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IMMUNOSUPPRESSIVE EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN IN STRAINS OF MICE WITH DIFFERENT SUSCEPTIBILITY TO INDUCTION OF ARYL HYDROCARBON HYDROXYLASE ENZYME .

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Short Title : AHH Inducibility and Antibody Production

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TOXICOLOGY AND APPLIED PHARMACOLOGY

A B S T R A C T

Mouse strains with different susceptibility to AHH enzyme induction and different levels and/or affinity of a specific cytosolic binding protein ("receptor") were used to investigate the immunosuppressive effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Humoral antibody production was strongly inhibited in C57Bl/6 and C3H/HeN mice (more susceptible strains) with very low, single doses of TCDD (1.2 ug/kg), while other strains (DBA/2 and AKR) required higher doses (at least 6 ug/kg) to be partially suppressed. Longer exposure (8 weeks) did not increase the sensitivity of DBA/2 mice.
A good correlation between the degree of enzyme inducibility and immunosuppression was observed in studies with B6D2F1 mice and backcrosses. Similar results were obtained with 2,3,7,8-tetrachlorodibenzofuran(TCDF), the most powerful competitor for TCDD "receptor" in vitro and in vivo. It is concluded that TCDD immunotoxic effects are associated with the presence of the specific cytosolic binding protein which mediates AHH enzyme induction.

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most toxic compounds known, formed as a byproduct during the commercial synthesis of 2,4,5 trichlorophenol. It induces marked thymic atrophy, a slow wasting syndrome, is teratogenic and carcinogenic (Vos et al., 1980; Gupta et al., 1973) in all the species investigated. In some species, hepatotoxicity, hyperkeratosis, chloracne and an edematous syndrome have been described (Gupta et al., 1973; McConnell et al., 1978). TCDD is the most powerful inducer of the aryl-hydrocarbon hydroxylase (AHH) enzyme system, evaluated in hepatic and extrahepatic tissues (Poland and Glover, 1974; Poland et al., 1974; Lee and Suzuki, 1980).

The sensitivity to TCDD induction differs in various mouse strains, those responsive to 3-methylcholanthrene being more easily induced than those non responsive (Poland and Glover, 1974). C57Bl/6 (B6) and DBA/2 (D2) mice have been extensively studied for their susceptibility to TCDD induction; about 10 times more TCDD is needed to induce D2 mice compared with B6 (Poland and Glover, 1975). These two strains have been widely used as representative of very sensitive (for example C3H/He and Balb/c) or poorly inducible strains (as AKR and SJL) respectively (Poland and Glover, 1975). Toxicity of halogenated aromatic hydrocarbon congeners has been shown to correlate very well with their potency in increasing AHH activity (Poland et al., 1979), this induction being mediated by a cytosolic protein which binds with high affinity to TCDD and then translocates to the nucleus (Okey et al., 1979; Greenlee and Poland, 1979). This cytosolic protein species is currently considered the receptor for TCDD (Poland et al., 1976; Gasiewicz and Neal, 1982) and is often reported as putative receptor; in this paper it will be called "receptor" for brevity, well aware of the meaning.

In mice the cytosolic "receptor," and hence enzyme induction is regulated by the Ah locus. Recent work in B6 and D2 mice and their progeny indicates that some toxic effects of TCDD (thymus involution, teratogenesis and porphyria) segregate with the Ah locus (Poland and Glover, 1980; Jones and Sweeney, 1980). The hypothesis that TCDD toxicity is mediated by the presence, amount and affinity of the specific cytosolic "receptor" has thus been put forward (Okey et al., 1979).

It is well known that TCDD affects immune responses (Vos et al., 1980). However nearly all these studies have been made in B6 mice or in their F_1 hybrid with the C3H/He strain. We have previously shown that in B6 mice TCDD induced a marked, lasting depression of antibody production at doses that did not affect cellular responses (Vecchi et al., 1980; Mantovani et al., 1980).

Because of the importance of the AHH enzyme system in the biotransformation of aromatic xenobiotics and the possible production or degradation of carcinogenic metabolites (Thorgeirsson and Nebert, 1977; Nebert, 1978), it was of interest to investigate the immunodepressive effects of TCDD on antibody production in strains of mice which differ in their susceptibility to AHH enzyme induction and hence, probably, in the expression of the specific cytosolic "receptor" (Okey et al., 1979; Poland et al., 1976).

MATERIALS AND METHODS

Animals: Male mice of the following strains: C57Bl/6, DBA/2, C3H/HeN, (C57Bl/6 x DBA/2)F1 and (C57Bl/6 x C3H/HeN)F1, hereafter called respectively B6,D2,C3, B6D2F1 and B6C3F1, were obtained from Charles River, Calco, Italy. AKR male mic were a gift from Dr. D. Collavo, University of Padua, Italy. Backcrosses were bred in our Institute, mating B6 and D2 females with B6D2F1 male mice. The percentages of males and females in backcrossed animals were 52% and 48% respectively. No differences were noted in the degree of TCDD induced immunosuppressi and results from both sexes were pooled. Mice were housed 4-6 per cage in a controlled-environment room with 12 hours of light per day and 60% relative humidity at 22±0.5 °C. Altromin M.T. diet (A. Rieper, Vandoies, Italy) and municipal tap water were provided at libitum. Animals were used at 8-10 weeks of age.

Chemicals: TCDD obtained from Kor Isotopes, Cambridge, MA, was given as a single i.p. injection in a volume of 0.1 ml/10 g body weight of an acetone : corn oil (1:6 v/v) solution. For repeated dosings, once a week for 5 or 8 weeks, it was given orally. Control mice always received the vehicle alone.

TCDF was a kind gift from Dr. C. Rappe, University of Umea, Sweden. It was dissolved and given i.p. as described for TCDD.

Response to sheep red blood cells :Mice (6-10 per group) were injected i.p. with $4x10^8$ sheep red blood cells (SRBC) 7 days after TCDD treatment, and spleen hemolytic plaque-forming cells (PFC) were counted 5 days later by Jerne's technique as previously reported (Vecchi et al., 1980). For repeated TCDD, SRBC were given 7 days after the last dose.

G.V.H. assay : Graft versus host (GVH) reaction was assayed by the popliteal lymph node weight gain assay (Vecchi et al., 1980). $5x10^6$ splenocytes from B6 and D2 treated mice were injected s.c. into the right hind foot-pad of $B6D2F_1$ or C3 mice respectively. C3 mice were treated with 100 mg/kg of Cyclophosphamide i.p. 24 hours before grafting.
An equal number of formol-inactivated cells was injected controlaterally. Seven days later both popliteal lymph nodes were removed and weighed. The ratio of the weight of the right to the left lymph node (lymph nodal index, L.I.) was taken as a measure of GVH reaction. Four mice per group were tested individually, injecting the splenocytes in 4 recipient mice per donor animal.

Statistical analysis : Results are presented as mean ± S.E. . Statistical significance was evaluated by Dunnett's test. Discriminant analysis was used to evaluate with what degree of probability values of backcrossed animals belong to the two parental groups. The basis for this decision was discriminant equation and significance of generalized Mahalanobis D-Square statistics, which gave significant values in all the comparisons.

R E S U L T S

Immunosuppressive effects of TCDD in different mouse strains

The immunodepressive effects previously observed in B6 mice were obtained with well tolerated doses of TCDD (Vecchi et al., 1980), body weights of control and treated animals being similar throughout the observation peric Body weight loss is in fact a commun sign of TCDD toxicity in animals. Weights of the mice of all the strains investigated are reported in Table 1.

No effects were observed 12 days after an intraperitoneal injection of the toxic substance. In good agreement with the results of Poland and Glover (1980) thymus weights were markedly reduced in B6, C3 and $B6D2F_1$ mice, while no effects were seen in D2 and AKR mice.

In previous works (Vecchi et al., 1980; Mantovani et al., 1980) we have shown that in adult mice humoral responses are more sensitive than cell-mediated reactivities to exposure to single low doses of TCDD. Thus the immunodepressive effect of TCDD was evaluated in different mouse strains on humoral antibody production. As shown in Table 2, B6 and C3 mice were highly susceptible to TCDD exposure, the degree of immunosuppression ranging from 70% at the dose of 1.2 ug/kg to more than 95% at 30 ug/kg. In D2 and AKR strains, a reduction of PFC/spleen less than 50% was seen only after treatment with 6 ug/kg. 1.2 ug/kg TCDD in B6 mice was as active as 30 ug/kg in D2, showing that this latter strain, needs higher doses of TCDD to produce suppression, as reported for other toxic effects (enzyme inducibility, porphyria, teratogenesis and carcinogenesis). When the test was performed on day 4, levels of immunosuppression identical to those on day 5 (Table 2) were induced in B6 and D2 mice by 6 ug/kg TCDD (data not shown).

To assess whether a longer exposure time was needed in the D2 strain, to induce suppression, repeated treatments were given for 5 or 8 weeks. As shown in Fig. 1, the total spleen count of PFC or of PFC per million splenocytes was significantly reduced in B6 mice with doses of 2 ug/kg for 5 weeks or 0.5 ug/kg for 8 weeks, but no effects were observed in D2 mice. G.V.H. reactivity of D2 mice was assessed 7 days after treatment with 30 ug, in order to see whether this strain, less susceptible than B6 mice to TCDD effects on humoral antibody production, was also sensitive as regards other types of immune responses. However, no significant modifications were observed in the lymphnodal indexes (L.I.). The L.I. of control and treated mice were respectively 2.5 ± 0.3 (mean $\pm$ S.E.) and 2.1 ± 0.1 for B6, and 2.9 ± 0.2 and 2.4 ± 0.2 for D2 mice.

Effect of TCDD in $B6D2F_1$ and backcrosses

When the effects of TCDD on AHH enzymes were studied in hybrids between B6 and D2 mice, an intermediate level of induction was observed when low doses (< 1 ug/kg) of TCDD were employed. At higher doses $B6D2F_1$ were indistinguishable from B6 (Niwa et al., 1975).

In our experiments the immunosuppression observed with the lower dose (1.2 ug/kg) was mid-way between the values observed in parental strains (Fig. 2). Results in C3 and $B6C3F_1$ are also reported in Fig. 2. Offspring of two very sensitive strains were as susceptible to TCDD immunosuppression as parental strains. Higher doses of TCDD (6 or 30 ug/kg) induced degrees of immunosuppression in $B6D2F_1$ mice superimposable to those in the B6 parent (Table 2).
The depressive effects of TCDD were also tested in backcross studies, with a limited number of animals. Fig. 3 shows the results in individual animals after treatment with 6 ug/kg; offspring of B6 x $B6D2F_1$ were as suppressed as their parents, while those of D2 x $B6D2F_1$ were heterogenous in their degree of immunosuppression. The discriminant analysis used to classify the results indicated that 6/11 of the latter offspring responded as D2 mice, while 5/11 was undistinguishable from $B6D2F_1$ or B6 mice. The same analysis showed that B6 x $B6D2F_1$ offspring and their parents belonged to the same groups as regards the degree of depression.

Effect of 2,3,7,8-tetrachlorodibenzofuran (TCDF) in B6 and D2 mice

It has been reported that TCDF is one of the most powerful competitors for the TCDD "receptor" in vitro and can bind to the same "receptor" in vivo (Greenlee and Poland, 1979). We investigated the TCDF suppressive effects in B6 and D2 strains. A dose of 180 ug/kg, found in preliminary experiments to be equiactive with 6 ug/kg of TCDD in B6 mice, was used. As reported in Table 3, this dose induced a significant decrease in thymus weight and inhibited antibody production by 85% in B6 mice. When D2 mice were

used, no effect on thymus weight was observed and only a 35% reduction in humoral responses was induced.

D I S C U S S I O N

In this study we investigated whether immunosuppression induced by TCDD parallels the susceptibility to AHH induction. B6 and C3 mice, very susceptible to AHH enzyme inducibility,were highly suppressed by 1.2 ug/kg TCDD which was totally inactive in D2 or AKR mice (less inducible). This difference reflects only a different degree of susceptibility, D2 mice not being totally unresponsive; in fact higher doses (6 and 30 ug/kg) caused significant immunodepression. In good agreement with Poland and Glover (1980), thymic involution paralleled the strains' sensitivity to AHH inductio The possibility that D2 mice, used as a prototype of less sensitive strains, need longer exposure to became susceptible to TCDD effects was explored in experiments in which TCDD was given repeatedly. However, no modifications were observed after 5 or 8 weeks of treatment, suggesting that the dose level, more than the time of exposure, is critical for D2 mice. Very recently Neal et al. (1982) reported LD50 for B6 and D2 as respectively 132 and 620 ug/kg and half-life of 17 and 37 days, indicating an intrinsically higher resistance of D2 mice to TCDD toxic effects, not simply a different susceptibility possibly due to faster elimination and relatively lower toxic concentrations in target organs. The same authors reported for $B6D2F_1$ mice a half-life of 17 days (the same as for B6) with an LD50 of 300 ug/kg. Thus crossing with D2 mice increased the resistance to TCDD toxicity but did not affect the half-life. As regards AHH inducibility , $B6D2F_1$ possesses an intermediate susceptibility that is seen more easily after low doses of TCDD (< 1 ug/kg) and when the inducing effect is expressed as a fractional response (Poland and Glower, 197

Our results showing that immunosuppression in F_1 and backcrosses parallels their expected susceptibility to AHH induction support the hypothesis that the presence of the TCDD "receptor" regulated by the Ah locus is relevant for the expression of TCDD immunosuppressive effect on antibody production.

The amount and/or affinity of the TCDD "receptor" have been studied in the liver of B6,D2 and B6D2F1 strains (Poland et al., 1976; Gasiewicz and Neal, 1982) and no data are available for the other strains here used, C3 and AKR. Moreover, there are no evidences, to our knowledge, of the presence of "receptor" in lymphoid organs in animals, with the exception of the thymus, where the highest concentration of "receptor" and a low inducibility of AHH enzymes have been reported. (Carlstedt-Duke, 1979; Poland and Glover, 1980). The immune response here considered has been evaluated in an organ, the spleen, not studied for the "receptor", thus it is impossible to directly correlate the level of immunosuppression and the amount and/or affinity of the binding protein.

It has been noted that the simple association of high AHH inducibility and TCDD toxic effects (for example porphyria, teratogenicity) does not imply that inducibility itself is a sufficient cause of these abnormalities (Jones and Sweeney, 1980). Similarly there are no indications that AHH enzyme induction is *per se* responsible for antibody production inhibition. Whether the strong induction of hepatic, and to a lesser degree, extrahepatic enzymes produces metabolic alterations which interfere to some extent with antibody production cannot at the moment be excluded. Rather, the presence of TCDD cytosolic "receptor" seems to play a relevant role in the immunological effects. In fact, Poland and Glover (1980) reported that liver and thymus cytosol from B6 mice specifically binds more TCDD *in vitro* than that from D2 mice, but the *in vivo* uptake of tritiated TCDD in the thymus at 24 hours is even higher in D2 than in B6 mice, while in the liver it is always lower.

These *in vivo* data on the TCDD level in the thymus are at variance with the marked atrophy observed in B6 but not in D2 mice, while the data on thymus "receptor" could better explain the effects observed *in vivo*.

Similar results have been reported for labelled TCDF; equal or even higher uptake was found in the spleen and thymus of D2 than B6 mice which have a faster elimination of TCDF (Decad et al., 1981). Thus the decrease in thymus weight and immunosuppression cannot simply be explained in terms of half-life and target tissue concentration for both compounds. In vitro (Poland et al., 1976) and in vivo (Greenlee and Poland, 1979) TCDF has been shown to be the most powerful competitor for the TCDD "receptor". Our results with TCDF in B6 and D2 mice, similar to those obtained with TCDD in the same strains, give further support to the hypothesis that the cytosolic "receptor" plays a role in TCDD toxicity.

D2 mice have been reported to have about 60-70% more adipose tissue than B6 mice and more TCDD could be stored in D2 mice fat, decreasing the amount available for a toxic effect. In fact, evidence have been reported that more TCDF accumulated in the adipose tissue of D2 mice compared with B6 (Decad et al., 1981). The authors suggested that the longer-life of TCDF in D2 mice was in part due to the storage in the fat and to the subsequent release. Whether the decreased susceptibility to TCDD toxic effects of D2 mice, including immunosuppression, could be, at least in part, due to the difference in the available amount of toxic, can not be excluded. If TCDD was in fact stored in the adipose tissue of D2 mice in greater amount, it would be possible that the longer release eventually causes a delayed increase in the TCDD levels with a shift of the toxic effect. Hepatic uptake and storage of (^{14}C)TCDD in B6 and D2 mice have been reported by Poland and coworkers (1976). Up to fourteen days after treatment the level of radioactivity found in the liver was always higher in B6 and D2 mice, reasonably excluding that the longer release of TCDD, if it really happens, could cause a late increase in the levels, at least in the liver. Moreover, as already mentioned at the beginning of the discussion, acute LD50 in B6D2F1 mice is higher than in B6, while the half-life of elimination value is identical and the amount of the adipose tissue is the same. (Neal et al., 1982).

The toxicity of TCDD can not thus be easily explained by the limited pharmacokinetics data.

The relevance of the cytosolic "receptor" for some toxic effects induced by TCDD (as for example the immunosuppression reported in this work) will be better understood when more pharmacokinetic data become available together with more information on the tissue distribution of the "receptor".

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Table 1 - Effect of TCDD on body and thymus weights

MOUSE STRAIN	TCDD (ug/kg)	BODY WEIGHT g	THYMUS WEIGHT mg	(% of control)
B6	0	22.7 ± 0.5	37.6 ± 2.6	(100)
	1.2	23.4 ± 1.2	33.6 ± 2.6	(89)
	6.0	24.0 ± 0.4	21.2 ± 2.5*	(56)
	30.0	21.8 ± 0.5	13.5 ± 1.6**	(36)
C3	0	21.8 ± 0.7	36.8 ± 2.5	(100)
	1.2	23.6 ± 0.5	29.3 ± 2.9	(80)
	6.0	21.8 ± 0.5	22.3 ± 2.1*	(60)
	30.0	19.1 ± 1.0	14.0 ± 2.2**	(38)
D 2	0	21.1 ± 0.5	27.7 ± 2.2	(100)
	1.2	22.3 ± 0.3	22.4 ± 2.9	(81)
	6.0	22.7 ± 0.8	23.4 ± 3.5	(84)
	30.0	21.0 ± 0.3	20.3 ± 4.5	(73)
AKR	0	27.2 ± 0.6	54.7 ± 3.5	(100)
	6.0	27.7 ± 1.2	40.0 ± 9.3	(73)
$B6D2F_1$	0	22.3 ± 0.6	38.0 ± 1.7	(100)
	1.2	23.3 ± 0.5	35.1 ± 2.8	(92)
	6.0	20.1 ± 0.9	29.0 ± 2.1*	(76)
	30.0	23.3 ± 0.5	21.7 ± 1.5*	(56)

TCDD was given i.p. 12 days before evaluation.
Results are means ± S.E.
* $p < 0.05$ by Dunnett's test
** $p < 0.01$ " "

Table 2 - Effect of TCDD on primary humoral response

MOUSE STRAIN	TCDD (ug/kg)	PFC/10^6 SPLENOCYTES	PFC/ SPLEEN	% OF CONTROLS
B6	0	242 (220 - 267)	36015 (32833 - 39506)	100
	1.2	54 (43 - 68)**	9642 (7848 - 11847)**	27
	6.0	38 (27 - 53)**	4900 (3561 - 6743)**	14
	30.0	7 (4 - 13)**	1080 (962 - 1189)**	3
C3	0	783 (692 - 886)	108239 (105177-113390)	100
	1.2	212 (174 - 258)**	37378 (30028- 46402)**	35
	6.0	35 (23 - 52)**	5383 (3562- 8137)**	5
D2	0	378 (328 - 436)	63127 (53141- 74989)	100
	1.2	309 (269 - 355)	54198 (46709- 62887)	86
	6.0	271 (225 - 326)	33806 (28541- 40043)*	54
	30.0	207 (160 - 251)	22473 (16413 - 25250)**	35
AKR	0	356 (323 - 391)	51185 (50176- 52213)	100
	6.0	197 (153 - 254)	31682 (26040- 38546)	62
$B6D2F_1$	0	504 (465 - 545)	71609 (63199- 81139)	100
	1.2	264 (236 - 296)*	36715 (34133- 39493)*	51
	6.0	93 (75 - 101)**	11458 (10032- 13450)**	16
	30.0	8 (5 - 15)**	896 (489- 1641)**	1

TCDD was given i.p. on day -7 , $4x10^8$ SRBC were injected i.p. on day 0 and test was performed 5 days later.
Logarithmic transformation of the data was used before statistical analysis. Results are antilogarithms of the means (± S.E.).

* $p < 0.05$ by Dunnett's test
** $p < 0.01$ " " "

Table 3 Effect of TCDF on primary humoral response.

MOUSE STRAIN	TCDF (ug/kg)	BODY WEIGHT g	THYMUS WEIGHT mg	PFC/10^6 SPLENOCYTES (°)		PFC/SPLEEN (°)	
B6	0	21.4 ± 0.9	31.2 ± 3.0	370	(301-454)	42647	(35074-51856)
	180	22.1 ± 0.9	18.6 ± 4.6*	61	(48- 77)**	7255	(5657- 9305)**
D2	0	25.8 ± 0.5	37.6 ± 4.6	120	(113-128)	25391	(23944-26926)
	180	26.3 ± 0.5	32.7 ± 2.4	98	(90-106)	15965	(14819-17199)*

TCDF was given i.p. On day -7, $4x10^8$ SRBC were injected i.p. on day 0 and test was performed 5 days later.

(°) Results are means ± S.E. as described in Table 2.

*$p < 0.05$
**$p < 0.01$

LEGENDS TO FIGURES

Fig. 1 - Effect of repeated oral TCDD on primary humoral response in B6 and D2 mice. Results are the means ± S.E.
0.5 ug/kg or 2 ug/kg were given once a week for 8 or 5 weeks respectively (0.5x8 - 2x5).

* $p < 0.05$

** $p < 0.01$

Fig. 2 - Effect of single TCDD doses (1.2 ug/kg) on primary humoral response in more sensitive (C3 , B6) and less sensitive (D2) mouse strains and their F_1 progeny $B6C3F_1$ and $B6D2F_1$. Results are means ± S.E.

* $p < 0.05$

** $p < 0.01$

Fig. 3 - Effect of single TCDD doses (6 ug/kg) in B6, D2 and $B6D2F_1$ strains and in back crosses. Responses of individual mice in each group . Horizontal bars are the means and the numbers in parentheses are means ± S.E.

FIG. 1

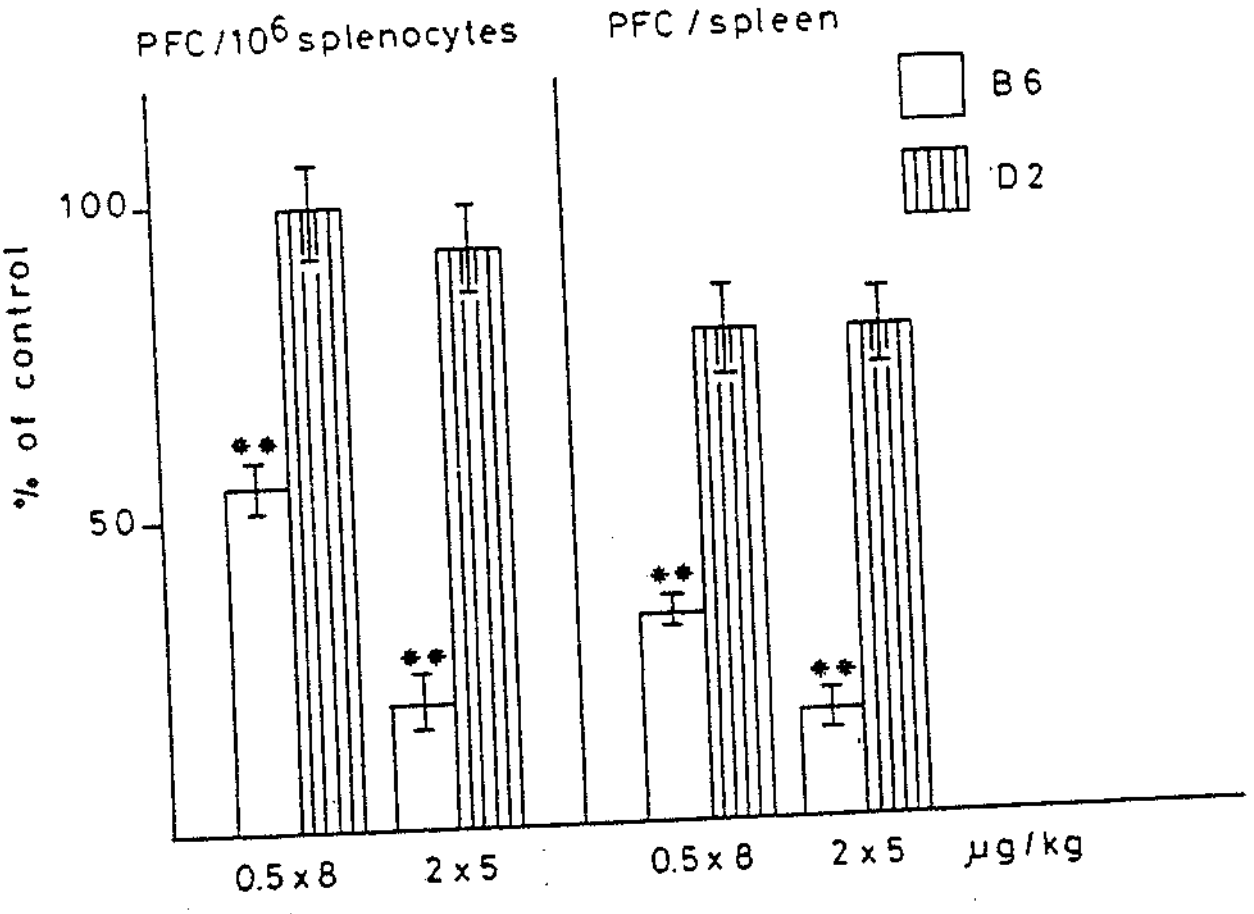

PFC/10⁶ splenocytes
PFC / spleen
B6
D2
100
50
% of control
**
0.5 x 8
2 x 5
0.5 x 8
2 x 5
µg/kg

FIG. 2

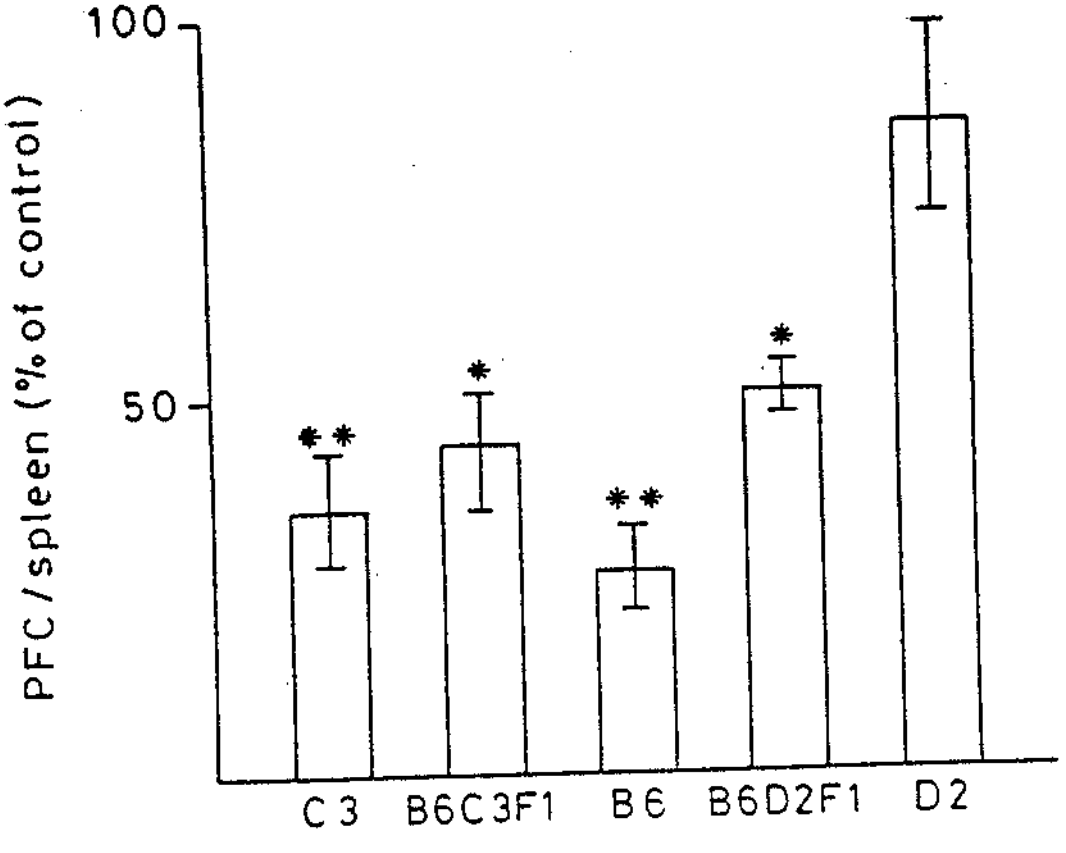
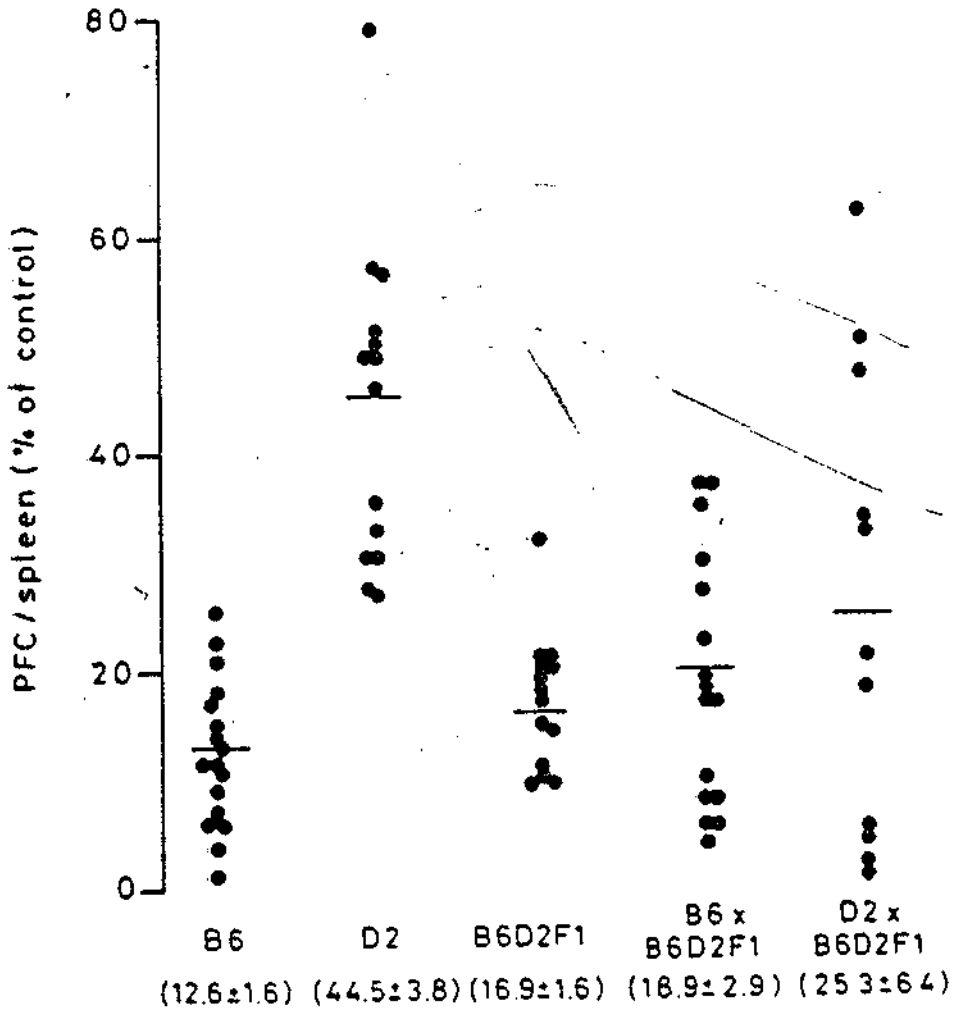

100
50
PFC / spleen (% of control)
**
*
**
*
C3
B6C3F1
B6
B6D2F1
D2

FIG. 3


80
60
40
20
0
PFC / spleen (% of control)
B6
D2
B6D2F1
B6 x B6D2F1
D2 x B6D2F1
(12.6±1.6)
(44.5±3.8)
(16.9±1.6)
(18.9±2.9)
(25.3±6.4)

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