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Monohydroxylated Fatty Acid Content in Peripheral Blood Mononuclear Cells and Immune Status of People at Long Times after the Chernobyl Accident

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The monohydroxylated fatty acid content of peripheral blood mononuclear cells from 23 cleanup workers and 16 unexposed individuals was studied in relation to their immune status after the Chernobyl accident. Men with absorbed doses below 0.32 Gy showed higher levels of free and esterified 12-hydroxyeicosatetraenoic acid (12-HETE) than unexposed men, whereas 15-HETE and the 17-hydroxy derivative of C22 fatty acid (17-OH 22), either free or esterified in phospholipids, were increased in a dose-dependent manner. The percentage of CD4-positive cells was also increased significantly in heavily irradiated men, whereas the percentage of CD8-positive cells tended to decrease with dose. Furthermore, the absolute count of CD4-positive cells was correlated positively with the amount of esterified 15-HETE in the phospholipid fraction of the mononuclear cells and with the total 15-HETE. These results show for the first time that the accumulation of autoxidized/lipoxygenase products of polyunsaturated fatty acids in the mononuclear cells of irradiated individuals was associated with immune imbalance. This may be the basis for certain late effects of radiation such as autoimmune disorders, somatic and neoplastic diseases, and early aging. © 2001 by Radiation Research Society

INTRODUCTION

The Chernobyl Nuclear Power Plant accident resulted in the accidental irradiation of an unprecedented number of people. The people most affected were those who participated in the cleanup activities at the Chernobyl plant (cleanup workers, also known as liquidators). Increased morbidity and mortality were observed in this group (*1*). Exposure of these individuals to ionizing radiation caused profound oxidative stress in radiosensitive tissues. Increased levels of oxygen free radicals lead to subsequent peroxidative damage of membrane polyunsaturated lipids, proteins and DNA. The biological effects of ionizing radiation are determined not only by the number of mutations that are induced, but also by other effects of oxidative damage on the biological system (*2–6*). The lesions produced by free radical-induced oxidation form the molecular bases of the diseases that occur at long times after exposure to ionizing radiation. It is well known that polyunsaturated fatty acids (PUFA) are good substrates for lipoxygenase- and cyclo-oxygenase-dependent oxidation. Oxidized lipids can alter membrane-associated processes (*7–9*) and the functional properties of cells (*10–14*). It has been shown that monohydroxylated derivatives of linoleic and arachidonic acids are important biologically active products of the lipoxygenase pathways (*15–17*). One of them, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), is the main metabolite of arachidonic acid (AA) produced by activated platelets. 12-HETE has been shown to accumulate in lymphocytes in an age-dependent manner and to inhibit the proliferative response to concanavalin A (*18*). However, the role of monohydroxylated fatty acids at both early and late times after exposure to ionizing radiation is unclear.

Injury to the immune system is a critical component of the syndrome that occurs soon after irradiation, and it also plays a crucial role in the late effects of exposure to radiation, such as leukemia, tumor development and early aging (*19*). Immune functions, especially proliferation and immunoglobulin output, are also closely related to lipid peroxidation. The immunological reactions of the heavily irradiated victims and cleanup workers were different during the period of the acute radiation syndrome and during the recovery period. An early period of immunodeficiency was

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TABLE 1
Pairs of Monoclonal Antibodies Used in Two-Color Analysis

Monoclonal antibody 1	Cluster of differentiation	Monoclonal antibody 2	Cluster of differentiation
Pan-leukocyte-FITC	CD45	Anti-LeuM3-PE	CD14
Anti-mouse IgG1-FITC		Anti-mouse IgG1-PE	
Anti-Leu4-FITC	CD3	Anti-Leu12-PE	CD19
Anti-Leu3a-FITC	CD4	Anti-Leu2a-PE	CD8
Anti-Leu4-FITC	CD3	Anti-HLA-DR-PE	
Anti-Leu4-FITC	CD3	Anti-Leu11-PE	$CD16^+$
		Anti-Leu19-PE	CD56

associated with increased lipid metabolism and modulation of neutrophil antioxidant status. Recovery occurred at the beginning of the 1990s (*20–22*). After recovery from the acute radiation injury, an inhibition of immune function remained; this was associated with changes in lymphocyte subsets, such as decreased $CD3^+$ and $CD4^+$ T-lymphocyte counts and increased numbers of somatic mutations at the T-cell receptor locus. Activation of antioxidant defense enzymes, an increase in cholesterol levels, and an enhancement of lipid peroxidation processes were also characteristic during this period (*23–27*). Early aging of the immune system was thought to be a result of radiation exposure. Lipid peroxidation due to radiation exposure might have induced changes in cellular monohydroxylated fatty acid content, which in turn could play a role in the development of the stable immune imbalance observed long after exposure. However, there is no information about the monohydroxylated fatty acid content in the peripheral blood mononuclear cells (PBMC) of the cleanup workers or about its relationship to the immune status of these individuals at long times after the Chernobyl accident.

The aim of this work was to study the monohydroxylated fatty acid content in the PBMC of irradiated cleanup workers relative to that of unirradiated controls and to evaluate the immune status of these men 12 years after the Chernobyl accident.

EXPERIMENTAL PROCEDURES

Chemicals

Lymphocyte separation medium (Histopaque), RPMI 1640 medium, dextran (MW 267000) and chemicals for the analysis of monohydroxylated fatty acids were purchased from Sigma-Aldrich Chemical Co. (La Verpillere, France). All were of analytical grade. Analytical-grade organic solvents and reagents for silica gel thin-layer chromatography were purchased from Merck (Darmstadt, Germany). The capillary column was from Hewlett Packard. Monoclonal antibodies were obtained from Becton Dickinson (Mountain View, CA) as mixtures of two or three monoclonal antibodies and used for immunophenotyping in two-color assays (Table 1). Polyethylene glycol-6000 was obtained from Loba Chemie Austranal Praparate (Austria). The anti-hepatitis C passive hemagglutination kit was from Dianabot Co. Ltd. (Tokyo, Japan), and the anti-cytomegalovirus and anti-toxoplasma particle agglutination kits were from Fudjirebio (Tokyo, Japan).

Selection of Patients

Thirty-nine subjects aged 25–69 years were selected randomly for the study. The study was approved by the Institutional Committee of the Research Center for Radiation Medicine of the Academy of Medical Sciences of Ukraine. Each subject gave informed consent and was protected in accordance with international regulations on human rights. The subjects were divided into three groups: 13 cleanup workers with absorbed ionizing radiation doses higher than 0.32 Gy, 10 cleanup workers with absorbed doses lower than 0.32 Gy, and 16 unirradiated controls (Table 2). All subjects were male. Absorbed doses were estimated primarily by direct physical dosimetry. For the subjects who had experienced and recovered from an acute radiation syndrome, the doses were confirmed by chromosome aberration studies. All subjects were observed at the Institute of Clinical Radiology of the Research Centre for Radiation Medicine of the Academy of Medical Sciences of Ukraine; individual medical histories were analyzed before the beginning of this study. All the subjects had similar dietary habits that reflected the diet common in Ukraine (potatoes, cabbage, milk, porcine meat).

Acquisition of Blood Samples

Venous blood (40–80 ml) was drawn into sterile tubes on CPD anticoagulant for lipid investigation. For flow cytometry, 300 μl was taken from each sample. Lysed blood samples were used for surface antigen analysis according to the Ukrainian National Committee for Clinical Laboratory Standards (1991). Immunoglobulins and infectious immunity studies were performed from 2 ml of blood.

Flow Cytometry Analysis

Staining was performed using the direct immunofluorescence assay. Blood was added as a 50-μl aliquot to six tubes. Then 20 μl each of undiluted reagents A–F was added to the appropriate tube. Cells were incubated for 20 min in the dark at room temperature. Two milliliters of 1× FACS lysing solution (Becton Dickinson) at room temperature was added to each tube for red blood cell lysis. The cells were then incubated for 10 min at room temperature. The supernatant was removed, and the cells were washed twice in PBS containing 0.1% sodium azide, with centrifugation at 200*g*. Analysis was performed within 12 h after staining. FACS analysis was also performed on mononuclear cell suspensions isolated on density gradients to obtain the percentages of lymphocytes and monocytes. Flow cytometry analyses were performed on a FACScan cytometer (Becton Dickinson) with a 488-nm 15-mW Cyonics laser. Thresholds for forward and side scatter were used. Adjusting of forward scatter, side scatter and fluorescence 1 and 2 (FL1, FL2) was performed with Calibrite beads (Becton Dickinson). Percentages of positive $FL1^+2^-$, $FL1^+2^+$ and $FL1^-2^+$ cells and fluorescence intensity were estimated for each sample. Electronic compensation coefficients for fluorescence spectra were 0.8% for FL1 and 18.4% for FL2. Mathematical analysis and subset discrimination were done on an HP340 workstation with LYSYS II software.

TABLE 2
Demographic Data and Radiation Doses for Exposed and Control Individuals

Irradiated individuals with high absorbed doses (>0.32 Gy)			Irradiated individuals with low absorbed doses (≤0.32 Gy)			Unirradiated individuals		
Subject no.	Age (years)	Absorbed dose (Gy)	Subject no.	Age (years)	Absorbed dose (Gy)	Subject no.	Age (years)	Absorbed dose (Gy)
1	69	1.8	14	59	0.32	24	62	0
2	43	1.7	15	52	0.25	25	34	0
3	53	1.5	16	52	0.25	26	33	0
4	43	1.4	17	52	0.25	27	30	0
5	51	1.0	18	50	0.25	28	45	0
6	34	0.80	19	47	0.25	29	35	0
7	35	0.70	20	34	0.25	30	35	0
8	46	0.65	21	36	0.20	31	51	0
9	45	0.64	22	54	0.10	32	60	0
10	52	0.54	23	45	0.05	33	40	0
11	58	0.50				34	45	0
12	46	0.50				35	50	0
13	39	0.42				36	25	0
						37	37	0
						38	43	0
						39	42	0
Means	47.2 ± 2.7	0.93 ± 0.13		48.1 ± 2.5	0.22 ± 0.02		41.7 ± 2.6	0

Studies of Humoral Immunity

The serum immunoglobulin concentrations of IgG, IgA and IgM were measured by simple radial immunodiffusion as described elsewhere (*28*). Estimation of infectious immunity was performed according to the instructions of the manufacturers.

Anti-hepatitis C antibodies. Twenty-five microliters of serially diluted serum from the blood of each subject was added to each well of a microplate containing 25 μl of a buffered suspension of red blood cells coated with hepatitis C antigens. Positive (high-titer anti-HCV antibodies containing serum) and negative (buffer) controls were used. Agglutination was evaluated after 1 h incubation at room temperature in a humidified chamber. Results were expressed as log2 of titers.

Anti-cytomegalovirus and anti-toxoplasma gondii antibodies. Twenty-five microliters of serially diluted serum from the blood of each subject was added to each well of a microplate containing 25 μl of a buffered suspension of latex particles coated with cytomegalovirus or toxoplasma antigens. Positive (high-titer anti-cytomegalovirus or anti-toxoplasma antibodies containing serum) and negative (buffer) controls were used. Agglutination was evaluated after 30 min of incubation at room temperature in a humid chamber. The results were expressed as log2 of titers.

Isolation of Lymphocytes

Lymphocytes were isolated as described previously (*18*). Briefly, whole blood was centrifuged for 20 min at 120*g*. Platelet-rich plasma was discarded. A solution containing 9% NaCl, 1 m*M* EDTA, and 5% dextran was added to the remaining blood cell suspension to a final concentration of 1% dextran. The suspension was mixed carefully and kept for 30 min at 37°C. The erythrocyte pellet was discarded. The supernatant was layered onto Histopaque 1077, and samples were centrifuged for 20 min at 600*g*. The PBMC-rich ring was collected, washed three times with RPMI 1640 culture medium, and finally resuspended in 1 ml of RPMI 1640 medium.

Lipid Extraction Procedure

Lipids were extracted with a mixture of ethanol:chloroform (1:2 by volume) containing 0.5 nmol ricinolejc acid and 1 nmol 2-ricinoleic acid-phosphatidylcholine used as internal standards as described previously (*29*). The mixture was acidified to pH 3 with 3 *M* HCl. To avoid artifactual monohydroxylated fatty acid formation and degradation, extracted lipids were immediately hydrogenated by hydrogen bubbling for 6 min in the presence of PtO_2 as a catalyst, which was then eliminated by centrifugation at 600*g* for 10 min. Hydrogenated lipid solutions were then evaporated to dryness and stored at −20°C until further analyses. Before GC-MS analysis, free monohydroxylated fatty acids and phospholipids were separated by silica gel thin-layer chromatography with the solvent mixture hexane:diethylether:acetic acid (60:40:1 by volume). The corresponding spots were scraped off and extracted by diethylether and by a mixture of ethanol:chloroform (1:2 by volume), respectively. Phospholipids were hydrolyzed at 100°C in a 5% KOH methanolic solution for 1 h, and the free monohydroxylated fatty acids were purified by thin-layer chromatography as described above. Finally, hydroxylated fatty acids were converted into methyl ester and trimethylsilyl ether derivatives as described previously (*29*).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed on an HP6890 System (Hewlett Packard) equipped with a 5973 Mass Selective Detector. Electron impact ionization was done at 25 eV. Compounds were quantified using the selected ion monitoring mode. For the estimation of free hydroxylated fatty acids, ricinoleic acid was used as the internal standard with ions at *m/z* 187 and 301 eluted at 17.6 min. 2-Ricinoleic acid-phosphatidylcholine was used as the internal standard for hydroxylated fatty acid esterified in phospholipids using the same characteristic ions. Quantification of hydroxylated fatty acids (either free or obtained after hydrolysis of phospholipids) was performed by comparing peak areas to those of the respective internal standards.

Statistical Analysis

Comparisons of the means ± SEM from the different groups were done using one-way analysis of variance and a two-tailed Student's *t* test. A P value <0.05 was considered as statistically significant. Microsoft Excel 97 for Windows 95 and ANOVA for PowerPC were used for statistical analysis of the data.

TABLE 3
Free Monohydroxylated Fatty Acid Content of Blood Mononuclear Cells

Monohydroxylated fatty acid	Monohydroxylated fatty acid content of blood mononuclear cells (pmol/10^6 cells)		
	Irradiated individuals with high absorbed doses (>0.32 Gy) $n = 13$	Irradiated individuals with low absorbed doses (≤0.32 Gy) $n = 10$	Unirradiated individuals $n = 16$
HHT	0.92 ± 0.37	1.96 ± 0.85*	0.41 ± 0.10
13-HODE	7.81 ± 3.73	4.48 ± 1.63	4.00 ± 1.48
5-HETE	0.44 ± 0.16*	0.28 ± 0.09	0.14 ± 0.02
8-HETE	0.26 ± 0.10*	0.15 ± 0.05*	0.07 ± 0.01
12-HETE	2.55 ± 1.59	5.65 ± 2.61*	1.20 ± 0.29
15-HETE	0.25 ± 0.10	0.36 ± 0.10	0.19 ± 0.05
Σ HETE	3.49 ± 1.69	6.44 ± 2.80*	1.59 ± 0.34
11-OH-22	0.05 ± 0.02	0.07 ± 0.02	0.03 ± 0.01
14-OH-22	0.44 ± 0.20	0.74 ± 0.31*	0.17 ± 0.04
17-OH-22	0.08 ± 0.03	0.05 ± 0.02	0.03 ± 0.01
ΣOH-22	0.58 ± 0.22	0.86 ± 0.34*	0.23 ± 0.05

* Significantly different from unirradiated individuals.

RESULTS

The monohydroxylated fatty acid content of PBMC from 23 men who were exposed to radiation as a result of the Chernobyl accident and 16 unirradiated controls was measured by GC-MS. As shown in Table 3, the most abundant free monohydroxylated fatty acids in the PBMC of both unirradiated controls and irradiated individuals were 13-HODE, derived from linoleic acid, and 12-HETE, derived from arachidonic acid. These species accounted for 64.3 and 19.2%, respectively, of the total amount of free monohydroxylated fatty acids in the PBMC of unirradiated controls. The other free monohydroxylated metabolites of arachidonic acid and of 22 carbon atom PUFA (OH-22) represented a minor part of total, and their individual amounts did not exceed 1 pmol/10^6 cells. In the PBMC of irradiated individuals with absorbed doses lower than 0.32 Gy, 13-HODE accounted for 32.6%, 12-HETE for 41.2%, and the other derivatives for 26.2% of the total free monohydroxylated fatty acids. In PBMC of irradiated individuals with absorbed doses higher than 0.32 Gy, the amounts of free 13-HODE, 12-HETE and other monohydroxylated fatty acids were 61.0, 19.9 and 19.1% of the total, respectively. The data shown in Table 3 indicate that the levels of free HHT, 8-HETE, 12-HETE, total HETEs, 14-OH-22 and total OH-22 were increased markedly and significantly compared to the controls (4.8-, 2.3-, 4.8-, 4.0-, 4.4- and 3.8-fold, respectively) in the PBMC of individuals with absorbed doses lower than 0.32 Gy. The levels of free 5-HETE and 8-HETE were also increased substantially in the PBMC of irradiated individuals with absorbed doses higher than 0.32 Gy compared to the unirradiated controls. Moreover, correlation analyses (Fig 1A, B) indicated a close relationship between the absorbed radiation dose and the levels of some specific monohydroxylated fatty acid in PBMC. Statistically significant positive correlations between the absorbed dose and the content of free 5-, 8- and 15-HETE, 11- and 17-OH-22, and total OH-22 derivatives in the PBMC of all the individuals studied were obtained (Fig. 1A).

The distribution of monohydroxylated fatty acids esterified in the phospholipid fraction of PBMC was similar to that of their free counterparts. As shown in Table 4, the main monohydroxylated fatty acids in the phospholipid fraction of PBMC from unirradiated controls were 13-HODE (66.2% of total amount) and 12-HETE (7.8% of total amount). These percentages were roughly similar in both groups of irradiated patients. However, when the individual hydroxy derivatives were considered, the levels of 13-HODE and 8- and 12-HETE were dramatically higher (3.6-, 4.7- and 4.3-fold, respectively) in the group of irradiated individuals with absorbed doses lower than 0.32 Gy than in the unirradiated group. It is noteworthy that the level of 15-HETE was significantly lower (2.7-fold) in irradiated individuals with doses below 0.32 Gy than in controls. In patients with absorbed doses exceeding 0.32 Gy, a significant increase in 15-HETE (2.3-fold) and 17-OH-22 (3.2-fold) was observed.

Thus the observed distributions of free and esterified lipoxygenase-derived monohydroxylated metabolites were different. It is noteworthy that the average amounts of 13-HODE, total HETEs and total OH-22 derivatives esterified in the PBMC phospholipids of unirradiated individuals were 2.4-, 2.0- and 6.6-fold higher, respectively, than those of their free counterparts. A similar asymmetrical distribution of free and esterified monohydroxylated fatty acids was found in irradiated individuals with absorbed doses lower than 0.32 Gy, with the exception of 5-, 12- and 15-HETE, which were found in similar amounts in both their free and esterified forms (Tables 3, 4).

The total monohydroxylated fatty acid content (free + esterified) of PBMC is presented in Table 5. The data shown in this table indicate that the levels of cyclo-oxy-

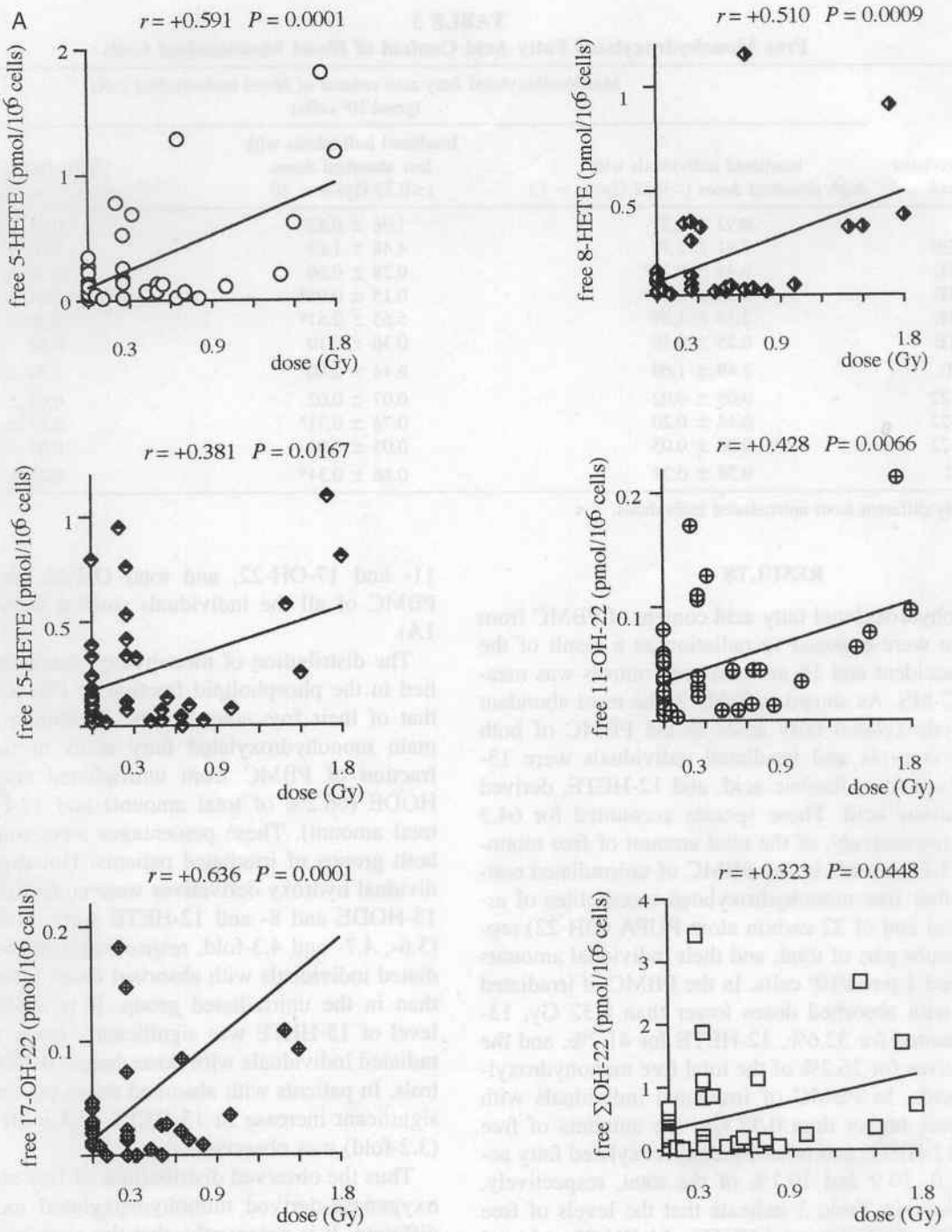

FIG. 1. Relationship between the absorbed radiation dose and the monohydroxylated fatty acid content of blood mononuclear cells. The monohydroxylated fatty acid content of PBMC was measured by GC-MS as described in the Experimental Procedures. Individual values are plotted as a function of absorbed dose; $n = 39$. Regression analyses were performed using Statview II. r and P values are indicated on each graph. Panel A: free monohydroxylated derivatives; panel B: esterified and total monohydroxylated derivatives.

genase-derived HHT and monohydroxylated metabolites of linoleic and arachidonic acids (13-HODE, 8- and 12-HETE, and total HETEs) were significantly higher in irradiated individuals with absorbed doses lower than 0.32 Gy than in unexposed controls. An increase in the 15-HETE content (2.1-fold) was found in the PBMC of irradiated individuals with absorbed doses higher than 0.32 Gy compared to unirradiated controls. Positive correlations between the absorbed dose and the amount of total 5-HETE, esterified and total 15-HETE, and esterified and total 17-OH-22 derivatives were also seen (Fig 1B).

Clinical examination of the blood samples from the control and exposed groups showed no differences between mean red cell, white blood cell or platelet counts. The rel-

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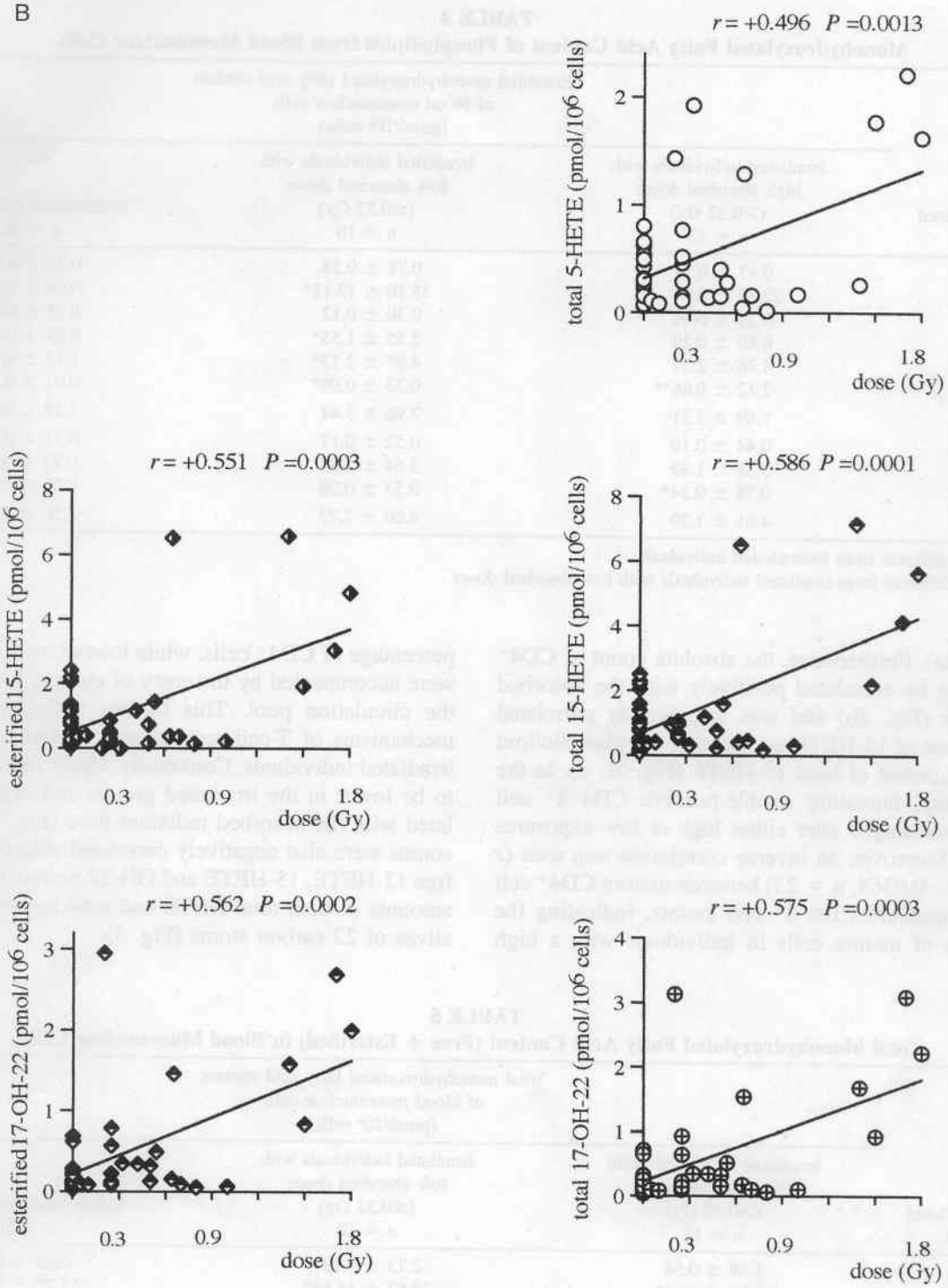

FIG. 1. Continued.

ative and absolute lymphocyte counts tended to be lower in the exposed group (35.6 ± 0.9% and 2.2 ± 0.1 × 10^9 cells/liter, $n = 23$) than in controls (38.9 ± 4.3% and 2.5 ± 0.3 × 10^9 cells/liter, $n = 13$), but the differences were not statistically significant. Further analyses of the different cell subsets revealed that the percentage of $CD3^+$ T cells tended to be higher in individuals with high exposures (71.2 ± 2.7%, $n = 13$) and lower in individuals exposed to less than 0.32 Gy (62.2 ± 4.6%, $n = 10$) than in controls (66.0 ± 2.0%, $n = 15$). However, the differences were not statistically significant. Further analyses of CD3 cell subsets showed that the elevation in the group with high exposure was related to a marked and significant increase in the proportions of T-helper/inducer cells compared to the other two

TABLE 4
Monohydroxylated Fatty Acid Content of Phospholipids from Blood Mononuclear Cells

Monohydroxylated fatty acid	Esterified monohydroxylated fatty acid content of blood mononuclear cells (pmol/10^6 cells)		
	Irradiated individuals with high absorbed doses (>0.32 Gy) $n = 13$	Irradiated individuals with low absorbed doses (≤0.32 Gy) $n = 10$	Unirradiated individuals $n = 16$
HHT	0.47 ± 0.17	0.78 ± 0.28	0.28 ± 0.08
13-HODE	22.88 ± 8.64	35.10 ± 13.12*	9.69 ± 2.14
5-HETE	0.22 ± 0.09	0.30 ± 0.12	0.25 ± 0.04
8-HETE	0.80 ± 0.20	2.35 ± 1.35*	0.51 ± 0.11
12-HETE	4.76 ± 2.57	4.97 ± 2.13*	1.15 ± 0.36
15-HETE	2.12 ± 0.66*#	0.33 ± 0.08*	0.91 ± 0.19
Σ HETE	7.89 ± 3.31	7.96 ± 3.44	3.18 ± 0.60
11-OH-22	0.44 ± 0.10	0.52 ± 0.17	0.31 ± 0.07
14-OH-22	3.38 ± 1.49	3.64 ± 2.39	0.94 ± 0.37
17-OH-22	0.78 ± 0.24*	0.53 ± 0.28	0.25 ± 0.05
ΣOH-22	4.61 ± 1.70	4.69 ± 2.77	1.50 ± 0.45

* Significantly different from unirradiated individuals.
\# Significantly different from irradiated individuals with low absorbed doses.

groups (Fig. 2a). Furthermore, the absolute count of $CD4^+$ cells tended to be correlated positively with the absorbed radiation dose (Fig. 2b) and was significantly correlated with the amount of 15-HETE esterified in the phospholipid and with the amount of total 15-HETE (Fig. 2c, d). In the irradiated group, immature double-positive $CD4^+8^+$ cell counts were unchanged after either high or low exposures (not shown). Moreover, an inverse correlation was seen ($r = -0.438$; $P = 0.0368$, $n = 23$) between mature $CD4^+$ cell counts and immature $CD4^+8^+$ cell counts, indicating the predominance of mature cells in individuals with a high percentage of $CD4^+$ cells, while lower levels of $CD4^+$ cells were accompanied by the entry of cortical thymocytes into the circulation pool. This finding indicates that normal mechanisms of T-cell reconstitution occur even in highly irradiated individuals. Conversely, $CD8^+$ cell counts tended to be lower in the irradiated groups and negatively correlated with the absorbed radiation dose (Fig. 3). $CD8^+$ cell counts were also negatively correlated with the amounts of free 12-HETE, 15-HETE and OH-22 products, and with the amounts of both total HETE and total hydroxylated derivatives of 22 carbon atoms (Fig. 3).

TABLE 5
Total Monohydroxylated Fatty Acid Content (Free + Esterified) in Blood Mononuclear Cells

Monohydroxylated fatty acid	Total monohydroxylated fatty acid content of blood mononuclear cells (pmol/10^6 cells)		
	Irradiated individuals with high absorbed doses (>0.32 Gy) $n = 13$	Irradiated individuals with low absorbed doses (≤0.32 Gy) $n = 10$	Unirradiated individuals $n = 16$
HHT	1.39 ± 0.54	2.73 ± 1.13*	0.69 ± 0.14
13-HODE	30.69 ± 12.11	39.57 ± 14.68*	13.70 ± 3.30
5-HETE	0.66 ± 0.21	0.58 ± 0.20	0.39 ± 0.05
8-HETE	1.05 ± 0.28	2.51 ± 1.38*	0.57 ± 0.11
12-HETE	7.31 ± 4.09	10.62 ± 4.57*	2.71 ± 0.52
15-HETE	2.37 ± 0.71*#	0.69 ± 0.09	1.10 ± 0.20
Σ HETE	11.38 ± 4.91	14.40 ± 5.99*	4.77 ± 0.79
11-OH-22	0.50 ± 0.11	0.58 ± 0.19	0.35 ± 0.07
14-OH-22	3.82 ± 1.68	4.38 ± 2.67	1.11 ± 0.38
17-OH-22	0.87 ± 0.27*	0.58 ± 0.30	0.27 ± 0.05
ΣOH-22	5.19 ± 1.91	5.54 ± 3.09	1.73 ± 0.46

* Significantly different from unirradiated individuals.
\# Significantly different from irradiated individuals with low absorbed doses.

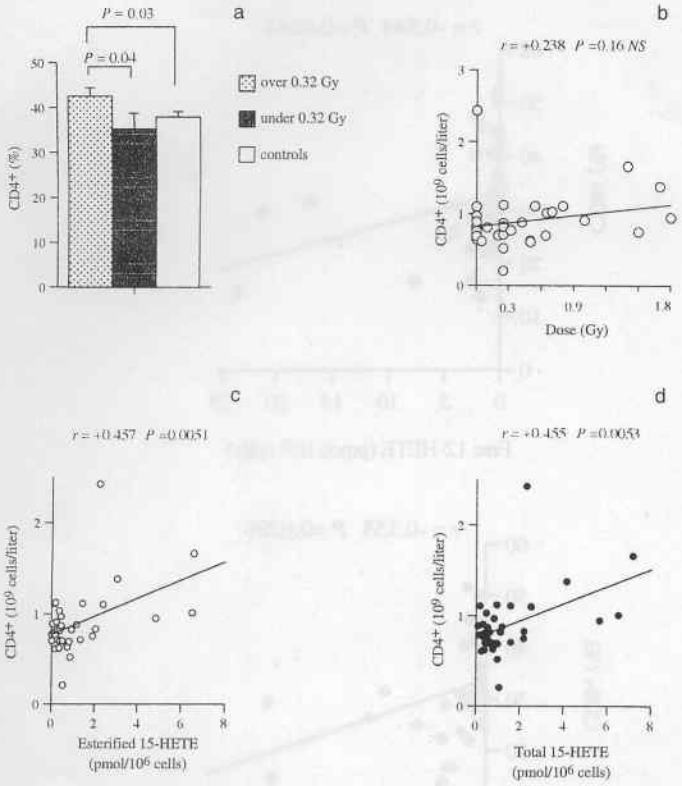

FIG. 2. Variation of CD4-positive cells with radiation dose and 15-HETE levels. CD4 subset analyses were performed by flow cytometry as described in the Experimental Procedures. In panel a, results are expressed as percentages and are means of 16, 13 and 10 determinations for control, absorbed dose over 0.32 Gy, and absorbed dose under 0.32 Gy, respectively. Data were analyzed by ANOVA, and the means were compared by Fisher's *t* test. Significance is indicated on the graph. In panel b, the scatterplot shows the dose dependence of the absolute $CD4^+$ count; in panels c and d, the scatterplots show the dependence of the absolute $CD4^+$ count on the level of esterified and total 15-HETE, respectively. Regression analyses were performed using Statview II. *r* and *P* values are indicated on each graph.

Another modification found in the T-lymphocyte population was the significantly higher absolute count of cytotoxic $CD3^+CD16^+CD56^+$ cells in individuals with low doses compared to those with doses greater than 0.32 Gy ($0.178 \pm 0.069 \times 10^9$ cells/liter compared to $0.069 \pm 0.009 \times 10^9$ cells/liter, $P = 0.05$).

Expression of activation antigens did not appear to be changed. HLA-DR expression (percentage of $CD3^+DR^+$ cells) was positively correlated with the percentage of $CD3^+$ cells ($r = +0.437$; $P = 0.0086$, $n = 35$) in the whole population, but there was no relationship between HLA-DR and $CD4^+$ or $CD8^+$ subsets. Levels of IL2 receptor-positive $CD4^+$ cells ($CD4^+25^+$) did not differ significantly from control levels in the exposed individuals. T-cell receptor mutation assays showed elevated counts of cells bearing somatic lymphocyte mutations in the group with doses higher than 0.32 Gy compared to the two other groups, but the differences were not significant.

Other findings included significantly higher IgA concentrations in individuals with doses below 0.32 Gy (2.59 ± 0.39 g/liter for exposed compared to 1.90 ± 0.41 g/liter for controls; $P = 0.001$). Exposed individuals also had higher frequencies of anti-hepatitis C, anti-Toxoplasma gondii, and anti-cytomegalovirus antibodies. Significant positive relationships were seen between the absolute counts of $CD3^+$ cells and the levels of anti-hepatitis C antibodies ($r = +0.72$), whereas the IgG concentration was negatively correlated with the levels of anti-hepatitis C antibodies ($r = -0.78$). The absolute counts of $CD3^+$ cells and the percentages of HLA DR^+ were positively correlated with the levels of anti-Toxoplasma antibodies ($r = +0.62$ and $+0.61$, respectively), whereas the percentages of $CD8^+$ and NK cells were negatively correlated with the levels of these antibodies ($r = -0.67$ and -0.64, respectively). All these changes indicate a role for specific immunity in the late effects of radiation on the immune system.

DISCUSSION

The late effects of the Chernobyl accident on the health of the people who were exposed to radiation have not been studied sufficiently. A high level of lipid peroxidation in cleanup workers was shown by investigators at the Research Centre for Radiation Medicine of the Academy of Medical Sciences of Ukraine. The levels of malondialdehyde and the antioxidant status of irradiated people were monitored for more than 10 years. It was shown that low doses of radiation induced lipid peroxidation in the blood cells. However, lipid peroxidation in cleanup workers was not evaluated as a function of the absorbed radiation dose. It is still unclear which peroxidative cascades are involved in the development of the late effects of radiation.

It has been shown that ionizing radiation can alter the enzymes of the arachidonic acid cascade (*30*). Lipoxygenases metabolize arachidonic acid and other unsaturated fatty acids to produce many different bioactive derivatives, the monohydroxylated products of linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6) being the most significant. Among the different constitutively expressed lipoxygenases (*31*), 12-lipoxygenase has been found mainly in platelets (*32*); 15-lipoxygenase has been found in monocytes (*33*), neutrophils and eosinophils (*34*). Linoleic acid, arachidonic acid and docosahexaenoic acid can serve as substrates for 15-lipoxygenase (*35*), resulting in the formation of 13-HODE, 15-HETE and 17-OH-22, respectively. Though these lipoxygenase metabolites are not synthesized in the lymphocytes themselves, they may accumulate in the lymphocytes of exposed individuals and may affect some of the functional properties of lymphocytes dramatically.

The immune system is one of the most radiosensitive systems in humans. Several reports have suggested that lipoxygenase metabolites may regulate lymphocyte proliferation (*15, 36*). However, there was no information about the effects of ionizing radiation on fatty acid peroxidation in immune cells at long times after irradiation. Only a few

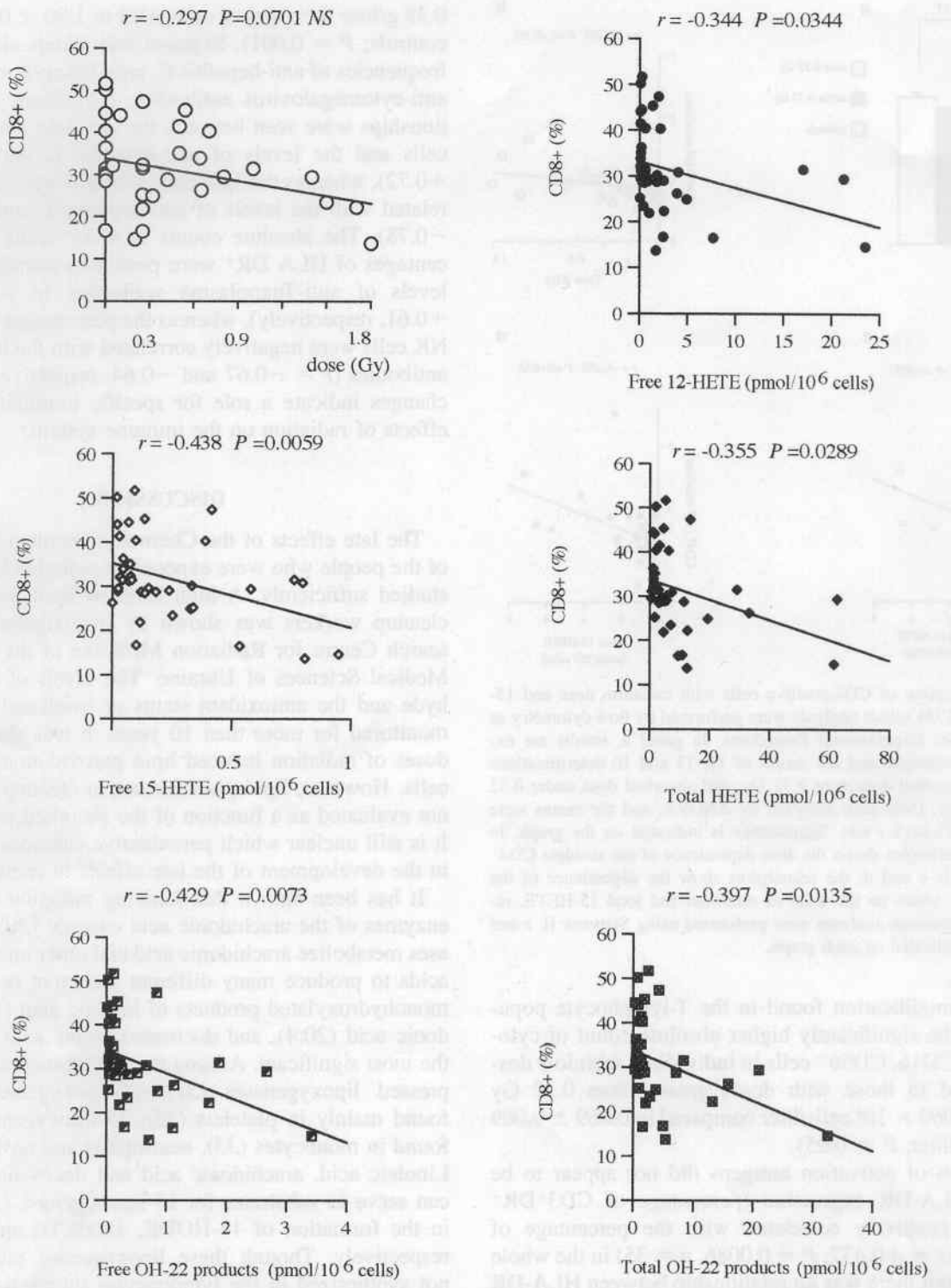

FIG. 3. Variation of CD8-positive cells with radiation dose and levels of some monohydroxylated fatty acids. CD8 subset analyses were performed by flow cytometry as described in the Experimental Procedures. The upper left scatterplot shows the dose dependence of the $CD8^+$ count; the other scatterplots show the dependence of the $CD8^+$ counts on the level of some monohydroxylated fatty acids. Regression analyses were performed using Statview II. *r* and *P* values are indicated on each graph.

reports deal with the effects of ionizing radiation on fatty acid peroxidation. Korystov *et al.* (*37*) have shown that irradiation of rat thymocytes with 6 Gy induced arachidonic acid metabolism and apoptosis, and that both processes were suppressed by the lipoxygenase inhibitor nordihydroguaiaretic acid. An accumulation of 15-HETE in the splenic lymphocytes of rats within 3–12 h after 1 Gy irradiation has also been reported (*38*). As observed in irradiated thy-

mocytes, accumulation of 15-HETE and DNA fragmentation were also blocked by nordihydroguaiaretic acid. These data suggest that 15-HETE and other lipoxygenase products may serve as early mediators of radiation-induced apoptosis. In contrast, many other reports have shown that lipoxygenase metabolites normally promote growth and inhibit programmed cell death in a variety of cells (*39*). Studies performed with 5-lipoxygenase-deficient mice suggested that leukotrienes may play a protective role in host defense against infection (*40*). In addition, 5-lipoxygenase inhibitors have been shown to reduce the proliferation of bone marrow cells *in vitro*; the inhibition could be partially reversed by the addition of 5-lipoxygenase metabolites (*39*). W256 cells transfected with 12-lipoxygenase-specific antisense oligonucleotides or antisense oligonucleotides directed against conserved regions of lipoxygenase gene sequences underwent time-dependent apoptosis; this could be blocked by exogenous 12-HETE or 15-HETE addition, but not by 5-HETE (*41*). These authors showed that inhibition of the lipoxygenase pathway down-regulates the expression of the anti-apoptosis protein BCL2. Also, 15-HETE has been shown to accumulate in the cells of patients with different inflammatory diseases (*42*) or bronchial asthma (*43, 44*), whereas 12-HETE accumulated in the cells of patients with neoplastic diseases (*45*), diabetes mellitus (*46*), and other diseases in which oxidative stress is involved.

In the present study, we found a dose-dependent accumulation of some monohydoxylated derivatives of arachidonic acid and docosahexaenoic acid in PBMC 12 years after irradiation. Thus the levels of free 5-HETE, 8-HETE and 15-HETE, of free 11-OH-22 and 17-OH-22, and of the total free 22-OH derivatives were increased significantly as a function of the absorbed radiation dose. In contrast, the amounts of free cyclo-oxygenase-derived HHT and of free 12-HETE were increased significantly in the PBMC of individuals with absorbed doses in the range of 0.05–0.32 Gy, but the changes were not significant when the absorbed doses exceeded 0.32 Gy. The levels of 15-HETE and 17-OH-22 esterified in phospholipids and of total (free + esterified) 5-HETE, 15-HETE and 17-OH-22 were also increased in a dose-dependent manner, whereas the amounts of esterified 13-HODE and 12-HETE were increased significantly only in the PBMC of individuals with absorbed doses lower than 0.32 Gy. Such incongruent dose responses have also been reported in previous studies examining the effect of ionizing radiation on various parameters. Exposure of rats to ionizing radiation activated cholesterol synthesis at a dose of 4 Gy, whereas a decrease was observed at higher doses (*27*). Sharetskii *et al.* (*22*) showed that exposure of mice to 0.1 Gy radiation increased the T-cell-dependent humoral immune response in lymph nodes, whereas 0.5 or 1 Gy caused dose-dependent immunosuppression. In a study of the structure and function of the immune system of mice within the 10-km zone of Chernobyl, it was shown that helper T lymphocytes were activated by doses less than 0.336 Gy and suppressor lymphocytes were activated at 0.336 Gy (*21*). Although the biological significance of the greater accumulation of 12-HETE at the lower radiation doses is unclear, it may be related to the hyper-radiosensitivity observed at very low doses in some cell lines (*47*). Although the variation in the levels of 15-HETE was clearly dependent on dose when the whole population was considered (Fig. 1A, B), it appeared that the group of individuals with doses less than 0.32 Gy had threefold less esterified 15-HETE in phospholipids than did the controls and that, conversely, the levels were twofold higher in the group with doses greater than 0.32 Gy. 15-HETE is a well-known inhibitor of 12-lipoxygenase (*48*), and it can serve as a substrate for 5-lipoxygenase (*49, 50*). This effect may explain the inverse changes in the contents of 12-HETE and 15-HETE in the lymphocytes of individuals with absorbed doses lower and higher than 0.32 Gy. Furthermore, since 5-lipoxygenase metabolites were also increased in a dose-dependent manner, it is tempting to speculate that the amount of 5-,15-diHETE might also have increased with increasing radiation dose. However, the procedure we used here did not allow us to measure dihydroxylated derivatives of PUFA.

At long times after irradiation, irradiated individuals tended to have increased levels of $CD3^+$ cells. Further analyses show that this elevation was related to the marked increase in the T-helper/inducer cell subset. It is noteworthy that the $CD4^+$ cell count was positively correlated with the level of 15-HETE esterified in phospholipids and with the amount of total 15-HETE. Significantly higher IgA concentrations as well as higher frequencies of anti-hepatitis C, anti-Toxoplasma gondii, and anti-cytomegalovirus antibodies were found in individuals with doses lower than 0.32 Gy compared to controls. This indicates a possible role of specific immunity in the late effects of radiation on the immune system. However, we cannot rule out the possibility that the signs of viral immunity observed in this group might be due to exposure to infectious agents and might not be related directly to the exposure to ionizing radiation.

High levels of monohydroxylated fatty acids have been shown to accompany the development of many pathological processes, such as the formation of atherosclerotic plaque, peroxidation of low-density lipoproteins, adhesion processes (*51–53*), and aging (*18*), most of which are also induced by ionizing radiation. Since the hydroxylated fatty acid derivatives are esterified rapidly into blood cells, including lymphocytes (*15*), such phospholipid remodeling could in turn alter phospholipid-dependent signaling processes (*54*). It has been shown that 15-HETE is rapidly esterified into neutrophil phospholipids *in vitro* to levels that are comparable with those of arachidonic acid, and that this phospholipid remodeling inhibits LTB_4-triggered stimulus–response coupling (*55, 56*). However, we did not find a clearcut dose dependence of the esterified-to-free ratio for any specific monohydroxylated fatty acid (data not shown), suggesting that ionizing radiation did not affect the exchanges between the two forms.

The present study shows for the first time an accumulation of monohydroxylated derivatives of polyunsaturated fatty acid in mononuclear cells of irradiated individuals at long times after irradiation. This suggests that ionizing radiation may have a long-lasting effect on hematopoietic cell progenitors. Phospholipids with monohydroxylated fatty acyl chains can alter the functional characteristics of blood cells. These changes may be part of the adaptive processes that allow recovery during the acute postirradiation period but in turn induce immune imbalance; this could explain some of the late effects of radiation such as viral persistence.

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