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ANTAGONISTIC EFFECTS OF INTERFERONS (IFNs) AND SODIUM ORTHOVANADATE ON RESPONSES PRODUCED BY TCDD IN SEVERAL CULTURE SYSTEMS

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ABSTRACT: *Several types of IFNs were tested for their ability to suppress TCDD-inducible P-450 dependent mixed function oxidase (MFO) system in mouse primary hepatocytes. Mouse IFN-gamma (IFN-G) markedly suppressed EROD activity when added at the same time as TCDD (10 nM). The antagonism of EROD activity by IFN-G exhibited both a dose-(10-100 U/ml) and time-dependence. In contrast, mouse IFN-A/B was only moderately suppressive and only at high concentrations (500 U/ml). Rat IFN-G was even more selective than mouse, whereas human IFN-G had no activity. The species and subtype specificities were confirmed using a monoclonal Ab against mouse IFN-G, which blocked the action of mouse IFN-G, but had no effect on the suppression by either mouse IFN-A/B or rat IFN-G. To confirm the direct effect of IFN-G, liver parenchymal cells were isolated from crude liver cell preparation and it was found that purified parenchymal cells were still sensitive to effects of IFNs. These results indicate that IFNs, particularly IFN-G, can antagonize TCDD-mediated enzyme induction in primary hepatocytes similarly in immune system. Sodium orthovanadate was known as a selective inhibitor of phosphotyrosine phosphatase. In view of protein tyrosine phosphorylation, vanadate was tested in several cell culture systems. Vanadate markedly suppressed EROD activity when added at the same time as TCDD dose- and time-dependently in mouse primary hepatocytes. TCDD-induced inhibition of T-dependent antibody formation in mouse* splenocytes was also reversed by sodium orthovanadate. As *another system, human malaria culture system in human RBC was tested. In both synchronized and unsynchronized malaria parasites, TCDD showed a growth stimulation even though RBC did not have nucleus and Ah receptor*

dependent gene turn-on. Sod. orthovanadate also compensated TCDD-induced stimulation of malaria growth. By above results in three different culture model systems, it could be assumed that sodium orthovanadate act as a real antagonist against TCDD through a common mechanism with TCDD.

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin(TCDD) is the prototype for a class of halogenated aromatic hydrocarbons (HAH) which elicit a variety of biological responses, including general and tissue-specific toxicity, changes in cell proliferation and differentiation, immunosuppression, and the induction of drug metabolizing enzymes (Polland and Knutson, 1982). Enzyme induction associated with TCDD exposure is believed to be mediated through the *Ah*(aromatic hydrocarbon) receptor (Whitlock, 1987). The role of this receptor in TCDD-induced toxicity has been widely accepted, but it has not been conclusively demonstrated and remains controversial. Especially for TCDD-induced humoral immune suppression there is increasing evidence that non-*Ah* locus-mediated mechanisms must play a significant role. In previous studies, we have observed that mouse interferon-gamma (IFNg) can reverse the suppression of *in vitro* antibody response in mouse splenocytes associated with the direct addition of TCDD (Snyder *et al.*, 1990). Commensurate reversal has been demonstrated with human and rat IFNg and elevated concentrations of mouse Type 1 (alpha and beta) IFN appear to have similar activity. The apparent interaction between mouse INFg and mouse Type 1 IFN suggests the involvement of the *Ah* locus. The present studies were initiated in part to determine if similar interactions occur for a non-immune TCDD-induced response which has been more clearly demontrated to be associated with the *Ah* receptor. The approach employed parallels that used in the immune function studies. Recombinant mouse and rat INFg, non-recombinant human IFNg, and non-recombinant mouse Type 1(alpha and beta) IFN(IFNA/B) were tested for their ability to suppress TCDD-mediated induction of the cytochrome P450-dependent mixed function oxidase (MFO) system in mouse primary hepatocyte cultures. Recently it was found that TCDD caused an increase in protein phosphorylation. The major kinases effected by TCDD were protein kinase C (serine, threonine phosphorylation) and tyrosine kinase (Bombick *et al.*, 1985; Kramer *et al.*, 1987; Madhukar *et al.*, 1988; Kawamoto *et al.*, 1989). Along with protein phsphorylation, the intracellular calcium concentration was also modified. Modified intracellular calcium concentration was related with TCDD induced immunotoxicity, cardiac toxicity and hepatotoxicity etc. (Canga *et al.*, 1988; McConkey and Orrenius, 1989). Sodium orthovanadate was known as an inhibitor of plasma membrane phosphotyrosine phosphatase and Ca-ATPase (Philosoph and Zilberstein, 1989; Ramponi *et al.*, 1989; Mustelin *et al.*, 1989). Intracellular tyrosine specific protein phosphorylation equilibrium is controlled by tyrosine kinases and phosphotyrosine phosphatase and the inhibition of

phosphotyrosine phosphatase can induce an increase of tyrosine phosphorylation. Sodium orthovanadate also cause an increase of intracellular calcium concentration by inhibition of Ca-ATPase. These two mechanisms of SO action might be related to two phenomena caused by TCDD treatment. In the present studies, we have examined the *in vitro* effects of SO on TCDD induced hepatotoxicity and immunotoxicity. The parameters used in the present studies were cytochrome P-450 related mixed function oxidase in hepatocytes and T-dependent antibody formation in lymphocytes which were effected by TCDD. Primary culture of mouse hepatocytes and primary culture of mouse splenocytes were used for this study. Exposure of TCDD induced immunosuppression, characterized by thymic atrophy, suppressed cell-mediated immunity, and increased susceptibility to infection, *in vitro B* cell suppression (Faith *et al.*, 1980; Vecchi *et al.*, 1980). Suppression of *B* cell-mediated or humoral immunity results in altered host resistance to the parasite *Plasmodium yoelii*, a malaria model (Tucker *et al.*, 1986). Human malaria parasite (*Plasmodium falcifarum*) can be maintained in continuous culture in human erythrocytes *in vitro* (Trager and Jensen, 1976). Human erythrocytes are anucleated cell and has no gene turn-on process. In the present studies, we have examined the *in vitro* effects of TCDD on malaria growth in human erythrocytes and compared the previously reported *in vivo* results. In addition, we included sodium orthovanadate to approximate the mechanism of TCDD action in this system. The direct effect of TCDD on malarial growth is discussed in corelation with *Ah* receptor and immuno-

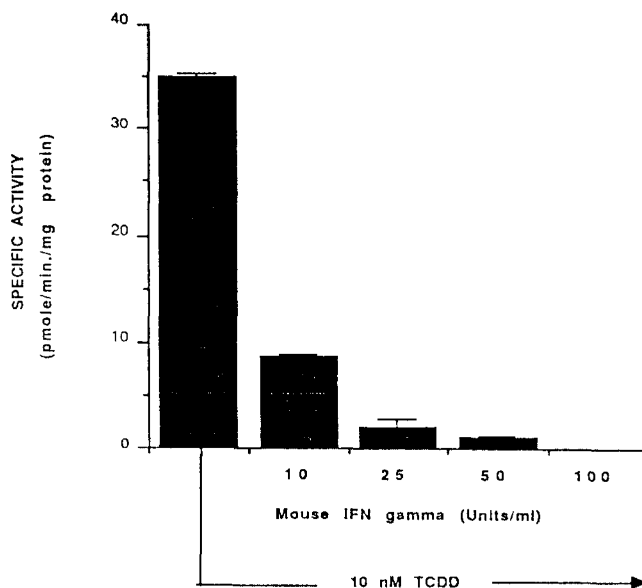

Fig. 1. EROD Specific Activity in primary hepatocytes after TCDD and recombinant mouse interferon gamma addition *in vitro*. The bars represent the mean±S E derived from 3 replicate cultures per treatment group.

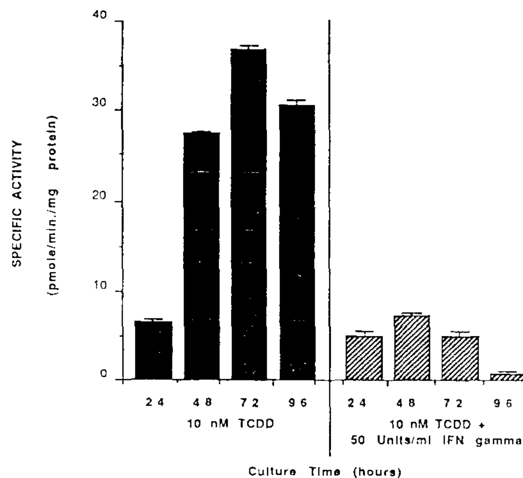

Fig. 2. Time course of EROD Specific Activity in primary hepatocytes after TCDD and recombinant mouse interferon gamma addition *in vitro*. The bars represent the mean±SE derived from 3 replicte cultures per treatment group.

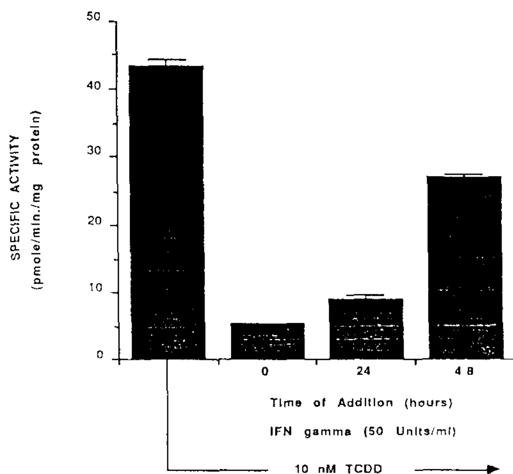

Fig. 3. Effect of time of addition of recombinant mouse gamma interferon on EROD Specific Activity in primary hepatocytes after TCDD addition *in vitro*. The bars represent the mean±SE derived from 3 replicate cultures per treatment group.

suppression.

Interferons and TCDD-inducible Cytochrome P-450

TCDD was an well known inducer of cytochrome P-450 related mixed function oxidases. Enzyme induction by TCDD is belived to be mediated through the *Ah* receptor. After initial binding to a soluble intracellular form of *Ah* receptor, the ligand-receptor complex is though to translocate to the nucleus and bind specific regulatory regions of DNA. The resulting pleiotrophic gene response includes increase in mRNAs specific for the induced enzymes. TCDD can show strong

inductive responses both *in vivo* and *in vitro*. When treated to primarily cultured hepatocytes, TCDD strongly induced ethoxyresorufin O-deethylase (EROD) activity. Mouse IFN-G suppressed TCDD-inducible EROD activity and showed a clear dose response if added commonly with TCDD (Fig. 1). The culture period used in this study was 72 hours. The time courses of effects of TCDD and mouse IFN-G were examined (Fig. 2). Induction of EROD activity in hepatocytes by treatment of 10 nM TCDD showed a clear time dependence and maximum level was obtained by 72 hours incubation. Because TCDD did not show a strong induction at 24 hours, the effect of interferon was not quite obvious. As culture periods were increased upto 96 hours, 50 Units/m*l* of IFN-G showed a good antagonistic effects on induction by TCDD. Even though cells were pretreated with TCDD prior to IFN-G, the antagonism could be shown (Fig. 3). Absolutely, the effect of mouse IFN-G was most significant in simultaneously treated case. But in the case of treatment of interferon from 48 hours, enzyme activity was decreased to about 60% of untreated control. This result showed that interferon could elicit antagonistic effect at any time point of TCDD action.

Subtype and Species Difference of Interferons

Mouse IFN-A/B was compared with mouse IFN-G in dose-response manner (Fig. 4). 50 Units/m*l* of IFN-G showed a near complete compensation for 10 nM of TCDD. But IFN-A/B showed no effects at the same concentration with IFN-G. If the concentration was increased to 500 Units/m*l*, IFN-A/B inhibited 60% of the action by TCDD. From these results, it could be assumed that mouse IFN-

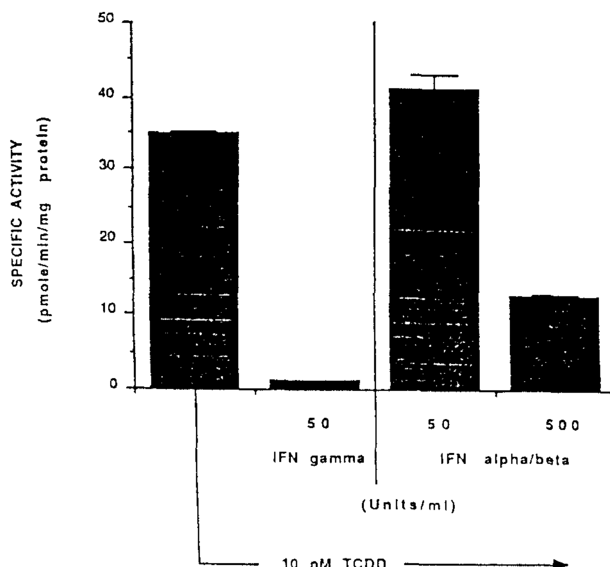

Fig. 4. EROD Specific Activity in primary hepatocytes after TCDD, recombinant mouse interferon gamma, and non-recombinant mouse alpha/beta interferon addition *in vitro*. The bars represent the mean±SE derived from 3 replicate cultures per treatment group.

G subtype was selective to antagonize TCDD in this system. Sources from which IFN-G was originated were also important for their effect. Fig. 5 showed species differences of IFN-G. Even though mouse hepatocytes were used for this studies, rat IFN-G was more potent than mouse IFN-G. When treated 50 and 500 Units/m*l*, human IFN-G did not elicited any effects on TCDD. This results showed that murine IFN-G was selective and species differences was obvious along with subtype of IFN.

Specificities of Interferons

IFN-G is a potent macrophage activation factor, and macrophage-like cells (*i.e.*, Kupffer cells) are likely present in primary hepatocyte cultures. Both IFN-G and macrophage-derived immunomodulatory products, most notably both alpha and beta interleukin 1 (IL-1) and tumor necrosis factor (TNF), have been reported to markedly depress hepatic cytochrome P-450 dependent MFO function (Szefler *et al.*, 1989; Duan *et al.*, 1988; Bertini *et al.*, 1989; Craig *et al.*, 1989). The mechanism of this depression is not clear. Moreover, most of the studies characterizing the actions of cytokines on drug metabolizing enzymes have used *in vivo* models and have evaluated effects on basal P-450 activity. In contrast, our experimental system utilizes primary hepatocytes exposed and cultured *in vitro*, and measures the effect of IFN-G on TCDD-mediated induction of P-450 activity. To confer the selectivity to the action of IFN and confirm the subtype and species differences, we introduced monoclonal antidody (MAb) to our studies. MAb which

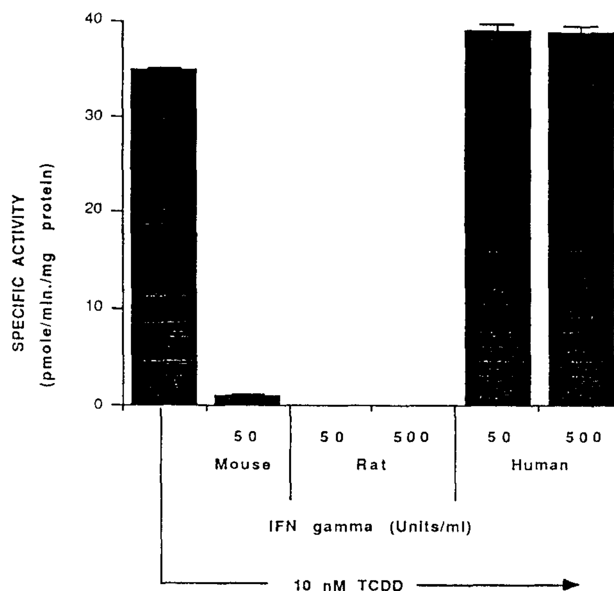

Fig. 5. EROD Specific Activity in primary hepatocytes after TCDD, recombinant mouse interferon gamma, recombinant rat interferon gamma, and non-recombinant human interferon gamma addition *in vitro*. The bars represent the mean±SE derived from 3 replicate cultures per treatment group.

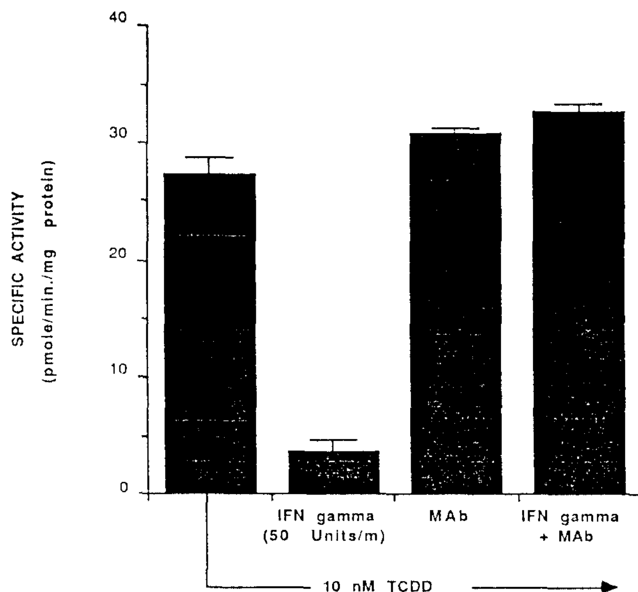

Fig. 6. Blockade of recombinant mouse interferon gamma (rMu-IFNg) antagonism of *in vitro* TCDD-induced EROD Specific Activity in primary hepatocytes after addition of rat anti-rMu-IFNg monoclonal Ab (MAb). The bars represent the mean±SE derived from 3 replicate cultures per treatment group.

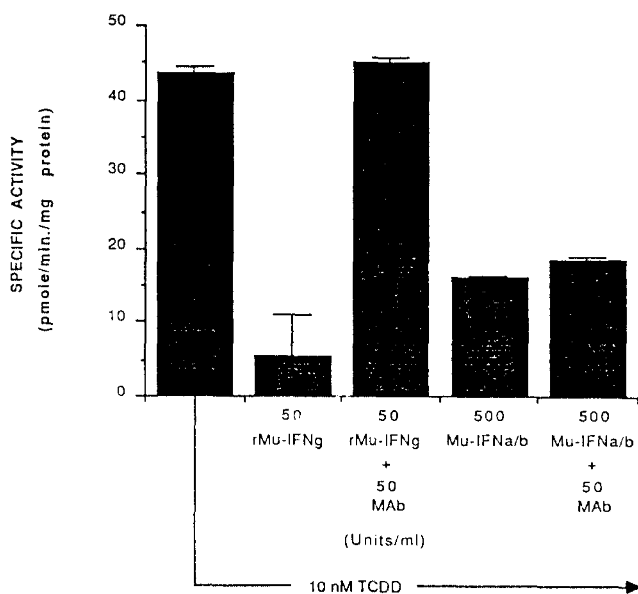

Fig. 7. Effect of rat anti-mouse recombinant gamma interferon (MAb) on EROD Specific Activity in primary hepatocytes after TCDD, recombinant mouse gamma interferon (rMu-IFNg), and mouse alpha/beta interferon (Mu-IFNa/b) addition *in vitro*. The bars represent the mean±SE derived from 3 replicate cultures per treatment group.

was used in this studies was rat anti-mouse recombinant IFN-G MAb (Lee BioMolecular). As could be expected, MAb completely blocked the effects of mouse IFN-G (Fig. 6). Any differnce could not be observed between TCDD and TCDD+IFN-G+MAb treated groups. But another subtype of IFN-A/B was not interfered by MAb (Fig. 7). By this results we could confirm that subtype difference was quite important for IFN action and result shown in Fig. 4 was well determined. In contrast with mouse, rat IFN-G was not vulnerable to MAb (Fig. 8). This results were somewhat stringent in view of above results. Because MAb was originated from rat, it could be assumed that MAb had a very narrow cross reactivity and antigenic determination site of mouse IFN-G for this MAb was quite different from that of rat IFN-G. Anyhow, the facts that mouse interferon-gamma was an ultimate effector molecule and did their action through binding to membrane receptor and signalling through cell membrane, could be confirmed. Additionally, we determined the ability of mouse IFN-G to antagonize TCDD's induction of EROD in purified liver parenchymal cells, to confirm that any antagonism due to a direct effect on the hepatocyte and not mediated by soluble factors released following the interaction of mouse IFN-G with accessory cells. Parenchymal cells were isolated by centrifugation over percoll (density=1.08 g/m*l*) (PHARMACIA) (Smedsrod and Pertoft, 1985). Fig. 9 showed the effect of mouse IFN-G in purified parenchymal cell. Mouse IFN-G antagonized TCDD-mediated induction of EROD specific

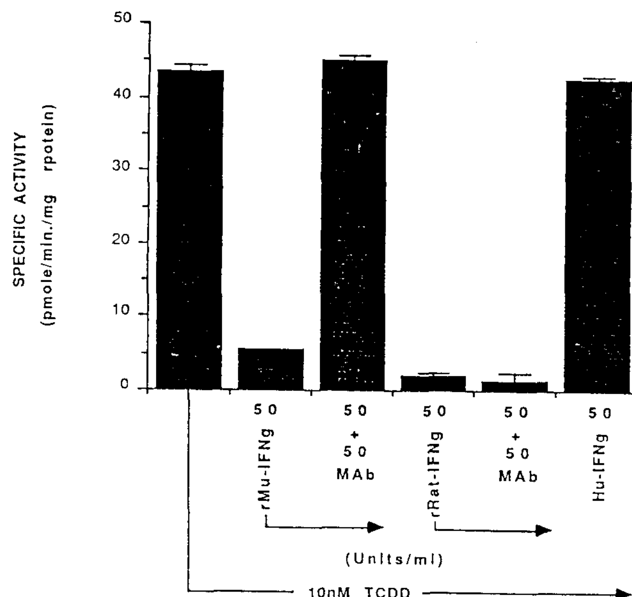

Fig. 8. Effect of rat anti-mouse recombinant gamma interferon monoclonal Ab (MAb) on EROD Specific Activity in primary hepatocytes after TCDD, recombinant mouse interferon gamma, recombinant rat interferon gamma, and non-recombinant human interferon gamma addition *in vitro*. The bars represnt the mean±SE derived from 3 replicate cultures per treatment group.

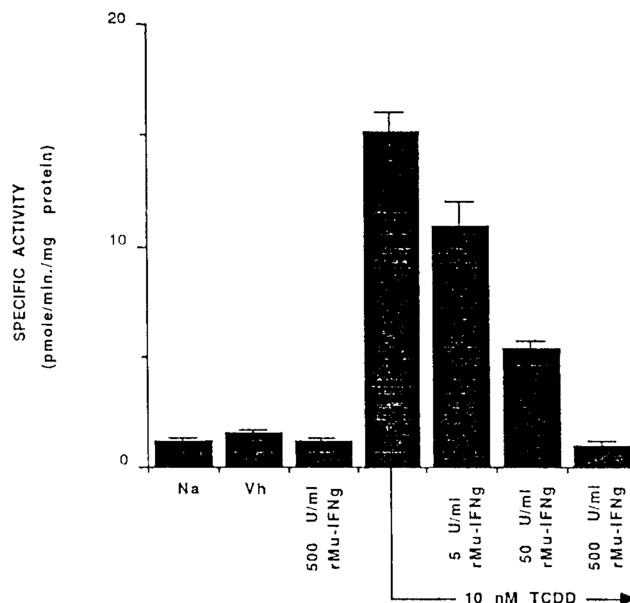

Fig. 9. EROD Specific Activity in purified mouse liver prenchymal cells after TCDD and recombinant mouse interferon gamma (rMu-IFNg), addition *in vitro*. The bars represent the mean±SE derived from 3 replicate cultures per treatment group.

activity in purified hepatic parenchymal cells (presumably hepatocytes free of Kupffer cells) from female B6C3F1 mice in a dose-related fashion.

Sodium Orthovanadate and TCDD-Inducible Cytochrome P-450

The results in Fig. 10 show the effects of orthovanadate on the EROD induction in hepatocytes by TCDD. The dose of TCDD was 10 nM and selected by previous studies. As shown in figure, TCDD potentially induced EROD activity by incubation during 72 hours after initial plating. Sodium orthovanadate showed the suppression effect on TCDD induced EROD activity by simultaneous addition with TCDD. The effect of orthovanadate was dose-dependent. At 1 μM and 10 μM concentration, orthovanadate suppressed 20% and 86% of TCDD action, respectively. When treated alone, orthovanadate showed no effects at all. The time dependent effects of orthovanadate were also examined and showed in Fig. 11. Hepatocytes were separately harvested at 24, 48 and 72 hours after cultivation, and EROD activities were examined. In untreated groups, the basal EROD activity decreased during incubation coincidely with previous studies. At 24 hours, EROD activities in TCDD or orthovanadate treated hepatocytes did not show large differences compared with untreated and vehicle-treated groups. At 48 hours, TCDD significantly induced EROD activity compared with controls. Sodium orthovanadate alone slightly suppressed EROD activity but when treated with TCDD, the enzyme level was nearly decreased to the untreated level. If the culture time was increased to 72 hours, the effects of TCDD and orthovanadate became

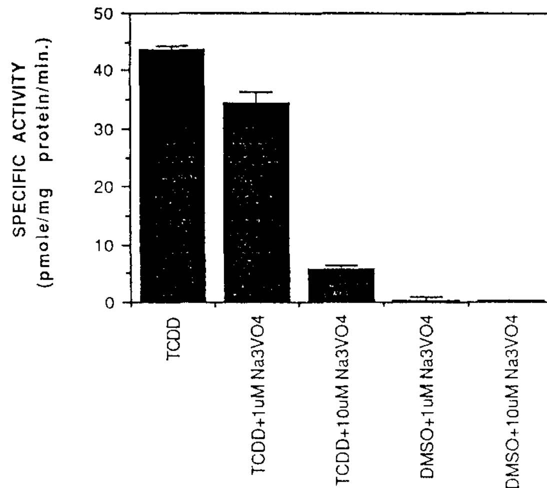

Fig. 10. Effect of sodium orthovanadate on TCDD-induced EROD activity in mouse primary hepatocyte culture. TCDD (10 nM) and sodium orthovanadate (1-10 uM) were added to culture medium from first medium change at 3 hours after initial plating. Cells were incubated for 72 hours and harvested. EROD activities were measured by the production of resorufin from ethoxyresorufin and expressed as specific activities by cellular protein amounts.

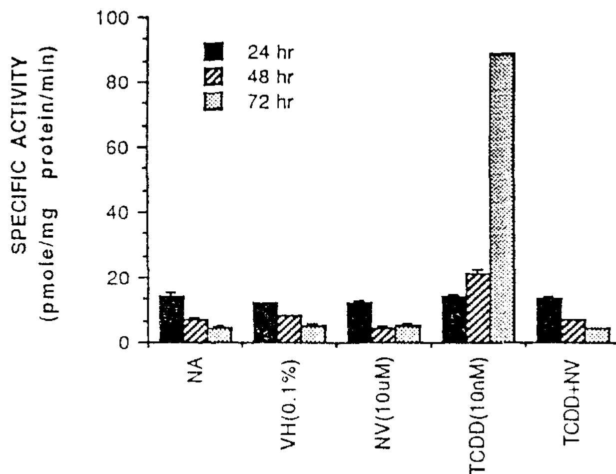

Fig. 11. Time course of action of sodium orthovanadate on TCDD-induced EROD activity in primary hepatocyte culture. TCDD (10 nM) and sodium orthovanadate (10 μM) were included from 3 hours after initial plating. Cells were separately harvested at 24, 48 and 72 hours and their EROD activities were measured. EROD activity was fluorimetrically measured by the production or resorufin from ethoxyresorufin and expressed as specific activity by cellular protein amount.

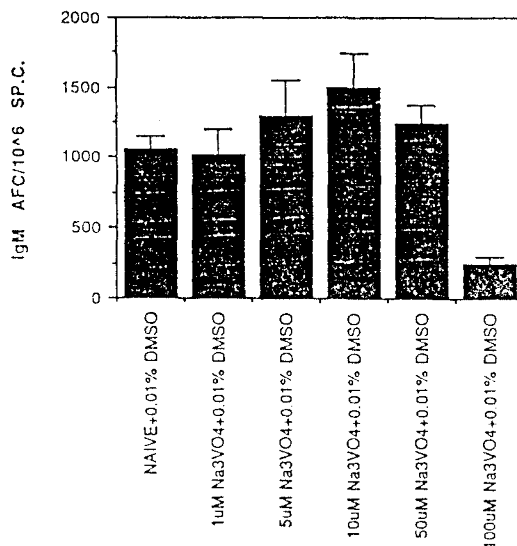

Fig. 12. Effects of sodium orthovanadate on T-dependent antibody response. Sodium orthovanadate was included in standard *in vitro* T-dependent antibody response assay. Mouse splenocytes were treated with sodium orthovanadate at concentration of 1 to 100 μM. Results were measured as IgM antibody forming cells and expressed as IgM AFC/10^6 splenocytes.

more obvious. 10 μM of sodium orthovanadate nearly perfectly neutralize the induction by 10 nM TCDD. These results indicated that the onset time of sodium orthovanadate action could be short enough to suppress TCDD action whenever TCDD showed its effect.

Sidium Orthovanadate and TCDD-induced Immunosuppression

As shown in previous results, mouse IFN-G showed an suppressive effect on TCDD induced EROD activity in mouse hepatocytes. The effect of mouse IFN-G was quite similar with sodium orthovanadate in view of dose and time-dependence characteristics. Along with in hepatocytes system, mouse IFN-G could reverse TCDD-induced immunosuppression in lymphocytes system (Snyder *et al.* 1990). By these backgrouns, we tested sodium orthovanadate in immune system. Sodium orthovanadate itself was examined in lymphocytes. Sodium orthovanadate alone was included in standard *in vitro* T-dependent antibody response assay for 5 days. The results were shown in Fig. 12. Orthovanadate elicited slight immuno-stimulation effects even though it is not significant in statistics. The reason of decrease at high doses was proved as a cell death (data not shown). This result was confirmed in IgM determination by ELISA as shown in Fig. 13. In both studies, the highest response could be observed at 10 μM of orthovanadate. The results in Fig. 14 show the effects of orthovanadate on the suppression of antibody formation by TCDD. As observed in previous results, TCDD showed inhibition of *in vitro* immunization against sheep RBC (Snyder *et al.*, 1990). When sodium

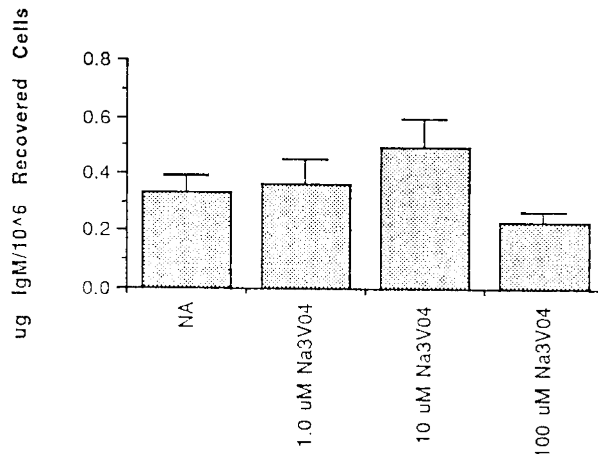

Fig. 13. Changes of IgM concentration in culture medium by sodium orthovanadate. Sodium orthovanadate (1-100 μM) was included in standard *in vitro* T-dependent antibody response assay, and culture supernatants were collected after 5 days treatment. IgM concentration was immunologically measured by ELISA technique and results were expressed as μg IgM/10^6 recovered cells.

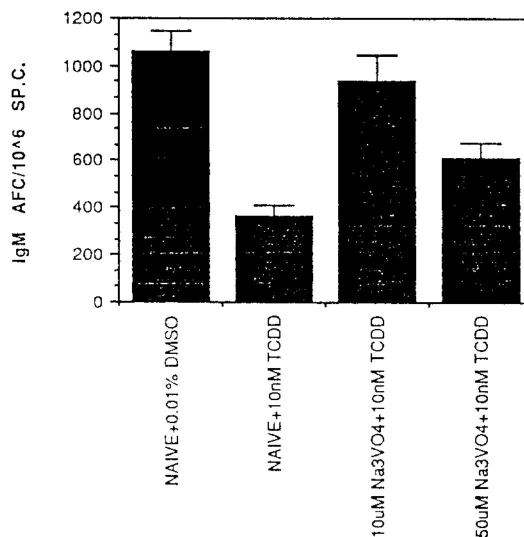

Fig. 14. Effects of sodium orthovanadate on TCDD-induced suppression of T-dependent antibody response. TCDD (10 μM) and sodium orthovanadate (10, 50 μM) were included in standard *in vitro* T-dependent antibody response assay. Results were measured by IgM antibody forming cells and expressed as IgM AFC/ 10^6 splenocytes.

orthovanadate treated commonly with TCDD, the effects of TCDD was partially reversed. At 50 μM concentration, orthovanadate decreased the cell viability and less reversal was observed. The doses of TCDD and orthovanadate at which nearly

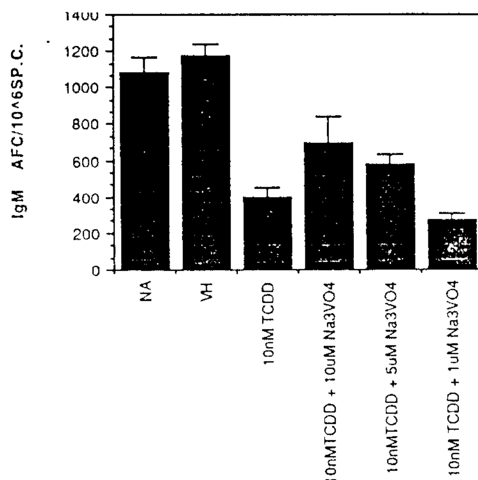

Fig. 15. Dose response of reversal of TCDD-induced immunosuppression by sodium orthovanadate. Standard *in vitro* T-dependent antibody response assay was used to measure the effect of sodium orthovanadate. TCDD (10 nM) and sodium orthovanadate (1-10 μM) were treated to splenocytes for 5 days and antibody forming cells were measured by Jerne plaque assay. Results were expressed as IgM AFC/ 10^6 splenocytes.

compensated each other were 10 nM and 10 μM respectively. These doses same in both hepatocytes and splenocytes. As lower doses, orthovanadate showed clear positive dose dependence (Fig. 15).

Effect of TCDD and Sodium Orthovanadate on *In Vitro* Malarial Growth

The effect of TCDD and sodium orthovanadate on growth of malarial parasite (*Plasmodium falciparum*) in human RBC is illustrated in Fig. 16. At 10 nM, TCDD stimulated that infectivity up to 50% compared with control. In constrast, sodium orthovanadate suppresed the growth about 30%. TCDD was known as a typical compound which depend on *Ah* receptor presence for their toxic action (Whitlock, 1987). The previous report compared the effect of TCDD on humoral immunity *in vivo* and *in vitro* using B6C3F1 splenic lymphocytes. It was described that TCDD suppressed *in vivo* humoral immunity. The direct effect of TCDD on cultured murine lymphocytes resulted in an inhibition of the differentiation of *B* lymphocytes to antibody-producing cells, which was further shown to correlate with *Ah* receptor levels by structure-activity studies and a comparison of congenic mice differing only at the *Ah* locus. The consequence of humoral immunosuppression was described in terms of altered resistance to malarial infection (Tucker *et al.*, 1986). These prior observations are different with present results at two points. First, the effect of TCDD on malarial growth did not require *Ah* receptor in target cells. Human red blood cells which were used in present study do not have nucleus. So these can not support any gene turn-on process. Second, the immunosuppression by TCDD may not be a primary reason for the stimulation of malarial

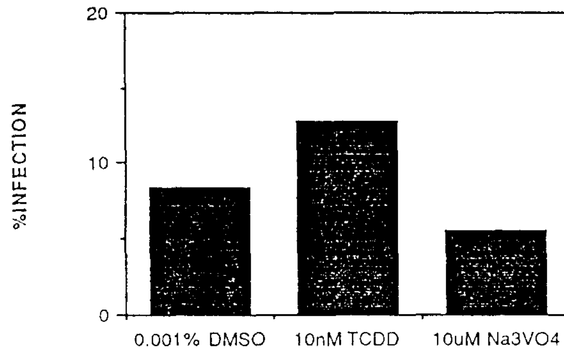

Fig. 16. Effects of TCDD and sodium orthovanadate on malarial growth in unsynchronized culture. The T9/96 strain of *Plasmodium falciparum* was maintained *in vitro* in human red blood cells. TCDD (10 nM) and sodium orthovanadate (10 μM) was included from parasitemia inoculation. After 48 hours incubation, samples were collected and parasitemia was checked by Giemsa staining.

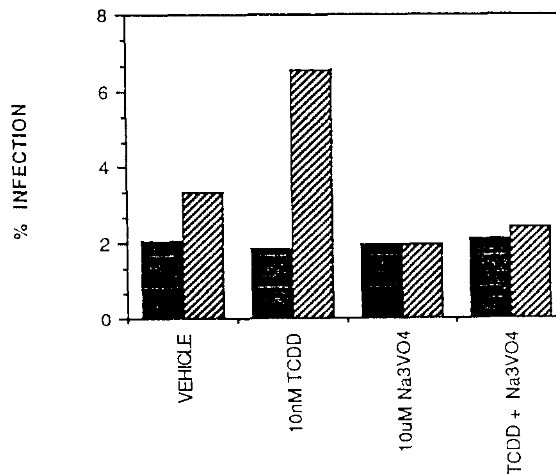

Fig. 17. Effects of TCDD and sodium orthovanadate on malarial growth in synchronized culture. Synchronized cells were obtained by the treatment of 5% D-sorbitol and Percoll density gradient centrifugation. TCDD (10 nM) and sodium orthovanadate (10 μM) was treated from inoclulation. After 24 and 48 hours, samples were collected and parasitemia was checked by Giemsa staining.

growth. Human red blood cell populations used in this study were nearly purified and had very little contamintion of immune cells. And the periods for this *in vitro* cell growth test were not enough to delіever *in vitro* immunization of malarial parasite antigens. The elevation of immune system involvement as a major mechanism of TCDD can be supported by indirect evidences. TCDD stimulate the release of tumor necrosis factor in immune cell (Kupffer cells) (Tayler *et al.*,

1990). Tumor necrosis factor has a cytotoxic effect on asexual blood-stage malaria parasites (Grau *et al.*, 1989). But in our observation, TCDD stimulated malarial growth oppositely with above observations. For the specific analysis, we synchronized parasitemia of malaria parasite. To establish synchrony, cells were treated with 5% D-sorbitol and infected cells were collected with percoll density gradient (Lambros and Vanderberg, 1979). In synchronized malarial parasites, a more clear result was obtained (Fig. 17). At 24 hours after treatment, the number of infected cell population was not changed by TCDD because of average cell phase span. But after 48 hours, TCDD showed two fold increase in infected cell number compared with control. But it can not be concluded whether malarial growth in RBC or infection to another cell was effectd by TCDD right now. In contrast, sodium orthovanadate showed a static effects on malarial growth. This result coincided with prior report (Mikkelson, 1986). In combined treatment, 10 μM of sodium orthovanadate compensated the effect of 10 nM TCDD. Even though *in vitro* malarial culture system was quite different in many aspects with primary culture of hepatocytes and splenocytes, antagonism by sodium orthovanadate responses by TCDD could be observed. These implied that orthovanadate interfered the common mechanism of TCDD in three different systems.

CONCLUSION

Recombinant mouse IFN-G antagonized TCDD-mediated induction of cytochrome P-450 dependent mixed function oxidase (measured as EROD specific activity) in primary hepatocytes isolated from female B6C3F1 mice in dose-related fashion. The observed antagonism was completely and specifically blocked by the addition of anti mouse IFN-G MAb. Recombinant mouse IFN-G-mediated antagonism was greastest when co-administered with TCDD. Although peak EROD induction by TCDD occured after 72 hours in culture, the magnitude of mouse IFN-G-mediated antagonism increased with increasing culture time up to 96 hours. Addition of IFN-G at later times during the culture period resulted in an more lessened antagonism. Mouse IFN-A/B showed an approximately 50-fold weaker activity than did mouse IFN-G. Rat IFN-G demonstrated an antagonism similar to that observed for the mouse species, while human IFN-G did not affect TCDD-induced EROD activity in our experimental system. Mouse IFN-G antagonized TCDD-mediated induction of EROD in purified parenchymal cells (presumably hepatocytes free of Kupffer cells) from B6C3F1 mice in a dose-related fashion. Sodium orthovanadate antagonized TCDD-mediated induction of EROD activity in dose-related fashion. Although the most significant result was observed after 72 hours in culture, same pattern of inhibition could be obtained at 48 hours. *In vitro* T-dependent antibody formation which was significantly suppressed by TCDD was also reversed by sodium orthovanadate in dose-dependent manner. Soidum orthovanadate did cause a little change in both hepatocyte and lymphocytes system by itself. In *in vitro* malarial culture, TCDD stimulated the growth or infectivity. More well defined result was observed in synchronized culture

state. Sodium orthovanadate suppressed malarial growth and antagonized TCDD-induced stimulation of malarial growth. The optimum dose of sodium orthovanadate was same in all three systems.

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