Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 329-335

USE OF [^{75}Se]-METHIONINE AS A TRACER OF THROMBOCYTOPOIESIS
II- Kinetics in normal rats and in platelet disorders in man: a new approach

E. DASSIN, N. ARDAILLOU, A. EBERLIN,
J. BOUREBIA and Y. NAJEAN

Formation associée n° 4, I.N.S.E.R.M.
Hôpital Saint-Louis, 2 place du Dr A.
Fournier, 75010 Paris, FRANCE

Received December 12,1977

SUMMARY: We have shown in the rat and in man that it is possible to determine the life span of autologous platelets and to quantify the production of these circulating cells by injecting [^{75}Se] -methionine and analysing the overall platelet radioactivity curves obtained: platelets and their precursors are labelled in vivo by adsorption of circulating plasma proteins onto their membrane, and in the marrow, principally during synthesis of thrombosthenin. This last phenomenon takes place 2 to 3 days before platelet release into the rat blood circulation and 5 to 6 days in the case of man. When there is a greatly increased production, the maturation time is slightly decreased.

INTRODUCTION

[^{75}Se] -methionine has been used as a cohort label of the platelet population, in order to quantitatively measure the platelet production. Several authors quantify thrombocytopoiesis in man and animals by measuring the rate of incorporation of this tracer in the circulating platelets, either when radioactivity is at a maximum (1) or at the 24th hour after its infusion (2).

We are presenting here a study of [^{75}Se] -methionine-labelled platelets in the circulation during their development and decay. The study has enabled us to distinguish between platelets labelled by one or both of the following two pathways: direct megakaryocytopoietic labelling from the injected amino-acid analogue, and delayed labelling from the radioactive plasma proteins. It permits us to determine the cell life span and to quantify the production of these cells.

MATERIALS AND METHODS

Male Wistar rats weighing between 250 and 300 g were used. They were fed on standard laboratory pellet food. [^{75}Se] -methionine (0.2-0.4 mCi/mM) or methyl [^{3}H] -methionine were purchased from C.E.A., Saclay, and injected through the tail vein in a dose of 5-10 µCi/100g body weight.

Blood samples were obtained by cardiac puncture under etner anaesthesia using plastic syringes containing acidified citrate solution (Aster's formula: Trisodium citrate 4.48g, Citric acid 2.73g, D-glucose anh. 2.00g, distilled water 100 ml). One volume of anticoagulant was used for 9 volumes of blood. 5 to 7 ml of blood was then transfused to plastic tubes and centrifuged at

0006-291X/78/0812-0329$01.00/0

400g for 10 minutes. The platelet containing plasma or supernatant was harvested slowly, leaving the lower part, after each centrifugation. Platelet-rich plasma was centrifuged at 1500g for 10 minutes and the platelet pellet washed twice in 0.154 M NaCl containing 10% acidified citrate solution. The haemoglobin concentration was inferior to 3.3 mg/ml and no leucocytes were visible by optical microscopy in any experiment.

Isolation of soluble platelet proteins: The platelet pellet was resuspended in 0.15 M Tris-HCl pH 7.5 and fragmented by freezing and thawing four times in liquid nitrogen. The homogenate was then centrifuged at 28,000g for 30 minutes to remove intact platelets and debris. Gel filtration of the supernatant or of plasma samples was carried out at 4°C with Sephadex G200 (Pharmacia, Uppsala, Sweden), using a column 35cm long and 2.5 cm in diameter; elution was performed with 0.1 M Tris-HCl pH 7.5, 1 M NaCl, at a rate of 15 or 20 ml per hour. Spectrophotometric absorption at 280 nm and the radioactivity of each 3.5 ml eluate fraction were measured.

Fibrinogen was isolated by thrombin precipitation: thrombin (2 U/ml) was added slowly to the first protein peak isolated from Sephadex chromatography or to plasma samples. The samples were incubated at 37°C for 30 minutes and the clot was removed with a glass rod, washed with distilled water and dissolved in alkaline urea (6.7 M urea, 0.2 M NaOH); the O.D. at 280 nm and the radioactivity were then determined.

Thrombosthenin, a specific platelet protein, was extracted by the technique described by Nachman et al. (3) and isolated by chromatography on Sephadex G200, using 0.015 M Tris-HCl pH 7.4, containing 0.6 M KCl, in a 35 cm long and 2.5 cm diameter column. The O.D. at 280 nm and the radioactivity of each 3.5 ml eluate fraction were measured.

In studies with human subjects 1.5 µCi/kg body weight of [^{75}Se] -methionine was injected. The procedure of platelet extraction was similar to that used after infusion of platelets labelled *in vitro* with [^{51}Cr] (4). The radioactivity of the pellets obtained daily for 20 to 25 days was measured and the appropriate corrections made for possible variations, from one preparation to another.

[^{75}Se] was measured in a well-type scintillation counter to an accuracy of at least ± 2% and [^{3}H] was measured by liquid scintillation with an accuracy of ± 3%.

RESULTS AND DISCUSSION

A- Rat studies: We have chosen to use methionine as a tracer of thrombocytopoiesis, since it is known that megakaryocytes synthetize a specific protein, thrombosthenin, which represents 15 to 20 % of the platelet proteins and is very rich in methionine (5); [^{75}Se] was used as a tracer, because of its physical properties (γ emission, half-life) which could enable its use in human pathology. However, it is known that an amino-acid and its analogue could not be incorporated in the same manner during protein synthesis, because the methionine-t-RNA synthetase does not have the same affinity for the amino-acid and its analogue (6). Using rats, we have compared the *in vivo* behaviour of [^{75}Se] -methionine and methyl [^{3}H] -methionine: the catabolism of the labelled proteins is the same, 5.5 to 6% per day, reflecting the renewal of the methionine pool in the organism (7). The rate of incorporation and the observed curves of platelet-bound radioactivity are similar (Fig 1). The concentration of the labelled plasma proteins in the intra- and the extra-vascular spaces

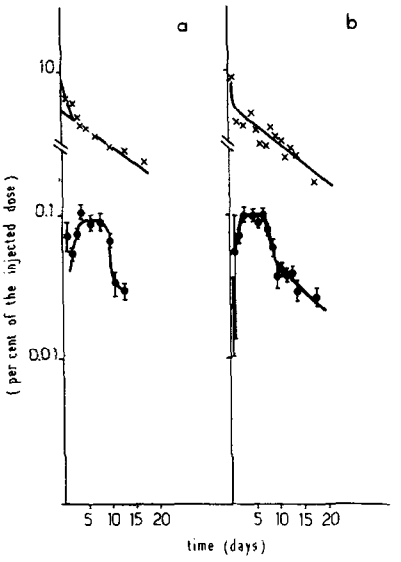

Fig 1. Changes in plasma (X - X) and platelet (. - .) radioactivity with time:
(a) Methyl [^{3}H] -methionine injected, (b) [^{75}Se] -methionine injected. Each point represents the mean obtained for 10 rats. (±1 s. d.).

is the same, as was calculated from the ratio of the levels of radioactivity determined from the exponential curves of radioactive decay (Fig 1). These results justify the use of [^{75}Se] -methionine as a tracer for the physiological study of thrombocytopoiesis.

Moreover, two hours after the injection of [^{75}Se] -methionine, 98.0±1.8% of the radioactivity is bound to plasma proteins (fibrinogen and principally albumin). After this, the specific plasma protein radioactivities decrease (Fig 2, a and b). Two of the three isolated platelet protein fractions have biochemical and immunochemical characteristics which identify them as fibrinogen and albumin (8). The loss of radioactivity from these two proteins (Fig 2, c and d), which are known to be adsorbed onto the plasma membrane of the platelets is identical to the loss of radioactivity from the two similar plasma fractions. However, the increase in specific radioactivity leads to a maximum observed only on the 3^{d} day after the tracer infusion, although the plasma protein radioactivity reaches a maximum as early as the 2^{d} hour; there is no decrease of platelet-bound albumin radioactivity after repeated washings of in vivo-labelled platelets.

These results suggest that the adsorption of labelled plasma proteins by the platelets occurs as the same time as liberation of these cells by the bone marrow.

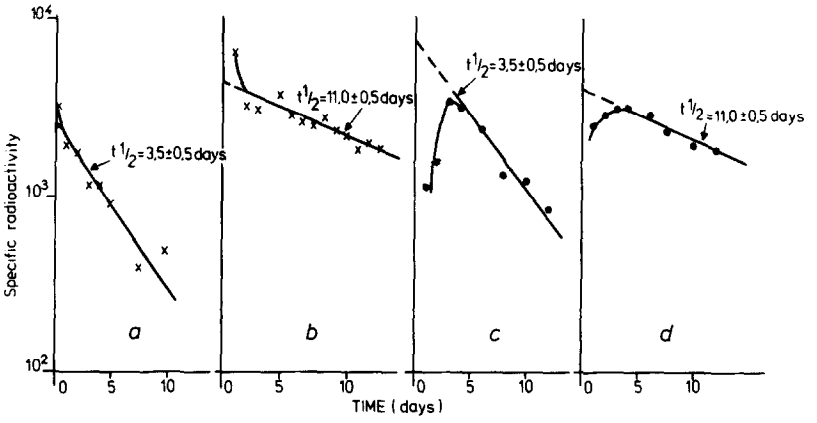

Fig 2. Changes in specific radioactivity of plasma proteins (X - X): (a) fibrinogen, (b) albumin, and of soluble platelet proteins (. - .): (c) and (d). Each point represents the mean obtained for three experiments (±1 s.d.).

We have isolated a third platelet proteic fraction, probably thrombosthenin (8), which is synthetized by the megakaryocytes. No radioactivity was found until 60 hours after infusion of the label, when an increase of radioactivity in this protein was noticed. The maximum value was attained at the 72^{d} hour and maintained until the 192^{d} hour, when radioactivity decreased, suggesting that the cells have a given life span which varies little.

These data suggest that some of the tracer is directly bound to the maturing megakaryocytes, liberated into the circulating blood, as platelet structural protein, and disappears at the time of cell death. Another fraction was radioactive plasma proteins, adsorbed onto the platelet membranes, the cells continously being released from the bone marrow. This coating of adsorbed proteins could possibly disappear randomly (exponentially), during the cell's life in the blood circulation.

Eight days after the tracer infusion, all the initially labelled cohort has disappeared, and the further decrease of the platelet radioactivity indicates the removal of plasma protein; the curve is described by an exponential equation:

$$A(t) = A\, e^{-\lambda t}$$

where λ, the catabolic rate of the radioactivity adsorbed to the platelets, can be experimentally determined, and A calculated.

B- Human studies: Fig 3 shows the fate of plasma protein radioactivity in one normal case (Fig 3,a) and in three pathological cases (Fig 3,b, c, d). It is possible, on the same manner, to reconstitute the curve of platelet radioactivity due to adsorption of plasma proteins onto the platelet membranes

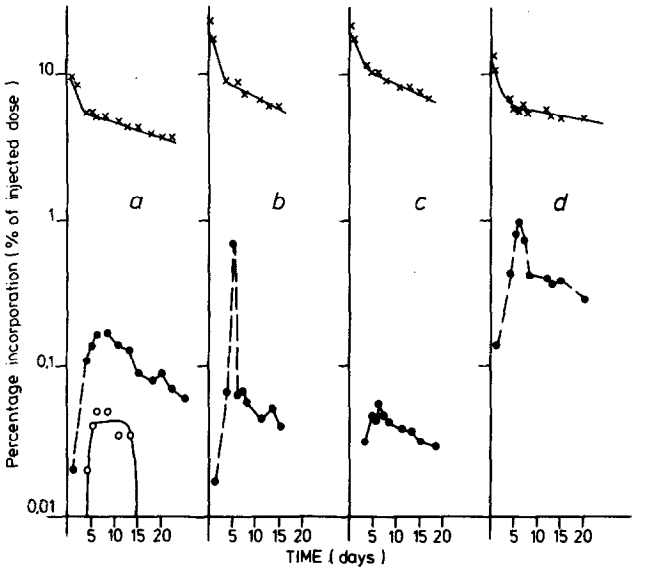

Fig 3. Fate of plasma protein radioactivity (X - X), circulating platelet bound radioactivity (. - .) and corrected platelet curve (o - o) in one normal case (a), and in three pathological cases: case n° 9 (b), case n° 15 (c) and case n° 20 (d).

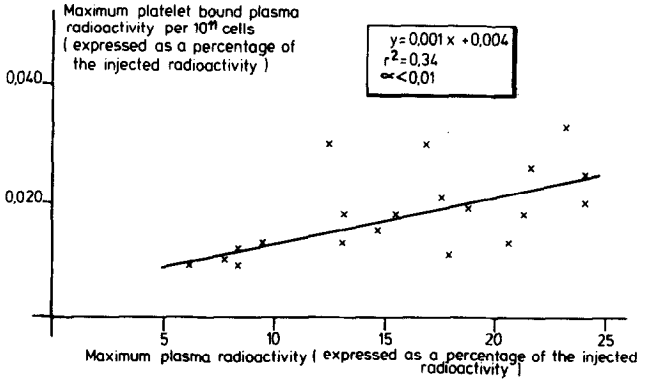

Fig 4. Correlation between maximum plasma radioactivity obtained 3 hours after [^{75}Se] -methionine injection and maximum plasma radioactivity adsorbed on to 10^{11} cells.

and we have observed that there is a strict correlation ($p \leqslant 0.01$) between the platelet-bound albumin radioactivity at its maximum and the specific radioactivity of the circulating plasma albumin at its maximum (Fig 4).

In 21 cases, using this method, we have found a good reproducibility

Table 1. Results obtained in 21 human cases. The diagnoses were: chronic granulocytopenia (cases n° 1 and 2), polycythaemia vera ([^{32}P]induced remission) (case n° 3), acute myelogenous leukaemia (cases n° 4, 5 and 14), intermittent thrombocytopenia (cases n° 6 and 7), primary chronic erythroblastopenia (case n° 8), idiopathic thrombopenic purpura (cases n° 9, 10, 12 and 13), constitutional thrombocytopenia (cases n° 11, 15 and 19), bone marrow tuberculosis (case n° 16), congenital thrombocytopenia (case n° 17), aplastic anaemia (case n° 18) and primary thrombocythaemia (case n° 21).
Life spans were determined by injected autologous (A) or isologous (I) platelets labelled *in vitro* with [^{51}Cr]CrO_4Na_2.

PATIENTS	PLATELET COUNT $\times 10^{-10}$/l	BONE MARROW MEGAKARYOCYTES	LIFE SPAN (days) of [^{51}Cr]CrO_4Na_2 labelled platelets	LIFE SPAN (days) of [^{75}Se]-Met labelled platelets	PLATELET PRODUCTION *
n° 1	27±3	N	-	8 - 9	0.04±0.01
2	28±4	N	-	9 -10	0.05±0.01
3	25±4	N	-	9 -10	0.05±0.01
4	30±3	↗	-	8 - 9	0.04±0.01
5	23±2	N	-	8 - 9	0.04±0.01
6	25±2	N	7±1 (I)	8 -10	0.04±0.01
7	30±4	N	-	9 -10	0.05±0.01
8	25±2	N	-	8 - 9	0.05±0.01
9	11±3	↗	2±1 (A)	1	0.73±0.03
10	1±0.5	↗	2±1 (I)	1	1.79±0.06
11	5±1	↗	8±1 (I)	1 - 2	0.14±0.06
12	10±2	N	2±1 (I)	1 - 2	0.16±0.05
13	11±3	↘	2±1 (I)	1 - 2	0.30±0.05
14	13±2	N or ↘	-	7 - 8	0.03±0.01
15	6±1	N	9±1 (I)	8 - 9	0.01±0.005
16	5±1	N	8±1 (I)	4 - 5	0.01±0.006
17	2±1	↘	6±1 (I)	4 - 5	0.01±0.005
18	7±2	↘	-	3 - 4	0.03±0.01
19	6±2	↘	7±1 (A)	7 - 8	0.01±0.006
20	76±4	↗	3±1 (A)	3 - 4	0.54±0.20
21	90±5	↗	6±1 (A)	6 - 7	0.32±0.12

* expressed as the percentage of the injected radioactivity.

of the results in the normal subjects (patients without haematological disease). Moreover, we have found a good correlation between the platelet number with their destruction and production rate (Table 1).

However, in 5 cases with splenomegaly, thrombocytopenia was associated with normal or subnormal platelet life span, and low methionine uptake, expressed as a percentage of incorporation into the circulating platelets; these facts cannot be interpreted as a production defect but as an abnormality of the ratio of those circulating to those in marginal platelet compartments, as has been clearly observed in hypersplenism (9). In such patients, in which the platelet concentration in the blood circulation could not be a true representation of the total platelet volume, an independant measurement of the platelet compartments is needed before interpretating the results given by the [^{75}Se] -methionine kinetic study.

The method also enables the measurment of the time elapsing between tracer infusion and the appearance of the labelled cohort in the circulation. From our preliminary results, this time appears to be constant at 5 to 6 days in normal subjects and in patients with production defects, but it decreased

in those cases in which a hyperdestruction was proved. Such an approach to the maturation defects of thrombocytopoiesis in man could be useful in pathology.

ACKNOWLEDGEMENTS: We are grateful to A. J. BARRETT, Leukaemia Research Fund Fellow, for English translation.

REFERENCES

1. Levin, J., Shreiner, D.P., and Evatt, B.L. (1971) Platelet kinetics, pp. 126-132, North Holland Publish., Amsterdam, London.
2. Penington, D.G. (1971) Platelet kinetics, pp. 143-147, North Holland Publish., Amsterdam, London.
3. Nachman, R.L., Marcus, A.J., and Safier, L.B. (1967) J. Clin. Invest. 46, 1380-1389.
4. I.C.S.H. (1977) Br. J. Haemat. in press.
5. Booyse, F.M., and Rafelson, M.E. (1971) Ser. Haemat. 4, 152-174.
6. Chapeville, F., and Rouget, P. (1972) The mechanism of protein synthesis and its regulation, pp. 5-32, North Holland Publish., Amsterdam, London.
7. Waterlow, J.C., Garrow, J.S., and Millward, D.J. (1969) Clin. Sci. 6, 489-504.
8. Dassin, E., and Najean, Y. in press.
9. Aster, R.H. (1966) J. Clin. 45, 645-657.