

Chapter XVI-2

IMMUNOLOGY

1. Introduction

Recent experimental data in animals have suggested that TCDD has deleterious effects on the immune system (Dean et al, 1984). As a result, the Science Panel Committee recommended that the immunotoxic potential of TCDD be evaluated during the physical examination portion of this study. Parameters selected for assessment included: (1) the enumeration of T-lymphocytes, T-lymphocyte subsets and B-lymphocytes using monoclonal surface marker analysis and (2) functional ability of lymphocyte to respond to selected antigen or mitogen stimuli in the lymphocyte transformation assay.

Five hundred ninety-two participants were randomly selected for this examination using the terminal digit of the participant's case number. This selection occurred during the time period March 1982 through September 1982. Of the 592 participants, 297 were Ranch Handers and 295 were comparisons. Of the 295 comparisons, 180 were original comparisons. The statistical testing presented in this chapter is all based on this basic set of 297 Ranch Handers and 180 original comparisons. However for each test performed, differing data deletions occurred. Specifically, data from professed homosexuals were removed from all analyses. Also, data were removed from all analyses if covariate information (age, smoking, alcohol use) was missing. Finally, data were removed from certain analyses (T_{11} , T_3 , T_4 , T_8 , T_4/T_8 , B_1 counts and percentages) if: (1) differential counts were unavailable, (2) if samples exhibited greater than 30% background fluorescence, or (3) if samples had a T_3 or T_{11} proportion of less than 10%.

Surface marker analysis and lymphocyte function studies were performed on purified mononuclear cells obtained from heparinized whole blood drawn at Kelsey-Seybold Clinic early on the second day of the examination period. Peripheral blood mononuclear leukocytes (PBL) were separated from erythrocytes and polymorphonuclear leukocytes using a density gradient centrifugation technique. Unfortunately, blood specimens were collected and processed in glass tubes with resultant variable loss of adherent PBL. White cell differential counts were not obtained on purified PBL so that the number of lymphocytes actually placed into functional assays could not be ascertained. Due to these laboratory difficulties, coupled with relatively small sample sizes, exposure index analyses are not provided in this chapter.

2. Analysis of Immunological Cell Count Data

Mouse monoclonal antibodies directed against various lymphocyte surface antigens were incubated with PBL. Following washing, fluorescent anti-mouse antibodies were added. After the cells had been stored for a variable period in paraformaldehyde, the presence or absence of fluorescent antibody on each PBL was determined and counted using a cytofluorograph. The percentage of cells positive for each surface marker is reported as the number of fluorescent

cells divided by the total number of lymphocytes in a given specimen. Since differential counts were not obtained on the purified PBL, a 250 cell differential count was performed at the recommendation of the Peer Review Committee on paraformaldehyde-fixed cells. These cells had been stored for 6 to 12 months. Although cell morphology was not optimal, determination of the percentage of lymphocytes in each specimen was possible. The number of surface marker positive cells per mm^3 was calculated by multiplying the percent marker positive cells by the total lymphocyte count.

The cells counted and analyzed for this report are classified as having T_{11} , T_3 , T_4 , T_8 , or B_1 cell surface markers. The T_{11} surface marker identifies thymus dependent lymphocytes which form rosettes with sheep erythrocytes (also called E^+ cells). The T_3 surface marker is found on nearly 100% of circulating T-lymphocytes cells (Reinherz and Schlossman, 1980). Cells with T_4 cell surface markers proliferate in response to soluble antigens and have an inducer or helper function in T-T, T-B and T-macrophage interactions (Reinherz and Schlossman, 1980). T_8 cells have cytotoxic and suppressor functions (Reinherz and Schlossman, 1980). B_1 cells, or bursa equivalent cells, are producers of immunoglobulins (David, 1979).

The number of T_{11} , T_3 , T_4 , T_8 , and B_1 positive cells per mm^3 are provided below by group, along with the T_4/T_8 ratio and total lymphocyte count. Additionally, percentages of T_{11} , T_3 , T_4 , T_8 , and B_1 positive cells are reported by group. The data were analyzed for statistically significant group differences using the Kolmogorov-Smirnov Two Sample Test. Also, crude group (Ranch Hand versus comparison) means were contrasted, and then the groups were contrasted while adjusting for age, smoking history in pack-years and alcohol intake measured as drink-years. The literature does not yet provide clear guidance to the selection of covariates for analysis as attempted here. Age, smoking and alcohol were chosen based on the observation that these variables frequently correlate with general measures of health and impact upon hematologic parameters. Group interactions with age, smoking or alcohol indicate group differences associated with these covariables. When group-covariate interaction is observed, group and associated covariate main effects are not reported, rather the interaction is detailed. The probability level used to indicate an interaction of interest is $P = 0.100$. In the absence of interaction, group and covariate main effects are reported in the usual manner. When $P > 0.100$ for all interactions, P values for the reduced model, consisting of main effects only, are provided.

Table XVI-2-1 provides the results of Kolmogorov-Smirnov testing of the number of surface marker positive cells per mm^3 . A borderline statistical difference is seen in the B_1 count with Ranch Handers having lower values. However, B_1 cells are an adherent set of cells. The purification process resulted in a variable loss of adherent cells, therefore, this data must be interpreted with extreme caution. Table XVI-2-2 provides the Kolmogorov-Smirnov testing of cell percentages and no statistically significant differences are observed. Table XVI-2-3 provides unadjusted means for the number of surface marker positive cells per mm^3 . No statistically significant group mean

differences are observed. Table XVI-2-4 provides unadjusted means for the cell percentages, and again no statistically significant group mean differences are observed. Both counts and percentages are provided to aid with interpretation.

Table XVI-2-1

KOLMOGOROV-SMIRNOV TESTING OF NUMBER OF SURFACE MARKER POSITIVE CELLS (THOUSANDS/mm³)

Variable	Group	N	Percentiles			P Value
			10%	50%	90%	
T ₁₁	COMP	144	0.77	1.23	2.02	0.74
	RH	235	0.70	1.25	1.96	
T ₃	COMP	144	0.73	1.28	2.13	0.39
	RH	233	0.70	1.27	1.96	
T ₄	COMP	147	0.48	0.78	1.42	0.81
	RH	231	0.398	0.794	1.251	
T ₈	COMP	147	0.277	0.604	1.168	0.34
	RH	235	0.296	0.569	0.985	
T ₄ /T ₈	COMP	147	0.64	1.38	2.62	0.78
	RH	231	0.64	1.41	2.70	
B ₁	COMP	147	0.022	0.071	0.247	0.097
	RH	235	0.023	0.071	0.188	
TLC	COMP	177	1.35	1.91	2.74	0.63
	RH	290	1.34	1.92	2.54	

COMP = comparison group
 RH = Ranch Hand group

Table XVI-2-2

KOLMOGOROV-SMIRNOV TESTING OF PERCENTAGE OF SURFACE MARKER POSITIVE CELLS
(THOUSANDS/mm³)

<u>Variable</u>	<u>Group</u>	<u>N</u>	<u>Percentiles</u>			<u>P Value</u>
			<u>10%</u>	<u>50%</u>	<u>90%</u>	
T ₁₁	COMP	144	42.0	66.0	87.5	0.90
	RH	235	41.6	68.0	88.4	
T ₃	COMP	144	48.5	66.5	83.5	0.79
	RH	233	48.4	66.0	83.6	
T ₄	COMP	147	26.8	42.0	58.0	0.45
	RH	231	23.0	44.0	61.0	
T ₈	COMP	147	17.8	31.0	47.0	0.82
	RH	235	16.6	29.0	49.0	
B ₁	COMP	147	1.0	3.0	13.2	0.48
	RH	235	1.0	4.0	10.4	

COMP = comparison group
RH = Ranch Hand group

Table XVI-2-3

UNADJUSTED MEANS FOR NUMBER OF SURFACE MARKER POSITIVE
CELLS (THOUSANDS/mm) AND P VALUES FOR TESTS BETWEEN GROUPS MEANS

<u>Variable</u>	<u>Group</u>	<u>N</u>	<u>Unadjusted Means</u>	<u>SEM</u>	<u>P Values</u>
T ₁₁	COMP	139	1.33	0.050	0.47
	RH	228	1.29	0.034	
T ₃	COMP	139	1.36	0.052	0.21
	RH	226	1.29	0.031	
T ₄	COMP	142	0.877	0.038	0.49
	RH	224	0.846	0.027	
T ₈	COMP	142	0.660	0.029	0.11
	RH	228	0.606	0.020	
T ₄ /T ₈	COMP	142	1.54	0.075	0.34
	RH	224	1.65	0.075	
B ₁	COMP	142	0.117	0.011	0.26
	RH	228	0.102	0.008	
TLC	COMP	171	2.00	0.046	0.14
	RH	280	1.92	0.028	

COMP = comparison group
 RH = Ranch Hand group
 TLC = total lymphocyte count
 SEM = standard error of the means

Table XVI-2-4

UNADJUSTED MEANS FOR PERCENTAGE OF SURFACE MARKER POSITIVE CELLS
AND P VALUES FOR TESTS BETWEEN GROUPS MEANS

<u>Variable</u>	<u>Group</u>	<u>N</u>	<u>Unadjusted Means</u>	<u>SEM</u>	<u>P Values</u>
T ₁₁	COMP	139	65.0	1.44	0.71
	RH	228	65.7	1.20	
T ₃	COMP	139	65.6	1.22	0.75
	RH	226	65.1	0.97	
T ₄	COMP	142	42.1	1.13	0.53
	RH	224	43.1	1.07	
T ₈	COMP	142	32.0	1.02	0.36
	RH	228	30.8	0.80	
B ₁	COMP	142	5.80	0.50	0.48
	RH	228	5.35	0.41	

COMP = comparison group

RH = Ranch Hand group

SEM = standard error of the means

Table XVI-2-5 provides the adjusted surface marker positive cell count means, along with P values for main (group, age, smoking and alcohol) and interaction (group by age, group by smoking, and group by alcohol) effects. No main or interaction effect associated with group is noted to be statistically significant.

The number of lymphocytes and T₈ positive cells per mm³ decreased with increasing age in both the Ranch Hand and comparison groups. The effect was -0.0043 thousand cells per mm³ per year of life for T₈ and was -0.0110 thousand cells per mm³ per year of life for the lymphocyte count. Smoking was observed to be associated with increased cell counts on all variables except for B₁ positive cells. Specifically, the slope was 0.0036 thousand cells per mm³ per pack-year for T₁₁; 0.0076 thousand cells per mm³ per pack-year for T₃; 0.0070 thousand cells per mm³ per pack-year for T₄; 0.0022 thousand cells per mm³ per pack-year for T₈; and 0.0083 thousand cells per mm³ per pack-year for total lymphocyte count.

Table XVI-2-5

ADJUSTED MEANS, PLUS MAIN AND INTERACTION P VALUES FOR
THE NUMBER OF MARKER POSITIVE CELLS (THOUSANDS/mm³)

Variable	Group (Gp)	N	Adj'd Mean	P Value for Adj'd Means	P Values for					
					Age Effect	Smkng Effect	Alco Effect	Gp x Age Effect	Gp x Smkng Effect	Gp x Alco Effect
T ₁₁	COMP	139	1.33	0.52	-	0.029	-	-	-	-
	RH	228	1.29							
T ₃	COMP	139	1.35	0.38	-	<0.001	-	-	-	-
	RH	226	1.30							
T ₄	COMP	142	0.864	0.82	-	<0.001	-	-	-	-
	RH	224	0.854							
T ₈	COMP	142	0.660	0.12	0.057	0.025	-	-	-	-
	RH	228	0.606							
T ₄ /T ₈	COMP	142	1.52	0.22	-	-	-	-	-	-
	RH	224	1.66							
B ₁	COMP	142	0.117	0.27	-	-	-	-	-	-
	RH	228	0.102							
TLC	COMP	171	1.99	0.20	<0.001	<0.001	-	-	-	-
	RH	280	1.92							

COMP = comparison group

RH = Ranch Hand group

- = P > 0.050 for main effects or P > 0.100 for interactions. When P > 0.100 for all interactions, P values for the reduced model, consisting of main effects only, are provided.

TLC = total lymphocyte count

Table XVI-2-6 shows adjusted means for percentage of surface marker positive cells. No statistically significant overall group differences are observed. The T₃ and T₄ percentages are influenced by smoking, but this effect is essentially the same in both study groups. The effect of smoking on the T₃ percentage is 0.124 percentage points per pack-year, while the effect of smoking on the T₄ percentage is 0.171 percentage points per year. A weak indication of a group specific alcohol intake effect was noted on the T₁₁ percentage. The association of alcohol use with the percentage of T₁₁ positive cells was 0.0980% per drink-year in the comparison group and -0.0042% per drink-year in

the Ranch Hand group. This pattern could reflect a diminished Ranch Hand immunological response to drinking in reference to the comparisons; the biological relevance of this borderline finding is uncertain at this time.

Table XVI-2-6

ADJUSTED MEANS AND OTHER MAIN AND INTERACTION EFFECTS FOR
PERCENTAGE OF SURFACE MARKER POSITIVE CELLS

Variable	Group (Gp)	N	P Value for			Age Effect	Smkng Effect	Alco Effect	Gp x Age Effect	Gp x Smkng Effect	Gp x Alco Effect
			Adj'd Mean	Adj'd Means							
T ₁₁	COMP	139	*	*	-	-	-	-	-	-	0.087
	RH	226									
T ₃	COMP	139	65.2	0.92	-	0.005	-	-	-	-	-
	RH	226	65.4								
T ₄	COMP	142	41.6	0.27	-	<0.001	-	-	-	-	-
	RH	224	43.4								
T ₈	COMP	142	32.0	0.34	-	-	-	-	-	-	-
	RH	228	30.7								
B ₁	COMP	142	5.79	0.52	-	-	-	-	-	-	-
	RH	228	5.36								

COMP = comparison group

RH = Ranch Hand group

* = that a group interaction effect was noted rendering overall group mean differences and the associated main effect not meaningful.

- = P > 0.050 or P > 0.100 per footnote in Table XVI-2-3.

In summary, the lymphocyte surface marker analyses reported in Tables XVI-2-5 and XVI-2-6 show no detectable differences between the Ranch Hand and comparison groups on these measures, except possibly for the borderline group difference in the T₁₁ percentage by alcohol use association.

3. T and B Cell Functional Studies

T and B lymphocyte function was determined by measuring the ability of these cells to transform in response to antigen or mitogen stimuli. Briefly, this assay is performed by culturing PBL in the presence of mitogens (plant lecthins which stimulate the cells to divide) or antigen. After a certain length of incubation time, the rate of DNA synthesis is estimated by adding tritiated thymidine (a radioactive DNA precursor). Thus, the counts per minute

of thymidine incorporated into the cell culture is a measure of the ability of those lymphocyte to proliferate in response to the added stimulus. Mitogens stimulate lymphocytes non-specifically. Phytohemagglutin (PHA) and concanavallin A (conA) stimulate T-lymphocytes to divide, while pokeweed mitogen (PW) stimulates B-lymphocytes through a T-lymphocyte. On the other hand, antigen require that lymphocytes recognize specifically antigen as a substance to which the host has been exposed. Tetanus toxoid (TT) is a T-lymphocyte dependent B-lymphocyte recall antigen.

Kolmogorov-Smirnov testing of the 4 stimulation and 2 control measurements are shown in Table XVI-2-7. No statistically significant group differences are noted. Unadjusted group mean net counts per minute for the stimulation studies and control measurements are shown in Table XVI-2-8. No statistically significant group differences are noted except in Control #1 where the Ranch Hand group was found to have a lower unstimulated proliferation rate. A comparable differential is also noted in Control #2, but is not statistically significant. The group differences noted are of unknown biological significance.

Table XVI-2-7

KOLMOGOROV-SMIRNOV TESTING OF T AND B CELL FUNCTIONAL STUDIES

Variable	Group	N	Percentiles			P Value
			10%	50%	90%	
Control #1	COMP	168	138	448	1483	0.20
	RH	279	140	374	1320	
After conA	COMP	168	13596	58394	99104	0.38
	RH	279	17741	54190	91724	
After PHA	COMP	168	30143	84339	135684	0.51
	RH	279	33027	79342	130064	
Control #2	COMP	168	142	404	1079	0.85
	RH	274	132	388	917	
After PW	COMP	168	12232	27916	53662	0.64
	RH	274	12700	29623	58288	
After TT	COMP	168	1001	3719	16058	0.81
	RH	274	866	3726	13979	

COMP = comparison group
RH = Ranch Hand group

Table XVI-2-8

UNADJUSTED MEANS FOR T AND B CELL FUNCTIONAL STUDIES BY GROUP, AND P
VALUES FOR TESTS BETWEEN GROUP MEANS

<u>Variable</u>	<u>Group</u>	<u>N</u>	<u>Unadjusted Means (nCPM)</u>	<u>SEM</u>	<u>P Value for Unadj'd Means</u>
Control #1	COMP	163	652	49.2	0.031
	RH	269	535	29.4	
After conA	COMP	163	57454	2248	0.31
	RH	269	54637	1658	
After PHA	COMP	163	83808	3048	0.37
	RH	269	80433	2244	
Control #2	COMP	163	523	37.1	0.31
	RH	264	480	23.9	
After PW	COMP	163	32092	1337	0.37
	RH	264	33710	1151	
After TT	COMP	163	6848	650	0.86
	RH	264	7051	787	

COMP = comparison group

RH = Ranch Hand group

nCPM = net counts per minute (stimulated CPM - control CPM).

SEM = standard error of the mean

Table XVI-2-9 shows adjusted net CPM means. A statistically significant group difference is noted in Control #1. Other group effects are noted as interactions with smoking and alcohol. Specifically, smoking was associated with a decreased proliferation rate to concanavallin A stimulation, (-113 nCPM per pack-year) in the comparison group, while smoking was associated with an increased proliferation rate in the Ranch Hand cohort (+169 CPM per pack-year). Two comparable group differences were observed as interactions of concanavallin A and phytohemagglutinin stimulation with alcohol use. Alcohol use was associated with an increased proliferation after concanavallin A stimulation in the comparison group (+212 CPM per drink-year), while an increase of 12 CPM per drink-year was found in the Ranch Hand cohort. Alcohol use in the comparison group increased proliferation after phytohemagglutinin by 167 CPM per drink-year, while alcohol use in the Ranch Hand group decreased proliferation by 76 CPM per drink-year. This alcohol effect has no known biologic explanation. The finding is of questionable significance and will need to be examined further in subsequent immunologic analyses.

In addition to these group specific effects, some effects not associated with group were observed. Age and smoking were covariates which were found to be highly statistically significant. Lymphoproliferative responses to phytohemagglutinin and concanavallin A decreased monotonically in both Ranch Hand and comparison groups with advancing age. Lymphocyte response to pokeweed mitogen increased with increasing pack-years in both Ranch Hand and comparison groups.

Table XVI-2-9

ADJUSTED MEANS, PLUS MAIN AND INTERACTION P VALUES FOR T AND B CELL FUNCTIONAL STUDIES BY GROUP

Variable	Group (Gp)	N	P Value for			Age Effect	Smkng Effect	Alco Effect	Gp x Age Effect	Gp x Smkng Effect	Gp x Alco Effect
			Adj'd Mean	Adj'd Means							
Control #1	COMP	163	657	0.023	-	-	-	-	-	-	
	RH	269	532								
After conA	COMP	163	*	*	<0.001	*	*	-	0.089	0.025	
	RH	269	*	*							
After PHA	COMP	163	*	*	<0.001	-	*	-	-	0.041	
	RH	269	*	*							
Control #2	COMP	163	518	0.41	-	-	-	-	-	-	
	RH	264	484								
After PW	COMP	163	31982	0.32	-	0.01	-	-	-	-	
	RH	264	33778								
After TT	COMP	163	6929	0.95	-	-	-	-	-	-	
	RH	264	7001								

COMP = comparison group

RH = Ranch Hand group

- = P > 0.050 or P > 0.100 per footnote in Table XVI-2-3.

4. Summary

The analysis of these data has provided a valuable insight into the rapidly changing area of clinical immunology. Analysis has revealed no statistically significant differences in mean T₁₁, T₃, T₄, T₈, T₄/T₈ ratio or B₁₁ counts between the Ranch Hand and comparison groups. Similarly, there were no statistically significant overall mean differences in PHA, conA, PW, or TT stimulation responses between the groups. There were significant differences in

unstimulated (control) thymidine incorporation ($P = 0.023$) with less activity in the Ranch Hand group. In both groups, lymphoproliferative responsiveness to PHA and conA decreased significantly with increasing age, and total lymphocyte counts were correlated with age and smoking. The subsets of T-lymphocytes (T_3 , T_4 , T_8 , and T_{11}) also were correlated with smoking.

From the clinical vantage point, the immunological findings do not present a picture indicative of immunological alteration in the herbicide-exposed group. However these data are of such quality that concern must be taken for a possibility of both false positive and false negative statements. Due to previously defined difficulties in surface marker analyses and lymphocyte stimulation assays, these data cannot be reliably referenced to other published data. Nonetheless, no gross adverse immunological effects were noted between the herbicide-exposed group and the comparison group.