PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 808–813 (1975)

Analysis of Tumor-Associated Alkyldiacylglycerols and Other Lipids During Radiation-Induced Thymic Leukemogenesis[1,2] (38904)

ROBERT C. BROWN[3,5], MERLE L. BLANK[4], JOEL A. KOSTYU[3], PAUL OSBURN[3], ALEXANDRA KILGORE[5], AND FRED SNYDER[4]

[3]*Department of Pathology, University of North Carolina School of Medicine, Chapel Hill, NC 27514,* [4] *Medical Division, Oak Ridge Associated Universities, Oak Ridge, TN 37830, and* [5]*Department of Pathology, Rutherford Hospital, Rutherfordton, NC 28139*

Significantly larger quantities of alkyldiacylglycerols have been detected in a variety of tumors of animals and man than in healthy tissues from the same sources (1, 2). This is explained by an increased activity of synthesizing enzymes (3) and a decreased activity of a key cleavage enzyme (4) for the ether-linked lipids; both enzymes are found in the microsomal fraction of tumor cells. However, the function of ether-linked lipids in tumors and other mammalian tissues is still not known. Also measurements of alkylglycerolipids in tissues during oncogenesis have not been done.

In the experiments described in this paper we have measured the quantity of alkyldiacylglycerols and certain other lipids in the thymus at 3 days after irradiation, when maximum thymic involution occurs, and in thymic leukemia developed in the thymus at 5 mo after irradiation. Our results show that lipid composition is altered in the leukemic thymus and indicate that lipid changes in the irradiated thymus before histologic recognition of thymic leukemia could be important in understanding early events that occur during oncogenesis.

Materials and Methods. Irradiation. Inbred 4- to 5-wk-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) received four weekly doses of 200 rads gamma radiation from a Theratron 80 Cobalt-60 Teletherapy Unit (Atomic Energy of Canada, Ltd., Ottawa, Canada) at a dose rate of 160 rads/min as measured by a Baldwin-Farmer Dosimeter (Nuclear Enterprises, Ltd., Beenham, Reading, England) placed within a mouse irradiation chamber. The C57BL/6J mouse was selected for the leukemogenesis model since involution and regeneration of the thymus occurs in a predictable manner and a consistently high incidence of thymic leukemia develops 3–5 mo after irradiation (5, 6). During irradiation, mice were in separate rubber-stoppered, ventilated polycarbonate tubes in radial position on a horizontal Lucite platform that was rotated in the radiation beams at 5–6 rpm. A Lucite sheet (0.625-cm thick) was placed between the tubes and the radiation source.

Both the control and experimental mice (six per cage) were given Purina Lab Chow and acid water (0.023% HCl solution) ad lib. The cages were covered with a Filter Bonnet (Filtek, Appleton, Wisconsin) for the duration of each experimental period.

Experimental procedure. After cervical dislocation, a section of thymus removed from each lobe was fixed in acetate-buffered 10% formalin for histologic study. The remaining portions of the lobes were immediately frozen at −180° in liquid nitrogen and then transferred to vials for storage in dry ice (−40°) until the lipids were extracted (7); all lipid extracts were stored at −23° in chloroform. For the 5-mo irradiated group, only neoplastic thymic lobes were pooled. The average wet weight of thymus glands for each experimental group was determined by averaging the weights of 12–35 thymuses before removing sections for histologic examination.

Thymic lobes were embedded in paraffin

[1] Research supported by the National Cancer Institute, Grant CA-12222; the United States Atomic Energy Commission; American Cancer Society, Grant BC-70D; Western Carolina Pathology-Associates Medical Laboratories, PA; and Rutherford Hospital, Inc.

[2–5] Portions of this research were presented at the 58th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, NJ, April 7–12, 1974 (Fed. Proc. 33, No. 3, Part 1, March 1974).

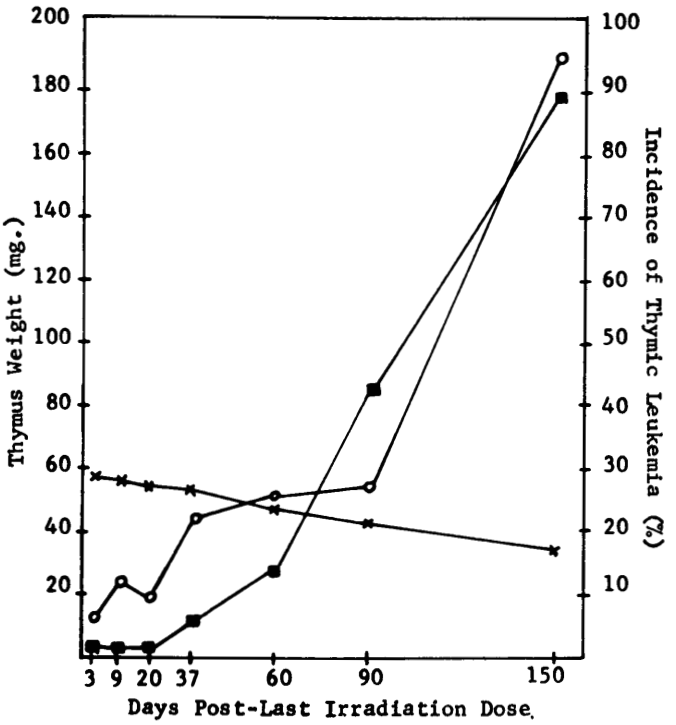

FIG. 1. Changes in thymus weight and incidence of thymic leukemia after irradiation. Symbols indicate thymus weights in control mice (×); thymus weights in irradiated mice (○); incidence of thymic leukemia in irradiated mice ■. No thymic leukemia occurred in the control mice.

and sections were stained with hematoxylin and eosin for evaluation. Histologic studies were performed on groups of irradiated mice sacrificed at 3, 9, 20, and 37 days and at 2, 3, and 5 mo after the last irradiation. Similar analysis was done on thymuses from nonirradiated mice of the same age that were maintained under the same conditions.

Silicic acid column chromatographic separation of neutral lipids and phospholipids (8) and the separation and densitometric measurements of the alkyldiacylglycerols on Silica Gel G layers (9) are reported elsewhere. Total ether lipids were determined after Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$, 70% in benzene] reduction (10). We used conditions for gas chromatographic analyses as previously described (11). Cholesterol and cholesterol esters were determined by using a modified Liebermann-Burchard reaction (12).

Results and Discussion. Morphologic changes in the irradiated thymus. Figure 1 gives the average weight of thymus glands and incidence of thymic leukemia in groups of mice sacrificed at various days after irradiation; values for the control groups of the same age mice are also shown. The thymic weights and tumor incidences after exposure to gamma radiation are similar to those previously reported for C57BL/6 mice exposed to X-radiation (6).

The following histologic groupings were recognized: normal; "thymocyte" depletion; slight, moderate, or marked "thymocyte" regeneration; and neoplasia. At 3 days after irradiation the glands characteristically exhibited thymocyte depletion. However, by 9 days, thymocyte regeneration was prominent; on the 20th day the average thymus weight was slightly less than that on the 9th day and thymus regeneration was diminished. At 37 days and 2 mo after irradiation, thymus regeneration was practically complete and most glands could not be distinguished from normal. We detected the first evidence of thymic leukemia (2 of 200 thymic lobes) at 20 days after irradiation.

TABLE I. ALKYLDIACYLGLYCEROLS IN THE THYMUS DURING RADIATION-INDUCED LEUKEMOGENESIS.

Experimental group	Lipid composition (%)		Alkyldiacylglycerols (%)	
	Neutral lipid	Phospholipid	Neutral lipid	μg/g Wet tissue
3-Day control	30 (6.3)[a]	70	0.10	6.3
	32 (6.0)	68	0.20	11.9
	34 (8·1)	66	0.17	13.7
	28 (6.3)	72	0.37	23.2
3 Days after irradiation	49 (14.8)	51	0.80	118.6
	46 (13.0)	54	0.30	38.9
	40 (10.6)	60	0·71	75.2
	44 (12.6)	56	0.38	48.0
5-Month control	39 (7.0)	61	0.20	14.0
	41 (9.7)	59	0.20	19.3
	39 (9.5)	61	0.31	29.5
5 months after irradiation (leukemia)	55 (16.2)	45	0.69	112.0
	41 (9.1)	59	1.10	100.5
	39 (8.5)	61	0.90	76.5
	36 (6.3)	64	1.30	82.3

[a] Values in parentheses are expressed as mg/g wet tissue.

The highest rate of leukemogenesis was initiated between the second and third months after irradiation and this continued at least until the fifth month when 89% of the thymic lobes were leukemic.

Alkyldiacylglycerols. Table I shows that the quantity of alkyldiacylglycerols is very small in thymuses at 3 days after irradiation and in thymic leukemia developed 5 mo after irradiation. However, there is consistently a larger amount of alkyldiacylglycerols (approximately 3- to 8-fold) in thymic leukemia at 5 mo after irradiation when compared with the thymuses from the same aged controls. Although the quantity of alkyldiacylglycerols in the thymuses from mice 3 days after irradiation varied considerably, there was a trend toward higher levels of this lipid class than that found in the corresponding controls. The quantity of alkyldiacylglycerols in thymic tumors of mice is similar to that found in chronic myelogenous leukemia and nonneoplastic lymph cells in man (2).

Analysis of total alkyl and alk-1-enyl glycerolipids after Vitride reduction also showed a greater amount of alkyl glycerolipids in thymic leukemia compared to 5-mo control thymuses; neutral lipids from three leukemic thymuses had 0.30, 0.46, and 0.27% alkyl glycerolipids (expressed as alkylglycerols) compared to 0.11% for neutral lipids from a single 5-mo control sample. In contrast, the total quantity of alk-1-enyl glycerolipids in thymic tumors was the same as that found for the thymuses of 5-mo control mice (3.4% of the phospholipids expressed as alk-1-enylglycerols); this is about the same level as that found in other tumors (1, 2).

The quantity of alkyldiacylglycerols in thymic leukemia is small compared to other cancerous tissues (1, 2) and thus does not provide a sensitive indicator for early detection of neoplastic transformation in the thymuses of irradiated C57BL/6J mice. However, the assay of the enzymes that synthesize the alkyldiacylglycerols de novo might provide a high degree of sensitivity. Preliminary studies done with tissue homogenates have demonstrated that [1-^{14}C]-hexadecanol, a precursor of the ether-linked chains, is incorporated into the alkyl lipids of the thymic tumors but not into alkyl lipids of thymuses of similar-aged control mice.

The small quantity of ether-linked lipids in thymic leukemia might be related to the relatively small amount of microsomal material in the "thymocyte" compared to that in the cells of tissues from which the other types of tumors originated because the syn-

TABLE II. TOTAL CHOLESTEROL AND CHOLESTEROL ESTERS IN THE THYMUS DURING RADIATION-INDUCED LEUKEMOGENESIS.

Experimental group	Total cholesterol		
	mg/g neutral lipid	mg/100 g wet tissue	% as cholesterol esters
3-Day control	289	183	16
	297	178	15
	456	287	33
	500	321	33
	500	307	22
	271	401	45
3 Days after irradiation	338	438	47
	335	355	37
5-Month control	237	166	15
	234	228	20
5 Months after irradiation (leukemia)	250	227	56
	231	197	35
	356	242	39

thesizing enzymes for the ether-linked glycerolipids are located in the microsomal fraction (3). Since the activity of the tetrahydropteridine-requiring alkyl cleavage enzyme was not determined, it cannot be excluded that a high activity of this catabolic enzyme could explain the low levels of alkyl lipids in thymic leukemic cells.

Cholesterol and cholesterol esters. The proportion of esterified cholesterol, Table II, is increased in the thymus at 3 days after irradiation and in thymic leukemia, when compared to corresponding controls. Three days after irradiation, the thymus contains more cholesterol than thymic tumors or 3-day or 5-mo control thymuses. This increase at 3 days after irradiation is due to an increase in the amount of the total lipid pool and not to an increase in the cholesterol/glycerolipid ratio. The cause of the elevated total lipids in the thymuses at 3 days after irradiation may be a direct effect of the higher lipid content of histiocytic cells, macrophages, fibrocytes, and epithelial cells that predominate in the thymuses at this time due to "thymocyte depletion" by irradiation.

The significance of the elevated level of cholesterol esters that is associated with thymic leukemia and the early postirradiation "thymocyte depletion" state is unknown. Similar elevations of cholesterol esters have been detected in certain other neoplastic conditions (13, 14). It is possible that the elevated sterol ester levels could be related to membrane synthesis associated with cell proliferation, but this seems unlikely in the thymus at 3 days after irradiation, since it exhibits very little cell replication at this time. Another possibility is that increased cholesterol esters could result from the relative increase in poorly differentiated "thymocytes" (neoplastic "thymocytes") in the tumors. However, we cannot exclude that the relative increase in stromal and reticular cells in the thymuses 3 days after irradiation and the increased numbers of degenerating cells in thymic tumors are reasonable for the elevated cholesterol esters.

Acyl composition of major lipid classes. Table III demonstrates that there is more 18:1[6] and 18:2 acids and less 14:0, 16:0, and 16:1 acids in the triacylglycerols from thymic leukemia than that from the thymus of the 5-mo controls; we have also observed less 16:0 and 16:1 acids in the triacylglycerols of Morris 7777 hepatomas than in normal liver (14). The distribution of fatty acids in triacylglycerols in the thymuses from mice 3 days after irradiation had slightly more 18:1 acid but distinctly less 18:2 acid than triacylglycerols from the thymuses of the 3-day controls. In contrast, triacylglycerols from the thymuses 3 days after irradiation show more 14:0, 16:0, and 16·1 and less 18:1 acids than triacylglycerols from the leukemic thymuses. It is possible that such differences in the fatty acid distribution of the triacylglycerols in thymic leukemia might be useful in detecting early stages of thymic leukemogenesis.

Although the acyl composition of the cholesterol ester fractions varied more than that of triacylglycerols, in general, it was similar in all groups tested except that in thymic leukemia there was about 25% more 18:1 acid in the cholesterol esters than that found in the thymus of 5-mo controls. We

[6] The number preceding the colon indicates the number of carbon atoms in the fatty acid whereas the number following the colon indicates the number of double bonds in the fatty acid.

TABLE III. FATTY ACID COMPOSITION OF TRIACYLGLYCEROLS IN THE THYMUS DURING RADIATION-INDUCED LEUKEMOGENESIS.[a]

Fatty acids	3-Day control		3 Days after irradiation		5-Month control			5 Months after irradiation (leukemia)		
Sample Number...	50	52	51	53	45	37	V731	48	29	V729
14:0	2.4	2.3	2.3	2.6	3·1	3.7	3.4	1.5	1.2	1.8
16:0	29.6	30·3	29.1	30.6	30.5	26.0	33.8	19.3	21.2	21.4
16:1	9.2	10.0	8.8	9.9	11.7	13.8	12.9	7.0	5.2	6.3
18:0	3.7	3.9	5.3	4.6	3.9	4.4	3.8	4.3	5.0	4.7
18:1	29.5	29.7	34.9	34.4	35.4	37.6	32·9	46.8	45.3	46.0
18:2	23.1	21.5	14.4	13.9	12.4	12.9	12.5	15.6	20.3	18.4

[a] Expressed as percentage of total fatty acid methyl esters measured. Peaks were identified by relative retention times of standards on a 10% EGSS-X column. Less than 5% of the fatty acids had retention times longer than 18:2.

found similar acyl composition for the total phospholipids from the thymic tumors and their corresponding controls.

Summary. Lipid composition of thymuses investigated during the development of thymic leukemogenesis induced by exposing C57BL/6J mice to gamma radiation led to the following conclusions.

1. Alkyldiacylglycerols, a class of lipids that are generally elevated in most neoplastic tissues, occurred only in small quantities (less than 1% of the total lipids) in the thymuses of both control and irradiated mice. However, we found a 3- to 8-fold increase in this fraction in thymic tumors of mice at 5 mo after irradiation when compared to controls of similar age. However, the small quantity of this lipid class in thymic leukemia and the fact that similar levels were found in some samples of involuted thymuses of mice 3 days after irradiation, suggests to us that the level of alkyldiacylglycerols is not sufficiently specific or sensitive for detecting early stages of thymic leukemogenesis.

2. Thymuses 3 days after irradiation and leukemic thymuses contain 2- to 3-fold greater quantities of cholesterol esters than control thymuses. No major differences were found in the distribution of acyl moieties in the cholesterol esters of the various thymus samples from the same aged mice except that in thymic tumors the quantity of 18:1 esters was increased by about 25% over that of the controls. The apparent lack of specificity of increased cholesterol esters for neoplasia indicates that its measurement would not provide a suitable indicator of early neoplastic transformation.

3. Acyl composition of the triacylglycerols of thymuses revealed an increase in the 18:1 and a decrease in the 18:2 acids at 3 days after irradiation when compared to the same aged controls. However, thymic tumors occurring at 5 mo after irradiation contained less 14:0, 16:0, and 16:1 acids and increased amounts of 18:1 and 18:2 acids esterified as triacylglycerols compared to controls. The fatty acid distribution in the phospholipid fraction of thymuses was not altered by the appearance of leukemia.

1. Snyder, F., and Wood, R., Cancer Res. **28,** 972 (1968).
2. Snyder, F., and Wood, R., Cancer Res. **29,** 251 (1969).
3. Snyder, F., Advan. Lipid Res. **10,** 233 (1972).
4. Soodsma, J. F., Piantadosi, C., and Snyder, F., Cancer Res. **30,** 309 (1970).
5. Jarplid, B., Acta Radiol. Suppl. **279,** 1 (1968).
6. Kaplan, H. S., and Brown, M. D., *in* "The Leukemias: Etiology, Pathophysiology, and Treatment" (J. W. Rebuck, F. H. Bethell, and R. W. Monto, eds.), p. 163, Academic Press, New York (1957).
7. Bligh, E. G., and Dyer, W. J., Can. J. Biochem. Physiol. **37,** 911 (1959).
8. Börgstrom, B., Acta Physiol. Scand. **25,** 101 (1952).
9. Privett, O. S., Blank, M. L., Codding, D. W., and Nickell, E. C., J. Amer. Oil Chem. Soc. **42,** 381 (1965).

10. Snyder, F., Blank, M. L., and Wykle, R. L., J. Biol. Chem. **246,** 3639 (1971).
11. Snyder, F., and Blank, M. L., Arch. Biochem. Biophys. **130,** 101 (1969).
12. "Hycel Cholesterol Determination" Manual No. 5002, 2nd revision, p. 1, Hycel, Inc., Houston, Texas (1964).
13. Grigor, M. R., Blank, M. L., and Snyder, F., Cancer Res. **33,** 1870 (1973).
14. Snyder, F., Blank, M. L., and Morris, H. P., Biochim. Biophys. Acta **176,** 502 (1969).

Received Nov. 25, 1974. P.S.E.B.M., 1975, Vol. 149.