Development of an ELISA That Detects Halogenated Aromatic Hydrocarbon Induced Immunosuppression.

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ABSTRACT
An enzyme linked immunosorbant assay (ELISA) was developed to detect immunosuppression caused by halogenated aromatic hydrocarbons in mice that were immunized with either a T-dependent antigen (trinitrophenyl haptenated sheep red blood cells, HAP-SRBCs) or a T-independent antigen (trinitrophenyl lipopolysaccharide, TNP-LPS). B6C3F1 female mice treated with 2,3,7,8-TCDD, benzo(a)pyrene (BaP), or a complex mixture of polynuclear aromatic hydrocarbons (PAHs) showed a dose-dependent decrease in anti-TNP IgM titer detected by ELISA which correlated with the established plaque-forming cell (PFC) assay. This novel ELISA is an efficient and inexpensive assay that may be used instead of the PFC assay to detect immunosuppression in acute and long term exposed animals.

INTRODUCTION
Immunosuppression is one of the most sensitive indicators of exposure to halogenated aromatic hydrocarbons. The commonly used PFC assay detects *in vivo* immunosuppression by determining the number of B-cells that are producing antibody to a particular antigen. B-cells may be stimulated to produce IgM antibodies in response to T-dependent antigens (requires T-helper cell) or T-independent antigens (does not require T-cell help). This paper describes the methods for an ELISA that detects B-cell production of IgM antibodies in response to trinitrophenyl haptenated sheep red blood cells (HAP-SRBCs, T-dependent antigen), or trinitrophenyl lipopolysaccharide (TNP-LPS, T-independent antigen) after treatment with 2,3,7,8-TCDD, benzo(a)pyrene, and a complex mixture of 2-, 3-, and 4-ring PAHs.

MATERIALS AND METHODS
Chemicals Sheep red blood cells (SRBCs) in Alsevars' solution were obtained from M.A. Bioproducts, Maryland, U.S.A. Guinea pig

complement, RPMI 1640 media, and Earls Balanced Salt Solution (EBSS) were purchased from GIBCO Laboratories, New York, U.S.A. Trinitrophenyl lipopolysaccharide (TNP-LPS, Sigma #T4020), picryl sulfonic acid and glycyl-glycine, goat anti-mouse IgM conjugated to alkaline phosphatase (IgM-AP, #A7784), and *p*-nitrophenyl phosphate (NPP, #104) were obtained from Sigma Chemical Co. All other chemicals used were of the highest grade commercially available.

Animals B6C3F1 female mice (6-8 weeks) received from an in house breeding colony were maintained on a 12 hour light/dark schedule with free access to food and water. All congeners were dissolved in corn oil and administered by i.p. injection in a total volume of 10 µL/g body weight.

The PFC Assay On day one the mice (5 per group) were treated with 2,3,7,8-TCDD, BaP, or the PAH mixture; on day 4 the mice were immunized with 50 µg of TNP-LPS or 4 x 10^8 HAP-SRBCs in a total volume of 200 µL phosphate buffered saline (pH 7.4) by i.p. injection; on day 8 the mice were bled from the tail vein and terminated by cervical dislocation. The "Cunningham" modification of the Jerne plaque-forming cell (PFC) assay was used.[1,2] HAP-SRBCs were previously prepared following the method of Rittenberg and Pratt[3] using EBSS instead of the modified barbital buffer. The number of viable spleen cells was determined by trypan blue staining.

ELISA Developement and Optimization

Blood Collection The blood was collected and allowed to coagulate overnight at 4°C before brief centrifugation to pack the red blood cells.

Sera Handling and Storage Sera was removed from the packed red blood cells and diluted 1:10 in phosphate buffered saline (PBS pH 7.4) containing 1 % bovine serum albumin (BSA) and 0.1% sodium azide. All samples were stored at 4°C until use.

ELISA Development A checker-board ELISA was performed to determine the optimal antigen concentration per well and sera dilution that would give the best and most consistent results. The optimized ELISA conditions are described as follows. Five µg of TNP-LPS in 100 uL PBS were added to each well of a Falcon (#3911) 96 well plate and incubated overnight at 4°C wrapped in aluminum foil to protect from light. The plate was then washed with PBS containing 0.05% TWEEN 20 (PBS/TWEEN) and the plate was blocked with PBS containing 1% bovine serum albumin (PBS/BSA) for 1 hr at room temperature. After washing the plate with PBS/TWEEN, 100 µL of diluted serum from each animal was run in duplicate and the plates were incubated overnight at 4°C. Serum from control mice containing anti-TNP IgM from was diluted 1:400. The plate was washed with PBS/TWEEN and 100 µL of a 1:300 dilution of IgM-AP in PBS/BSA was added to each well. The plate was incubated with the secondary antibody for 2-3 hours at room temperature. In order to generate IgM units, a standard curve of IgM-AP in serial dilutions ranging from 1:300 to 1:64000 were run on each plate in duplicate. Just prior to visualization with NPP, 50 µL of each standard dilution of IgM-AP was added to each well. The ELISA was visualized by incubating 100 µL of NPP in 1M tris buffer containing 0.3 mM $MgCl_2$ (pH 9.8) at 37°C for 30 to 60 minutes. The reaction was stopped with 1 N NaOH. The plates

were read on a MR600 Dynatech Microplate Reader at 405 nm. The anti-TNP-LPS IgM titer for each mouse was quantitated after conversion to relative IgM units using the standard curve of IgM-AP vs. optical density.

RESULTS

Ideally, serum serial dilution curves should not extend beyond a 2 to 3 log range. Serial dilutions of control serum containing Anti-TNP IgM cover a 2 log range and are linear within the optical density cutoff points of 0.2 to 2.0 (Figure 1). In addition, a standard reference curve of anti-IgM-AP was run on each plate to generate IgM units. All of the standard curves had correlation coefficients > 0.99 (Figure 2).

Figure 1
Dilutions of Serum Containing Anti-TNP IgM

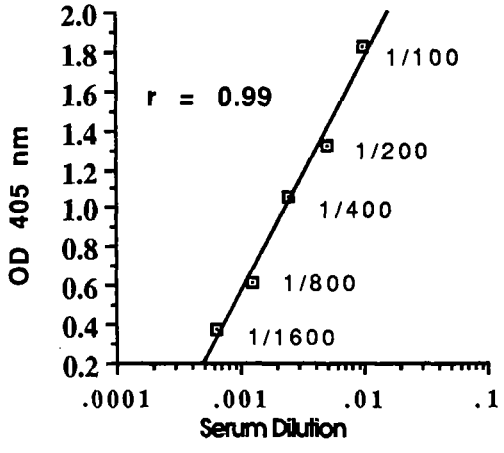
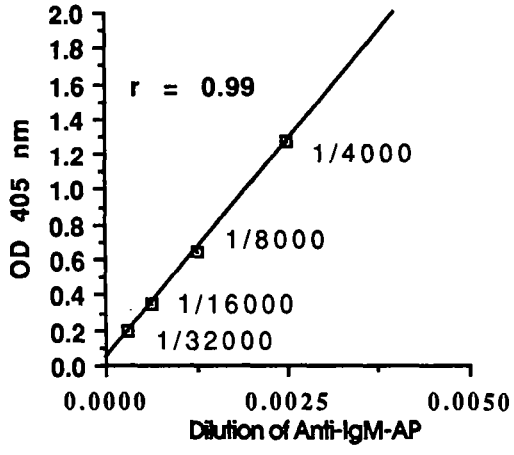


Figure 2
Standard Curve Used to Generate IgM Units



After treatment with 2,3,7,8-TCDD, BaP, or the PAH mixture, and immunization with HAP-SRBCs, the ELISA detects a dose-dependent decrease in anti-TNP IgM titer which correlates with a dose-dependent decrease in the number of B-cells producing antibody detected by the PFC assay. Likewise, for the B6C3F1 mice immunized with TNP-LPS, there was a dose-dependent decrease in anti-TNP IgM which also correlated with the PFC assay (Table 1). After probit coversion, the effective dose required to decrease 50 % (ED-50) of the anti-TNP IgM titer or 50 % of the number of plaque-forming B-cells was determined. The anti-TNP IgM ED-50 values are similar to the PFC assay ED-50 values generated (Table 1). These results show that the ELISA can detect immunosuppression caused by PAHs as well as the PFC assay.

DISCUSSION

The PFC assay is commonly used to detect immunosuppression in chemically exposed animals. The shortcomings of this assay are that 1) the animals must be sacrificed to get the results, 2) the assay is time consuming and requires experienced recognition of plaques, and 3) the amount of data generated in one day is limited to approximately 50 animals. This ELISA

was developed so that 1) immune function could be monitored *in vivo* in animals exposed to chemicals in acute and long term studies; 2) the assay is relatively quick and simple, and 3) this assay can be automated so that many plates can be read in one day; each plate represents duplicate samples from 40 mice. In addition, the ELISA can monitor immune function in the same animal over time since blood collection is a relatively non-invasive procedure. This novel ELISA can potentially replace the plaque-forming cell assay in detecting immunosuppression caused by individual and complex mixtures of polyhalogenated and polynuclear aromatic compounds

Table 1

Comparison of the ELISA and the PFC Assays in B6C3F1 Female Mice Treated with 2,3,7,8-TCDD, BaP, or PAH Mixture

		HAP–SRBCS		TNP–LPS		
Treatment	Dose (mg/kg)	Anti-TNP IgM % Control	PFC/1E6 Cells % Control	Anti-TNP IgM % Control	PFC/1E6 Cells % Control	Correlation Coefficient (r) IgM v. PFC
2,3,7,8-TCDD	0	100	100	100	100	HAP-SRBC
	0.0006	--	--	84	98	r = 0.98
	0.0012	--	--	77	70	
	0.0036	79	84	66	59	TNP-LPS
	0.0072	47	41	53	48	r=0.94
	0.0144	51	34	--	--	
(ED-50)		(9.7 µg/kg)	(8.2 µg/kg)	(6.3 µg/kg)	(4.7 µg/kg)	
BaP	0	100	100	100	100	HAP-SRBC
	10	75	65	89	94	r = 0.99
	50	40	28	79	86	
	100	41	--	71	57	TNP-LPS
	200	36	25	67	63	r = 0.90
(ED-50)		(61 mg/kg)	(44 mg/kg)	(203mg/kg)	(250 mg/kg)	
Complex	0	100	100	100	100	HAP-SRBC
PAH	19.5	--	81	79	95	r = 0.94
Mixture	39	57	62	59	89	
	78	34	47	55	53	TNP-LPS
	312	41	29	44	59	r= 0.82
(ED-50)		(57 mg/kg)	(52 mg/kg)	(114mg/kg)	(177 mg/kg)	

REFERENCES

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