fatty tissues. The quantitative significance of these findings remains to be evaluated.

*Summary*. Rats were fed a cholesterolemic diet which contained either 20% lard or corn oil. The blood lipid and cholesterol levels were appreciably lower in the rats fed diets containing an excess of PUFA. The livers of these rats had a higher cholesterol content but equal lipid and appreciably lower cholesterol activities than the group ingesting saturated fatty acids. Results suggest that a decreased rate of cholesterol synthesis occurs only after sufficient cholesterol has accumulated in the liver to inhibit its formation. The greater activities in the lipids of the intestinal and fatty tissue apparently were not promoting higher blood lipid levels. The activities of the cholesterol of both intestinal and fatty tissues were lower in the PUFA fed rats. Thus a decreased synthesis of cholesterol occurs in the liver as well as the intestinal tissue along with a slightly decreased content in the fatty tissues of rats fed a cholesterolemic diet containing large amounts of PUFA.

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1. Kinsell, L. W. Michaels, G. D., Friskey, R. W., Splitter, S., Fourth International Conference on Biochemical Problems of Lipids, Academic Press, New York, 1957, p125.

2. Hegsted, D. M., Gandy, R. B., Myers, M. L., Stare, F. J., Am. J. Clin. Nutrition, 1965, v17, 281.

3. Bloomfield, D. K., J. Lab. Clin. Med., 1964, v64, 613.

4. Srere, P. A., Chaikoff, I. L., Treitman, S. S., Burstein, L. S., J. Biol. Chem., 1950, v182, 629.

5. Harper, P. V., Jr., Neal, W. B., Jr., Hlavecek, G. R., Metabolism, 1953, v2, 69.

6. Hotta, S., Chaikoff, I. L., Arch. Biochem., 1955, v56, 28.

7. Sperry, W. M., Webb, M., J. Biol. Chem., 1950, v187, 97.

8. Harlen, J. W., Atomlight, 1961, v19, 8.

9. Siperstein, M. D., Guest, M. J., J. Clin. Invest., 1960, v39, 642.

10. Okey, R., Lyman, M. M., Harris, A. G., Einset, B., Hain, W., Metabolism, 1959, v8, 241.

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## Irradiated and Preserved Leukocytes in Mixed Leukocyte Cultures.* (32090)

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After several days' incubation, cultures of mixed leukocytes from 2 individuals contain immature mononuclear cells which probably are derived from small lymphocytes. These cells incorporate thymidine into DNA, and measurements of $H^3$-thymidine uptake have been used to quantitate the blastogenic reaction in mixed leukocyte cultures(1,2). The disadvantage of the mixed leukocyte reaction has been that the reaction is not "one-way". Various attempts have been made to develop a technique of unidirectional measurement. Red blood cells and platelets were ineffective (1). Frozen and thawed leukocytes have been used by some workers(3,4,5), but we and others(6-9) have failed to demonstrate consistent blastogenesis with frozen and thawed cells. We have reported that X-irradiated cells could be used for unidirectional stimulation in mixed leukocytes cultures(9). Homologous macrophages(10) and cells treated with mitomycin C(11) or nitrogen mustard(12) have been used to achieve one-way stimulation.

Techniques for low temperature storage of peripheral blood leukocytes have been published by Ashwood-Smith(13), Cavins(14), and Pegg(15). We decided to study the possible application of these techniques to the

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preservation of intact and X-irradiated leukocytes for use in mixed leukocyte cultures.

*Materials and methods.* Leukocyte suspensions were prepared from the blood of healthy donors, as described previously(1). In the experiments involving the preservation of irradiated leukocytes, Falcon plastic tissue culture flasks (30 ml) containing these leukocyte suspensions in plasma were subjected to 2,000 r (106 r/min) of irradiation (Picker Vanguard, 280 KVP, cone size 15 × 20 cm). It has been shown that 10% is the optimal concentration of dimethylsulfoxide for the preservation of blood leukocytes(13-15). Two parts of the plasma containing either non-irradiated leukocytes or irradiated leukocytes were added to one part dimethylsulfoxide and 7 parts of tissue culture medium. The suspension, totalling 50 ml, was transferred to a 150 ml Fenwal plastic bag. Each bag was then placed between copper plates and frozen to ensure a uniform freezing rate throughout the cell suspension. The freezing rate was approximately 1°C per minute to —20°C and somewhat faster to the final storage temperature of —70°C. Storage periods of individual samples were 2 weeks. At the end of this time, specimens were thawed by gentle agitation in a 37°C water bath and centrifuged for 10 minutes at 1,000 r.p.m. The supernatant was decanted, and the leukocytes were resuspended in medium 199 containing 20% fresh pooled normal plasma. Cell counts were done on each suspension and the percentage of viable cells was determined by a trypan blue dye exclusion test after mixing one drop of the leukocyte suspension with one drop of 0.2% trypan blue(16). Ten minutes after mixing, the percentage of unstained cells (viable cells) was determined. In each experiment, the amount of medium added was adjusted so that the concentration of viable non-irradiated cells was between 1,000 and 1,500/mm$^3$ and that of irradiated cells was between 800 and 1,500/mm$^3$ in the final suspensions. On the day of thawing the stored leukocytes, a fresh leukocyte suspension from the original donor was prepared. The leukocyte suspension obtained from the original donor of stored irradiated cells was subjected to 2,000 r of X-irradiation. In each experiment the concentrations of fresh and stored viable cells were adjusted so that they were approximately equal. The concentrations of fresh irradiated and stored irradiated cells were similarly made equal.

The leukocytes were incubated at 37°C in 17 × 100 mm disposable plastic culture tubes (Falcon). Each culture contained 4 ml of cell suspension. The mixed leukocyte cultures were incubated for 5 days and those using phytohemagglutinin were incubated for 3 days. All cultures were set up in duplicate or triplicate.

Cultures consisted of 1:1 mixtures of (1) fresh leukocytes and irradiated fresh allogeneic leukocytes, (2) stored leukocytes and irradiated fresh allogeneic leukocytes, (3) stored leukocytes and irradiated fresh syngeneic leukocytes, (4) fresh leukocytes and stored irradiated allogeneic leukocytes, and (5) fresh leukocytes and stored irradiated syngeneic leukocytes. Unmixed fresh and stored leukocytes containing 0.1 ml of phytohemagglutinin (Difco, Detroit, Mich.) were cultured at the same time. At the end of the incubation period, $H^3$-thymidine (Thymidine-6-T, sp. act. 5 c/mM) was added to the culture tubes to give a concentration of 1 $\mu$c/ml. After one hour at 37°C, the cells were washed three times with cold physiological saline and their radioactive content was measured by a liquid scintillation counter as previously described(2). Smears were made from one of the culture tubes in some experiments. The $H^3$-thymidine count correlated well with the number of lymphoblastoid cells found in the smears.

*Results. Effect of irradiation* in vitro *on mixed leukocyte cultures and on leukocyte cultures with phytohemagglutinin.* Leukocyte suspensions were exposed to various doses of X-irradiation as described under *Methods,* and 30 minutes later, mixed leukocyte cultures were set up as illustrated in Table I. Unmixed intact leukocytes both with and without PHA were cultured at the same time. When leukocytes from only one donor received less than 600 r, there was no inhibition of the mixed leukocyte reaction. When 600 r was administered to the leukocytes from both donors, $H^3$-thymidine uptake was inhibited, but was not abolished. After 1,000 r,

TABLE I. Effects of X-irradiation *in vitro* on Mixed Leukocyte Cultures and on Single Leukocyte Cultures Containing Phytohemagglutinin.
Counts/min./culture

| Dose of X-ray | A* | B | A + B† | Ax + B‡ | Ax + Bx | Ax + PHA | A + PHA |
|---|---|---|---|---|---|---|---|
| 600 r | 632 | 448 | 32,146 | 11,779 | 2,872 | 15,754 | 40,561 |
| 800 r | 739 | 539 | 30,923 | 17,088 | 1,828 | 18,574 | 49,574 |
| 1,000 r | 1,026 | 732 | 27,011 | 16,078 | 2,062 | 10,375 | 40,342 |
| 1,200 r | 843 | 125 | 35,446 | 19,243 | 1,060 | 13,393 | 36,576 |
| 1,500 r | 378 | 863 | 36,374 | 9,356 | 739 | 13,048 | 41,243 |
| 1,800 r | 945 | 564 | 29,451 | 19,062 | 223 | 10,031 | 48,356 |
| 2,000 r | 778 | 562 | 22,690 | 9,539 | 522 | 11,041 | 44,710 |
| 3,000 r | 748 | 697 | 14,288 | 10,842 | 314 | 10,394 | 48,182 |
| 6,000 r | 1,201 | 866 | 20,033 | 9,553 | 398 | 565 | 55,498 |

* For simplicity, the leukocytes of each member of any pair of subjects are labeled A and B, respectively.

† Cultures of mixed leukocytes from A and B subjects.

‡ Cultures of irradiated cells (Ax) and intact allogeneic cells (B). The cells were cultured 30 minutes following irradiation of the cells.

there was very questionable enhancement of DNA synthesis in the mixed cultures of irradiated cells as compared with cultures of non-irradiated leukocytes from a single donor. After application of 1,500 r, or more, to leukocytes of only one donor, these cells still caused blastogenesis of non-irradiated leukocytes from a second donor in mixed leukocyte cultures. When this dose was applied to leukocytes of both donors, the $H^3$-thymidine uptake of their mixed leukocyte cultures was no greater than that of the control cultures of non-irradiated cells from a single donor. Thymidine uptake was actually depressed below control values when doses above 1,500 r were administered. When cultures of irradiated leukocytes with PHA were compared with similar cultures of the same donor's non-irradiated leukocytes with PHA (Table I), $H^3$-thymidine uptake was inhibited but not abolished even after application of 5,000 r (not shown in the Table). When leukocyte suspensions were treated with more than 5,000 r and then cultured with PHA, blastogenesis was completely inhibited.

The experiments depicted in Table II were performed to determine whether the stimulatory activity of leukocytes is altered by exposing them to 2,000 r of X-irradiation. In all of these experiments, the sum of the one-way (Ax + B) and (A + Bx) reactions was approximately equal to the 2-way (A + B) reaction (Table II).

*Reactivity of leukocytes stored at low tem-*

TABLE II. Relationship Between "One-Way" and "Two-Way" Reactions in Mixed Leukocyte Cultures.
$H^3$-thymidine uptake Counts/minute/4 × $10^6$ cells

| Exp. No. | A + B*,† | Ax + B*,‡ | A + Bx*,‡ |
|---|---|---|---|
| 1 | 24,708 ± 1,853§ | 12,277 ± 1,026 | 10,683 ± 487 |
| 2 | 21,754 ± 1,941 | 11,236 ± 420 | 10,467 ± 796 |
| 3 | 14,349 ± 197 | 7,587 ± 831 | 7,349 ± 651 |
| 4 | 22,988 ± 665 | 7,307 ± 524 | 19,029 ± 169 |
| 5 | 13,891 ± 804 | 9,986 ± 1,162 | 5,474 ± 599 |

* For simplicity, the leukocytes of each member of any pair of subjects are labeled A and B, respectively. Different donors were used in separate experiments.

† Cultures of mixed leukocytes from A and B subjects ("two-way" reaction).

‡ Cultures of irradiated cells and intact allogeneic cells. The cells were cultured 30 min. following irradiation (2,000 r) of the cells ("one-way" reaction).

§ Mean values from triplicate cultures ± S.D.

TABLE III. Blast Transformation of Stored Leukocytes *in vitro* When Stimulated by X-irradiated Allogeneic Leukocytes.

Counts/min./culture

| Exp. No. | Control cultures of unmixed cells from A* | Cultures of irradiated allogeneic cells from B* and intact cells from A* | stored cells from A* | Cultures of irradiated cells from A* and stored cells from A* |
|---|---|---|---|---|
| 1 | 1,333 | 19,843 | 20,680 | 870 |
| 2 | 474 | 8,419 | 6,225 | 195 |
| 3 | 603 | 6,360 | 5,537 | 158 |
| 4 | 1,041 | 13,283 | 12,203 | 144 |
| 5 | 579 | 7,973 | 6,958 | 216 |
| 6 | 1,139 | 6,284 | 5,988 | 163 |
| 7 | 1,016 | 6,254 | 5,147 | — |
| 8 | 1,098 | 7,816 | 6,620 | — |
| 9 | 791 | 19,158 | 16,092 | — |
| 10 | 870 | 15,395 | 14,514 | — |

* For simplicity, the leukocytes of each member of any pair of subjects are labeled A and B, respectively.

*perature.* The data in Table III showed that stored leukocytes exhibited blastogenic transformation *in vitro* (expressed by $H^3$-thymidine uptake) when cultured with X-irradiated allogeneic leukocytes. The loss in total cell count after storage varied from 10% to 20%. The percentage of viable cells (unstained cells with trypan blue) ranged from 78% to 95% with a mean of 85%. After storage at —70°C for 2 weeks, the reactivity of stored leukocytes to allogeneic irradiated cells in mixed leukocyte cultures was of similar magnitude to that of leukocytes freshly prepared from the same donor.

*Antigenicity of stored X-irradiated leukocytes.* Table IV illustrates the ability of stored irradiated leukocytes to enhance DNA synthesis and mitosis of allogeneic intact leukocytes in mixed leukocyte cultures. In most instances, the stimulatory activity of fresh irradiated leukocytes was greater than that of stored irradiated cells. Storage of irradiated cells resulted in 40-50% cell loss. This was 2 to 3 times as great as that which occurred after storage of intact cells. Stored irradiated leukocytes did not enhance DNA synthesis of syngeneic leukocytes in leukocyte cultures.

TABLE IV. Effect of Low Temperature Storage on Ability of X-irradiated Leukocytes to Stimulate Blastogenesis of Normal Allogeneic Lymphocytes *in vitro*.

Counts/min./culture

| Exp. No. | Control cultures of unmixed cells (Average of A, B)* | Cultures of intact cells from A* and fresh irradiated cells from B* | stored irradiated cells from B* | Cultures of intact cells from B* and stored irradiated cells from B* |
|---|---|---|---|---|
| 1 | 1,333 | 19,843 | 11,386 | 735 |
| 2 | 474 | 8,419 | 4,986 | 133 |
| 3 | 603 | 6,360 | 4,293 | 420 |
| 4 | 1,041 | 13,283 | 17,775 | 630 |
| 5 | 1,139 | 6,284 | 3,968 | 167 |
| 6 | 579 | 7.973 | 4,765 | 654 |
| 7 | 1,016 | 6,254 | 9,986 | 824 |
| 8 | 1,098 | 7,816 | 4,395 | 715 |
| 9 | 791 | 19,158 | 5,730 | 1,254 |
| 10 | 870 | 15,395 | 9,757 | 291 |

* See legend for Table III.

Table V. Reactivity of Stored Leukocytes to Phytohemagglutin.

Counts/min./culture

| Exp. No. | Control cultures of unmixed leukocytes from A | 0.1 ml Phytohemagglutinin added to Stored leukocytes from A* | Fresh leukocytes from A* |
|---|---|---|---|
| 1 | 763 | 58,880 | 129,883 |
| 2 | 841 | 48,094 | 38,368 |
| 3 | 625 | 41,461 | 40,915 |
| 4 | 915 | 40,881 | 34,309 |
| 5 | 1,042 | 34,717 | 38,770 |
| 6 | 826 | 83,132 | 149,243 |
| 7 | 528 | 24,528 | 62,091 |
| 8 | 462 | 30,752 | 74,148 |
| 9 | 508 | 37,943 | 55,473 |
| 10 | 412 | 32,437 | 78,137 |

* See Legend for Table III.

In Table V, it may be seen that stored leukocytes underwent blast transformation when cultured with PHA, but reacted less strongly than fresh leukocytes in 6 of 10 cultures.

*Discussion. In vitro* irradiation of leukocytes with 1,500 r or more destroyed their capacity for DNA synthesis and mitosis in mixed leukocyte cultures (Table I, Ax + Bx). However, their ability to stimulate intact leukocytes was not destroyed because blastogenesis occurred in mixed cultures in which leukocytes from only one subject had been irradiated (Table I, Ax + B). Under these conditions, the stimulatory activity of irradiated leukocytes was not modified, since the sum of the undirectional (Ax + B) and (A + Bx) reactions were equal approximately to the two-way (A + B) reaction (Table II). These results show that properly irradiated leukocytes can be used for unidirectional quantitation of the mixed leukocyte reaction.

Blastogenesis in mixed leukocyte cultures was abolished by 1,500 r, but the response to phytohemagglutinin was not completely inhibited by 5,000 r. In other experiments, we have observed that uremic plasma inhibits the reactivity of normal cells in mixed cultures, but does not inhibit the normal reaction to PHA. The difference in response to these two blastogenic stimuli may be due simply to the greater strength of the PHA stimulus or it may be that stimulation by allogeneic leukocytes requires initial steps (such as processing of antigen by macrophages) which are circumvented by the action of PHA.

The magnitude of the effect on PHA cultures was found to vary only slightly over the range of doses from 600 to 3,000 r, and 6,000 r were required before blastogenesis was completely inhibited. This indicates that there may have been two discrete lymphocyte populations: a sensitive population, which was completely inhibited by 600 r, and a resistant population, which could still be stimulated by PHA after receiving 3,000 r, and which was completely inhibited only after 6,000 r.

Intact leukocytes stored at low temperature for 2 weeks lost almost none of their capacity to respond to irradiated allogeneic leukocytes. Irradiated leukocytes were more labile, and following low temperature storage, they lost a significant portion of their ability to stimulate blastogenesis. This loss of activity cannot now be explained, but may be prevented by technical improvements.

Further investigation is required to define fully the possible applications of leukocyte preservation at low temperatures to tissue matching. Preserved leukocytes could be exchanged between institutions, allowing the comparison of results from different research groups. Exchange of leukocytes would aid in the standardization of leukocyte typing and culture techniques, and it might improve our ability to select donors for kidney and other transplants.

*Summary and conclusions.* When leukocytes received 1,500 r or more *in vitro,* they were rendered incapable of DNA synthesis and mitosis in mixed leukocyte cultures, but did not lose their ability to stimulate the blastogenesis of allogeneic intact leukocytes. The sum of the "one-way" (Ax + B) and (A + Bx) reactions was approximately equal to the "two-way" (A + B) reaction. Therefore, irradiated leukocytes can be used for unidirectional quantitation of the mixed leukocyte reaction. When leukocytes were irradiated with less than 6,000 r and cultured with PHA, some transformation to blasts occurred. Leukocytes stored at −70°C for 2 weeks retained their capacity for blast transformation when cultured in the presence of

X-irradiated allogeneic cells. Frozen storage of irradiated leukocytes reduced their blastogenic effect variably up to 30% of that of fresh controls.

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1. Bain, B., Vas, M. R., Lowenstein, L., Blood, 1964, v23, 108.

2. Bain, B., Lowenstein, L., Science, 1964, v145, 1315.

3. Bach, F. H., Hirschhorn, K., ibid., 1964, v143, 815.

4. Hashem, H., Rosen, F. S., Lancet, 1964, v1, 201.

5. Moynihan, P. C., Jackson, J. F., Hardy, J. D., ibid., 1965, v1, 453.

6. Gordon, J., MacLean, L. D., Nature, 1965, v208, 795.

7. Lowenstein, L., in Histocompatibility Testing, Publication 1229, Nat. Acad. Sci., Washington, D.C., 1965, p139.

8. Ceppellini, R., ibid., p138.

9. Kasakura, S., Lowenstein, L., in Histocompatibility Testing, Series Haematologica 11, Munkgaard, Copenhagen, 1965, p203.

10. Marshall, W. H., Rigo, S. J., Melman, S., Lancet, 1966, v1, 730.

11. Bach, F. H., Voynow, N. K., Science, 1966, v153, 544.

12. Ceppellini, R., Franceschini, P., Miggiano, V. C., Tridente, G., Series Haematologica, 1966, v11, 225.

13. Ashwood-Smith, M. J., Blood, 1964, v23, 38.

14. Cavins, J. A., Scheer, S. C., Thomas, E. D., Ferrabee, J. W., Blood, 1964, v23, 38.

15. Pegg, P. J., Brit. J. Haemat., 1965, v11, 586.

16. Sampson, J. J., Arch. Int. Med., 1924, v34, 490.

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## A Sensitive *in vitro* Method for Prolactin Determination.* (32091)

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The most common bioassays for prolactin are of rather low sensitivity. The most sensitive *in vivo* test is the crop-sac proliferation after intradermal injection; it is sensitive to 0.05 I.U. of prolactin.

It would seem that the most accurate method for prolactin assay would be one which brings the hormone in direct *in vitro* contact with its target organ, the mammary gland (1-5). Various technics of tissue culture for prolactin assay have shown that for differentiated development of mammary tissue 5 different hormones are required: estradiol, progesterone, hydrocortisone, insulin and prolactin(6-9). Rivera(10) added aldosterone and growth hormone. The shortcomings of the method devised by Prop(7,9,15,16) and its sensitivity (0.01-0.001 IU) will be described in the discussion.

In this work, the proper requirements for the use of the rat mammary gland and the optimal hormone concentrations, as well as their specificity *in vitro*, will be reported. The quantitative aspects will be reported separately.

*Materials and methods.* Mammary gland slices were obtained from female virgin, mature, non-primed albino rats of the "Sabra" strain of the Hebrew University, weighing 270 $\pm$ 10 g when in metestrus. The rats were sacrificed by a blow on the head. The abdomen was then shaved and swabbed with 70% alcohol. A mid-ventral incision was made, the 2 inguinal glands were exposed and transferred *in situ* into a sterile Petri dish containing a few ml of medium M-199(11), a synthetic, protein-free medium. The gland was then stretched, the frontal and caudal parts rejected, and the middle part cut into six 2 mm longitudinal strips. This precaution had to be taken since the frontal part of the gland is less sensitive, and the caudal part is over-developed. This difference in sensitivity required the running of parallel control pieces in the culture. Each of the 6 strips was cut into 2 mm long pieces, yielding ten 2 mm

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