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The *in vitro* incorporation of acetate-1-C^{14} into normal and leukemic leukocyte lipids

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SUMMARY

White blood cells and plasma from normal and leukemic subjects were incubated in the presence of acetate-1-C^{14}. The distribution of radioactivity in six lipid fractions of cells and plasma was measured. Leukemic leukocytes incorporated the highest percentage of radioactivity into the phospholipid fractions whereas normal leukocytes incorporated the highest percentage into the glyceride fractions. There is no conclusive evidence that the differential white cell count had an influence on these observations. The difference may be related to a more active lipid metabolism in leukemic cells.

Recent studies (1, 2, 3) have shown that leukocytes are responsible for most of the radioactivity incorporated into blood lipid components when whole blood is incubated with acetate-1-C^{14}. Previous work of this laboratory had demonstrated a higher uptake of radioactivity into the phospholipid fraction of the blood of leukemic patients compared with the blood of normals. These studies, however, were performed using whole blood (4).

The effect of cytostatic agents on lipid metabolism of leukemic leukocytes has also been studied (5).

Further information is required on the composition of the lipid fractions derived from the different types of leukocytes and the relative incorporation of radioactivity into these fractions. To pursue this aim and also to investigate possible differences between different types of leukocytes, blood from normal subjects and from patients with nonleukemic leukocytosis, chronic lymphatic leukemia, and chronic myelogenous leukemia was studied.

METHODS AND MATERIALS

Separation of Leukocytes. Whole blood or separated leukocytes were used for incubation. Separation of leukocytes, whether carried out before or after incubation, was performed as follows:

Whole blood (4 volumes) was added to 1 volume of A.C.D. solution (1.32% sodium citrate, 0.48% citric acid hydrated, 1.47% dextrose). Polyvinylpyrrolidone (in 0.84% NaCl and 3% dextrose solution) was added to the mixture to produce a final concentration of polyvinylpyrrolidone of 1.5%. The mixture was allowed to stand at 10° for about 1 hour to allow sedimentation of erythrocytes. The supernatant solution was siphoned off and the leukocytes were separated by centrifugation at $50 \times g$ for 15 minutes; plasma and platelets were decanted. This procedure yielded leukocytes contaminated with fewer than 4 red blood cells per 1 white blood cell when the leukocyte count was normal. When the leukocyte count was high, on the other hand, separation was almost complete. It must be emphasized here that the contaminating red cells do not contribute significantly to incorporation of activity (1, 2, 4, 5). Sterile conditions and plastic or siliconized (6) glassware were used throughout the separation and incubation of leukocytes.

Incubation. Whole blood or separated leukocytes mixed with plasma from the same patient, free of polyvinylpyrrolidone, were shaken for 6 hours at 37° in an atmosphere of 5% CO_2 and 95% O_2 with added acetate-1-C^{14} (specific activity 48.9 μc per mg) in a concentration of 0.5 μc per ml and in the presence of 100 units per ml of penicillin and 100 μg per ml of streptomycin. When preseparated leukocytes were used, the final volume for incubation was 4 ml with 2 to 4×10^5 leukocytes per mm^3. The incubated leukocytes were washed once with 2 ml of isotonic NaCl and examined microscopically.

Prior to extraction of plasma lipids, plasma was centrifuged at 200 × *g* for 10 minutes and the sediment, containing platelets and the remaining leukocytes, if any, was discarded.

Extraction of Lipids. Leukocytes were lysed by freezing and thawing. Lipids were extracted from both leukocytes and plasma using a mixture of chloroform—methanol 2:1 as described by Folch *et al.* (7). After the removal of the solvent at reduced pressure, the dry residue was re-extracted three times with petroleum ether and the combined extracts were centrifuged to remove any polyvinylpyrrolidone present. Negligible amounts of free acetate radioactivity remained in the extract after this procedure; this was verified by experiments in which only 0.01% of added acetate-1-C^{14} could be extracted from a plasma—leukocyte mixture by this procedure.

Chromatography. Total lipids were separated by silicic acid chromatography into six fractions using a procedure based on the method of Fillerup and Mead (8, 9). A 1-cm i.d. column containing 5 *g* of silicic acid (Mallinckrodt Chemical Works, St. Louis, Missouri, 100 mesh, preactivated for 12 hours at 115°) was used. The column was successively washed with 20 ml of each of the following solvents: methanol, acetone, ethyl ether, and petroleum ether (b.p. 50°–70°). The extract containing the total lipids was applied to the column dissolved in 1 ml of petroleum ether, and lipid fractions were eluted with solutions of ether in petroleum ether as follows:[1] cholesterol esters and hydrocarbons with 50 ml of 1% (v/v) solution; triglycerides and free fatty acids (FFA) with 140 ml of 5% (v/v) solution; free cholesterol with 180 ml of 10% (v/v) solution; diglycerides with 60 ml of 25% (v/v) solution; monoglycerides with 40 ml of ethyl ether; phospholipids with 60 ml of methanol. All reagents were analytical grade, redistilled in glass. Ethyl ether was treated with sodium, distilled, and stored over sodium at 5°.

The eluted fractions were evaporated to dryness at reduced pressure in a rotary flash evaporator and gravimetrically estimated when the experiment involved a leukocyte preparation relatively free of red blood cells. The radioactivity of each fraction was measured by the direct technique of Entenman *et al.* (10) in a flow Geiger-Mueller counter (Nuclear Chicago Corp., Chicago, Illinois, type C-110B, operated in conjunction with scaler type 186 and printing timer type C-1113). This system had an efficiency of 14% and a background of 14 cpm. No self-absorption corrections were necessary since the amounts of material applied to the planchets were extremely small. Duplicate results (from four experiments) of incubations of the same bloods agreed to within ±4.3% to 12.5%. Results were expressed as the percentage of total radioactivity recovered from the column; specific radioactivities were also calculated as microcuries per milligram of lipid whenever leukocytes were separated relatively free of red blood cells, as in the studies of blood from patients with leukemia. Free fatty acids were usually removed from triglycerides by passage through a short column of alumina or by stepwise elution on the silicic acid column. Phospholipids were occasionally eluted into 10 fractions. Cholesterol determinations were made by the Liebermann-Burchardt reaction (11) and lipid phosphorus was determined as described by King (12). Free fatty acid analyses were performed by titration. The radioactivity of the free cholesterol fraction was assayed after digitonin precipitation of cholesterol; more than 80% of the radioactivity was found in the precipitate after four washes with ether.

[1] A mixture of known percentage of cholesterol esters, triglycerides, free cholesterol, and lecithin, kindly supplied by Dr. J. F. Mead, was used for the standardization of elution conditions. Radioactive tripalmitin, sodium palmitate, and free cholesterol were used for controls.

RESULTS

Preliminary Experiments. A number of preliminary experiments were performed to resolve certain questions of technique.

Figure 1 shows the results of an experiment in which increasing the concentration of the acetate-1-C^{14} added to the incubation medium, under the same conditions of volume and leukocyte counts, caused a proportional increase in the incorporation of radioactivity into the leukocyte lipids. Previous studies (5) have shown that the rate of incorporation remains almost linear over the 6-hour period.

Table 1 shows the lipid pattern of leukocytes and shows that no significant changes had taken place in lipid weight distribution after a 6-hour incubation.

Table 2 shows that no alteration was observed in the distribution of radioactivity in the lipid fractions whether the incubation took place before or after the separation of leukocytes from erythrocytes. It also shows that leukocyte concentration was without effect on radioactivity distribution in the experiment summarized. This has been examined in greater detail in previous work (4). On the other hand, as has been previously reported (5), the concentration of leukocytes during incubation seems to affect greatly the total amount of radioactivity incorporated into their lipids when it is calculated on a per cell basis,

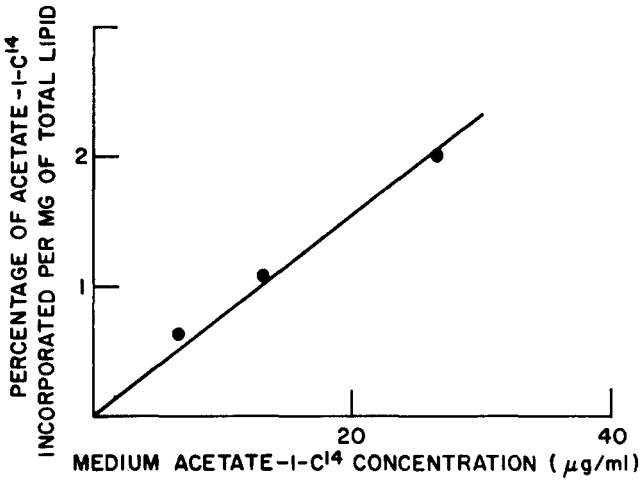

FIG. 1. Incorporation of labeled acetate as a function of medium acetate concentration. Acetate-1-C^{14} (50 μc/mg) was added in varying concentrations to incubation mixtures containing 1.07×10^5 leukocytes per mm^3 in 3 ml of plasma. The mixture was incubated for 6 hours and then the total lipids were extracted from the separated leukocytes.

and it has been shown elsewhere (2) that incubation after separation of leukocytes markedly depresses the total acetate incorporation into lipids.

Composition of Lipid Fractions. Figure 2 shows results of silicic acid chromatography of the lipids in case No. 10. Most of the radioactivity of the first fractions of leukocyte lipids belongs to the hydrocarbons and not to the cholesterol esters (Fig. 2a). The contrary is true for plasma lipid radioactivity of those fractions (Fig. 2b). Furthermore, 25% to 46%[2] of the radioactivity of the second fraction of leukocyte lipids occurs in FFA. Up to 95% of the

TABLE 1. EFFECT OF INCUBATION ON LEUKOCYTE LIPID FRACTIONS

	Percentage of Total Recovered Weight	
	Incubated*	Non-incubated†
Cholesterol esters + hydrocarbons	2.8	2.7
Triglycerides + FFA	4.7	3.7
Free cholesterol	13	11.7
Diglycerides	1	1.6
Monoglycerides	1.3	3.0
Phospholipids	77	77

* After 4 ml of a plasma-leukocyte mixture was incubated for 6 hours, the leukocytes were separated and the extracted lipid (15.5 mg) chromatographed.

† The leukocytes of 4 ml of the same mixture were separated immediately without incubation and the lipids (16.8 mg) extracted and chromatographed.

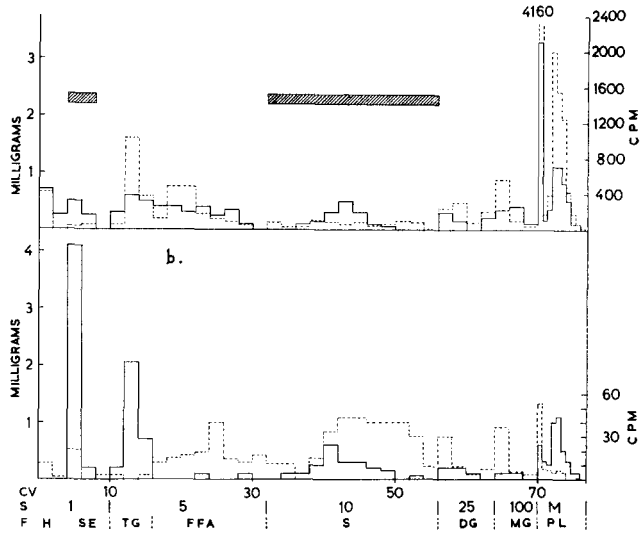

FIG. 2. Chromatographic separation of lipids; a. from leukocytes; b. from plasma.

Abscissa: First line of numbers designates column volumes (CV). Solvents (S) are: per cent ether in petroleum ether (second line of numbers) and methanol (M). Lipid fractions (F) are: hydrocarbons (H); sterol esters (SE); triglycerides (TG); free fatty acids (FFA); sterols (S); diglycerides (DG); monoglycerides (MG); and phospholipids (PL).

(- - -) = cpm; (——) = weight in mg; ▨ indicates fractions giving a positive Liebermann-Burchardt reaction.

Notes on PL fraction: (*a*) phosphorus content was estimated and full coincidence was observed between phosphorus and weight and radioactivity; (*b*) total area of PL fraction must not be compared with the areas of the other fractions because PL were eluted with smaller volumes of eluent.

TABLE 2. EFFECT OF SEPARATION OF LEUKOCYTES FROM ERYTHROCYTES ON INCORPORATION OF RADIOACTIVITY*

	Percentage Distribution of Radioactivity	
	Pre-separated†	Post-separated‡
Cholesterol esters + hydrocarbons	9.5	8
Triglycerides	11.8	13.8
FFA§	5.4	4.4
Free cholesterol	7.8	5.3
Diglycerides	2.3	3.9
Monoglycerides	4.5	3.6
Phospholipids	58	61

* Results from subject No. 6 (Table 3).

† Incubation of leukocytes after separation from erythrocytes. Concentration of leukocytes during incubation—5.3×10^5 cells/mm^3.

‡ Separation of leukocytes took place after incubation. Concentration of leukocytes during incubation—2.8×10^5 cells/mm^3.

§ Separation of FFA achieved by stepwise elution on the silicic acid column.

TABLE 3. PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN SEPARATED FRACTIONS OF LIPIDS FROM NORMAL AND LEUKEMIC LEUKOCYTES AFTER INCUBATION WITH ACETATE-1-C^{14}*

Subject No.	Subject's Age, Sex, Diagnosis, Treatment	Differential Count and Number of Leukocytes per mm^3	Percentage Distribution of Radioactivity					
			HC + SE	TG + FFA	S	DG	MG	PL
1†	Normals 31 to 51/♀	Normal 5,700–6,970	2.2	58	5.3	3.1	3.6	27
2	64/♀ Polycythemia vera with leukeoid reaction‡	P = 85, L = 7, B = 1, E = 3, MN = 4 38,900	0.8	58	11	1.5	4.1	25
3	50/♀ Pulmonary tuberculosis‡	P = 84, L = 9, MN = 6 12,400	1.3	65	6.5	2.9	3.2	21
4	55/♂ Bronchial asthma, cor-pulmonale, secondary polycythemia§	P = 90, L = 4, MN = 6 16,000	0.5	41	3	12	3.5	40
5	55/♀ Chronic lymphatic leukemia‡	L = 72, P = 20, E = 2, MN = 4, IL = 2 39,400	0.7	23	4.4	8.6	7.4	56
6	71/♂ Chronic lymphatic leukemia‡	L = 99, P = 1 782,000	3.4	21	11	2.7	7.3	54
7	65/♂ Chronic lymphatic leukemia§ leukeran 1 month ago	L = 97, P = 2, MN = 1 145,000	2.4	23	3.5	5.3	2.6	63
8	65/♂ Chronic lymphatic leukemia§ leukeran 1 month ago	P = 2, L = 98 95,700	1.6	25	16	5.5	3.4	48
9	54/♀ Chronic myelogenous leukemia§	L = 3, E = 3, MN = 1, M = 4, P = 89, (65% MF, 35% IM) 150,000	0.3	34	11	4.2	5.4	45
10	47/♂ Chronic myelogenous leukemia§ irradiation of spleen	P = 27, L = 4, E = 6, Bas = 9, MN = 1, B = 2, M = 14, PM = 1, MM = 36 (3 blasts/100 leukocytes) 143,000	0.3	43	7.6	0	4.7	45
11	28/♀ Chronic myelogenous leukemia‡ irradiation of spleen	P = 34, L = 3, E = 2, Bas = 6, MN = 1, B = 8, M = 10, MM = 36 (2 blasts/100 leukocytes) 189,000	1.2	23	5.6	3.5	3	64

* Abbreviations: P—Polymorphonuclear; L—Lymphocytes; MN—Monocytes; B—Blasts; Bas—Basophile; E—Eosinophile; M—Myelocyte; PM—P Myelocyte; MM—M Myelocyte; I—Immature cells; YF—Young form; BF—Band form; MF—Mature form.

† Mean values of results from three normal subjects.

‡ Preseparated leukocytes.

§ Postseparated leukocytes.

TABLE 4. PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN SEPARATED LIPID FRACTIONS FROM LEUKOCYTES OF NONLEUKEMIC AND LEUKEMIC SUBJECTS AFTER INCUBATION WITH ACETATE-1-C^{14}

	Normal*		Nonleukemic Leukocytosis†		Chronic Lymphatic Leukemia‡		Chronic Myelogenous Leukemia§	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Cholesterol esters + hydrocarbons	2.2	(1.4–2.9)	0.8	(0.5–1.3)	2	(0.7–3.4)	0.5	(0.3–1.2)
Glycerides + FFA	65.0	(59–68)	63.4	(56–71)	33.5	(30–38)	40.0	(29–47)
Free cholesterol	5.3	(2.6–9.2)	7.0	(3.0–11)	8.9	(3.5–16)	8.2	(5.6–11)
Phospholipids	27.4	(26–30)	28.6	(21–40)	55.6	(48–63)	51.5	(45–64)

* Results from subjects 1 (Table 3).
† Results from subjects 2, 3, 4 (Table 3).
‡ Results from subjects 5, 6, 7, 8 (Table 3).
§ Results from subjects 9, 10, 11 (Table 3).

radioactivity of the second elution fraction of plasma lipids may be in FFA.[2] The phospholipid fraction could be separated into two main subfractions that may consist largely, according to the standard mixture of lipids used, of cephalins (the first eluted) and lecithins (the second). Radioactivity was negligible in the second plasma phospholipid fraction. More suitable methods for phospholipid fractionation will be used to evaluate these findings further.

Distribution of Radioactivity in Separated Lipid Fractions. Table 3 shows the results obtained when leukocytes from normals and from patients with various forms of leukocytosis were incubated with acetate-1-C^{14}.

The results from Table 3 are summarized in Table 4 with the subjects classified into four groups according to their leukocyte picture: normal; nonleukemic leukocytosis, mainly polymorphonuclear; chronic lymphatic leukemia; chronic myelogenous leukemia. Radioactivities of mono-, di-, and triglycerides and of FFA are included in one fraction.

The distribution of radioactivity among the various fractions is similar in the normal and nonleukemic leukocytosis; it is also similar in the two leukemic groups. The greatest uptake of radioactivity is observed in the phospholipid fractions of the leukemic groups, but in the glyceride fractions of the nonleukemic groups. We have no evidence that the distribution of radioactivity among leukocyte lipid fractions shows any correlation with leukocyte type.

Table 5 shows the distribution of radioactivity among plasma lipid fractions of some of the subjects. Lipid exchange between leukocytes and plasma is affected by factors other than leukocyte type and origin. Not all lipid fractions appear to have the same ability to exchange between plasma and cells but specific radioactivity data must be available for further evaluation of these observations. The relative sizes of the different lipid pools are obviously an important consideration.

Weight Distribution. Table 6 shows the results of experiments in which fractions of lipids from leukocytes, free of red cells, were determined gravimetrically. The relative phospholipid content of the cells from case No. 4 (nonleukemic) is lower than that in the cells from patients with chronic lymphatic leukemia, and the relative glyceride content is higher. These results are to be compared with those of Table 4. A general correspondence between radioactivity distribution and weight distribution can be deduced from these results.

Table 7 shows that no significant alteration was observed in radioactivity distribution before, during, and after cessation of treatment with cytostatic agents with alkylating action as long as the disease was still in an active state.

DISCUSSION

Several investigators have reported that human leukocytes are able to incorporate radioactivity from acetate-1-C^{14} into their lipids (1, 2, 3). Evidence from the present study proves that radioactivity is distributed among almost all fractions of the leukocyte lipids studied.[3]

There are, however, large differences in the distribution of radioactivity among the various lipid fractions when leukocytes from nonleukemic and leukemic subjects are compared. In nonleukemic

[2] Result from seven studies by stepwise elution or absorption on alumina.

[3] Most of the cholesterol ester radioactivity was found in the plasma fraction. It is uncertain whether labeled plasma cholesterol esters have a cellular origin or are synthesized by esterification of FFA—C^{14} or cholesterol—C^{14} released from the cells during incubation. Etienne and Polonovski (13) reported esterification of free cholesterol in the course of incubation of human serum at 37° for 36 hours.

TABLE 5. PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN SEPARATED FRACTIONS OF LIPIDS FROM NORMAL AND LEUKEMIC PLASMA AFTER INCUBATION OF LEUKOCYTES AND PLASMA WITH ACETATE-1-C^{14}

Subject* No.	Percentage Distribution of Radioactivity					
	HC SE	TG + FFA	S	DG	MG	PL
1†	6.5	54	11	9.2	6.7	16
3	0	36	33	16	3.8	8.5
4	1.0	52	8.5	8.5	11	19
5	1.2	6.3	51	9.5	10	21
9	1	23	30	3.7	6	38
10	0	12	19	11	5.9	46

* Subject numbers are the same as those in Table 3.
† Mean values of results from three normal subjects.

subjects, whether normal or with an increased polymorphonuclear count, glyceride radioactivity exceeded that of phospholipids. These results, as well as those of plasma lipid radioactivity distribution, are in agreement with data reported by Marks *et al.* (2) for normal bloods. The contrary was observed when leukemic blood was used for incubation regardless of whether or not the donors were irradiated or treated with a cytostatic agent, at least in the first stages of the treatment. Similar differences in weight distribution of leukocyte lipids were found between nonleukemic subjects with increased polymorphonuclear counts and patients with lymphatic leukemia. It appears that the lipid composition of the leukemic leukocyte in the cases studied differs from the normal primarily in showing an increased phospholipid content.

The distribution of radioactivity in the separated plasma lipid fractions is different from that of the cell lipids. This suggests that plasma lipid radioactivity is attributable to the release of lipids from living cells rather than lysis of leukocytes during incubation. There is also evidence that not all leukemic white-cell phospholipids have the same ability to exchange with plasma phospholipids. High phospholipid content of leukemic leukocyte lipids could be due to such exchange differences. However, similar differences have been reported by Rowe (14), who studied the exchange of phospholipids between plasma and cells in normal bloods. There is also a possibility that the high *in vivo* ratio of leukocytes to plasma influences the observed high phospholipid content of leukemic leukocytes, although leukemic cases have been reported (4) with low leukocyte count and radioactivity content of the phospholipid fraction of whole blood higher than normal. Thus, the available data do not permit us to attribute the observed high phospholipid content of leukemic leukocytes to any exchange reaction. It seems more likely that some special metabolic changes take place in leukocyte lipids of leukemic patients.

TABLE 6. LIPID COMPOSITION OF NONLEUKEMIC LEUKOCYTES AND OF LEUKOCYTES IN LYMPHATIC LEUKEMIA

	Composition of Lipids, % of Total*	
	Nonleukemic† Leukocytes	Lymphatic‡ Leukemia
Cholesterol esters + hydrocarbons	7.4	5
Glycerides + FFA	35.8	14.9
Free cholesterol	17	14.4
Phospholipids	40	65.7

* Expressed as percentage of total weight recovered from the column. Overall recovery was 88 to 102%.
† Results from Subject 4 (Table 3).
‡ Mean of results from Subjects, 5, 6, and 7 (Table 3).

It has been generally accepted that a larger proportion of phospholipid is found in those organs in which active metabolic changes in fat are presumably taking place (15); the observed high phospholipid content of leukemic leukocytes may be similarly explained. Whether or not any one of the different phospholipid fractions is preferentially increased will be the subject of future research.

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TABLE 7. EFFECT OF A CYTOSTATIC AGENT ON DISTRIBUTION OF RADIOACTIVITY*

	I†	II‡	III§
Cholesterol esters + hydrocarbons	3.5	5.3	9.5
Glycerides + FFA	31	30	24
Free cholesterol	11	7.8	7.8
Phospholipids	55	57	58

* Results from subject No. 6 (Table 3) expressed as percentage of total recovered radioactivity.
† I. Untreated (leukocyte count 7.82×10^5 cells/mm^3).
‡ II. After treatment with 12.5 mg of TEM (triethylenemelamine) received during 26 days (leukocyte count 6.4×10^5 cells/mm^3).
§ III. Twenty days after cessation of the treatment (leukocyte count 5.8×10^5 cells/mm^3).

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