Impaired In-Vitro Lymphocyte Responses in Patients with Elevated Pentachlorophenol (PCP) Blood Levels

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ABSTRACT. Immune parameters were examined in 188 patients who were exposed for more than 6 mo to pentachlorophenol-containing pesticides. Blood levels of pentachlorophenol, lymphocyte subpopulations, in-vitro responses to mitogenic and allogeneic stimulation, plasma neopterin levels, and plasma cytokine and cytokine receptor levels were determined. Impaired in-vitro lymphocyte stimulation responses were impaired in 65% of the patients. The likelihood of impaired lymphocyte stimulation increased significantly with levels of pentachlorophenol that exceeded 10 μl/l ($p < .05$). Patients who had high blood levels of pentachlorophenol and abnormal lymphocyte stimulation also had increased proportions of blood monocytes in blood ($p < .05$), as well as increased IL-8 serum levels ($p < .02$). Eleven patients who had abnormal mitogen stimulation experienced decreased CD4/CD8 ratios of < 1.0; 5 of these patients had decreased $CD4^+$ lymphocyte counts of < 500/μl, and 3 patients had increased plasma neopterin of > 15 nmol/l. These results indicate that increased levels of pentachlorophenol in blood can lead to severe T lymphocyte dysfunction.

THE FUNGICIDAL SUBSTANCE pentachlorophenol (PCP) was used commonly for wood protection in Germany until 1989. In 1989, use of PCP-containing pesticides was proscribed by law. PCP is a lipophilic substance. Residues of PCP were found in human testes, kidney, prostate gland, liver, and adipose tissue.[1] PCP is a potent inhibitor of the Ca^{++}-transport-ATPase, as measured by inhibition of calmodulin-sensitive enzyme activity.[2] PCP is suspected to induce (a) neurotoxicity; (b) DNA damage that may lead to cancer, including leukemia[3-10] and aplastic anemia[11]; and (c) immune dysfunctions, such as decreased antibody production and decreased T lymphocyte responses and IL-2 production.[12-17] T and B lymphocyte defects were observed in animals exposed to PCP, as well as in in-vitro experiments in which lymphocytes of healthy human subjects were incubated with PCP.[15,18-20] In the present study, we examined lymphocyte stimulation responses, phenotypic lymphocyte subsets, plasma neopterin, and plasma cytokines in patients who were exposed to PCP.

Materials and Method

Patients. A total of 188 patients who were exposed for more than 6 mo to PCP-containing pesticides were studied. The mean age of the patients was 42.7 ± 13.5 y (range = 6-81 y), and 57% of the patients were male and 43% were female. PCP exposure was verified by measurement of PCP blood levels. Patients with chronic diseases, such as persistent hepatitis B virus infection,

renal failure, diabetes mellitus, or rheumatic diseases, were excluded from the study. The most frequent clinical symptoms observed in the patients were as follows: lack of mental concentration (45%), bronchitis (34%), frequent common cold diseases (29%), general fatigue (27%), rapid exhaustion (27%), sleeplessness (24%), headache (23%), nausea (20%), and irritation of mucous membranes of the throat and nose (18%). These clinical symptoms accorded with other reports of PCP-exposed patients.[21,22]

Lymphocyte subpopulations were determined in 157 patients, in-vitro mitogenic and allogeneic stimulation responses in 163 patients, plasma neopterin levels in 118 patients, and plasma cytokine and cytokine receptor levels in 100 patients.

Determination of lymphocyte subpopulations. Lymphocyte subpopulations were analyzed in whole blood by flow cytometry. Heparinized blood (100 µl) was incubated with 10 µl of anti-CD3 (OKT3, pan T [Ortho; Raritan, NJ]); anti-CD4 (OKT4, inducer/helper [Ortho]); anti-CD8 (OKT8, suppressor/cytotoxic [Ortho]); OKIa1 (B lymphocytes, monocytes, activated T lymphocytes [Ortho]); anti-CD11b (OKM1, monocytes [Ortho]); anti-CD16 (NK cells, Ortho); or anti-CD25 (OKT26a, anti-IL-2 receptor [Ortho]) monoclonal antibody for 30 min at 4 °C. Erythrocytes were lysed with NH_4Cl for 15 min. The remaining cells were washed and incubated with 50 µl goat-anti-mouse-Ig FITC-conjugated antibody (dianova; Hamburg, Germany), diluted 1:40, for an additional 30 min at 4 °C. The cells were washed and resuspended in phosphate-buffered saline (PBS), and lymphocyte counts/µl and percentages of fluorescence-labeled subpopulations were determined with an Ortho Cytoron flow cytometer. CD4/8 ratios of < 1.0 and $CD4^+$ cell counts of < 500/µl were considered abnormally low, based on measurements in healthy individuals.[23,24]

In-vitro stimulation of lymphocytes. In-vitro stimulation of lymphocytes was performed as described elsewhere.[25] Briefly, patient mononuclear cells (MNCs) were isolated from heparinized whole blood by density gradient centrifugation, and 105 cells (100 µl) were added to each well of a microtiter tray (Nunc; Roskilde, Denmark). Pokeweed mitogen (PWM), concanavalin A (ConA), phytohemagglutinin (PHA), anti-CD3 monoclonal antibody (OKT3), or pooled allogeneic stimulator cells from five healthy volunteers were used as stimulating agents. Each mitogen was tested in three different concentrations in triplicate. A total of 100 µl of each mitogen dilution was added to the cells, followed by incubation for 3 d at 37 °C. Mixed-lymphocyte cultures were incubated for 5 d. The cells were pulsed with ^3H-thymidine for 8 h and harvested with a cell harvester (Inotech, - Dunn [Asbach, Germany]). ^3H-thymidine incorporation was measured with an automatic-filter counting system (Inotech) and was recorded as counts per minute (cpm). MNCs from healthy volunteers were tested in parallel, and ^3H-thymidine incorporation of patient lymphocytes was compared with ^3H-thymidine incorporation of healthy controls. Relative responses were calculated as cpm of patient lymphocytes cultured with mitogen or stimulator cells minus cpm of patient lymphocytes in medium, divided by cpm of control lymphocytes cultured with mitogen or stimulator cells minus cpm of control lymphocytes in medium. Because each mitogen was tested at three concentrations, we calculated three relative responses for each mitogen. The highest of the three relative responses was used for statistical analysis. Relative response values that were less than 0.66 were considered impaired. The in-vitro response of an individual was considered to be impaired if the test result with at least one of the five stimulating agents was abnormal.

Determination of plasma neopterin levels. Plasma neopterin was measured with the Neopterin-RIAcid assay (Hennig; Berlin, West Germany). A measurement that exceeded 15 nmol/l was considered abnormally high, based on measurements in 70 healthy individuals.[26]

Determination of plasma cytokine and soluble cytokine receptor levels. Serum interleukin-1-alpha (IL-1α), interleukin-2 (IL-2), soluble interleukin-2-receptor (sIL-2R), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon-gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were determined in enzyme-linked immunosorbent assays (ELISA).[27,28] IL-1α, IL-2, IL-3, IL-4, IL-6, and IL-8 were measured with Quantikine kits (Biermann [Bad Nauheim, Germany]); sIL-2R was measured with Immunotech kits (Dianova [Hamburg, Germany]); and GM-CSF was measured with MRL kits (Biermann).

Determination of PCP levels in blood. Blood levels of PCP were determined in the laboratory of Dr. Bauer (Saarbrücken, Germany), using gas chromatography (Hewlett Packard-GC 5890).

Statistical analysis. The Wilcoxon rank sum test, Fisher's exact test, and linear regression were used for statistical analysis.

Results

In-vitro responses to four different mitogens and allogeneic stimulator cells were tested in 163 patients. A total of 106 (65%) of the patients exhibited impaired lymphocyte stimulation (relative response < 0.66, with at least one stimulating agent) (Table 1). The likelihood of impaired lymphocyte stimulation was significantly increased when blood levels of PCP blood exceeded 10 µg/l. Of the 30 patients whose blood levels of PCP were ≤ 10 µg/l, 15 had impaired and 15 had normal lymphocyte stimulation responses. In contrast, of 133 patients with blood levels of PCP ≥10 µg/l, as many as 91 (68%) showed impaired stimulation (Fisher's exact test: $p < .05$ [Table 1]). The rate of abnormal stimulation results was 71% in patients with blood levels of PCP > 20 µg/l (Table 1).

In 8 patients with detectable PCP levels, we examined whether impaired lymphocyte function persisted over time. The time interval between the earliest and latest tests ranged from 9 to 36 mo. In 1 patient (CV), lymphocyte stimulation returned to normal after 17 mo; 1 patient (BH) had 2 normal and 2 impaired stimulation

responses within a 26-mo period; and the remaining 6 patients had consistently impaired test results (Table 2).

PCP-exposed patients with impaired mitogen stimulation had an increased proportion of peripheral blood monocytes (Wilcoxon rank sum test: 14.7 ± 7.0% versus 12.5 ± 6.1% [$p < .05$]) and increased IL-8 serum levels (Wilcoxon rank sum test: 48.0 ± 72.3 pg/ml versus 15.1 ± 44.5 pg/ml [$p < .02$]), compared with patients with normal lymphocyte responses, thus indicating additional immune defects in these patients (Table 3). Increased IL-8 serum levels were associated with increased levels of blood PCP (linear regression test: $p < .05$). Importantly, 11 patients with impaired mitogen stimulation had abnormally low CD4/CD8 ratios of < 1.0. In 5 of these patients, $CD4^+$ T lymphocytes were decreased to < 500/µl, and 3 patients had increased plasma neopterin of > 15 nmol/l. All 11 patients had PCP levels that exceeded 10 µg/l (Table 4).

Table 1.—Mitogen Stimulation and Levels of PCP in Blood*

Mitogen stimulation	No. patients with PCP blood levels of 0–10 µg/l	11–20 µg/l	> 20 µg/l
Impaired (n = 106)	15	41	50
Normal (n = 57)	15	22	20

*Fisher's exact test: PCP ≤ 10 µg/l versus > 10 µg/l; $p < .05$.

Discussion

Immune defects in animals and humans exposed to PCP and abnormal in-vitro responses in cell cultures of PCP-treated lymphocytes of healthy human individuals have been reported.[15,18-20,29-33] PCP-exposed rats had decreased antibody titers to bovine serum albumin, decreased delayed-type hypersensitivity (DTH) responses, and increased numbers of peritoneal macrophages that displayed hyperphagocytic activity.[20] Contaminants of technical-grade PCP (PCP-T; 86% PCP), such as chlorinated dioxin and furans, appeared to potentiate the immunosuppressive effect of PCP. Mice treated with a single oral dose of chlorinated dioxin/furan and challenged with sheep erythrocytes as antigenic stimulus exhibited a dose-related suppression of the anti-sheep-erythrocyte antibody response; in contrast, mice that received analytical-grade PCP (PCP-A; > 99% PCP) showed normal responses.[18] After an 8-wk PCP-T dietary exposure, mice showed a reduced response in the mixed-lymphocyte culture.[19] It has been demonstrated in recent studies that PCP-A, as well as PCP-T suppressed T and B lymphocyte functions in-vitro.[15]

The current study provides evidence that exposure of humans to PCP can induce moderate to severe immune dysregulation. Some patients had stimulation defects accompanied by abnormalities of other immune parameters, such as decreased CD4/CD8 ratios, decreased $CD4^+$ cell numbers in the blood, and elevated serum neopterin levels.

McConnachie and Zahalsky described elevated $CD26^+$ T cells, elevated $CD10^+$ B cells, increased or

Table 2.—Immune Defects and PCP Blood Levels in Patients (Pat.) Tested Repeatedly

Pat.	Date tested (mo/y)	Stimulation defect*	PCP (µg/l)	CD4/8	CD4/µl	Lym/µl	Neo
AT	08/90	++	67	3.1	858	1 588	6
	05/91	+	21	2.4	1 654	3 243	9
BB	05/91	++	16	2.1	1 129	2 689	5
	03/92	++	28	1.4	456	1 452	4
BE	04/91	+	13	2.0	1 042	2 542	9
	03/92	+	21	2.2	796	1 810	8
BH	08/90	0	11	0.9	1 211	3 106	5
	12/90	++++	15	1.5	1 161	2 831	6
	03/92	0	22	1.3	845	2 346	4
	10/92	+	10	1.4	1 112	3 006	7
CV	09/90	+++	15	1.0	452	1 130	ND
	03/91	+	10	1.7	720	2 322	10
	02/92	0	ND	1.3	604	1 776	5
GH	04/90	+	21	2.9	2 092	4 752	5
	04/93	++	5	2.0	502	836	7
HG	10/90	++	29	1.3	245	661	6
	03/92	+++	16	1.4	308	881	7
	08/92	+	ND	0.8	598	1 811	ND
LE	06/92	++	4	2.2	553	1 084	12
	03/93	++	ND	3.0	528	851	6

Notes: Neo = neopterin; normal ≤ 15nmol/l; CD4/8: normal > 1.0; CD4/µl: normal > 500; and Lymphocytes/µl (Lym/µl): normal > 1 000.
*Stimulation defect: + = impaired response to one of the four mitogens or alloantigen; 0 = normal.

Table 3.—Immune Parameters (% ± *SD*) of Patients with Impaired or Normal Mitogen Stimulation

Immune parameter	Mitogen stimulation		*p**
	Impaired	Normal	
CD3 (%)	65.2 ± 9.6	66.1 ± 8.1	NS
CD4 (%)	41.7 ± 8.3	43.4 ± 7.2	NS
CD8 (%)	25.8 ± 7.3	24.8 ± 6.9	NS
OKIal (%)	14.4 ± 11.5	12.0 ± 4.6	NS
CD11b (%)	14.7 ± 7.0	12.5 ± 6.1	< .05
CD16 (%)	7.8± 5.8	6.7 ± 5.2	NS
CD4/CD8 ratio	1.7 ± 0.7	2.0 ± 0.9	NS
Total lymphocytes/µl	1 948.3 ± 867.2	1 881.7 ± 665.6	NS
CD3/µl	1 266.5 ± 547.8	1 222.9 ± 423.8	NS
CD4/µl	804.4 ± 380.3	790.5 ± 256.3	NS
CD8/µl	498.9 ± 257.4	463.1 ± 217.0	NS
OKIal/µl	276.0 ± 242.6	219.9 ± 101.2	NS
CD11b/µl	276.7 ± 200.1	230.4 ± 138.6	NS
CD16/µl	148.4 ± 131.8	123.2 ± 113.0	NS
Neopterin (nmol/l)	9.6 ± 9.9	8.5 ± 3.8	NS
sIL-2R (pg/ml)	2 471.5 ± 2 383.8	2 504.3 ± 1 865.4	NS
IL-1α (pg/ml)	3.9 ± 17.1	4.5 ± 14.7	NS
IL-2 (pg/ml)	2.2 ± 10.5	2.6 ± 7.0	NS
IL-3 (pg/ml)	2.2 ± 4.4	10.7 ± 27.1	NS
IL-4 (pg/ml)	0	0	NS
IL-6 (pg/ml)	12.1 ± 23.9	9.0 ± 17.8	NS
IL-8 (pg/ml)	48.0 ± 72.3	15.1 ± 44.5	< .02
GM-CSF (pg/ml)	28.7 ± 60.0	17.6 ± 46.5	NS
IFN-γ (pg/ml)	704.8 ± 2 018.0	230.4 ± 492.6	NS

Note: NS = not significant.
*Wilcoxon rank sum test.

Table 4.—PCP Levels in Blood, Lymphocyte Stimulation Responses, Serum Neopterin, and $CD4^+$ Cell Counts in Patients (Pat.) with CD4/8 Ratios < 1.0

Pat.	CD4/8	CD4/µl	Lym/µl	Neo	Stimulation defect*	PCP (µg/l)
A	0.7	480	1 654	11	+	11
B	0.7	780	2 136	22	+	22
C	0.8	737	2 106	8	++	326
D	0.6	656	2 626	9	+	266
E	0.9	548	1 662	8	++	17
F	0.8	354	907	8	++	32
G	0.9	449	1 321	25	+++	25
H	0.9	244	787	33	+++	33
I	0.6	75	394	7	+++++	13
J	0.9	1 492	5 146	5	++	33
K	0.8	984	3 080	12	+++	15
L	0.8	384	1 239	10	0	14

Notes: Neo = neopterin; normal ≤ 15nmol/l; CD4/8: normal > 1.0; CD4/µl: normal > 500; and Lymphocytes/µl (Lym/µl): normal > 1 000.
*Stimulation defect: + = impaired response to one of the four mitogens or alloantigen; 0 = normal.

decreased serum immunoglobulins, and anti-smooth-muscle antibodies in 38 individuals who were exposed to PCP in manufacturer-treated log houses.[12] In accordance with our results, these authors also found impaired in-vitro lymphocyte responses. The current study, however, provides the first evidence that in-vitro lymphocyte responses are associated with the patient blood levels of PCP.

Results from PCP-containing lymphocyte cultur showed that PCP-induced immunosuppression w mediated by a downregulation of IL-2 production. Lymphokine production, as well as Ig secretion in-vit

were suppressed in a dose-dependent fashion after exposure to PCP-T or PCP-A occurred. The two PCP preparations caused a similar suppression of lymphocyte functions.[15] The mode of immunosuppression mediated by PCP shows interesting similarities with the mode of immunosuppression mediated by immunosuppressive drugs, such as steroids, ciclosporin, or FK506. Steroids inhibit primarily IL-1ß and IL-2 production, whereas ciclosporin and FK506 suppress IL-2 and IL-2 receptor expression.[34,35] Transplant patients who receive long-term immunosuppression have an increased risk of infection and lymphoma, and one might speculate that the persisting levels of PCP in blood may also promote infections—or even malignancies—in some individuals with increased chemical sensitivity.[36]

Our data and the published results in the literature enable us to conclude that PCP inhibits the function of T and B lymphocytes[15] and that patients with high levels of PCP in blood are at risk of developing lymphocyte dysfunctions. Immune dysfunctions, especially lymphokine abnormalities, may explain some of the clinical symptoms observed in the patients, e.g., chronic infection, chronic fatigue, or hormonal dysregulation.

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