The Relationship of Age, Strain and Species to the Reducing Activity of Leukocytes

By Albina Ann Yakaitis

GRANULES of formazan resulting from the reduction of neotetrazolium have been observed in leukocytes by Wachstein.[1] Studies were made on rats, mice, guinea pigs, rabbits and on normal and leukemic humans. In normal human material, formazan formation was noted principally in the mature myeloid elements and in varying degrees in lymphocytes and monocytes. In myelocytic leukemia, mature myeloid cells reduced neotetrazolium, but immature cells were negative in this regard. In chronic lymphocytic leukemia, most of the cells of the polymorphonuclear series and varying percentages of the lymphocytes were positive.

For the present study, 2- (p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) was used because of its rapid reduction and its large crystal formation. Although these crystals preclude precise intracellular localization, they serve as convenient markers for "counting" positive cells. Thus a semi-quantitative measure of enzyme activity may be made.

Materials and Methods

Included in this study were 80 $C3H_f$/An/Sp mice, 21 $BALB_f$/cAn/Sp mice, 15 AKR/J/Sp mice and 11 Fisher 344/a/Sp rats (table 1 and fig. 1). The two former strains of mice are relatively resistant to leukemia while the AKR strain has a high incidence of the disease. Animals varied in age from less than three days to 14 to 16 months.

Peripheral blood was procured in the three day old and two week old mice from the stump of an amputated upper extremity; in the older mice from the tip of the tail, and in the rats from the femoral vessels. The infant mice and rats were anesthesized with chloroform. Blood was drawn into a white blood cell counting pipette to the 0.4 mark. An incubating solution consisting of 6 mg. INT° dissolved in 2 ml. of N,N-dimethylformamide† and 2 ml. of M/15 phosphate buffer at pH 7.5 was then drawn into the pipette to fill the expanded portion. The pipette was placed on a shaker and gently agitated in a horizontal plane for an incubation period of 15 minutes at approximately 25 C. After discarding the first two or three drops of the mixture, the pipette, while held at an acute angle, was drawn lengthwise across a glass slide 3 to 5 times forming interflowing rows of approximately 0.1 ml. of the incubated mixture. The slide was gently rocked to get an even distribution of cells. To facilitate drying, excess fluid was drained by tipping the slide and allowing some of the mixture to flow onto absorbent paper.

Two slides were prepared from each sample. One of the slides was immediately chilled on crushed ice and then stored overnight in the cold (4 C. to 11 C.) for drying. The preparation was counterstained with a 50 per cent solution of Harris' hematoxylin for 20 to 25 minutes, rinsed in tap water and mounted in glycerogel. The percentage of leukocytes

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°Obtained from Dajac Laboratories, Leominster, Mass.

†Obtained from Eastman Organic Chemicals, Rochester, N. Y.

TABLE 1.—*Reducing Activity of Leukocytes*

Group	Type of Animal	Age	No. and Sex	Range of Positive Reaction (% of 700)	Mean of Positive Reaction (% of 700)
I	$C3H_f$ mice	6–7 mo.	6M + 9F	59.2 — 73.6	66.5 ± 3.88*
	$C3H_f$ mice	<3 days	8M + 8F	11.6 — 27.2	20.7 ± 4.28
	$C3H_f$ mice	2 wks.	8M + 3F	24.8 — 44.8	35.8 ± 5.57
	$C3H_f$ mice	2 mo.	2M + 4F	59.8 — 69.6	64.6 ± 3.96
	$C3H_f$ mice	4 mo.	6M + 6F	49.4 — 69.0	63.5 ± 6.42
	$C3H_f$ mice	14–16 mo.	8F	50.2 — 63.2	55.1 ± 4.11
	$C3H_f$ mice	14–16 mo.	12M	33.6 — 58.4	48.8 ± 7.64
II	$BALB_f$ mice	6–7 mo.	5M + 5F	48.0 — 74.8	63.3 ± 9.01
	$BALB_f$ mice	<3 days	8M + 3F	13.8 — 36.8	28.2 ± 7.81
III	AKR mice	6–8 mo.	11M	67.4 — 83.8	77.1 ± 5.20
IV	Fisher rats	6–7 mo.	5M + 6F	47.8 — 68.6	54.7 ± 6.03

*Mean with standard deviation.

showing crystalline formation was determined under oil immersion from a 700 cell count.

The second slide was allowed to dry at room temperature. The state of the reaction was noted microscopically, and photographs were taken at different time intervals during the drying process.

Simultaneously, white blood cell counts and smears were done for the adult animals. Blood from abnormal adult animals was not included in the experiment. Counts and smears were done for three day old mice and two week old animals when sufficient blood was available.

All glassware was chemically clean. White blood cell pipettes were dried in a hot oven rather than with rapidly evaporating solvents.

In additional studies, 10 per cent formalin was used as a fixative. The incubated material was placed on slides as described above and approximately 0.1 ml. of formalin was added to the mixture. The slide was agitated to get a thorough mixing of the cells and the fixative. Excess fluid was drained and the slide was dried at room temperature.

The significance of differences noted was determined by the method described by Goulden[2] and by using the table of "t" values given by Fisher and Yates.[3]

RESULTS

Counts of cells positive for reducing activity.—Cells on slides which had been left to dry at room temperature showed either no evidence of tetrazolium reduction or varying amounts of diminished reduction. Figures 2 to 5 demonstrate the gradual disappearance of formazan crystals from a slide allowed to dry at room temperature. This disappearance did not occur when slides were chilled and dried in the cold. Therefore the latter technic was used routinely. Late in the study, it was found that formazan crystals remained stable if the incubated cells were fixed with 10 per cent formalin.

Group I consisted of $C3H_f$ mice varying in age from less than three days to 14 to 16 months (table 1 and fig. 1). The youngest mice had the lowest percentage of leukocytes showing evidence of tetrazolium reduction (20.7 per cent). Nucleated red blood cells of infant blood did not contain formazan. The two week old mice had a greater percentage of active cells (35.8 per cent), whereas the 6 to 7 month old mice had the highest percentage for the group (66.5 per cent). All the above differences were statistically significant ($P < .001$). A difference in the percentage of active leukocytes between the blood

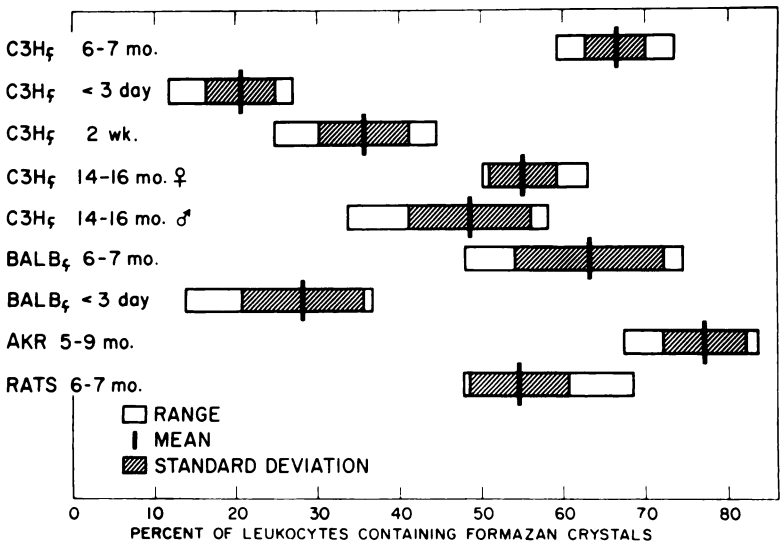
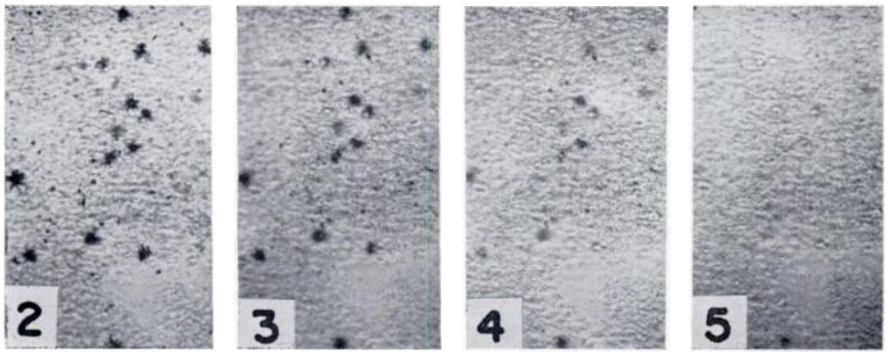


FIGURE 1



FIGS. 2–5.—Disappearance of formazan crystals (× 300).

of older female (55.1 per cent) and older male mice (48.8 per cent) was observed ($P < .05$). The amount of leukocytic reduction of the older animals, male or female, was lower than the amount noted for the 6 to 7 month old animals ($P < .001$, $< .001$). Mice of 6 to 7 months or less did not exhibit a sex difference; for example, 65.8 per cent of the leukocytes of the 6 to 7 month old $C3H_f$ males and 67.0 per cent of the cells of the females showed reduction.

Group II consisted of animals belonging to the $BALB_f$ strain. The standard deviation from the mean for this group was greater than that for the $C3H_f$ mice of similar age. The results, however, were basically similar for the two groups; infant blood had fewer leukocytes with the ability to reduce tetrazolium than did adult blood (28.2 per cent and 63.3 per cent, $P < .001$).

Group III consisted of normal mice belonging to the AKR strain. Leukocytes of this strain showed the greatest amount of tetrazolium reduction (77.1

per cent). This amount of leukocytic activity for the AKR animals was significantly greater than the amount noted for any of the other animals ($P < .001$).

Group IV was comprised of young adult Fisher rats. The percentage of leukocytes (54.7 per cent) having the ability to reduce tetrazolium was close to that noted for old female mice of the $C3H_f$ strain (55.1 per cent).

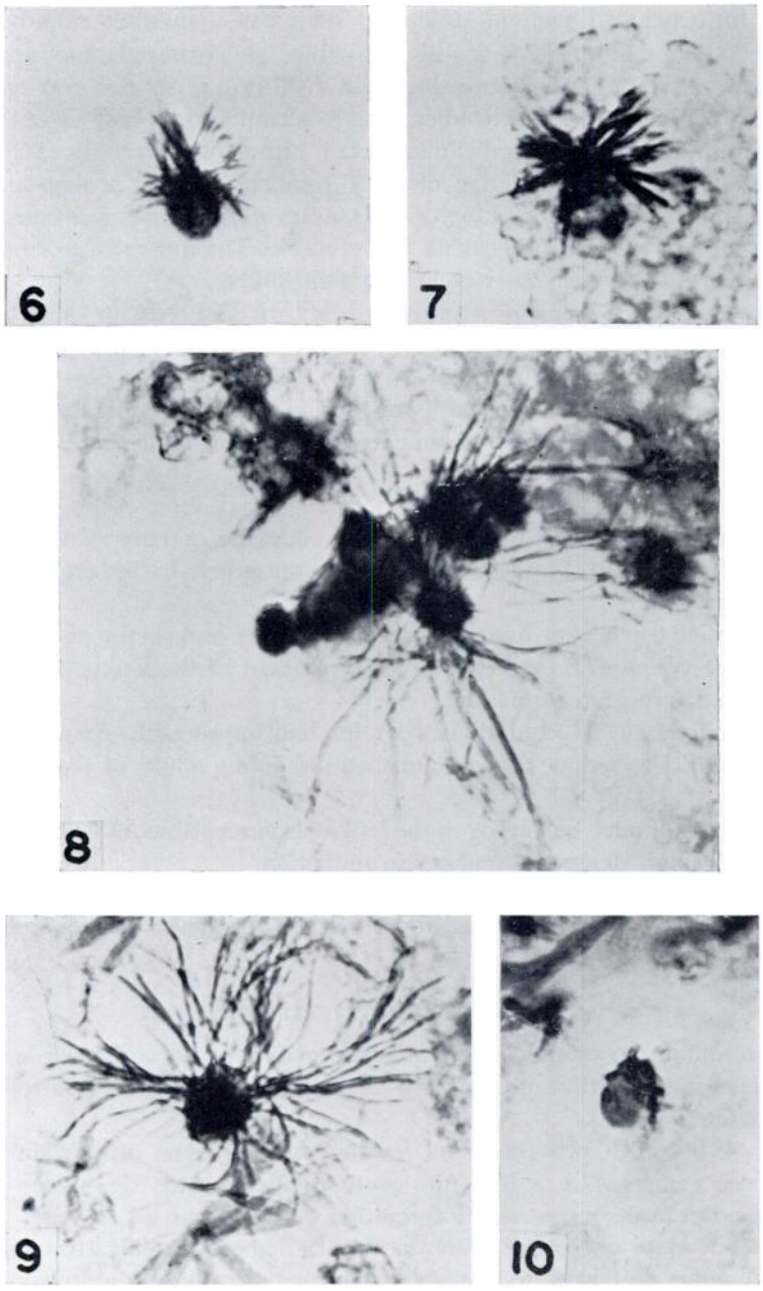
Microscopic appearance.—The formazan crystals in the reacted blood of young adult mice and rats were long and slender, and dark red in color. Crystals were not restricted to one type of cell but were found in both multilobular and monolobular cells. No further identification of leukocytes for computation was made in this study. Figures 6 and 7 represent typical reactions. Some cells contained such an abundance of needles that the shape of the nucleus was obscured. In such cases, however, the blueness of the hematoxylin-stained nucleus could be seen in the midst of the crystals thereby verifying the association of crystals with cells. The majority of the formazan crystals in the blood of infant mice were microscopically similar to those of the adults. In addition, however, to these typical needles, crystals of a different appearance were also found in practically all the infant blood. These crystals were longer, finer and lighter red in color than the needles of the adult animals (fig. 9). Many of these long crystals were not associated with cells. When such crystals were associated with cells, the cells were usually lymphocytes. Occasionally, lymphocytes of some adult animals showed a few such crystalline formations. Another feature of the blood of some of the infant mice was the aggregation of lymphocytes (fig. 8). Crystals were occasionally encountered in the leukocytes of older animals which were different in appearance from those of young adults. Instead of appearing as long needles protruding from the cell the crystals were in the form of sparse nodular chains which sometimes partially or wholly encircled the cell (fig. 10).

Discussion

The present observations show that leukocytes of some of the lower animals possess the ability to reduce a tetrazolium salt and also that this ability depends upon the strain, age and species.

Leukocytes from the two strains of mice which are relatively resistant to leukemia, $C3H_f$ and $BALB_f$, had a lower capacity for reducing a tetrazolium salt than did leukocytes from mice of the AKR strain, a strain with a high incidence of spontaneous leukemia. Whether the difference between the strains has a genetic or extrachromosomal basis is not established by the data of this experiment. Wachstein,[4] however, observed that leukocytes of patients with infectious diseases showed an increased amount of tetrazolium reduction.

Age affected the amount of tetrazolium reduction. The percentage of leukocytes showing reducing ability was especially low in infant mice, was highest in young adult mice and then decreased in old age. This decrease was more pronounced in males than in females of the strain. Less formazan deposition may be due to a lower oxidative activity in the newborn and the old animal.



FIGS. 6–10.—Formazan in leukocytes. Counterstained with Harris' hematoxylin (× 1300). 6, Lymphocyte from AKR male—reactivity 82.0 per cent; 7, polymorphonuclear cell from $C3H_f$ male—reactivity 68.6 per cent; 8 and 9, lymphocytes of infant $C3H_f$ female approximately 66 hrs. old—reactivity 18.8 per cent; 10, lymphocyte of $C3H_f$ female, 12 to 14 months old—reactivity 48.4 per cent.

The histochemical method described here has a number of advantages. The necessary equipment is readily available. The manipulations are simple and easily mastered. The tetrazolium salt INT is rapidly reduced and gives rise to large, highly colored needles. The localization, however, is not precise. For studies of precise intracellular detail nitroneotetrazolium (NNT) has been used[5-6]; but the reduction of NNT requires a long incubation period, and the small size of the resulting crystals makes positive cells less conspicuous. Therefore, for rapid counting, INT is preferable. The method can be adapted further for the study of other cell-containing fluids.

This work differs from most previous work on reduction in that the tissue is not prefrozen and also in that no specific substrate is provided. Thus it becomes a general measure of an over-all "reductase" reaction and has no claim for specificity. Additional work using varied temperatures, specific substrates, and inhibiting and enhancing agents could be devised.

Summary

A method is presented for studying the reducing activity of leukocytes of mice and rats using 2-(p-iodophenyl)-3-)p-nitrophenyl)-5-phenyltetrazolium chloride (INT).

Between 48.0 per cent and 83.8 per cent of the leukocytes of young adult mice and between 47.8 per cent and 68.0 per cent of the leukocytes of young rats showed formazan formation.

Infant mice of $C3H_f$ and $BALB_f$ strains had appreciably fewer leukocytes with the ability to reduce tetrazolium than did young adults of their respective strains.

Healthy adult mice belonging to the leukemia susceptible AKR strain showed a relatively high degree of leukocytic reduction.

Old mice of the $C3H_f$ strain showed less formazan formation than young adult animals. This decrease was more marked for the male members of the species.

Summario in Interlingua

Es presentate un methodo pro studiar le activitate reductori del leucocytos de muses e rattos, utilisante chloruro de 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium.

Inter 48,0 e 83,8 pro cento del leucocytos de juvene adultos in le caso del muses e inter 47,8 e 68,0 pro cento del leucocytos de juvene adultos in le caso del rattos monstrava le formation de formazan.

Muses neonate del racias $C3H_f$ e $BALB_f$ habeva significativemente plus basse numeros de leucocytos capace a reducer tetrazolium que juvene adultos del mesme racias.

Normal muses adulte del racia (AKR (que es susceptibile de contraher leucemia) monstrava un relativemente alte grado de reduction leucocytic.

Muses de etate avantiate in le racia $C3H_f$ monstrava minus formation de formazan que juvene adultos del mesme racia. Iste diminution esseva plus marcate pro masculos.

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