Studies on the Platelet Iso-antibody

by Using C-serotonin.

by

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1) Introduction

It is well known that the blood platelet contains serotonin\(^1\). Interestingly enough, however, no enzyme which synthesizes serotonin (5HT) can be found in the platelet so far. Consequently, it is now considered that platelets can only concentrate 5HT actively by taking it up from the blood under the influence of platelet ATP. (Fig. 1).

As it is shown by many investigators both in vitro and in vivo\(^2\), 5HT uptake by the platelet can be greatly influenced by many conditions and by many agents. For instance, 5HT uptake by platelets suspended in plasma shows great differences according to the temperature, incubation time and the concentration of 5HT in plasma. The uptake can be suppressed by many kinds of metabolic inhibitors such as azide, fluoride and also by reserpine and thrombin\(^3\).

It has already been reported from our laboratory\(^4\) that the platelet antibody can also suppress the 5HT uptake by the normal platelets in various degrees. The hetero-specific antibody in the rabbit serum prepared by injecting human platelets into rabbit shows strong suppression of 5HT uptake by normal human platelets. (Fig. 2).

The existence of platelet antibodies in the serum of patients can be detected by many methods, such as by agglutination and lysis test\(^5\), the anti-human globulin consumption test, the indirect coombs test, the complement fixation test, the
Fig. 2. Invitro uptake of $^{14}$SHT by normal platelet

2) Methode and material

a) Preparation of the platelet rich plasma (PRP) and platelet poor plasma (PPP).

Blood is taken by vein puncture into the syringe containing 3.1% sodium citrate solution. The ratio of the blood and the citrate solution should be around 4:1. The well mixed citrated whole blood is centrifuged for 10 minutes at 1,000 rpm in 4°C. The PRP is then pipetted off and stored in 4°C until use. The PRP is desirable to contain around 3-6 x 10^5 platelets per cumm. If it contains less platelets, the data becomes less reliable.

The test should be set up as soon as possible, because the viability of platelets is very easy to undergo changes, even if they are stored in 4°C. The PPP can be prepared by spinning the citrated blood for 30 minutes at 3,000 rpm in 4°C.

When serum is to be tested, it should be prepared as follows. 100 mg of BaSO4 is added to each 1.0 ml serum and mixed well. After 15 minutes of mixing it is centrifuged for 30 minutes at 3,000 rpm in 4°C. As for its inactivation, the supernatant is incubated in the water bath for 30 minutes at 56°C.

b) Preparation of C$^{14}$5HT

C$^{14}$5HT used in this experiment is 5-hydroxytryptamine-3-creatine sulfate made...
by Nuclear Chicago Cooperation. Each one vial contains 1,800 micrograms of 5HT and its radio-activity is 0.05 mc. In use, we add 10 ml saline to each one vial and stored in 4°C until use. This stock solution is diluted with saline so as to contain 50 micrograms per ml saline.

c) Procedure

PPP or treated serum in the amount of 1.0 ml is added to each aliquot of 1.0 ml normal human PRP. After the subsequent addition of 0.2 ml C\textsuperscript{14}-5HT solution, samples are incubated at 37°C for 30, 60, 90 and 120 minutes respectively, then centrifuged at 3,000 rpm for about 45 minutes in 4°C. The platelet bottom thus obtained are washed with 2.0 ml saline by centrifugation, then resuspended in exactly 2.0 ml distilled water. The platelets are subsequently disrupted by freezing and thawing four times, then the platelet debris is spun down. 1.5 ml aliquots of the supernatant are transfused to steel planzets. After they dry completely their radio activity is measured by an end-window gas flow counter. (Fig. 3)

In this paper, the % uptake is calculated, 1) by comparing the uptake of a given sample to that of an autologus system incubated 120 minutes (relative uptake), 2) and the % of the uptake of the C\textsuperscript{14}-5HT to the absolute dosis which was added to the platelet samples (absolute uptake). (Table 1)

d) Patients and controls

Patients are consist of one PNH patient, five patients with ITP, one aplastic anemia and two neonatal thrombocytopenic purpura. They are all hospitalized to the New England Center Hospital. Controls are consists of more than eighty normal, healthy adults who have never been transfused or pregnant before.
Table 1.
Relative & absolute uptake system of C$^{14}$ 5 HT

(Relative)

<table>
<thead>
<tr>
<th>PRP</th>
<th>PPP or (Serum)</th>
<th>C$^{14}$ 5HT</th>
<th>30'</th>
<th>60'</th>
<th>90'</th>
<th>120'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Donor</td>
<td>.2ml</td>
<td>4,504</td>
<td>42.0</td>
<td>6,160</td>
<td>61.6</td>
</tr>
<tr>
<td>Donor</td>
<td>patient</td>
<td>.2ml</td>
<td>462</td>
<td>4.3</td>
<td>784</td>
<td>7.3</td>
</tr>
</tbody>
</table>

(Absolute)

<table>
<thead>
<tr>
<th>PRP</th>
<th>PPP or (Serum)</th>
<th>C$^{14}$ 5HT</th>
<th>30'</th>
<th>60'</th>
<th>90'</th>
<th>120'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>Donor</td>
<td>.2ml</td>
<td>4,504</td>
<td>13.6</td>
<td>6,160</td>
<td>18.7</td>
</tr>
<tr>
<td>Donor</td>
<td>patient</td>
<td>.2ml</td>
<td>462</td>
<td>1.4</td>
<td>784</td>
<td>2.4</td>
</tr>
</tbody>
</table>

PNH patient. (count in control 33,100)
C......count

III) Results

a) Normal range of C$^{14}$ 5HT uptake

According to my data summerized from more than eighty normal human specimens, the normal range of uptake in the homologous platelet system by 60, 90, and 120 minutes is $59.8 ± 14.4\%$ ($19.1 ± 6.8\%$), $83.8 ± 16.4\%$ ($25.3 ± 8.6\%$) and $101.2 ± 19.5\%$ ($30.4 ± 10.5\%$) respectively. Whereas the % of uptake in the autologous platelet system is $59.0 ± 16.9$ ($19.0 ± 6.9\%$), $84.0 ± 14.2\%$ ($25.3 ± 8.1\%$) and $100.0\%$ ($31.5 ± 8.4\%$) respectively. When normal PPP from 23 donors are combined with platelets from one donor, the average uptake was $97.4 ± 9.8\%$ ($29.5 ± 6.1\%$).

b) Relationship between ABO blood type and platelet antigen type

In order to investigate this problem from our experimental point of view, we tested normal PRP and PPP from known A and B blood donors (each group consists of more then ten donors), who have never been transfused or pregnant. The uptake of each combination is classified and summerized in the table 2.

As it is shown in the table 2, there is not too much difference in each group. Statistically, correlation coefficient of between AA and BB is 0.089, AA and AB is 0.074 and BA, BB is 0.109, respectively. Thus we can not get any significant relationship between AB blood type and platelet antigen types.
Table 2. Relationship between A, B blood type and 5HT uptake by the platelet.

<table>
<thead>
<tr>
<th>PPP</th>
<th>blood type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>PRP</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>99.0 ± 15.4%</td>
</tr>
<tr>
<td>(27.3 ± 6.6)</td>
<td>(25.0 ± 6.1)</td>
</tr>
<tr>
<td>B</td>
<td>95.4 ± 8.2%</td>
</tr>
<tr>
<td>(28.6 ± 6.3)</td>
<td>(28.1 ± 7.8)</td>
</tr>
</tbody>
</table>

Notice: A, B show PPP and PRP from A and B blood type.
( ) shows absolute uptake.

We also tried to determine whether there are differences of uptake between platelets from known A and B type blood donors, when they are combined with anti-A and anti-B sera. Typing sera and sera from A and B blood donors are absorbed with BaSO₄ (100 mg of BaSO₄ are added to each 1.0 ml serum) then heated for 30 minutes at 56°C.

The combination of platelets from the blood of type A and anti-A serum, platelets from the blood of type B and anti-B serum showed the lowest uptake in each group, respectively, as it is shown in the column 1 of the table 3.

Whereas, when both typing sera are absorbed with red cells from type A and type B, respectively. Red cells are washed three times with saline and packed red cells are suspended in the corresponding serum so as to contain approximately 5×10⁶/cumm cells and stored overnight at 4°C before centrifugation. These absorption are repeated until they show no visible red cell agglutination.

Table 3. Relationship between anti-A, anti-B sera and platelet antigen types.

<table>
<thead>
<tr>
<th>PRP (1.8ml)</th>
<th>sera (0.2ml)</th>
<th>% uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>100.0 (29.0)</td>
</tr>
<tr>
<td>A</td>
<td>anti-A</td>
<td>79.6 (20.3)</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>97.6 (28.3)</td>
</tr>
<tr>
<td>A</td>
<td>anti-B</td>
<td>85.2 (24.7)</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>100.0 (33.5)</td>
</tr>
<tr>
<td>B</td>
<td>anti-B</td>
<td>76.9 (25.8)</td>
</tr>
<tr>
<td>B</td>
<td>A</td>
<td>102.5 (34.4)</td>
</tr>
<tr>
<td>B</td>
<td>anti-A</td>
<td>89.0 (29.9)</td>
</tr>
</tbody>
</table>

( ) shows absolute uptake.

In the column II, typing sera were absorbed three times with corresponding red cells.
Anti-A and Anti-B sera were from Hyland Laboratories in Los Angeles, California.
According to our repeated experiments, it might be able to assume that platelet antigens seems to have some relationship with red cell antigens. Since after the absorption with red cells, typing sera lost their ability to suppress the 5HT uptake by the platelets.

c) 5HT uptake in various diseases with thrombocytopenia

I. Paroxysmal nocturnal hemoglobinuria

Mrs. Fullerton; 41 years old white house wife. She has been suffering from PNH for many years. Because of anemia and thrombocytopenia (50,000—117,600), she was transfused several hundred times before admission. It might be reasonable to assume that in such a patient, thrombocytopenia may suggest the presence of platelet iso-antibody resulting from repeated blood transfusions.

We tested her PPP against platelets from 20 normal donors in order to verify the presence and the strength, if possible, of the iso-antibody by using our method described above.

The mean value of uptake was 27.6 ± 20.5% (6.0 ± 2.4%). From this data, I think, we can readily assume the presence of very strong iso-antibody which suppressed the 5HT uptake by normal platelets. (Fig. 4)

Furthermore, we could confirm this by the Dausset test and the complement fixation test (308 units in this patient while it was 54 units as an average in normal people).

Interestingly enough, we could use the platelet from her healthy identical twin sister. The uptake by using her twin sister's PRP was 89.9% (20.5%), a value which is considered within almost normal range according to our standered range.

2. Idiopathic thrombocytopenic purpura

There may be little doubt that the cause of thrombocytopenia in ITP patients may

<table>
<thead>
<tr>
<th>Patients</th>
<th>diagnose</th>
<th>platelet count</th>
<th>% uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mrs. London</td>
<td>ITP</td>
<td>7,420–24,300</td>
<td>94.8 (36.2)</td>
</tr>
<tr>
<td>Mr. Maderos</td>
<td>ITP</td>
<td>90,000</td>
<td>96.9 (30.1)</td>
</tr>
<tr>
<td>Mrs. Rogers</td>
<td>ITP (Drug?)</td>
<td>18,300</td>
<td>99.4 (26.7)</td>
</tr>
<tr>
<td>Miss Byrne</td>
<td>ITP (Drug?)</td>
<td>7,000</td>
<td>108.0 (42.7)</td>
</tr>
<tr>
<td>Mrs. Finkle</td>
<td>ITP</td>
<td>50,000–73,000</td>
<td>93.5 ± 5.8 (28.4 ± 3.8)</td>
</tr>
<tr>
<td>Miss Donovan</td>
<td>Normal</td>
<td>200,000–300,000</td>
<td>97.4 ± 9.8 (29.5 ± 6.1)</td>
</tr>
</tbody>
</table>
possibly be due to the presence of platelet auto-antibody in the patients serum, even though no definite mode of detection is yet available. We tested five patients diagnosed as the ITP, yet none of them showed significant suppression of uptake below normal range (Table 4).

As it is shown in the table 4, Mrs. Finkle's PPP was 93.3±5.8% (28.4±3.8%) in the average when combined with PRP from fourteen normal donors. There is no significant difference between this and that of the normal average. (Fig. 4)

3. Aplastic anemia

Mrs. Briggs; 59 years old white house wife has been transfused many times in recent years because of anemia. Her platelet count was in the range of 43,540—59,200. We tested patient's PPP against PRP from nine normal donors. The maximum uptake was 100.0% (40.2%), the minimum was 43.3% (13.8%) and the average was 81.9±20.1% (23.7±9.7%). This data shows slight suppression of uptake according to our normal standard range. Thus, we can assume there might be some weak iso-antibody due to repeated transfusions, and this antibody played some role on the development of thrombocytopenia in the patient. (Fig. 4)

Fig. 4. Comparison of C$^{14}$-5HT uptake between normal and various diseases

Fig. 5. Changes of % uptake of C$^{14}$-5HT in NTP during pregnancy

4. Neonatal thrombocytopenic purpura

Mrs. Wyse, a healthy looking white house-wife with almost normal platelet count, is a mother of three children. Her second and third child showed severe throm-
bocytopenic purpura when they were born. She came to us because of the fourth pregnancy. At the very beginning of the pregnancy her serum showed no suppression of uptake, that is 122.6% (32.0%), while at around the fifth month of pregnancy the uptake dropped to 62.0% (12.4%), by the end of the sixth month it was 43.5% (8.5%) then it dropped to 20.0% (4.6%) by the seventh month and it became only 4.2% (0.8%) before delivery. From these data we strongly suspected the development of maternal iso-antibody against the fetal platelets. Unfortunately, however, we could not confirm this with the new born child because her family removed to a city far from my hospital, therefore, the uptake of 5HT by the platelet from this new born is suppressed or not by the serum from the mother could not be studied. (Fig 5)

I also studied Mrs. Correa, a white house wife now showing around 110,000 platelets/cumm, who delivered a child with thrombocytopenic purpura before. She is now sixth month of her second pregnancy and her PPP tested against her healthy husband and normal donor showed 61.4% (12.2%) and 55.7 (11.4%), respectively.

IV) Discussion

In mammalian tissue, serotonin is localized chiefly in the mucosa of gastro-intestinal tractus, the brain, the spleen and blood platelets. The concentration of 5HT in plasma is usually very low in comparison with that of platelets.

The infusion of 5HT into a dog results a marked increase in the platelet 5HT level. On the other hand, the administration of a single injection of reserpin in the dose of 5 mg per kg body weight to animals depletes the serotonin contents of the brain and platelets almost completely.7)

Humfery, Jaques7) and Code8) found that during in vitro reaction of antigen with partially purified antibody, serotonin and histamine are liberated from platelets in heparinized rabbit, dog or guinea pig blood. They postulated that this might be due to the protease reaction of antigen-antibody complex to the platelet. Although we did not have a definite proof that the suppression of 5HT uptake is due to the direct influence of this antigen-antibody complex, yet the platelets combined with antigen show platelet agglutination and lysis, positive fluorescein conjugation, short survival of infused platelets, positive complement fixation test, inhibition of clot retraction and reduction of thromboplastin generation.

From these data above, we might easily assume that the platelet undergo some modifications by the presence of the platelet antibody and this modification of the platelet might possibly results suppression of 5HT uptake by the platelet.
Baldini and Bridges\(^4\) have already reported that the heterospecific antibody in the rabbit serum prepared by injection of human platelets into rabbit shows strong suppression of 5 HT uptake by normal human platelets.

As it is already mentioned above, not only hetero-specific platelet antibody but also platelet iso-antibody can show suppression of 5 HT uptake by normal human platelets.

According to my observations, 5 HT uptake by normal human platelets after 120 minutes incubation is \(101.2 \pm 9.5\%\) (30.4±10.5\%) in homologus platelet system, whereas absolute uptake in autologus platelet system is 100.0\% (31.5±8.4\%), when the donors are normal and healthy and never be transfused or pregnant before. As it is shown above, the 5 HT uptake by normal platelets shows no significant differences between autologus and homologus system.

There might be little doubt about the existence of platelet antigenicity. However, many different opinions\(^{10}\)\(^{11}\)\(^{12}\)\(^{13}\) still exist whether human platelets possess the A and B antigen identical to those of red cells. The presence of the A and B antigen in platelets seems to have been well established at one time. However, since the existence of individual platelet groups and types independent from red cell antigens was reported by Harrington and Sprague\(^{14}\), and more recently their findings are favoured either by the estimation of life span of transfused platelets or by the complement fixation test.

According to my experiment, the uptake of 5 HT in various combinations of PRP and PPP from known A and B blood type donors in homologous platelet system shows no significant differences statistically. However, when anti-A and anti-B typing sera are combined with platelets from known A and B blood type donors, the uptake in the combination of platelet in type A (donor) and anti-A serum shows lowest uptake than any other combination. Almost same tendency is noticed in the combination of platelets from type B donor and anti-B serum. Furthermore, when these typing sera are absorbed with red cells from A and B type blood respectively, the differences of uptake disappeared completely. In another words, the substance which suppressed the 5 HT uptake was absorbed completely with red cells, and this was confirmed by repeated experiments thereafter.

It might be able to conclude that platelets may have some relationship with red cell antigens, even though most of them are platelet group specific.

The development of platelet iso-antibody may chiefly depends upon the frequency of transfusions given previously. It might not be difficult to assume the presence
of strong isoantibody in the serum of Mrs. Fullerton (PNH), who has been transfused several hundred times before. I was able to demonstrate very strong iso-antibody in her serum by the $^{14}$C-5 HT uptake system.

The pathogenesis of the neonatal thrombocytopenic purpura may be classified into three groups; the first is caused by congenital absence of megakaryocytes, the second is found in the children born to mother with ITP and in the third thrombocytopenia occurs in infants born to normal mother. The neonatal thrombocytopenic purpura in the second and third case, may be due to placental transfer of maternal auto- or iso-antibody formed against fetal platelets$^{14)15)}$.

I studied two cases of neonatal thrombocytopenic purpura. I couldn't demonstrate any iso-antibody in the serum of the mother (Mrs. Wyse) by our method when she was very beginning of her pregnancy, but her serum showed steady increase of platelet isoantibody. By the end of the pregnancy the uptake was only 4.2% (0.8%) which suggested the development of strong platelet iso-antibody in her serum during the course of her pregnancy.

V) Conclusion

As it is reported above, I studied the presence of platelet iso-antibody by the $^{14}$C-5 HT uptake system originated in our laboratory (Dept. of Hematology, New England Center Hospital. Director Dr. William Dameshek). I am now comparing my data with that of Dausset test, complement fixation test and platelet life span. As the consequence, I do believe that this method is worthwhile to try to find out platelet iso-antibody in the thrombocytopenic patients who have been transfused or pregnant before. As it was mentioned already, $^{14}$C-5 HT uptake by the platelet is influenced by many agents and conditions, however, the details of it's mechanism is not clear in some points, and I'm now trying to go further to solve this problem.

VI) References

C-14 HT による血小板抗体の新診断法

関西医科大附属病院
第二内科 守屋 邦男

免疫血液学の発展に伴い、各種血液疾患中その発生機序に免疫機構の関与する場合が少なくない事が注目されているが、著種血小板減少症の場合においても免疫血液学との関連において多くの研究が進められている。

血小板抗体の存在が、血小板寿命の短縮、血小板凝集・溶解、血小板破壊の少ない、T G T抑制、発光抗体法、補体結合反応法等によって診断される事はよく知られているが、なお充分とは云い難い。

私は米国留学中、この問題の解明を Dameshek 教授から仕業され、Baldini 博士と 1 年間共同研究を行った。その結果血小板中の Serotonin 含量は、血小板抗体と抗元の反応の場において鋭敏に影響される事実を知り、種々実験を重ねた結果、血小板中の Serotonin 含量を指標とする血小板抗体測定法を突発した。

この方法は、論文中に詳述した如く、患者血液、正常人血液、C-14 HTの三者を利用して、血小板抗体反応時における血小板中残存する Serotonin の量から、逆に患者血清中の抗体の有無を診断する方法であって、臨床上輸血後の血小板減少症や新生児貧血症等の血小板同種抗体の診断に役立つものであり、実験的には異種抗体の診断上極めて有力であると信じる。

【なお本論文要旨は、Boston 血液集談会、第45回関西医科大学医学集談会、大阪市立大学医学部、大阪血液学会講演、第60回日本内科学会総会等で演題した】