during culture is consistent with that seen in antigen stimulated cultures (11).This effect of TCDD was not accompanied by enhanced cellular proliferation as determined by ^{3}H-thymidine incorporation (11). Therefore, the increased antibody production seen with TCDD probably represents an effect on early events in B cell activation leading to differentiation. As mentioned in the introduction, TCDD has been shown to alter differentiation in several cell types (2,3). Some of the early events in B cell activation/differentiation have recently been characterized. Bijsterbosch, et al. (17) demonstrated that during B cell proliferation stimulated by anti-Ig, breakdown of phosphatidylinositol-4,5-bisphosphate and an increase in cytosolic calcium occur. These results imply that stimulation of membrane receptors on B cells leads to activation of phospholipase C, leading to PKC activation and calcium mobilization. Recently, Rothstein, et al. (18), showed that treatment of murine splenic B cells with PMA and calcium ionophore stimulated entry of B cells into S phase. These agents together can mimic the activation of PKC by diacylglycerol and the increase in cytosolic calcium caused by inositol-1,4,5-trisphosphate and therefore bypass receptor mediated phosphatidylinositol turnover. Guy, et al. (8), have recently demonstrated that treatment of human B cells with mitogens or PMA and the calcium ionophore ionomycin leads to redistribution

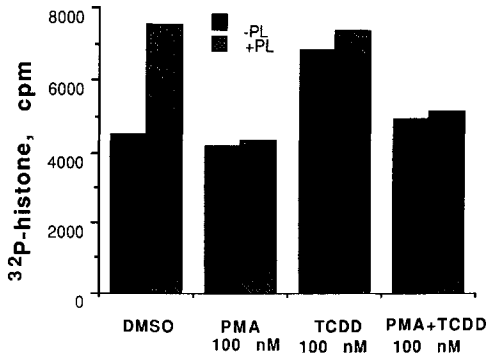
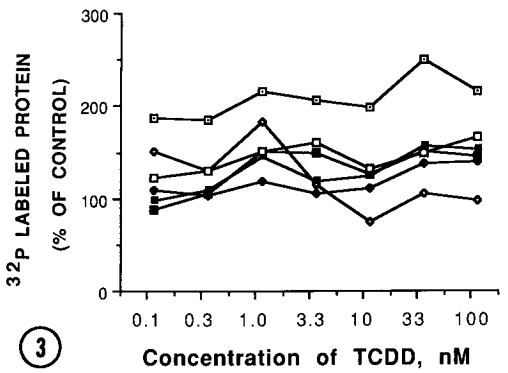


FIGURE 2. EFFECT OF PMA AND TCDD ON KINASE ACTIVITY IN B CELL CYTOSOL. Purified B cells were incubated with 100 nM TCDD, 100 nM PMA or 100 nM each of PMA and TCDD for 30 min. at 37°C., then homogenized and cytosol prepared as described as in Methods. Kinase activity was measured in the presence or absence of added phospholipids (PL) as described in Methods. Data are from one of three similar experiments performed in triplicate.

of PKC and cleavage to its proteolytically activated form, protein kinase M. Several B cell proteins are phosphorylated in response to PMA, anti-IgM, mitogens or the combination of PMA and ionomycin (9,10). Since these phosphorylation events presumably are linked to early activation of the B cell, we studied both kinase activation and protein phosphorylation in B cells when stimulated by TCDD. PMA was included in these studies for comparative purposes. We used a highly purified population of murine B cells rather than splenocytes in order to specifically study early events taking place in the B cell.

Murine B cells contain a measurable amount of PKC in their cytosol (Figure 2), and this activity is abolished in cells which were treated with 100 nM PMA. This most likely represents binding of PKC to the membrane, as shown by Guy, et al. (8), in human cells. We occasionally observed reduced phospholipid-stimulated (i.e., PKC) kinase activity in DMSO treated cells as compared to cells treated with medium alone (not shown), which may represent an effect of trace amounts of DMSO on phospholipids used to measure PKC. Therefore, effects of PMA and TCDD are compared to DMSO. Treatment of B cells with 100 nM TCDD resulted in an increase in kinase activity in the absence of added phospholipids, with no increase in kinase activity in the presence of phospholipids. This increased activity was partially calcium dependent (not shown), and therefore probably does not represent



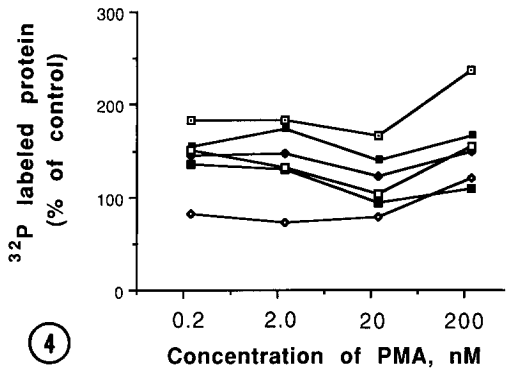


FIGURE 3. CONCENTRATION RELATIONSHIP FOR TCDD STIMULATED PROTEIN PHOSPHORYLATION IN B CELLS. Purified B cells were prelabeled with $^{32}PO_4$ for 60 min, stimulated with TCDD for 30 min. at 37° C., then proteins were separated by SDS electrophoresis and labeling of specific protein bands described as in Methods. Data shown are from one of three similar experiments.

12.2 KDa
14.6 KDa
29.2 KDa
45.2 KDa
52.3 KDa
62.7 KDa

FIGURE 4. CONCENTRATION RELATIONSHIP FOR PMA STIMULATED PROTEIN PHOSPHORYLATION IN B CELLS. Purified B cells were prelabeled with $^{32}PO_4$ for 60 min, stimulated with PMA for 30 min. at 37° C., then proteins were separated by SDS electrophoresis and labeling of specific protein bands described as in Methods. Data shown are from one of three similar experiments.

12.2 KDa
14.6 KDa
29.2 KDa
45.2 KDa
52.3 KDa
62.7 KDa

protein kinase M. This supports our earlier finding that TCDD does not act on PKC (7), but suggests that TCDD does activate some other protein kinase. To further explore a possible interaction between TCDD and the PKC pathway, we examined the combination of TCDD and PMA. In combination, the effects of TCDD and PMA were nearly additive, suggesting that the two agents are acting on different pathways.

Phosphorylation of endogenous proteins was measured in purified B cells which were prelabeled with $^{32}PO_4$ and then stimulated by TCDD, PMA or the combination, for 30 minutes (Figures 3 and 4). TCDD (0.1-100 nM) stimulated phosphorylation of several proteins, with molecular weights of 12.2, 14.6, 29.2, 45.2, 52.3 and 62.7 KDa (Figure 3). The concentration-response curves for protein phosphorylation are relatively shallow, a finding which we have observed in other cell types using whole cell lysates run on one dimensional gels. We would expect concentration-response curves to be steeper for fractionated cells run on two dimensional gels, due to enhanced resolution. Those experiments are currently in progress. The 45.2 and 52.3 KDa proteins are close in molecular

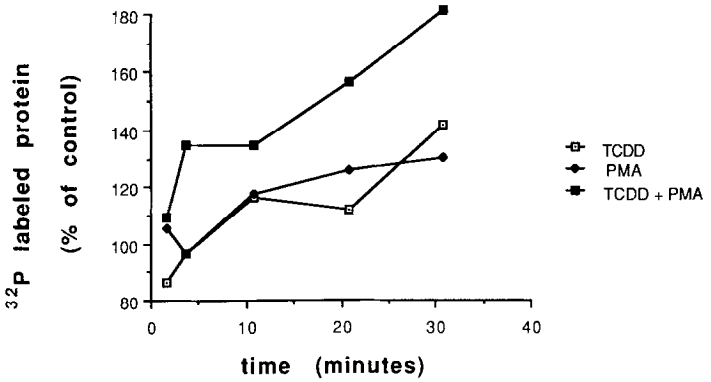


FIGURE 5. TIME COURSE OF PHOSPHORYLATION OF 12.2 KDa PROTEIN BY PMA AND TCDD. Purified B cells were prelabeled with $^{32}PO_4$ for 60 min, stimulated with 10 nM TCDD, PMA or 10 nM each of TCDD and PMA for the times indicated. Labeling of the 12.2 KDa protein was determined as described in Methods. Data shown are from one of four similar experiments.

weight to proteins reported by others to be phosphorylated during B cell mitogenesis (9,10). However, phosphorylation of the other proteins was not observed in those studies, so that the 12.2, 14.6, 29.2 and 62.7 KDa proteins may represent differentiation related responses, or proteins which may have a role in early events leading to antibody production. Other B cell proteins with molecular weights similar to these proteins and with important roles in B cell differentiation include IL-2, 15.5 KDa (19), BCGF-II, 60 KDa (20), and Ia antigen, 60 KDa (21). A similar set of proteins was also phosphorylated by PMA (Figure 4), the major difference from results with TCDD being a lack of effect of PMA on the 45.2 KDa protein.

The time course of phosphorylation of the 12.2 KDa protein by 10 nM TCDD roughly paralleled that of 10 nM PMA (Figure 5). When TCDD and PMA were given in combination, phosphorylation of the 12.2 KDa protein was more than additive. This additivity was also observed with the 62.7 KDa protein, but not with any of the other phosphoproteins (not shown). One interpretation of these results, taken together with the kinase data in Figure 2, is that PMA and TCDD may act via different kinases to phosphorylate at least some of the same proteins.

These effects of TCDD on early events in B cell stimulation may represent a mechanism by which TCDD drives the B cell into

a more differentiated, antibody producing state. The suppression of stimulated antibody production (i.e. stimulated by the B cell mitogen, lipopolysaccharide and measured after two days in culture) by TCDD requires addition of TCDD during the first three hours of culture with the stimulus, which implies that TCDD is affecting early activation or maturation of the B cell. Our results presented here support this possibility. Recently, Hoffman, et al., speculated that conditions which induce polyclonal antibody synthesis may block the subsequent production of antigen-specific antibody (22). The exact relationship between the TCDD-induced increase in unstimulated antibody production (shown here) and the TCDD-induced decrease in stimulated antibody responses (11,12) is currently under study.

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REFERENCES

1. Jones, P.B.C., Durrin, L.K., Galeazzi, D.R. and Whitlock, J.P., Jr. (1986) Proc. Nat. Acad. Sci. 83: 2802-2806.
2. Knutson, J.C. and Poland, A. (1980) Cell 22:27-32.
3. Osborne, R. and Greenlee, W.F. (1985) Toxicol. Appl. Pharmacol. 77:434-437.
4. Knutson, J.C. and Poland, A. (1984) Molecular and Cellular Approaches to Understanding Mechanisms of Toxicity, A.H. Tashjian, ed., Harvard School of Public Health.
5. Gasiewicz, T.A. and Neal, R.A. (1982) Anal. Biochem. 124:1-11.
6. Bombick, D.W., Madhukar, B.V., Brewster, D.W. and Matsumura, F. (1985) Biochem. Biophys. Res. Comm. 127:296-302.
7. Kramer, C.M., Sando, J.J. and Holsapple, M.P. (1986) Biochem. Biophys. Res. Comm. 140:267-272.
8. Guy, G., Gordon, J., Walker, L., Michell, R.H. and Brown, G. (1986) Biochem. Biophys. Res. Comm. 135:146-153.
9. Monroe, J.G. and Gaulton, G.N. (1985) Surv. Immunol. Res. 4:192-199.
10. Hornbeck, P. and Paul, W.E. (1986) J. Biol. Chem. 261:14817-14824.
11. Holsapple, M.P., Dooley, R.K., McNerney, P.J. and McCay, J.A. (1986) Immunopharm. 12:175-186.
12. Dooley, R.K. and Holsapple, M.P., J. Immunol, submitted.
13. Johnson, K.W., Munson, A.E. and Holsapple, M.P. (1987) Immunopharmacology, in press.
14. Jerrels, T.R., Dean, J.H., Richardson, G.L. and Herberman, R.B. (1980) J. Immunol. Methods 32:11-29.
15. Rittenberg, M.B. and Pratt, K.L. (1969) Proc. Soc. Exp. Biol. Med. 132:575-580.

16. Witt, J.J. and Roskoski, R. (1975) Anal. Biochem. 66:253-256.
17. Bijsterbosch, M.K., Meade, C.J., Turner, G.A. and Klaus, G.G.B.(1985) Cell 41:999-106.
18. Rothstein, T.L., Baeker, T.R., Miller, R.A. and Kolber, D.L. (1986) Cell. Immunol. 102:364-373.
19. Smith, K. (1984) Ann. Rev. Immunol., 319-333.
20. Howard, M., Nakanishi, K. and Paul, W. (1984) Immunol. Rev. 78:185-210.
21. Klein, J., Figuiroa, F. and Nagy, Z. (1983) Ann. Rev. Immunol. 1:119-142.
22. Hoffman, M.K., Gilbert, K.M., Hirst, J.A. and Scheid, M. (1987) J. Mol. Cell. Immunol. 3:29-36.