Discussion

A causal relationship between methysergide treatment and otherwise unexplained retroperitoneal fibrosis—a rare condition—has now been widely accepted. In conditions as common as pleural effusions and fibrosis the diagnosis of drug-induced disease is more difficult to prove and rests mostly on circumstantial evidence. In both of our cases pleural effusions and fibrosis developed while the patient was receiving methysergide, and rapid improvement followed when the drug was stopped.

The most striking feature in these cases was the degree of radiological clearing of pleural fibrosis after cessation of methysergide therapy, particularly in Case 2. This is an uncommon occurrence in pleurisies of other origins but in accordance with the regression observed in retroperitoneal fibrosis due to methysergide.

The number of people treated for migraine is considerable and presumably many of them receive methysergide. In cases of otherwise unexplained "pleurodynias," pleural effusions, and fibrosis the possibility of drug induction by methysergide should be considered.

We wish to thank Dr. D. S. Freestone for his valuable advice on the properties and hazards of methysergide.

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Preliminary Communications

Isotope Bioassay for "Thrombopoietin"

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Summary: An assay system has been developed in mice for the humoral agent "thrombopoietin." This is based on utilization in newly-formed platelets of the radio-amino-acid ^{75}Se selenomethionine 24 hours after intravenous injection. The possibility that the results could be due to the effects of species antibody, to foreign protein, or to an effect primarily on selenomethionine metabolism, has been explored, but the findings are consistent with the presence of a physiological humoral factor. Thrombopoietin has been shown to be present in the plasma of patients suffering from severe thrombocytopenia of varied aetiology.

Introduction

Evidence suggesting the existence of a new humoral agent, "thrombopoietin," has been reveiwed in earlier publications (de Gabriele and Penington, 1967a, 1967b). This agent, which is analogous to the physiological regulator of red cell production, "erythropoietin," appears to subserve a physiological role in the regulation of platelet production.

Hitherto attempts to demonstrate the presence of the hormone in plasma have rested on finding a raised platelet count in recipient animals, the increases being relatively small, of the order of 20 to 25% over the resting platelet count (Kelemen *et al.*, 1958; Linman *et al.*, 1959; Rák *et al.*, 1959; Odell *et al.*, 1961; Spector, 1961; Schulman *et al.*, 1965; McClure and Choi, 1968). Errors inherent in all visual methods of counting platelets are considerable even under the most carefully controlled conditions (Hellem, 1960), and in many of the studies referred to above the technique used for counting platelets is not quoted; the possibility of observer bias in visual counting methods is always present unless specific precautions are taken to avoid this.

The results reported have been very far from consistent, with claims of raised thrombopoietin in polycythaemia with thrombocytosis (Linman *et al.*, 1959) and in both polycythaemia vera and essential thrombocythaemia (Kelemen *et al.*, 1961); these are reminiscent of the early conflicting reports of the role of erythropoietin in similar conditions using assays dependent on visual counting of red cells and reticulocytes. Many of these findings were subsequently disproved once an objective and quantitative assay system for erythropoietin was available, using the isotope ^{59}Fe. Abildgaard *et al.* (1967), working in Schulman's laboratory, noted difficulty in reproducing their earlier demonstration of thrombopoietin based on platelet counting in rats, and there is clearly a need for a reproducible and quantitative method of assay for thrombopoietin. Until such a method is available little progress can be made in studying the chemical and physiological nature of the hormone, and confusion will remain regarding its role in the genesis of human disease.

The radio-amino-acid, ^{75}Se selenomethionine, has been shown to label in vivo the formed elements of the blood, including platelets (Cohen *et al.*, 1965; Penner, 1966), and Evatt and Levin (1968) have reported, in abstract form, that plasma from thrombocytopenic rabbits increased the percentage utilization of the label in the circulating platelets of recipient animals. This technique has now been applied to establish a bioassay for thrombopoietin in human plasma.

Subjects and Methods

The techniques of platelet counting, sampling, labelling with ^{75}Se selenomethionine, and calculation of utilization of the isotope were as previously described (Penington, 1969). Male C57 mice weighing 20-26 g. were injected with 0·002 ml. of an antiserum to mouse platelets (prepared in rabbits as described previously) one week before beginning the assay. The animals were injected subcutaneously with varying doses of plasma from thrombocytopenic donors. The *maximum* dose for a single injection was 0·5 ml., given subcutaneously under light anaesthesia. The timing of injections of plasma and ^{75}Se selenomethionine varied in the different studies as indicated. In a small group of animals the assay was carried out without previous injection of antiplatelet serum.

The *routine assay procedure* consisted of twice-daily injections of 0·1 or 0·5 ml. of plasma into groups of five animals for two days, the animals having been prepared with antiplatelet serum a week before the first injection. On the third day 2 μCi of ^{75}Se selenomethionine was injected intravenously, and determination of the utilization of the isotope in platelets was performed 24 hours later by the method previously described (Penington, 1969).

Human subjects were bled into acid-citrate-dextrose anticoagulant in the proportion of 1 ml. to 9 ml. of blood. The plasma was separated by centrifugation and stored at −10°C. until required for assay. The donors with thrombocytopenia all had platelet counts of less than 40,000/cu.mm.; the causes of the thrombocytopenia are shown in Table I. Plasma from five normal donors was similarly separated and stored; these subjects had platelet counts between 215,000 and 360,000/cu.mm. All samples were assayed within three weeks of their being obtained from the donor except where otherwise indicated.

RESULTS

Timing of Plasma Injection.—Plasma from a thrombocytopenic patient with acute myeloid leukaemia was used in this study. Groups of six mice received two daily injections of 0·5 ml. of plasma on the first, second, and third days (group 1), on the second and third days (group 2), and on the second, third, and fourth days, (group 3). ^{75}Se selenomethionine was administered on the fourth day, and it was found that utilization of the isotope after 24 hours in the three groups did not differ significantly, suggesting that injection over two days gave satisfactory results. Injection of the same daily dose of plasma for one day only (group 4) gave a significantly lower utilization of the isotope ($P<0·01$), but this smaller total dosage gave the same stimulation of isotope utilization when spread over two days—that is, four injections of 0·25 ml. (group 5). The results are summarized in Fig. 1.

Dose-response Relationship with Human Plasma.—With the injection schedule described above for the routine assay procedure, a dose-response relationship was found between the total volume of human plasma administered and isotope utilization (Fig. 2). The results with each dose represent mean and standard deviation in groups of 8 to 12 mice. The stimulation achieved after a total dose of 0·4 ml., 1 ml., and 2 ml. of plasma differed significantly from that observed in uninjected animals ($P<0·01$ for the lower doses and $P<0.001$ for the greatest dose).

Group	Injection Schedule				24-hr. ^{75}Se Utilization *	Animals
1	■■	■■	■■		5·83 ± 0·75	6
2		■■	■■		6·06 ± 0·83	6
3		■■	■■	■■	5·40 ± 0·65	5
4		■■			4·34 ± 0·88	5
5		■■	■■		4·27 ± 0·93	6
6	no injection				2·01 ± 0·83	6
Day	1	2	3	4	5	

FIG. 1.—Timing of injections and thrombopoietic response. (*Utilization % × 10^{-2}.)

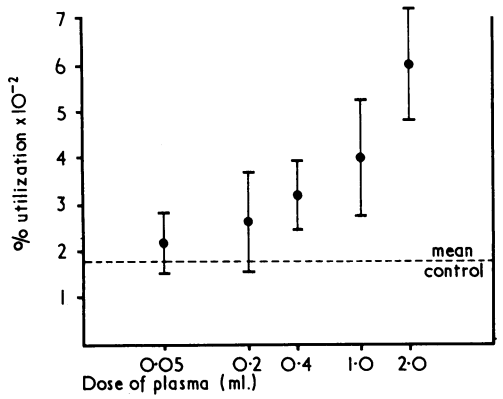

FIG. 2.—Dose-response curve with standard assay procedure.

Effect of "Thrombocytopenic Plasma" on Plasma ^{75}Se Selenomethionine Activity.—Plasma radioactivity was unaffected by administration of plasma from thrombocytopenic patients. Counts per minute per millilitre of plasma on completion of the assay in animals receiving a total dose of 2 ml. of "thrombocytopenic" human plasma compared with animals receiving no injection were:

Plasma counts in "stimulated" animals: $2·448 \times 10^6 \pm 0·409$ (mean and standard deviation in 11 animals).
Plasma counts in uninjected animals: $2·461 \times 10^6 \pm 0·229$ (mean and standard deviation in 12 animals).

No correlation was observed between plasma radioactivity and platelet radioactivity in any of the assay studies.

Effect of "Thrombocytopenic Plasma" on Platelet Count and Haematocrit in Assay Animals.—Platelets counted from leg veins during the course of the assay and on completion of the assay showed no reduction following the administration of plasma from any of the donors listed in Table I. Plasma in a dose of 0·1 ml. (four injections) produced no significant fall in packed cell volume in the recipient animals, but in a dose of 0·5 ml. per injection from both normal donors and thrombocytopenic donors it caused a significant fall in the recipients—a mean level of 42·6% in uninjected animals compared with 35·1% in injected animals. In none of the animals studied was there a significant rise in platelet count compared with control animals at the completion of the assay. Mean platelet count in these animals was 1,060,000/cu.mm. (standard deviation ± 180,000).

TABLE I.—*Thrombopoietin Assay in Human Plasma*

Diagnosis	Ratio % utilization stimulated: control	
	0·4 ml.	2·0 ml.
Idiopathic thrombocytopenia	1·07	2·15
Idiopathic thrombocytopenia	0·90	2·42
Aplastic anaemia	1·40	2·32
Hodgkin's disease, hypersplenism	1·65	2·61
Myeloblastic leukaemia	1·87	3·56
Normal subject 1	1·20	1·31
" " 2	1·35	1·40
" " 3	1·10	1·31
" " 4	1·23	1·33
" " 5	0·86	1·55

Effect of Mouse Red Cells on Plasma Thrombocytopenic Activity.—The possibility that the apparent thrombopoietin effect was due to species antibody to mouse cells in human plasma was tested by incubation of both "active" and normal human plasma with washed mouse red cells at 37°C. for one hour. No diminution in thrombopoietic activity was noted following this procedure with either the "active" plasma used in establishing the dose-response curve or in the slight activity present in normal human plasma.

Comparison of Assay in Antiserum-Treated and Normal Mice.—Plasma from the same subject used in the above study and normal human plasma were assayed in groups of normal mice and mice treated with antiplatelet serum (A.P.S.). The results are shown in Table II.

TABLE II.

Material assayed	Percentage Utilization × 10—2	
	Normal Recipient Mice	A.P.S.-Treated Mice
"Thrombocytopenic" plasma	6·45 ± 2·19	6·06 ± 0·83
Normal plasma	3·36 ± 0·79	1·99 ± 0·57
Uninjected animals	2·42 ± 0·67	2·01 ± 1·04

Stability of Thrombopoietin in Plasma on Storage.—Despite storage of plasma at −10°C. consistent loss of activity was noted over periods of one to three months. The same plasma giving a utilization of 6·21 x 10^{-2} per cent. in the first three weeks of storage gave a utilization of only 4·32 x 10^{-2} per cent. after eight weeks. A second plasma giving a utilization of 5·96 x 10^{-2} per cent. initially gave a figure of 3·15 x 10^{-2} per cent. after an interval of 10 weeks' storage.

Assays of Plasma from Thrombocytopenic and Normal Human Subjects.—All samples were assayed at two dose levels, with a total dosage of 0·4 ml. and 2 ml. in four injections over two days, as described in the routine assay procedure above. The results have been expressed in terms of a ratio of mean percentage uptake in the assay animals to mean percentage uptake in five uninjected mice used as controls in each assay. The findings are shown in Table I and in Fig. 3 (those in the latter being recorded as percentage utilization). The stimulation produced by plasma from thrombocytopenic donors was highly significant ($P<0.01$) at a total dose of 2 ml. of plasma and was significant also ($P<0.02$) in three instances in a dose of 0·4 ml. Normal human plasma failed to produce significant stimulation in any instance in a dose of 0·4 ml., while in the higher dosage the slight increase in utilization of the isotope was less in each instance than that produced by plasma from thrombocytopenic donors and differed significantly from the uninjected controls in only two of the five plasma samples tested ($P<0.02$).

Discussion

The possibility that foreign protein might induce stimulation of platelet production was raised by Odell *et al.* (1964). This led to serious doubts about the significance of assays of thrombopoietin in human plasma, but the present findings strongly suggest that human plasma in states of thrombocytopenia does indeed contain a specific stimulus to platelet production. Thus plasma from subjects with thrombocytopenia stimulates platelet production to a greater degree than is the case with plasma from normal donors, and the fact that the biological activity is not removed by mouse red cells and is lost on storage over several months at low temperatures further suggests that the phenomenon is due to a specific factor rather than an effect of species antibody or foreign protein administration.

The optimal time conditions for assay of thrombopoietin appear to be very similar to those which have been used in studies of erythropoietin, and the dose-response relationship is also similar to that seen with low dosage of the hormone when erythropoietin is assayed by means of iron utilization at 24 hours (White *et al.*, 1960). Larger doses of plasma were not administered in the present study, as at at the highest dose moderate anaemia developed in the assay animals; this was presumed to be due to haemodilution rather than haemolysis since previous incubation of plasma with mouse red cells had no effect on this phenomenon. Further studies providing more complete evidence concerning the dose-response relationship suitable for mathematical analysis must await the availability of concentrates of thrombopoietin.

In the standard assay procedure the assay animals were treated with antiplatelet serum a week before beginning the assay in order that they could be used as recipients following the peak of the reactive thrombocytosis, at a time when platelet production was slowed (Penington, 1969). On comparison of the response of animals prepared in this manner with that of normal animals to administered thrombopoietin, no difference was noted, but animals used as controls showed a lesser utilization of isotope if prepared with antiserum, and these animals were less responsive to the effects of normal human plasma. Further investigation will be required to ascertain whether the slight stimulation of isotope utilization by normal human plasma is the result of a non-specific rather than a physiological stimulus.

The results of assay of plasma from patients suffering from thrombocytopenia indicate that thrombopoietin concentration in the plasma of these subjects must be at least five times that found in normal human plasma, and indeed it is possible that the titre of the hormone is many times greater than this. The results presented are the first in which a dose-response relationship has been established, and in each instance the higher dose of plasma from a thrombocytopenic donor has given a significantly higher utilization than seen at the lower

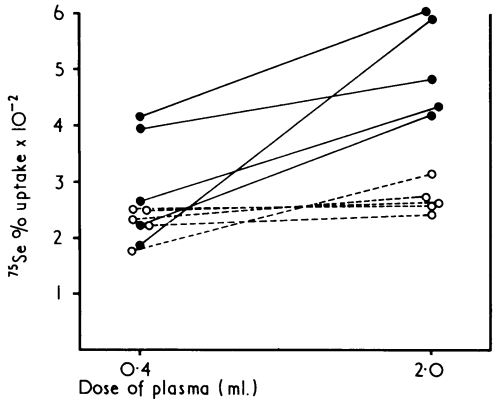

FIG. 3.—Assays of thrombopoietin. Solid lines represent thrombocytopenic plasma; and dotted lines represent plasma from normal donors.

dose, further confirming the physiological significance of the findings. The present assay method opens the way for studies of the significance of thrombopoietin in human disease, and provides a tool for the investigation of the chemical nature, origin, and biological significance of this humoral agent.

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