A Case of Thrombasthenia with a Study of Platelet Aggregation by Hydrogen Peroxide (H_2O_2)

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HIGASHI, O., KIKUCHI, Y. and KONNO, K. *A Case of Thrombasthenia with a Study of Platelet Aggregation by Hydrogen Peroxide (H_2O_2)*. Tohoku J. exp. Med., 1972, 106 (4), 399-409 — A six-year-old girl with a congenital hemorrhagic disorder characteristic of thrombasthenia or Glanzmann's disease was reported. A special study on platelet aggregation was performed. It was found that patient's platelets failed to aggregate in response to hydrogen peroxide (H_2O_2), while normal platelets were readily aggregated by this agent. This H_2O_2 induced aggregation of normal platelets can be inhibited by adenosine, suggesting a possible mechanism that H_2O_2 might stimulate platelets to release ADP, which in turn causes platelet aggregation. ——— thrombasthenia; platelet aggregation by hydrogen peroxide

Thrombasthenia or Glanzmann's disease is the hemorrhagic state which is characterized by a congenital bleeding tendency with normal platelet count, long bleeding time, variably deficient clot retraction and absent platelet aggregation.

The fundamental defect in this condition appears to be the inability of platelets to aggregate in response to adenosine diphosphate (ADP) (Hardisty *et al.* 1964, Caen *et al.* 1966, Zucker *et al.* 1966, and Corby *et al.* 1971).

The purpose of the present paper is to describe a case with thrombasthenia and to report the results of a study on the platelet aggregation induced by a new aggregating agent, hydrogen peroxide (H_2O_2).

CASE REPORT

The present case (S.C.) was referred to the Akita University Hospital for the study of her hemorrhagic tendency. The early history revealed that a prolonged umbilical bleeding was encountered at birth. At 3 years of age, she had a profuse bleeding from mouth following trauma. Since then she had had frequent petechiae, ecchymoses and epistaxis, sometimes necessitating blood transfusions.

The family history revealed that the parents are unrelated. Neither parents nor two younger sisters of the present patient had symptoms suggesting hemorrhagic diathesis. A cousin of the paternal grandfather died of a hemorrhagic diathesis of unknown nature.

The physical examination on admission revealed a well developed and well nourished 6-year-old girl with new and old petechiae scattered over the entire

body, but with no other marked findings.

During a period of about 3 months of hospitalization, she has had frequent episodes of nose bleed, which were controlled by local hemostatic measures.

Laboratory Findings

RBC was 3.96 millions per mm³, hemoglobin 11.2 g per 100 ml, hematocrit 34%, reticulocytes 12‰, WBC 5,800 per mm³. Differential counts and morphology of leucocytes were normal. Other normal laboratory data included serum protein, serum electrolytes, serum GOT and GPT, alkaline and acid phosphatases and lactic dehydrogenase. A microscopic hematuria and a positive fecal occult blood test were encountered sometimes.

Bleeding, coagulation and platelet studies

The bleeding time (Duke) was prolonged, being 32 minutes. The Rumpel-Leede test was positive. The platelet count (Rees and Ecker) was normal, being 270,000 per mm³. In stained blood smears, almost no platelet clumping was seen. The nuclear segmentation of megakaryocytes in bone marrow (Higashi *et al.* 1971) was within normal limits, the lobe average being 1.05.

The blood coagulation studies, including recalcification time, partial thromboplastin time (PTT), Kaolin-PTT, prothrombin time (Quick) and thrombotest (Owren) were all within normal limits. The thromboplastin generation tests (Biggs and Macfarlane 1957) using patient's own platelets, deprothrombinized plasma and serum, also gave rise to the entirely normal results. The activity of Factor XIII (Barrey *et al.* 1965) was normal. Plasma fibrinogen was 260 mg per 100 ml. The clot retraction (Didisheim 1967) appeared to be within normal limits, being 75. In the thromboelastogram, however, a prolongation of *k* and decreases of *ma* and *mε* values were evident (cf. Table 1). Adhesiveness of platelets on glass surface (Tanaka 1957) was reduced (cf. Table 1).

The results of the platelet aggregation studies (Corby *et al.* 1971) were grossly

TABLE 1. *Evaluation of platelet status of the present patient and her parents*

	Patient	Mother	Father	Normal control
Bleeding time (min)	32	3	2	1-6
Platelet count/cmm	270,000	177,000	213,000	150,000-450,000
Platelet adhesion, %	0	27	40	26-34
Platelet aggregation, %*				
APD	5-10	41-93	62-51	69-97
Collagen	2	37	55	67
H ₂ O ₂	2-4	37-50	13-18	20-67
Thrombelastogram				
<i>r</i> (min)	10.5	10.5	10.8	9-13
<i>k</i> (min)	10.0	4.5	4.5	4-6
<i>ma</i> (mm)	38.0	55.5	52.5	49-61
<i>mε</i> (%)	61.3	124.5	110.5	96-156

* The maximum per cent aggregation of platelets observed during a period of six minutes after the addition of the aggregating agent.

Fig. 1. Platelet aggregation by ADP in the present patient, her parents and two normal controls.

abnormal. The platelets of patient failed to aggregate normally in response to ADP* or collagen (Bovine Achilles tendon*) (cf. Fig. 1 and Table 1).

Family studies: The parents of the present case were studied with respect to platelet status and the results were normal except for the H_2O_2 induced aggregation of father's platelets as shown in Table 1 (cf. Table 1).

Special Investigation

Platelet aggregation induced by hydrogen peroxide (H_2O_2)

MATERIALS AND METHOD

Blood specimens were collected by venipuncture through a disposable needle into a plastic syringe and was immediately transferred to a silicone-coated centrifuge tube, containing 10% by volume of 3.8% sodium citrate solution, capped with parafilm and was mixed by inversion. Platelet-rich plasma (RPR) was prepared by centrifuging blood for 8 to 10 minutes at about 1,200 rpm and platelet-poor plasma (PRP) by centrifuging for 30 minutes at 3,000 rpm at 4°C.

Platelet counts were done by the use of Rees-Ecker diluting fluid. The PRPs of

* ADP and collagen were acquired from Tokyo Kasei Kogyo Co.

various platelet counts were prepared by diluting the original PRP with the PPP.

In the standard method, the PRP containing about 300,000 of platelets per cmm was used, unless indicated otherwise.

The erythrocyte content, if any, in the original PRP was less than 4,000 per cmm.

Imidazole buffer was prepared by the method described by Mustard *et al.* (1964).

The solutions of hydrogen peroxide (H_2O_2), adenosine*, reduced glutathione (GSH†), N-ethyl maleimide (NEM†) and disodium ethylenediaminetetraacetate (EDTA) were made up in the imidazole buffer solution, pH 7.3 (IBS).

The platelet aggregation was measured in a platelet aggregation meter, EEL 169 (Evans Electroselenium Ltd., Halstead, Essex, England) and recorded on a recorder (Model N-14, Rikadenki Kogyo Co., Tokyo).

In the standard method used in the present investigation, 0.03 ml of IBS or the test substance was added to 0.6 ml of PRP and platelet aggregation was initiated by the addition of 0.07 ml of 0.3% H_2O_2 solution. H_2O_2 solution was added by the use of a microsyringe (Hamilton, Type 1710).

The percent change in platelet aggregation at various time intervals were determined by the method described by Corby *et al.* (1971).

Platelet aggregation was also observed macroscopically and microscopically. In this case the same reaction mixture as described above in a siliconized test tube was vigorously shaken manually and was observed macroscopically. Aliquotes of the reaction mixture were taken out at time intervals and drawn into the leucocyte pipet up to the mark 0.5 and then the Rees-Ecker fluid was drawn up to the mark 101, mixed well, and then transferred to a counting chamber and subjected to a microscopic observation.

RESULTS

Normal platelets did aggregate very readily in response to H_2O_2 , while the platelets from the present patient did not (cf. Table 1 and Figs. 2-4).

Platelets from the mother of the present case showed a normal aggregation by H_2O_2 , while platelets from the father tended to show a low normal or slightly decreased aggregation (cf. Table 1 and Fig. 4).

The H_2O_2 induced aggregation of normal platelets reached at maximum levels during a period of time between one and one and a half minute after addition of H_2O_2 and then, in most cases, partly dispersed or disaggregated, as was shown in Fig. 2.

The maximum degree of aggregation by H_2O_2 of platelets from 11 normal control cases ranged from 20 to 67% and $46 \pm 3\%$ in average (Table 2).

The aggregation and disaggregation of normal platelets were also confirmed by serial macroscopic and microscopic observation, by the use of method described above, of the mixture of PRP and H_2O_2 during a period of time between one and five minutes following the addition of H_2O_2 .

Relation between platelet aggregation and concentration of H_2O_2

When the concentration of H_2O_2 was increased from 0.1% to 0.3%, (or from 0.01% to 0.03% in the final concentration), the extent of platelet aggregation was also increased. But when the concentration of H_2O_2 was further increased from 0.3% to 0.5% or 1%, the maximum percentage of platelet aggregation was not

* Merck Co.

† Tokyo Kasei Kogyo Co.

Fig. 2. A record by the aggregation meter of a normal platelet aggregation by H_2O_2 in a control case.

Fig. 3. A record by the aggregation meter of the absent platelet aggregation by H_2O_2 in the present patient.

Fig. 4. Platelet aggregation by H_2O_2 in the present patient, her parents and two normal controls.

TABLE 2. *The maximum levels of platelet aggregation by H_2O_2 in control cases*

No.	Name	Age	Sex	Clinical conditions	Maximum platelet aggregation by H_2O_2 (%)
1.	N.J.	15 y	F	Apparently healthy	48
2.	H.O.	48 y	M	Apparently healthy	42
3.	N.K.	9 y	M	Apparently healthy	20
4.	S.A.	30 y	F	Apparently healthy	54
5.	O.K.	13 y	M	Upper respiratory infection	32
6.	S.Y.	21 y	F	Apparently healthy	30
7.	H.Y.	20 y	F	Apparently healthy	62
8.	W.K.	20 y	F	Apparently healthy	54
9.	I.M.	20 y	F	Apparently healthy	46
10.	I.Y.	20 y	F	Apparently healthy	67
11.	H.T.	24 y	F	Apparently healthy	56

Mean $\pm$ S.D. = 46 ± 3

always increased and could conversely be decreased as shown in Fig. 5.

Relation between platelet count and platelet aggregation by H_2O_2

As is shown in Fig. 6, there is a tendency that as the platelet count in PRP decreases the maximum per cent aggregation of platelets also decreases.

On the basis of such observation it was decided, as described already, to use PRP with a constant platelet count of about 300,000 per cmm of PRP, whenever possible, for the present H_2O_2 induced platelet aggregation test.

Fig. 5. Platelet aggregation by H_2O_2 , varying 0.01 to 0.1% in a final concentration.

Effect of adenosine on the platelet aggregation by H_2O_2

0.03 ml of adenosine solutions of various concentrations, *i.e.* 0.2, 0.4 and 1 mM, respectively, was added to 0.6 ml of PRP and the mixture was incubated at $37^\circ C$ for 5 minutes, and then 0.07 ml of 0.3% H_2O_2 solution was added to initiate platelet aggregation.

The inhibitory effect of adenosine on the platelet aggregation by H_2O_2 was clearly demonstrated as was shown in Fig. 7.

Effect of N-ethylmaleimide (NEM) on the platelet aggregation by H_2O_2

To 0.6 ml of PRP, either 0.03 ml of IBS or 0.03 ml of 70/3 mM-NEM or 0.015 ml of 140/3 mM-GSH and 0.015 ml of 140/3 mM-NEM, or 0.015 ml of 140 mM-GSH and 0.015 ml of 140/3 mM-NEM was added, and the mixture was incubated at $37^\circ C$ for 1 minute and then 0.07 ml of 0.3% H_2O_2 solution was added.

The results showed that platelet aggregation by H_2O_2 was inhibited by NEM in a final concentration of 1 mM and this inhibition by NEM was prevented by the addition of GSH in a final concentration of 3 mM (Fig. 8).

Effect of disodium ethylenediaminetetraacetate (EDTA)

Effects of EDTA on the aggregation of platelets in citrated PRP induced by H_2O_2 were shown in Fig. 9. EDTA exerted its inhibitory effect on platelet aggregation in final concentrations of 1 to 10 mM.

Fig. 6. Relationship between platelet count in PRP and platelet aggregation by H_2O_2 .

Fig. 7. The inhibitory effect of adenosine, of various concentrations in the solution added, upon the platelet aggregation by H_2O_2 . For details, see text.

Fig. 8. Effects of N-ethyl maleimide (NEM), with or without addition of reduced glutathione (GSH), upon the platelet aggregation by H_2O_2 . For details, see text.

Fig. 9. The inhibitory effect of disodium ethylenediaminetetraacetate (EDTA), varying 1 to 10 mM in a final concentration, upon the platelet aggregation by H_2O_2 .

DISCUSSION

The present case has shown a congenital bleeding tendency with normal platelet count, long bleeding time, decreased platelet adhesiveness to glass, abnormal thrombelastogram and absent platelet aggregation after the addition of ADP and collagen, and thus fulfills the diagnostic criteria of thrombasthenia (Hardisty *et al.* 1964, Zucker *et al.* 1966, Corby *et al.* 1971 and Hathway 1971).

The basic defect in this disease appears to be the failure of platelets to aggregate in the presence of ADP. It is generally accepted that ADP plays a central role in platelet aggregation (Gaarder *et al.* 1961, Born 1962 and Käser-Glanzmann *et al.* 1962).

A variety of substances, including trypsin, serotonin, epinephrine, norepinephrine, and such particulate or semiparticulate substances as long-chain saturated fatty acids, protein-coated polystyrene particles, antigen-antibody complexes, heat-denatured plasma proteins, uric acid crystals, certain bacteria and viruses and glass beads, are known to be able to induce platelet aggregation. It has been postulated that ADP provides a final common pathway for all these agents (Marcus 1969).

The platelet aggregation brought about by ADP can be inhibited by adenosine (Born *et al.* 1963, Mustard *et al.* 1964 and O'Brien *et al.* 1966).

To our knowledge, there is no literature dealing with the platelet aggregation induced by hydrogen peroxide (H_2O_2), and the present paper may be a first to describe such a phenomenon.

The exact mechanism underlying the phenomenon of platelet aggregation induced by H_2O_2 is obscure. But, the fact that the normal platelets preincubated with adenosine failed to aggregate in response to H_2O_2 , as demonstrated in the present investigation, has suggested that H_2O_2 might stimulate the platelets to release ADP, which in turn made platelets aggregate.

If this is the case, it seems conceivable that the platelets of the present patient of thrombasthenia could not be aggregated not only by ADP but also by H_2O_2 . The problem that how H_2O_2 does affect, if any, ADP metabolism in platelet remains to be elucidated.

Platelet aggregation by ADP is dependent on calcium (Born and Cross 1963, Harrison, Emmons and Mitchell 1966) and thiol groups of platelets are involved in the process of aggregation (Robinson, Mason and Wagner 1963, Harrison, Emmons and Mitchell 1966). In these respects, the platelet aggregation by H_2O_2 appears to be similar to that induced by ADP, as suggested by the results of present experiments using a chelating agent EDTA and a sulfhydryl inhibitor MEM. The possible role of calcium in the ADP platelet aggregation was discussed by Skålhegg *et al.* (1964).

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