Histochemical Demonstration of 5-Hydroxytryptamine in Platelets and Megakaryocytes

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Rand AND Reid \(^1\) were the first to show that circulating 5-hydroxytryptamine (5-HT) is concentrated in the blood platelets. Under normal conditions, there is no detectable 5-HT in the circulating blood components other than platelets. Clark et al.\(^2\) and Gaddum and Giard\(^3\) showed that both spleen and bone marrow are lacking the enzyme, 5-hydroxytryptophan (5-HTP) decarboxylase, and that a large amount of 5-HT is stored in the platelets without accompanying 5-HTP decarboxylase.\(^*\)

It is well established that platelets take up 5-HT from the surrounding fluid, both in vitro and in vivo, and that the portal blood contains more 5-HT than the blood of other areas. Furthermore, the observation of patients with malignant carcinoid tumors revealed an elevated level of 5-HT in platelets and an increased urinary excretion of 5-hydroxyindoleacetic acid.\(^4\) These findings suggest that platelet 5-HT originates from the enterochromaffin cells of the intestinal mucosa, not from the cell components of spleen or bone marrow.

It has been shown that the amount of 5-HT in the cells other than platelets in peripheral blood and bone marrow is too small to be detected biochemically. The highly specific fluorescence method developed by Falck and co-workers\(^5-7\) enables one to visualize the cellular localization of the biogenic monoamines. Thus there arises the possibility that 5-HT of bone marrow cells can be demonstrated by this method. The purpose of the present investigation is to demonstrate histochemically the biogenic monoamines in peripheral blood and bone marrow cells.

METHODS AND MATERIALS

The fluorescence technic of Falck and co-workers\(^5-7\) was used for demonstrating the cellular localization of biogenic monoamines. Although the technic was originally used for other tissues, it has now been extended, in the present experiments, to smear preparations of peripheral blood and bone marrow.

Peripheral blood and bone marrow of male rabbits weighing 1.8 to 2.2 Kg. and also
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those of humans were used. The peripheral blood was sampled in rabbits from the carotid artery and in humans from the cubital vein. The bone marrow was aspirated from the rib or femur in rabbits and from the sternum in humans. All glassware and needles used for collecting blood and preparing platelet suspensions were siliconized. Heparin (100 mg per cent in normal saline) was used as an anticoagulant. Platelet-rich plasma was prepared by differential centrifugation of whole blood at the low speed of 100 g for 20 minutes. A quantity of 0.02 ml of platelet-rich plasma was smeared on the nonfluorescent glass slide. The smears of peripheral blood, bone marrow, and platelet-rich plasma were allowed to dry in vacuo at a temperature below 10 C for 30 minutes, or in an air flow by the aid of an electric fan for 2 hours. The smears were then treated with formaldehyde vapor at 80 C for 1 hour in a closed glass vessel containing paraformaldehyde granules. During this treatment, the monoamines condense to yield a product, 6-hydroxy-3, 4-dihydro-β-carbol ine, which fluoresces intensively.1 The optimal reaction occurred when the paraformaldehyde had a humidity of about 60-70 per cent. Finally, the treated smears were mounted in liquid paraffin.

Microscopic analysis was performed with a binocular fluorescence microscope (Carl Zeiss). The exciting light was provided by an Osram HBO 200 high-pressure mercury lamp, and was filtered through a Schott BG 12. The secondary filter was a Zeiss “50.” Usually a Zeiss dark-field condenser was used. For microphotography, Kodak Tri X film was employed.

Quantitative estimation of 5-HT in blood was carried out spectrophotofluorimetrically after purification on a column of cation exchange resin (Amberlite CG 50-Type 1). Protein was estimated colorimetrically by measurement of the blue color produced by addition of Folin-Ciocalteu phenol reagent to an alkaline solution of protein.10

RESULTS

Cellular Localization of 5-HT in Peripheral Blood and Bone Marrow

Whole Blood. Formaldehyde treatment of blood smears of rabbits produced a bright yellow fluorescence in platelets but not in leucocytes and erythrocytes. The platelets were visible separately and/or in clumps among the strands of fibrin network, which showed only a dull autofluorescence. On human blood smears, platelets showed medium-intense yellow fluorescence, which was weaker than that of rabbit platelets, and occasionally a very weak but definite fluorescence of separate platelets was detected.

Platelet-Rich Plasma. In order to obtain specific fluorescence of individual platelets more distinctly than with whole blood, smears of platelet-rich plasma were processed. An intense yellow fluorescence developed in rabbit platelets which were seen abundantly in the smears (Fig. 1). The fluorescent substances were found packed to their full extent in the platelets. Due to this, the fine structure of the fluorescent substances could scarcely be seen. The background showed a very weak greenish-yellow fluorescence, probably due to the 5-HT released from the platelets. When rabbit platelet-rich plasma was incubated with reserpine (10^-6 Gm./ml.) for 2 hours at 37 C., the degree of fluorescence decreased sharply (Fig. 2). In human platelet-rich plasma a fairly intense yellow fluorescence was also seen in the platelets. After microscopic observation, the cover-glass was removed and the liquid paraffin was dissolved with xylene. The smear was then stained with May-Grünwald-Giemsa, confirming that the fluorescent particles were platelets.

Bone Marrow. Treatment of bone marrow smears with formaldehyde vapor produced a distinctive yellow fluorescence in the platelets and megakaryocytes.
Fig. 1.—Specific monoamine fluorescence in normal rabbit platelets; × 600 (enlarged 3 times).

Fig. 2.—The effect of reserpine on rabbit platelets in vitro is shown by the decrease of platelet size and of fluorescence intensity, which is evident by the change of color from yellow to greenish-yellow; × 600 (enlarged 3 times).

The fluorescence was diffused in the cytoplasm of megakaryocytes, partly sparing the noticeable nuclear shadow. Other cell components in the bone marrow aspirates showed no fluorescence at all or a dull green fluorescence. After microscopic observation, megakaryocytes were identified by May-Grunwald Giemsa staining.

Numerous yellowish fluorescent granules in rabbit megakaryocytes bore almost the same size, shape, and intensity or tone as those of platelets. The granules were seen packed in the megakaryocyte cytoplasm (Fig. 3). The
Fig. 3.—Specific monoamine fluorescence in a normal rabbit megakaryocyte; × 600 (enlarged 5 times).

Fig. 4.—Specific monoamine fluorescence in a control human megakaryocyte; × 600 (enlarged 5 times).

The intensity of fluorescence of human megakaryocytes was not so distinct as that of rabbit, but it was strong and specific enough to be distinguishable from the other cell components (Fig. 4).

The intensity of fluorescence in the cytoplasm of individual megakaryocytes was variable in degree, according to the morphologic maturation stage of the megakaryocytes. The fluorescence of the intermediate forms with no evidence of platelet budding was not as intense as that observed in the platelets and in the mature megakaryocytes with evidence of platelet production. The fluorescence of the intermediate megakaryocytes was seen diffusely in the
Specific fluorescence of a rabbit megakaryocyte was identified as 5-HT by treatment with sodium borohydride. The greenish-yellow fluorescence of the megakaryocyte disappeared after this treatment, but autofluorescence due to other cell components remained unchanged; × 600 (enlarged 3 times).

The effect of reserpine on rabbit megakaryocytes in an in vivo experiment is shown here by decrease of the intensity of fluorescence in the megakaryocyte cytoplasm; × 600 (enlarged 3 times).

cytoplasm. There was a tendency to an increase in the intensity of fluorescence along with the maturation of the megakaryocytes from intermediate to mature —i.e., platelet-producing—forms. No megakaryoblasts or promegakaryocytes showed any characteristic fluorescence in the preparations examined.

The Specificity of Fluorescence

The specificity of the yellow fluorescence of platelets and megakaryocytes
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was examined by the following histochemical and pharmacologic criteria:
(1) To obtain a highly specific fluorescence, optimal reaction time in formaldehyde vapor was 1 hour at 80 C. Optimal humidity was also an important factor. Dry formaldehyde gas could yield very weak or no fluorescence even if a large amount of 5-HT existed in the specimen. Conversely, an increased humidity of the gas above 80 per cent brought about only indistinct fluorescence, probably due to an increased diffusion of 5-HT. (2) With appropriate filters, the specific yellow fluorescence of 5-HT was observed. (3) Even in the course of the microscopic observation, the yellow fluorescence of human platelets and of immature megakaryocytes decreased rapidly on exposure to UV light. The fluorescence of the human specimens was too sensitive to UV light to be demonstrated in microphotographs. The strong yellow fluorescence of rabbit platelets and mature megakaryocytes also decreased considerably in several minutes. (4) When the preparations were moistened with water, the yellow fluorescence faded. (5) If the preparations were treated with 0.1-1 per cent sodium borohydride in 90 per cent isopropanol, which reduced 5-HT to nonfluorescent 1, 2, 3, 4- tetrahydroβ-carboline, the yellow fluorescence disappeared. As is shown in Figure 5, the fluorescence of rabbit megakaryocytes was not observed after sodium borohydride treatment. This fluorescence reappeared after dehydration and formaldehyde treatment. (6) Biochemical determination of 5-HT was performed using the spectrophotofluorometric method. Platelet-rich plasma of normal rabbits was found to contain 5.5 μg/ml of 5-HT. When this was incubated in vitro with reserpine 10⁻⁶Gm./ml for 2 hours at 37 C., the level of 5-HT decreased to about a half. Such release of platelet 5-HT by reserpine was demonstrated histochemically (Fig. 2). The photograph shows that the intensity of specific fluorescence of 5-HT in some platelets decreases, but other platelets remain resistant to the reserpine effect. In in vivo experiments, the preparations of rabbit blood platelets and of bone marrow megakaryocytes were obtained 16 hours after intravenous injection of reserpine (1 mg. per Kg. body weight). The content of 5-HT in platelets decreased from 2.51 to 0.01 μg/mg. protein. Histochemically, the fluorescence of rabbit megakaryocytes was decreased in intensity (Fig. 6). These findings indicate that the yellow fluorescence observed was due to 5-HT.

DISCUSSION

The histochemical fluorescence method of Falck and associates permits the direct demonstration at the cellular level of biogenic monoamines in various tissues in paraffin sections or stretch preparations. Recently, this histochemical technic has been applied to single neoplastic mast cells and to isolated nerve ending particles on smear preparations. We attempted to apply this technic to blood and bone marrow cells in smear preparations. Although some autofluorescence of erythrocytes and leukocytes was seen, the specific fluorescence of 5-HT was easily distinguishable by histochemical and pharmacologic criteria. The results obtained here indicate that the yellow fluorescence is due to 5-HT.

Although it is well known that platelets contain a large amount of 5-HT
without 5-HTP decarboxylase, it remained to be settled whether such enzyme and the amine are contained in megakaryocytes. A sensitive method for demonstrating the cellular localization of biogenic amines had not been available prior to the development of the fluorescence technic used here and also the demonstration of 5-HTP decarboxylase in single cells has not yet been achieved. Thus, it is of great interest that 5-HT was demonstrated here in smear preparations of both platelets and megakaryocytes.

The results obtained here indicate that there is a large amount of 5-HT in the platelets and in the mature megakaryocytes with presence of platelet budding, but only a relatively small amount of it is present in the intermediate forms of megakaryocytes which do not have evidence of platelet formation. The serotonin may be derived from (1) transport to the platelets and mature megakaryocytes of 5-HT formed elsewhere, and/or (2) 5-HT formation from 5-HTP during maturation of the megakaryocytes. However, the latter possibility that megakaryocytes would be able to synthesize 5-HT from 5-HTP, and that the 5-HT would be transferred to newly formed platelets, still remains unsettled, pending the demonstration of the enzyme 5-HTP decarboxylase in megakaryocytes.

Since the biochemical and physiologic significance of endogenous 5-HT in the platelets has not yet been elucidated, the demonstration of endogenous 5-HT in the megakaryocytes defined as precursors of platelets by histochemical, immunochemical, electron microscopic, and cinematographic means may give rise to stimulation for further research in this field.

SUMMARY AND CONCLUSION

A histochemical fluorescence method for the demonstration of biogenic monoamines was applied to the smear preparation of peripheral blood platelets and bone marrow megakaryocytes of rabbits and humans. The fluorescence obtained was identified as 5-HT by histochemical and pharmacologic criteria. With this technic, the following results were obtained: (1) A large amount of 5-HT was present in platelets and in mature platelet-forming megakaryocytes. (2) Only a small amount of 5-HT was demonstrable in the intermediate maturation forms of megakaryocytes with lack of platelet budding.

The possibility that the 5-HT detected was derived from (a) transport of 5-HT formed elsewhere, and/or (b) 5-HT formation from 5-HTP in the megakaryocytes themselves during their maturation was discussed.

SUMMARIO IN INTERLINGUA

Un metodo histochemic a fluorescentia pro le demonstration de monoaminas biogene eseva applicate a frottis de plachettas de sanguine peripheric e de megakaryocytos de medulla ossee de conillos e humanos. Le fluorescentia eseva identificate como 5-HT per criterios histochemic e pharmacologic. Con le technica le sequente resultatos eseva obtenite: (1) Un grande quantitate de 5-HT eseva presente in plachettas e in matur plachetti-formante megakaryocytos. (2) Solmente un micre quantitate de 5-HT eseva demonstrabile in le formas intermedie de maturation de megakaryocytos sin germination plachettal.

Es commentate le possibilitate que le 5-HT observate eseva derivate (a) ab 5-HT in transitio e formate alterubi e/o (b) ab le formation de 5-HT ab 5-HTP in le megakaryocytos mesme durante lor maturation.
REFERENCES

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