HUMAN SEROTONECTIN: A BLOOD GLYCOPROTEIN THAT BINDS SEROTONIN AND IS ASSOCIATED WITH PLATELETS AND WHITE BLOOD CELLS

H. TAMIR, R. F. PAYETTE, Y. L. HUANG, K. P. LIU AND M. D. GERSHON

New York State Psychiatric Institute, Division of Neuroscience and Columbia University, College of Physicians and Surgeons, Departments of Psychiatry and Anatomy and Cell Biology, New York, N.Y. 10032, U.S.A.

SUMMARY

A glycoprotein that circulates in blood, binds to the surface of platelets, and also binds serotonin with high avidity and specificity, has previously been found in rats. This glycoprotein has been called serotonectin. We now report the purification and characterization of a similar circulating glycoprotein in human blood that we propose be called human serotonectin, to distinguish it from the rat protein. Human serotonectin binds serotonin with high affinity ($K_d = 36 \text{nM}$; $K_{d2} = 1-1 \mu M$). Monospecific antisera were raised in rabbits to purified human serotonectin. These antibodies were used to locate human serotonectin immunocytochemically, for quantitative estimation of the glycoprotein, and for rapid preparation of material purified by affinity chromatography. Evidence was obtained that indicated that human serotonectin circulates in plasma and also binds to the surfaces of white blood cells and platelets but not to red blood cells. In bone marrow it is found on megakaryocytes and on developing white cells of the eosinophil line. The protein can be completely removed by washing with isotonic sucrose or salt solutions from the surfaces of white cells but similar treatment only partially (63 % sucrose wash/73 % salt wash) removes human serotonectin from platelets. Antibodies to human serotonectin antagonize the uptake of serotonin by platelets but do not inhibit platelet aggregation. These data show that humans, like rats, have a circulating serotonin-binding glycoprotein that is also present as a peripheral membrane protein on platelets. The human also differs from the rat serotonectin in binding to white cells. The material may function in platelet serotonin uptake in both humans and rats; however, its function, if any, with respect to white cells is obscure.

INTRODUCTION

As a result of the effectiveness of ketanserin, an antagonist of the action of serotonin (5-hydroxytryptamine; 5-HT) on vascular 5-HT receptors, in the treatment of hypertension (DeCree et al. 1982; Symoens, 1982), considerable interest has recently been directed to vascular 5-HT. The major source of blood 5-HT is the gastrointestinal tract, where 5-HT is produced, stored, and released from mucosal enteroendocrine cells and intrinsic enteric neurons (for a review, see Gershon, 1982). 5-HT reaching the blood is actively taken up and stored within platelets in specific serotonin-storage organelles or granules (DaPrada & Pletscher, 1968). In addition, some of the vascular 5-HT is cleared by hepatic metabolism and more by

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the transport into, and subsequent metabolism by, endothelial cells of the lung (Steinberg & Das, 1980). In a previous study of rat platelets (Tamir, Bebirian, Muller & Casper, 1980) we identified a glycoprotein that specifically binds 5-HT with high affinity ($K_D = 42 \text{nm}$ and $0.8 \mu \text{M}$). The glycoprotein was originally found in the 100000 $g$ supernatant fraction of homogenates of platelets; however, it was soon found that the protein was not an intracellular component of platelets, but instead was a peripheral membrane protein that readily dissociated from the outer surface of the platelet plasma membrane (Tamir, Kupsky, Huang & Gershon, 1983). The rat glycoprotein was purified to homogeneity and monospecific antibodies were raised against it. This antibody was used for further characterization of the glycoprotein and for light and electron-microscopic immunocytochemical localization. The glycoprotein was associated only with the vascular surface of the platelet plasma membrane and not with the surface-connecting system. It could be removed reversibly from platelet membranes by washing platelets in physiological salt solution. Subsequent work showed that the protein circulates in the blood and apparently is in equilibrium with the bound material on platelet surfaces. Because of its ability to bind both to platelet membranes and to serotonin, the glycoprotein was named serotonectin. Serotonectin of rats does not bind to red blood cells, white blood cells, or to endothelial cells. Using incubation with radioactive amino acids and immunoprecipitation of homogenates of various organs, the source of circulating serotonectin was found to be blood-forming organs, such as haematopoietic rat spleen and bone marrow.

A suggested functional property of serotonectin came from the observation that anti-serotonectin inhibited the uptake of 5-HT by rat platelets. Serotonectin thus may participate in this process; however, it is not the 5-HT uptake site itself, since serotonectin does not bind [3H]imipramine, an uptake site marker (Langer et al. 1980), and washing the protein off platelet membranes does not impede the binding of [3H]imipramine to the membranes (Tamir et al. 1983).

It is possible that serotonectin plays a role in the interaction of vascular elements with 5-HT. If so, a defect in this protein could be linked to abnormal vascular effects of 5-HT and thus to a 5-HT contribution to hypertension. In the current experiments, therefore, we have attempted to determine whether human blood contains a protein similar to rat serotonectin and, if so, to purify it, produce antibodies against it and determine its properties in comparison to the rat material. Pignatti & Cavalli-Sforza (1975) have reported two human platelet glycoproteins that bind 5-HT; however, they neither purified nor fully characterized either protein (Pignatti & Cavalli-Sforza, 1975).

**MATERIALS AND METHODS**

**Assay of serotonin-binding protein**

The assay used to detect human serotonectin was carried out as previously described for the rat (Tamir et al. 1980). The assay involves incubation in 0.02M-potassium phosphate buffer (pH 7.5) of purified or partially purified (see below) protein with [3H]5-HT (12 Ci/mmol; Amersham Co., Arlington Heights, Ill.; 1 $\mu \text{M}$) in the presence of Fe$^{2+}$ (0.1 $\text{mM}$). The resulting protein–Fe–[3H]5-
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HT complex was then separated from free \(^{[3H]}5\text{-HT}\) by molecular-sieve chromatography on a column of Sephadex G-50. Protein-bound \(^{[3H]}5\text{-HT}\) was identified by counting samples of the fractions eluted from the column. Radioactivity was determined using a liquid scintillation spectrometer and was counted with an efficiency of 35\%. Protein content was measured by absorption at 280 nm. When needed for identification, a sample of 0·1 ml was applied to 7·5 % sodium dodecyl sulphate (0·05 % SDS)/polyacrylamide gels and subjected to electrophoresis (PAGE). The binding of \(^{[3H]}5\text{-HT}\) to serotonecin is sufficiently tight that the radioactivity of \(^{[3H]}5\text{-HT}\) migrates as a complex with protein and serves to identify the serotonin-binding protein in the gels following PAGE (Tamir et al. 1980; Tamir, 1982).

Purification of human serotonecin

Two methods were used. Initial purification was accomplished by several procedures previously used for the purification of rat serotonecin. Subsequently, a newer procedure was devised to take advantage of antibody raised against human serotonecin. Because of the proteolytic activity of plasma, a mixture of proteolytic enzyme inhibitors was present throughout the steps of purification from the time of homogenization onward. The mixture included: EDTA (1·0 mm); EGTA (0·1 mm); phenylmethylsulphonyl fluoride (0·3 mm); pepstatin A (1 mm) and toluene sulphonyllysylchloromethyl ketone (0·1 mm) (obtained from Sigma Chem. Co., St Louis, Mo.) dissolved in potassium phosphate buffer (0·02 m, pH 7·5). This solution was called buffer A.

Method I. The purification was carried out 15 times using blood from different healthy donors of either sex. Venous blood (20 ml) was used as the starting material. Platelet-rich plasma was prepared (Tamir et al. 1980). Subsequently, the platelets were disrupted by freezing and thawing three times followed by homogenization in a tight Teflon—glass homogenizer. Unbroken cells and debris were then removed by centrifugation (100 000 g; 60 min). The high-speed supernatant was saved and subjected to fractionation with (NH\(_4\))\(_2\)SO\(_4\) (0·30 % saturation). The material sedimented by (NH\(_4\))\(_2\)SO\(_4\) was re-dissolved in buffer A and further fractionated according to size by molecular-sieve chromatography on Sephadex G-200 that had previously been equilibrated with buffer A. The fractions that displayed \(^{[3H]}5\text{-HT}\) binding capacity emerged close to the void volume, and were further purified by application to a column of Affi-Gel R-Blue (BioRad, Rockville, N.Y.; 100—200 mesh, 1 cm \(\times\) 5 cm, equilibrated with buffer A) to remove albumin and some proteolytic enzymes. The column was washed and eluted with the same buffer. Fractions that bound \(^{[3H]}5\text{-HT}\) were pooled and applied directly to an affinity column constructed of 10 mg/ml concanavalin A (ConA) covalently bound to Sepharose (Pharmacia, Piscataway, N. J.). Material binding to this column was eluted with \(\alpha\)-methylmannoside (0·5 m in 20 mM-potassium phosphate buffer, pH 7·5) and concentrated by ultrafiltration on Amicon filters (XM—100). A final step in the purification consisted of preparative electrophoresis on 7·5 % SDS/polyacrylamide slab gels. The single band with an \(R_p\) of 0·15 that stained by the periodic acid-Schiff procedure (Zacharius, Zell, Morrison & Woodlock, 1969) was eluted from the gels as previously described (Tamir et al. 1983) and used for immunization (see below).

Method II. Antibody to serotonecin (10 mg/ml of the immunoglobulin G (IgG) fraction obtained following DEAE Affi-Gel B Blue fractionation (Tamir et al. (1983)), was coupled to cyanogen-bromide-activated Sepharose 4B (Cuatrecasas, Wilchek & Anfinsen, 1968). Following the coupling reaction, excess ligand was washed away and remaining active sites were blocked by treatment with 0·2 m-glycine buffer (pH 8·0). The anti-serotonecin affinity gels were then poured into glass columns (0·9 cm \(\times\) 5 cm) equilibrated with buffer A. Crude serotonecin (25 mg of material obtained after (NH\(_4\))\(_2\)SO\(_4\) fractionation described above) was applied to the anti-serotonecin affinity column. Inert proteins that do not bind 5-HT were eluted by washing the column with 0·1 m-Tris-HCl (pH 8·0). The column was subsequently washed with 1·0 m-sodium acetate buffer at pH 5·5. Serotonecin was finally eluted from the column with 1·0 m-N\(_2\)H\(_4\)OH. The pH of the eluted, protein-containing solution was either neutralized immediately with 5 m-HCl, or the eluate was passed directly through a column of Sephadex G-50 to separate serotonecin from N\(_2\)H\(_4\)OH. This material was subjected to SDS/PAGE (7·5 %) and stained with either Coomassie Brilliant Blue R-250 or PAS to establish its purity.
Preparation and characterization of antisera to serotonectin

Purified human serotonectin, electrically eluted from preparative acrylamide gels (see above), was injected into rabbits with Freund’s complete adjuvant. The specificity and affinity of the antisera obtained from these rabbits was tested by rocket and crossed-rocket immunoelectrophoresis as described previously (Tamir et al. 1983). Further characterization was done by subjecting whole plasma to 7.5% SDS/PAGE slab gels, transfer to nitrocellulose sheets (Towbin, Staehelin & Gordon, 1979), and immunostaining the nitrocellulose blots with the unlabelled antibody-peroxidase/anti-peroxidase bridge technique (Sternberger. 1974).

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was done according to a standard procedure (Weeks, 1973; Tamir et al. 1983). Plasma proteins were first separated by electrophoresis in a 1% agarose gel containing 0.5% Triton X-100. Immediately thereafter, electrophoresis was done perpendicularly to this sizing gel in 1% agarose gel containing antiserum to serotonectin (6% antiserum with 0.1% Triton X-100).

Immunohistochemical localization of serotonectin

Specific antiserum, characterized as described above, was used to locate serotonectin in a variety of preparations. These included smears of human bone marrow, whole human blood, platelet-rich plasma, and blood cells separated by centrifugation on density gradients. Two gradients were used, whole blood layered over Lymphoprep® (Gallard-Schlesinger, Carle Place, N.Y.), and cells that had first been sedimented at 150 g for 10 min layered over Lymphoprep® containing 30 mg/ml of sodium diatrizoate (Boyum, 1968). Both gradients were centrifuged for 20 min at 1500 g. The first gradient was useful in obtaining an enriched preparation of granulocytes and the second in obtaining a white cell mixture well separated from red blood cells. The separated white cells obtained by the above two methods were carefully removed, washed with Krebs solution, and finally resuspended in plasma cleared of cells by centrifuging at 2000 g for 20 min, before using them for smears. For experiments designed to evaluate the removal and re-binding of serotonectin by blood cells, smears were made from buffy coat preparations that had been washed three times in 3 ml of Krebs solution to remove adherent serotonectin. Rebinding was assessed by allowing the cells to incubate for 30 min at room temperature with fresh human plasma or plasma diluted 1:10, 1:100, 1:1000 and 1:10000.

Smears of blood cells were allowed to air-dry and then were hydrated briefly for 1 min in 0.1M-phosphate-buffered saline (PBS) at pH 7.4 before fixation with the lysine-periodate-4% formaldehyde fixative of McLean & Nakane (1974) for 20 min. Smears were then washed extremely with several changes of 0.1M-Tris buffer (pH 7.6) for 1 h. They were then incubated in the same Tris buffer containing 10% (v/v) horse serum (GIBCO; Grand Island, N.Y.) for 30 min at room temperature. Note that horse serum does not contain material reacting with antibody to human serotonectin, and does not by itself impart any specific immunofluorescence to washed cells or platelets. Anti-serotonectin antiserum was diluted with 0.1M-Tris buffer and used at concentrations of 1:500 to 1:5000. Slides remained in contact with this primary antiserum overnight at 4°C in a humid chamber. The unbound primary antibody was removed by washing in 0.1M-Tris buffer for 30 min with several changes, followed by incubation with affinity-purified biotinylated goat anti-rabbit IgG (1:500; Vector Labs, Burlingame, Ca.) washed and then incubated with avidin–rhodamine (1:400; Vector Labs.). Slides were then washed in 0.1M-Tris buffer for 30 min and incubated with bisbenzamide (Sigma Chemical Co., St Louis, MO.) diluted in 0.1M-PBS to a final concentration of 0.1 mg/ml for 15 min at 37°C to act as a nuclear fluorochrome. After staining, the slides were washed extensively with PBS, dipped in distilled water, mounted in Gelvatol (Monsanto Chemical Co., Indian Orchard, Mass.) or glycerol and examined by fluorescence microscopy. Suitable filter and dichroic mirror combinations permitted sequential visualization of bisbenzamide (Leitz D cube) or TRITC (Leitz N2 cube).
Solid-phase quantitative immunoassay of serotonectin

Platelet-rich plasma or the purified white cells from the crude buffy coat were used for the quantitative estimation of serotonectin. The crude buffy coat was layered over Lymphoprep® fortified with 50 μg/ml of sodium diatrizoate and centrifuged at 600 g for 15 min. The interface layer, which contains most white cells, was carefully removed, washed with PBS and collected by centrifugation (2 min; 1500 g). Enzyme-linked immunoabsorbent assay (ELISA) was used for this purpose. Cells of either preparation were fixed by exposure to 20 vol. of ice-cold formaldehyde solution (4%, diluted in PBS at pH 7.4) at 4°C for 30 min. Following fixation the cells were washed twice with PBS containing 0.3% gelatin and pelleted by centrifugation (1500 g, 4°C, 2 min). The platelet and white blood cell pellets were resuspended in PBS containing 0.3% gelatin. A 180 μl sample of the platelets (1.7×10^6 cells) or white blood cells (1.0×10^7 cells) was placed into a small test tube (12 mm × 75 mm) and incubated with 250 μl of diluted anti-serotonectin serum. The cell suspensions were shaken occasionally at room temperature for 2 h followed by washing with 1 ml of PBS-gelatin solution. The cell suspensions were centrifuged at 1500 g for 2 min and the supernatant was discarded. After washing twice with PBS-gelatin solution, 200 μl of alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:400 in PBS-gelatin, Miles-Yeda, Israel) were added and the suspensions were incubated for a further 2 h at room temperature. Excess secondary antibody was then washed off the cells and the amount of bound alkaline phosphatase was determined. Alkaline phosphatase activity was assayed by adding 0.5 ml of 0.05 M sodium carbonate buffer (pH 9.8) containing 1 mg/ml of p-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO) as substrate and 1 mM MgCl₂. The reaction was stopped after 10 min by adding 0.1 ml of 1M NaOH and the developed colour was measured at 400 nm (Engvall & Perlmann, 1972).

Effect of anti-serotonectin antiserum on platelet aggregation

Platelet aggregation was evaluated by a modification of the method of Weiss, Tschopp & Rogers (1974). Platelet-rich plasma was prepared in siliconized vessels and 1 ml was added to the cuvette of a dual channel aggregation module (Payton Associates, Buffalo, N.Y.). The suspension was stirred and platelet aggregation was recorded continuously as the increase in light transmittance (relative to a blank of platelet-poor plasma) on a pen recorder. Aggregation was initiated by adding epinephrine (1 μM final concentration) or a mixture of epinephrine (1 μM) and 5-HT (5 μM). To test the effect of anti-serotonectin antiserum on platelet aggregation, 0.9 ml of platelet-rich plasma were pre-incubated with 40–80 μl of the antiserum (or a partially purified IgG fraction) for 20 min at room temperature. Preimmune serum (or a partially purified IgG fraction derived from it) served as a control.

Effect of anti-serotonectin serum on uptake of 5-HT by platelets

Human platelet-rich plasma (0.5 ml; 7.5×10⁷ cells/ml) was preincubated (30 min; 37°C) with either an IgG fraction (1–2 mg protein) prepared from preimmune rabbit serum or with anti-serotonectin serum (1:20), or with PBS. Platelets were centrifuged at 3000 g for 15 min and the supernatant (0–30 ml) was removed. Cell counts indicated that over 95% of the platelets were pelleted by this treatment. [³H]5-HT (final concentration 2×10⁻⁷ M) was then added to the platelets and the samples were incubated for 5 min at 37°C. Under these conditions, the uptake of [³H]5-HT is linear for up to 10 min. In control experiments the platelets were kept on ice. The uptake of [³H]5-HT was stopped by immersing all tubes in ice and centrifuging the platelets (10000 g; 5 min). The supernatant was removed and the unstirred surface of the platelets was washed twice with Tyrode’s buffer. The wash was discarded and the pellet dissolved in tissue solubilizer (TS-1; 0.5 ml; Research Product Inc., Mt Prospect, 111.) and counted. No control was included for radioactivity trapped in the intercellular spaces of the platelet pellet; however, the minimal amount of radioactivity in the pellet of platelets incubated at 0°C indicated that extracellular radioactivity was negligible. In addition, the radioactivity in the platelet pellet from the control experiments (on ice) was subtracted from the values obtained from material incubated at 37°C in order to correct for intercellularly trapped or surface-bound [³H]5-HT.
RESULTS

Identification of protein that binds $[^3H]5$-HT in human platelet-rich plasma

Platelet-rich and platelet-poor plasma were assayed for the presence of proteins able to bind $[^3H]5$-HT. A crude protein preparation was obtained by ammonium sulphate fractionation (0–30%) and incubated with $[^3H]5$-HT in the presence of Fe$^{2+}$. The radioactive mixture was passed through a Sephadex G-50 column to separate bound from free $[^3H]5$-HT and then applied to 7.5% polyacrylamide tube gels containing 0.05% SDS. A protein was detected in both starting materials that bound $[^3H]5$-HT sufficiently tightly to migrate with $[^3H]5$-HT as a complex on the gels. The molecular weight of the complex corresponded to approximately $200 \times 10^3$ ($R_c = 0.15$). Following electrophoresis, parallel gels were either sliced and counted, or stained with Coomassie Brilliant Blue or PAS. Only one distinct band on the gels was labelled by $[^3H]5$-HT. This band was also PAS-positive (Fig. 1). Other proteins stained by Coomassie Blue did not bind $[^3H]5$-HT. In the absence of SDS all radioactive protein was confined to the origin of both 7.5% and 5% polyacrylamide gels. The inability of human plasma protein(s) that bind $[^3H]5$-HT to enter polyacrylamide gels in the absence of SDS may be due to protein aggregation and stands in marked contrast to the situation encountered when similar proteins of rats were studied (Tamir et al. 1980). Rat serotonectin enters 7.5% polyacrylamide gels even in the absence of SDS. In addition, the complex of $[^3H]5$-HT with rat serotonectin is not, as is true of the human material, resistant to dissociation by SDS.

Fig. 1. Identification of human serotonectin by PAGE. Protein (50μg) from a crude platelet preparation was incubated with Fe(NH$_4$)$_2$(SO$_4$)$_2$ (0.1 mM) and $[^3H]5$-HT (1.0 μM). The complex formed between $[^3H]5$-HT and protein was separated from free $[^3H]5$-HT by molecular-sieve chromatography and then applied to SDS (0.05%)/polyacrylamide gels (7.5%). Gels were run at a constant current. Gels were either sliced and counted or stained with PAS. A glycoprotein that binds $[^3H]5$-HT (PAS positive peak) was detected.
Fig. 2. Electrophoretic purification of human serotonectin. Material eluted from a column of ConA-Sepharose was subjected to electrophoresis on a 7.5% polyacrylamide gel with SDS. When stained with Coomassie Blue B, at least five bands of protein can be discerned (lane a); however, only one of these is also stained by the PAS reaction (lane b). The band that stained with PAS was cut out, electroeluted and subjected again to electrophoresis but on a 10% polyacrylamide gel with SDS. A single band is now seen upon staining with Coomassie Blue (lane c).

Purification and partial characterization of human serotonectin

Venous blood was used as the starting material with which to purify the glycoprotein of platelet-rich plasma that was found electrophoretically to bind [3H]5-HT. This protein will subsequently be referred to as human serotonectin. Platelet-rich plasma was prepared, the platelets were disrupted, and high-speed centrifugation of
the homogenate yielded 515–764 mg of protein with a $[^3H]5$-HT binding capacity of $5\cdot9\times10^5$ c.p.m./mg protein. This value represents total $[^3H]5$-HT binding and includes the binding of $[^3H]5$-HT by both human serotonectin and albumin. Ammonium sulphate fractionation of the high-speed supernatant was done to remove albumin. The $[^3H]5$-HT binding capacity of the almost albumin-free (<10%) 0–30% fraction was $3\cdot2\times10^5$ c.p.m./mg protein. Further steps of purification were done essentially as described previously for rat serotonectin (Tamir et al. 1980).

Fractionation of a Sephadex G-200 column resulted in recovery of 23% of the applied protein in the void volume. This protein-bound $[^3H]5$-HT with a capacity of $5\times10^5$ c.p.m./mg protein. Remaining traces of albumin and some of the proteolytic enzyme activity of the mixture were removed by passage through a column of Affi-Gel$^R$-Blue. Human serotonectin, recovered in the wash that contained 60% of the protein applied to the Affi-Gel$^R$-Blue column, was purified further by chromatography on an affinity column of ConA-Sepharose. All $[^3H]5$-HT binding activity was removed by passage through this column. None of the protein passing through this column bound $[^3H]5$-HT; however, when the ConA affinity column was eluted with a-methyl-D-mannoside, the $[^3H]5$-HT binding activity was recovered from the column. This eluted fraction contained 14% of the originally applied protein and had a $[^3H]5$-HT binding capacity of $7\cdot5\times10^5$ c.p.m./mg protein. Finally, the eluate was subjected to preparative gel electrophoresis (Fig. 2, lane a). Several bands were still detectable by Coomassie Blue staining. Only one of these also stained by the PAS procedure (Fig. 2, lane b) and migrated with bound $[^3H]5$-HT (apparent molecular weight 200 $\times$ 10$^3$). This band was cut out, eluted from the gel, and used to raise antibodies in rabbits as previously described (Tamir et al. 1983). When re-run on a 10% polyacrylamide gel and stained with Coomassie Blue, the material present in this band yielded a single band (Fig. 2, lane 2).

In subsequent experiments the antisemurum raised in rabbits against the serotonectin band eluted from the gels was used to purify the protein. These antibodies were covalently bound to Sepharose beads and the immobilized antibodies were used for affinity chromatography. Crude human serotonectin (material obtained after $(\text{NH}_4)_2\text{SO}_4$ fractionation in the procedure outlined above) was applied to the affinity column. The protein fraction that did not bind to the column contained proteins unable to bind $[^3H]5$-HT. Bound serotonectin was eluted from the column with 1-0 M-$\text{NH}_4$OH and yielded a single band when subjected to 7-5% PAGE (stained either with Coomassie Blue or PAS). The immuno-affinity column method of purification of human serotonectin provided material with a $[^3H]5$-HT binding capacity of $2\cdot4\times10^6$ c.p.m./mg protein.

**Binding properties of human serotonectin**

The binding of $[^3H]5$-HT to purified human serotonectin was found to be dependent upon the concentration of the amine and was saturable. Scatchard analysis revealed a complex pattern of binding (Fig. 3). A non-linear Scatchard plot
Fig. 3. Scatchard analysis of the binding of \(^{3}H\)5-HT by human serotonectin. Incubation of 25 \(\mu\)g of purified serotonectin (obtained by elution from ConA-Sepharose columns) was carried out in presence of \(Fe\textsubscript{2+}\textsubscript{(NH}_4\textsubscript{2})\textsubscript{SO}_4\textsubscript{2} (0·1 mM) with the indicated amount of \(^{3}H\)5-HT for 30 min at 30°C. The ratio of bound to free \(^{3}H\)5-HT \([S]\textsubscript{b}/[S]\textsubscript{f}\) was plotted against the concentration of bound \(^{3}H\)5-HT \([S]\textsubscript{b}\). The equilibrium dissociation constants \((K_D)\) were calculated from the slope of the lines (slope = \(-K_D^{-1}\)). Two dissociation constants were determined for serotonectin: \(K_D\textsubscript{1} = 36 nM\) and \(K_D\textsubscript{2} = 1·1 \mu M\). The experiment was carried out twice.

was obtained with a Hill coefficient less than unity. These data could be accommodated by assuming two \(^{3}H\)5-HT binding sites with equilibrium dissociation constants \((K_D)\) of 36 ± 9·5 nM and 1·1 ± 0·2 \(\mu M\), respectively. Alternatively, the data are also compatible with a hypothesis of negative cooperativity for binding by a single population of 5-HT binding sites. If one assumes two binding sites, the ratio of the high to the low affinity site is about 1:6. There is also some non-saturable binding of \(^{3}H\)5-HT by the protein. The total binding \(^{3}H\)5-HT (both sites saturated) to serotonectin was enhanced two- to threefold in the presence of \(Fe\textsubscript{2+}\).

| Table 1. Effect of drugs on the binding of \(^{3}H\)5-HT to serotonectin |
|-----------------------------|-----------------|-----------------|
|                             | 5 \(\mu M\)     | 10 \(\mu M\)    |
| 5-HT receptor antagonist    |                 |                 |
| Ketanserin                  | 26·1 ± 4        | 29·5            |
| Methysergide                | 0               | 0               |
| 5-HT depleters              |                 |                 |
| Reserpine                   | 0               | 10 ± 5          |
| Tetrabenazine               | 19·5 ± 3        | 15 ± 6          |
| 5-HT uptake inhibitors      |                 |                 |
| Fluoxetine                  | 0               | 0               |
| Imipramine                  | 15 ± 5          | 16 ± 3          |
Effect of drugs on the binding of [3H]5-HT by human serotonectin

A number of compounds that influence the receptor binding, storage or uptake of 5-HT were tested for their ability to affect the binding of [3H]5-HT by human serotonectin. These compounds included reserpine and tetrabenazine, both of
which affect 5-HT storage; the receptor antagonists, ketanserin and methysergide; and the uptake blockers, fluoxetine and imipramine (Table 1). None of these compounds significantly influenced the binding of $[^3H]5$-HT by serotonectin at drug concentrations below 10 $\mu$m.

**Fig. 5. Crossed-rocket immunoelectrophoresis of human serotonectin.** Plasma (3 $\mu$l) and SDS (10%) were placed in the well. The first dimension of electrophoresis was performed at 10 mA for 50 min in 1% agarose containing 0.5% Triton X-100. The second dimension was performed at 10 mA for 17 h into agarose containing anti-serum (6%) and Triton X-100 (0.1%). The plasma serotonectin formed two rockets. Note that the rocket on the right (lower molecular weight) is immunochemically identical to the original material, human serotonectin (no crossed rockets). The rocket on the right may be a degradation product.

**Preparation and characterization of rabbit antisera to serotonectin**

Human serotonectin, injected into rabbits in the presence of SDS, was found to be an effective antigen. Analysis of the resultant antiserum by rocket immunoelectrophoresis yielded a single rocket with a sharp profile, indicating a high affinity of the antibody for human serotonectin. When plasma proteins were fractionated by SDS/PAGE (slab gels) and transferred by blotting to nitrocellulose paper, only one of the several bands that could be stained with Amido Schwartz was immunoreactive with the antiserum to human serotonectin (Fig. 4). This band migrated identically to purified human serotonectin ($M_r 200 \times 10^3$). Occasionally, when plasma was stored for a period of time, a second protein with a slightly lower molecular weight than that of human serotonectin was also immunoreactive. The inconsistent appear-
ance of this smaller protein and its absence from fresh plasma indicates that this second immunoreactive protein probably results from proteolysis of human serotonectin. Crossed-rocket immunoelectrophoresis was also done. Again, evidence of a single antibody class recognizing a common antigenic domain was found (Fig. 5). A further test of antibody specificity was done by examining potential cross-reactivity with human fibronectin. None was found by rocket immunoelectrophoresis or ELISA.

**Immunocytochemical localization of human serotonectin**

When smears of whole human blood or platelet-rich plasma were examined with anti-serotonectin antibody, immunofluorescence was found on platelets and white blood cells (Fig. 6). Red blood cells did not stain. Control slides exposed to preimmune sera or serum absorbed with serotonectin showed no staining. The observation that white blood cells as well as platelets were immunoreactive when exposed to antiserum to human serotonectin was surprising because these elements in rats were not stained by antiserum to rat serotonectin (Tamir et al. 1983). In order to determine if all classes of white blood cells were immunoreactive, preparations of human blood cells fractionated by centrifugation through gradients of Lymphoprep® or Lymphoprep®-diatrizoate were processed for immunofluorescence. All mononuclear cells and granulocytes were found to be immunoreactive (Fig. 7). Permeabilization of cells with Triton X-100 or ethanol had no effect on the appearance of the immunoreactivity in any of the cells. Human serotonectin could not be immobilized by ethanol fixation alone; such fixation resulted in loss of specific cellular immunofluorescence. Formaldehyde fixation, especially with added lysine-periodate, a fixative designed to cross-link glycoproteins (McLean & Nakane, 1974), preserved the cellular localization of immunoreactivity.

When blood cells were washed with Krebs solution before fixation and processing for immunocytochemistry the antigen was removed from both platelets and white

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**Fig. 6.** Peripheral blood smears fixed and processed for the immunocytochemical demonstration of human serotonectin. A. Field exposed to anti-serotonectin primary antibody (1:2000) showing the specific TRITC fluorescence of a field of platelets. ×375. B. Field exposed to anti-serotonectin antiserum (1:100) absorbed with purified human serotonectin. Immunofluorescence was abolished. ×375. C. Field exposed to anti-serotonectin primary antibody (1:1000). In addition to platelets a single white blood cell shows specific TRITC fluorescence. ×400. D. The same field as illustrated in c; however, the microscope’s dichroic mirror and filter system has been set to demonstrate bisbenzamide fluorescence. The nuclear staining reveals the immunofluorescent white cell shown in c to be a granulocyte. ×300.

**Fig. 7.** Buffy coat smeared on a slide and processed to demonstrate immunoreactivity of human serotonectin. ×1100. A. Four white blood cells and a platelet (arrow) show specific TRITC-fluorescence after exposure to antiseraum to human serotonectin. B. The same field as illustrated in A; however, the microscope’s dichroic mirror and filter system has been set to demonstrate bisbenzamide fluorescence. The nuclear staining reveals that two of the white cells are lymphocytes (l) and two have the multilobed nuclei of granulocytes (g). The platelet has no chromatin and does not stain with bisbenzamide.
Figs 6 and 7
blood cells. Preparations of washed cells were incubated with various dilutions of human plasma. A dilution of 1:10,000 was sufficient to restore the minimal immunoreactivity to both types of cell. These observations are consistent with the hypothesis that human serotonectin is present in plasma and binds to the external face of the plasma membranes of white blood cells and platelets. This hypothesis is strengthened further by the observation that human serotonectin can be purified from platelet-poor plasma (about 35-fold). This establishes that free plasma serotonectin must exist and that the free material is not all derived from material artifactually liberated from platelets during processing.

Bone marrow smears, examined with anti-serotonectin antibody, revealed immunofluorescent labelling of eosinophilic myelocytes and metamyelocytes as well as megakaryocytes (Fig. 8). (Cell types were identified by staining with Wright’s stain, after photographing and marking the location of immunofluorescent cells with a diamond marker.) No other precursors to developing blood cells were stained.

![Fig. 8. A bone-marrow smear fixed and processed for the immunocytochemical demonstration of human serotonectin. A. Field exposed to anti-serotonectin primary antiserum (1:1000). Specific TRITC-fluorescence is shown by a large and a small cell (arrow and arrowhead, respectively). ×500. B. The same field as illustrated in A; however, the microscope’s dichroic mirror and filter system has been set to demonstrate bisbenzamide fluorescence. The multilobulated nucleus (arrow) confirms that the giant cell with human serotonectin immunoreactivity shown in A is a megakaryocyte. The indented nucleus (arrowhead) of the serotonectin-immunoreactive small cell can be discerned. ×500. C. The two cells that displayed serotonectin immunoreactivity in A have been stained with Wright’s stain and are shown at somewhat higher magnification (×900). The small immunoreactive cell (arrowhead) was identified as an eosinophilic metamyelocyte. Its specific granules can be discerned in the micrograph.]

**Solid-phase quantitative immunoassay of serotonectin**

Formaldehyde fixation was used to link bound serotonectin irreversibly to cell surfaces. This permitted cell-bound serotonectin to be estimated by ELISA. Optical
density of the alkaline phosphatase reaction in arbitrary units was measured. Using this technique, 1000 purified white blood cells yielded the same optical density measurement (0.23) as 8000 platelets from platelet-rich plasma. Cell size was estimated by direct microscopic measurement of the apparent enclosed areas of the formaldehyde-fixed cells. For this purpose cells were embedded in 1.5% agarose films formed between glass slides in order to provide a monolayer of cells not flattened by air drying on glass. The agarose films containing the purified cells of the buffy coat or platelet-rich plasma were examined with Nomarski differential interference optics and cell areas were measured by tracing cell outlines on a digitizing tablet with the aid of a camera lucida attachment. An Apple II plus computer with a Leitz-Bioquant® stereology program was used for computation. The mean area of platelets was \(8.4 \pm 0.3 \mu m^2\) (\(n = 103\)), of lymphocytes was \(39.9 \pm 1.4 \mu m^2\) (\(n = 51\)), and of granulocytes was \(62.9 \pm 1.7 \mu m^2\) (\(n = 50\)). The area of white cells is thus five to eight times as great as platelets. White blood cells, therefore, bind more serotonectin per cell than do platelets; however, the relative binding capacity of each is approximately the same when considered as a function of area. Moreover, since a unit volume of normal blood contains about 66 times as many platelets as leucocytes, the total amount of serotonectin associated with platelets would be expected to be approximately eightfold higher than that associated with white blood cells.

Some of the platelet-bound serotonectin, but not that of leucocytes, seems to be resistant to removal by washing. When unfixed platelets are washed with Krebs solution or 0.32M-sucrose, 72.5% and 63.2%, respectively, of the serotonectin initially present is removed from platelet surfaces and can be recovered in the wash. This leaves 27.5–36.8% of the material still bound to platelets. In contrast, a similar washing of white blood cells in either Krebs solution or 0.32M-sucrose removes all of the bound serotonectin. No serotonectin remains bound to the washed leucocytes.

**Effect of anti-serotonectin antiserum on platelet aggregation**

5-HT has been shown to induce platelet aggregation (for a review, see Weiss, 1982). This activity of 5-HT is synergistic with that of epinephrine. Since serotonectin bound to platelets is located on their surfaces and binds 5-HT, we examined the relationship of serotonectin to the 5-HT receptors involved in mediating the aggregation phenomenon. Anti-serotonectin antibody was used for this purpose. Preimmune serum was used as a control. Both the purified \(\delta\)-globulin fraction obtained by \((NH_4)_2SO_4\) fractionation of antisera and crude antisera were tested for their ability to antagonize the aggregation of platelets induced by 5-HT. No effect of either the purified or crude antisera was found on the rate or extent of aggregation induced either by 5-HT alone or by 5-HT in the presence of epinephrine.

**Effect of anti-serotonectin antiserum on uptake of 5-HT by platelets**

Antiserum to rat serotonectin inhibits the uptake of \([^{3}H]5\text{-HT}\) by rat platelets (Tamir et al. 1983); therefore, we examined the effect of antiserum to human serotonectin on the uptake of \([^{3}H]5\text{-HT}\) by human platelets. Preimmune serum was
used as a control. This serum itself inhibited serotonin uptake by platelets; however, removal of excess human plasma from suspensions of platelet-rich plasma permitted this problem to be overcome. Platelet-rich plasma was preincubated with either preimmune or immune sera and then centrifuged gently to sediment platelets and bound antibody. Most of the clear plasma was removed and discarded. This discarded material contained less than 5% of the platelets originally present in the platelet-rich plasma. The sedimented platelets were then resuspended in their residual plasma and incubated with [3H]5-HT. Under these conditions, preimmune sera failed to inhibit the uptake of [3H]5-HT, while an equal amount of immune sera reduced the uptake of [3H]5-HT by 70% (Fig. 9). Incubation of platelets with [3H]5-HT at 4°C caused a 90% reduction of the amount of [3H]5-HT associated with the platelet pellet. Anti-serotonectin antibody, therefore, strongly inhibits uptake of 5-HT by human platelets.

**DISCUSSION**

In previous studies we found that rat blood contains serotonectin, a glycoprotein with a molecular weight of approximately $200 \times 10^3$ that binds 5-HT and also binds to the external face of platelet plasma membranes. The present study was done to determine if human blood contains a similar material and if so, to purify and...
Human serotonectin

characterize it. We have found a similar protein in human blood, which we have
called human serotonectin; however, our experiments reveal significant differences
as well as similarities between the rat and human proteins.

Human serotonectin, like that of rat, circulates in the blood. It binds $[^3H]5$-HT
with two apparent dissociation constants ($36 \pm 9.5 \text{ nM}$ and $1.1 \pm 0.2 \text{ \mu M}$) that are
very close to those of the rat material ($42 \text{ nM}$ and $0.8 \text{ \mu M}$; Tamir et al. 1980). Both
the human and rat serotonectins are glycoproteins with a rich mannose core, so that
both bind to ConA columns. The human protein has a similar apparent molecular
weight to that of rat ($200 \times 10^3$). Human serotonectin, like that of rat also binds to
platelets; however, unlike rat serotonectin, there is binding of human serotonectin
by white blood cells as well. Platelets and leucocytes bind human serotonectin in
approximately equal amounts as a function of cell area, but the greater number of
platelets in the circulation probably means that the total binding of serotonectin to
platelets is normally about eightfold greater than that to white cells. As with rat
serotonectin, evidence was obtained that is consistent with the production of human
serotonectin in the bone marrow. Immunocytochemical studies revealed human
serotonectin on megakaryocytes as well as on eosinophilic precursor cells in human
bone marrow. It is possible that the serotonectin receptor on other white cells is
masked or absent from their membranes until the cells mature.

We purified the human serotonectin and raised antibodies against it in rabbits.
The antiserum against human serotonectin did not react with rat serotonectin and
antibodies to rat serotonectin did not react with the human material. The two
glycoproteins are thus antigenically different molecules. Nevertheless, the antibodiess
human serotonectin demonstrated the localization of bound human
serotonectin on platelet and leucocyte surfaces when used for immunofluorescence,
and also could be used for preparation of purified protein by affinity chromatography.
Use of the antisera to human serotonectin provided no evidence to
support a possible interaction between human serotonectin and the receptors on
platelet surface that are responsible for mediating the aggregation of platelets
induced by 5-HT. On the other hand, anti-serotonectin was found to be a potent
inhibitor of the ability of platelets to take up $[^3H]5$-HT. This observation suggests
that in humans, as has previously been found for rats (Tamir et al. 1983),
serotonectin may play a role in the platelet 5-HT uptake mechanisms. Again,
however, as with rats, serotonectin does not seem to be the 5-HT transport molecule
itself. This latter molecule binds imipramine (Langer et al. 1980) and fluoxetine
(Wong et al. 1974) and neither of these compounds is antagonistic to the binding of
$[^3H]5$-HT by human serotonectin. It seems likely, therefore, that the serotonectin
binding sites on platelet membranes are close to the transport molecule. At the least,
the presence of antibody bound to these sites interferes with the transport process;
however, the observation that Krebs-washed platelets do not take up $[^3H]5$-HT as
well as platelets from which serotonectin has not been removed suggests (but does not prove) that the interaction between serotonectin and the 5-HT transporter may be of physiological significance.

Nothing can be said at this time about the binding of human serotonectin by white
blood cells. Red cells do not bind the protein, so the binding is not a general property of all of the cellular elements of the blood. This property of the human protein is not shared by rat serotonectin but resembles fibronectin, a plasma glycoprotein of the blood, which also binds both to platelets and white blood cells (Bevilacqua, Amrani, Mosesson & Bianco, 1981; Pommier et al., 1982). The greater resistance of platelet-bound human serotonectin to removal by washing than that of leucocytes suggests that platelets have a higher affinity than white cells for the protein or that a fraction of the platelet-bound material is internal or an integral membrane protein. No functional information with respect to white blood cells and serotonectin was obtained.

The finding of similar serotonectin-like molecules in human and rat blood suggests that this kind of molecule may be a constituent of the blood in most mammals. Its function, other than the one postulated for platelet uptake of 5-HT, can only be surmised. 5-HT is a vasoactive amine (for a review, see Erspamer, 1966; Osborne, 1982). It may be involved in immune phenomena and act on white cells (Askenase, Burstar, Gershon & Gershon, 1980). Conceivably, the circulating material serves to regulate or limit the free 5-HT that is available in the blood for binding to endothelial or leucocytic receptors. It should be noted that this is not the first observation of protein binding of 5-HT by circulating-blood proteins. Two such proteins were reported by Pignatti & Cavalli-Sforza (1975), but were not characterized, while we find only one. Since these authors did not take precautions to prevent proteolysis, it is possible that degradation of serotonectin led to the two proteins they described. In any case, the only saturable 5-HT-binding protein we could identify in blood, in addition to serotonectin in either rat or human, was albumin.

It should be noted that serotonectin of human and rat is one of a family of proteins that bind 5-HT with high affinity. Others are intracellular proteins found in cells that store 5-HT. Of these, the best characterized is serotonin-binding protein or SBP. This protein is found in central (Tamir & Kuhar, 1975; Tamir, Klein & Rapport, 1976) and enteric serotonergic neurons (Jonakait, Tamir, Gintzler & Gershon, 1979) and in the parafollicular cells of the thyroid gland (Bernd, Gershon, Nunez & Tamir, 1981; Tamir & Gershon, 1981). These cells are all of neuroectodermal origin. Within neurons, SBP is stored as a complex with 5-HT in synaptic vesicles (Tamir & Gershon, 1979; Gershon, Liu, Karpak & Tamir, 1983). Additional 5-HT binding proteins have been identified in mast cells (Tamir, Theoharides, Gershon & Askenase, 1982) and enteroendocrine cells (Gold, Gershon & Tamir, 1982). None of these other proteins resembles serotonectin. The serotonectin-binding proteins found in mammals, therefore, seems to be specific to cell lines derived from different germ layers and to have different functions. That of the intravesicular proteins such as SBP, may be to reduce the osmotic pressure within vesicles by forming a macromolecular complex with many molecules of 5-HT (Gershon & Tamir, 1981). The function of serotonectin is not yet entirely clear but, despite the ability of platelets to store 5-HT, serotonectin is not intravesicular and thus clearly plays no role in the storage mechanism.
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REFERENCES


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