Evaluation of Nialamide on the Coagulation of Blood

Introduction of a New Technic for Measuring Adhesiveness of the Platelets

By CHAPOUR MASCHOUF, ROGER W. ROBINSON AND RAOUL J. LEBEAU

SINCE 1960, Shimamoto and his associates have reported in several publications1 on the antithrombotic properties of nialamide,* a monoamine oxidase inhibitor. When the internal surface of arteries and veins of the rabbit’s ear were injured, the authors found a “powerful antithrombotic effect” in animals pretreated with nialamide by “preventing intravascular thrombosis without inhibition of extravascular clotting.” Nialamide was observed to prevent the swelling of the endothelial cells, the appearance of stickiness of these cells to platelets and leukocytes, and the formation of white thrombi following the intravenous injection of serotonin, epinephrine or norepinephrine. These findings prompted the present investigation of the antithrombotic effect of this drug in human beings. The present work supports the findings of Shimamoto and shows that the inhibitory action of nialamide on thrombosis is presumably achieved by diminishing the platelet’s contribution to this process.

MATERIALS AND METHODS

Patient Material

All tests on the patients or healthy volunteers were carried out in a fasting state in the morning. They were requested to abstain from smoking2 until the examination was completed. The patients were selected from a group of the hospital population with “minor disorders,” who were not acutely ill and were not receiving medication. They had an age range of 22–78 with a mean age of 56 years. Nialamide was given by mouth.

Eighty-eight patients were divided into four groups for different types of study. In addition, 40 healthy volunteers were selected for the introduction of a new technic for measurement of the adhesiveness of the platelets, which has been applied to the present study.

(1.) Ten ambulatory patients received 75–200 mg. of nialamide daily in divided doses (1–3 mg./Kg. body weight/day). The duration of treatment was from 1–8 months. The following determinations were carried out before treatment and subsequently at different intervals, ranging from 1 week to 1 month, depending on the duration of the therapy: tourniquet test, red cell count, white cell count, differential white blood count, hemoglobin, hematocrit, sedimentation rate, bleeding time, Lee-White clotting time, silicone clotting time, clot retraction and prothrombin time.

(2.) Seventeen patients received a single oral dose of 100 mg. of nialamide (1–2 mg./Kg.

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*N-N-[2-(benzylcarbamyl) ethylamino]-isonicotinamide, Niamide(R), Pfizer Laboratories, Brooklyn, New York.

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body weight). Just before and 2 hours after medication, the same clinical and laboratory examinations were completed as in the first group.

(3.) Thirty-two patients received a single oral dose of 100 mg. of nialamide (1–2 mg./Kg. body weight). Platelet adhesiveness was determined before and 2 hours after medication. For this group, the method of Moolten and Vroman was used.

(4.) Twenty-nine patients received a single dose of 150–200 mg. (average 3 mg./Kg. body weight) of nialamide. The adhesiveness of the platelets was determined by the technic presented in this paper. Fifteen of these patients also had determination of prothrombin consumption, and in 10 patients of the latter group the thromboplastin generation was measured. All tests were done before and 3 hours after the therapy.

Methods

Quick's one-stage prothrombin time was determined, using undiluted plasma with Simplastin as the thromboplastic extract, which was also utilized in the prothrombin consumption test. Ivy bleeding time was done with a disposable lancet 3 mm. in length. The technic of Wintrobe was followed for the tourniquet test and cell counts. For clot retraction a modification of MacFarlane's method was used. Prothrombin consumption was measured by the method of Sussman, Cohen and Gittler, and Vroman's technic of platelet adhesiveness was applied in one group of the patients. For the modified thromboplastin generation test, as described by Hicks and Pitney, reagents of Warner-Chilcott Laboratories were used.

In the absence of sufficient knowledge of the phenomenon of the adhesiveness of platelets, it was not possible to be reasonably certain whether the results of the measurement of the adhesiveness with the method of Moolten and Vroman reflected the pharmacological property of nialamide. After reviewing and evaluating all available methods for the measurement of the adhesiveness of the platelets (Rowntree and Shionoya, Johnson, Best et al., Morawitz and Jürgens, Wright, Moolten and Vroman, Revol, Marx and Derlath, Hellem, Borchgrevink, O'Brien, Garvin, Nestel, Swank) which have been briefly presented by Hellem and Biggs and MacFarlane, the authors concluded that most of the available methods have one or several disadvantages. The addition of an anticoagulant introduces an unknown factor. The surface area to which blood is subjected is not standardized. Isolation of blood from its circulating state creates an unusual environment. Multiple handling of the blood increases the chance of hemolysis with the possible induction of platelet aggregation. Delayed counting of the platelets and the resulting data may not represent the true adhesiveness. The present technic which has been developed after considering these facts, eliminates many of these possible sources of error.

Method for Measuring the Adhesiveness of the Platelets: Essential Materials for Construction and Preparation of the Unit (Fig. 1)

A 45 cm. and 25 cm. PE tubing (C and I) are attached to needles (A and K) via adapters (B and J). The filter column (F) contains 10 Gm. of non-silicone coated glass beads (G). Two 6.6 mm. stoppers (E) are put on either end and the sleeves are inverted. A piece of nylon chiffon (H) separates the stoppers from the beads. The PE tubes are connected to the filter by two needle shafts (D) (needles cut off from heel and tip).

* (1) Siliconized sterile needle, gauge 20, a 5 ml. aspiration syringe, 3-way stopcock (Becton and Dickinson). (2) Polyethylene tube PE-100 and male Luer-lock polyethylene tubing adapter (Clay Adams). (3) Sleeve type rubber stoppers, 6.6 and 7.6 mm. and glass tubing 5 mm. inside diameter (Howe & French). (4) Glass beads 0.5 mm. in diameter (Dragon-Werk, Beyreuth, W. Germany). (5) Parafilm (Marathon, A Division of American Can Company, Menasha, Wisconsin.)

†All letters in parenthesis refer to those in figure 1.
Fig. 1.—Schematic drawing of the apparatus used for measuring the adhesiveness of the platelets.

The test tubes (O and T) each contain 1 mg. ethylenediamine tetra-acetate (EDTA) by evaporating 0.5 ml. of a 0.2 per cent solution in siliconized test tubes and leaving them in a dust-free warm chamber. The level of the fluid is marked on the test tube (0.5 ml.) for future estimation of the amount of collected blood. The tube (O) is covered with a 7.6 mm. sleeve type rubber stopper (M). The Brecher's method31 was utilized for platelet counting, using a phase microscope and a rotating pipet shaker.

Performing the Test

The adapters are sealed with melted paraffin and all joints are covered heavily with grease. A blood pressure cuff is put on the patient's arm and inflated to 50 mm. Hg. The plunger of the syringe is kept at 0.6 ml. level, the stopcock (B) is closed toward the syringe (S). The needle (A) is inserted in the vein. The plunger is pulled back against the resistance of the vacuum pressure within the syringe and locked in the metal clip (U). The stopcock is opened and a stop watch is started. The vacuum effect is transferred via R, Q, P, L, O system to the filter unit. As soon as blood is sighted in the upper tube (I), the watch is stopped. This measured time is referred to as "flow time." When about 0.5 ml. of blood is collected in the test tube (O), the needle (K) is pulled out and put in the tube (N). The PE tube (C) is clamped and cut for collecting the control specimen in the test tube (T). The tubes are immediately covered with Parafilm(R) and inverted five times gently to dissolve the anticoagulant. The samples are
immediately prepared for platelet counting. The index of adhesiveness in the per cent of platelets lost after filtration, with a flow time of 20 seconds. *

"Normal" Value of Adhesiveness with the Present Technic

Before applying the present technic to the clinical pharmacology of nialamide, the test was tried on 40 healthy volunteers with an average age of 29 years. Two filter units of different size were used and also the flow time was randomly influenced by producing different amounts of vacuum pressure in the syringe. Figure 2 shows the result graphically. Table 1 is a summary of the data. Since the flow time through the filter under the prescribed technic will usually fall within 17.5 to 21 seconds, the following formula will convert any obtained result within the range of 17.5-21 seconds, to a standard 20 second time:

\[
\text{per cent adhesive platelets (for 20 seconds flow time)} = \text{per cent of observed adhesive platelets} \left(\frac{20}{\text{observed time in seconds}}\right) - \left[(20 - \text{observed time in seconds})^{0.5}\right]
\]

The technic was carried out on 16 normal subjects and repeated 3 hours later to check the reproducibility of the test. There was a mean variation of 5 per cent in the adhesiveness of the platelets. The mean value for platelet adhesiveness was 63 ± 3 per cent in the initial determinations. The mean value of the determinations done after three hours was 68 ± 4 per cent. The values decreased in 5 patients, increased in 9, and showed no change in 2. A further trial of the technic on 60 normal individuals showed the average value for platelet adhesiveness to be 66 ± 2 per cent with a Standard Deviation of 15.6.

RESULTS

(1) The data obtained from 10 patients treated with 75-200 mg. of nialamide daily in divided doses (1-3 mg./Kg. body weight/day) for a duration of 1 to 8 months showed neither a persistent change nor a difference from the control value in the tourniquet test, red cell, white cell and differential count, hematocrit, hemoglobin, sedimentation rate, platelet count, bleeding time, clot retraction, clotting time, silicone clotting, and prothrombin time. (2) Seventeen patients treated with a single dose of 100 mg. of nialamide (1-2 mg./Kg. body weight) exhibited no difference from the control value, 2 hours after the drug intake, when the same determinations were carried out as in the above-mentioned first group. (3) The mean index of adhesiveness of the platelets, using the method of Moolten and Vroman on 32 patients, decreased from a control level of 1.33 ± 0.05 (S.D. 0.25) to 0.94 ± 0.03 (S.D. 0.17) after a single oral dose of 100 mg. of nialamide which was a significant decrease in the adhesiveness of the platelets (p < 0.001). (4) In Table 2, the adhesiveness of the platelets, when applying the authors' method in 29 patients, demonstrated similarly a significant decrease after a single oral dose of 150-200 mg. (average 3 mg./Kg. body weight) of

*All tubular parts which have come in contact with blood should be rinsed immediately after the test to avoid clotting. By means of a vacuum, water should be aspirated through them. The needle shafts can be connected serially with the help of polyethylene tubes to simplify cleaning. The polyethylene tubes, the rubber stoppers and the glass beads may be discarded after each test. The adapters are soaked in xylene solution for removal of paraffin. The syringe, three-way stopcock and the tube attached to it require only occasional cleaning.
Fig. 2.—Adhesiveness of platelets in normal persons. Each two dots, connected with a line, present two values of the adhesiveness of the platelets in one person. The two values are obtained by the separate, but simultaneous, determinations of the adhesiveness by varying the rate of blood flow through the filter. The two lines parallel with abscissa separate the area between 17.5–21 seconds, which is the range of flow time under described conditions.

The adhesiveness of platelets decreased significantly after nialamide (p < 0.001). The average decrease of the adhesiveness of the platelets was 14.9 per cent ± 1.3. The platelet count, also in this group, showed no significant change though it decreased about 1 per cent. The thromboplastin generation test (using a commercial “platelet” factor, rabbit brain cephalin) produced no change. The prothrombin consumption time revealed significant shortening after nialamide. The mean control value of 44.9 seconds decreased to 30.5 seconds (p < 0.001) with a mean difference of 14.4 ± 0.45 seconds.

**Discussion**

In the present work the normal thromboplastin generation test, when uniformly active commercial platelet substitute was used (rabbit brain cephalin), indicated that nialamide did not alter the function of the clotting factors in stage I of clotting when platelets were excluded. The decreased prothrombin consumption after treatment with nialamide did not correlate with an unchanged platelet count since no changes were observed in any of the accessory clotting factors. Thromboplastin generation tests were within
### Table 1

<table>
<thead>
<tr>
<th>Flow Time, Seconds</th>
<th>Mean % of* Adhesive Platelets (% of Platelets Lost in the Filter) with S.E.M.</th>
<th>S.D.</th>
<th>Linear Correction</th>
<th>Mean corrected value for 20 seconds as % adhesive platelets</th>
<th>Corrected by</th>
<th>Mean corrected value for 20 seconds as % adhesive platelets</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Correction factor</td>
<td>Correction factor</td>
<td></td>
</tr>
<tr>
<td>17.5</td>
<td>55.7 ± 2.24</td>
<td>14.2</td>
<td>$x_{20} = x_0 \left( \frac{20}{t_0} \right) - \left[ \frac{(20 - 17.5)0.5}{17.5} \right]$</td>
<td>63.6</td>
<td>62.35</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>62.6 ± 1.94</td>
<td>12.3</td>
<td>1</td>
<td>62.6</td>
<td>62.6</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>64.9 ± 2.1</td>
<td>13.4</td>
<td>$x_{20} = x_0 \left( \frac{20}{t_0} \right) - \left[ \frac{(20 - 21)0.5}{21} \right]$</td>
<td>61.8</td>
<td>62.3</td>
<td></td>
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*Read from the junction of the individual slope or its extension, when the given time was not the actual measurement.

†$x_{20} = $ per cent adhesive platelets for 20 seconds flow time; $x_0 = $ per cent adhesive platelets observed; $t_0 = $ flow time in seconds observed.

A summary of graphically presented data in Fig. II. It presents the development of a correction factor of the adhesiveness of the platelets with a “flow time” of 20 seconds, when the flow time varies within 17.5–21 seconds, in 40 healthy volunteers with an average age of 29 years.
EVALUATION OF NIALAMIDE ON BLOOD COAGULATION

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Mean Values</th>
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<tbody>
<tr>
<td></td>
<td>Before Rx</td>
</tr>
<tr>
<td>Adhesiveness of platelets</td>
<td>58.6% ( \pm ) 3.6</td>
</tr>
<tr>
<td>Platelet count</td>
<td>278,000</td>
</tr>
<tr>
<td>Prothrombin consumption in seconds</td>
<td>44.9 ( \pm ) 5.6</td>
</tr>
<tr>
<td>Thromboplastin generation</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Values of adhesiveness of the platelets, platelet count, prothrombin consumption and thromboplastin generation before and after nialamide administration.

normal limits when commercial platelet factor substitutes were used in the determinations. This finding suggests that the platelet is the only remaining blood component to be affected by this treatment. The findings propose further that the effect on the platelet is not one of diminution of numbers, but a change in the physiological activity of the platelet or of one of the platelet factors.

The avoidance of an anticoagulant could not have affected the thromboplastin evolution since the 20 second "flow time" is too short for the complete formation of thromboplastin. The effect of nialamide on the adhesiveness was not secondary to the prothrombin change since prothrombin activity was not altered by the therapy.

The possibility exists that the platelet agglutination action of nialamide is due to its effect on thrombin. However, this work and the recent work of Baumgartner et al. does not appear to support this view. No evidence of platelet aggregation was seen during the microscopic estimation of platelet numbers, either in the filtered or in the control samples. The control blood should not have been significantly altered during the short collection time since all surfaces except the glass beads were silicone-treated, and no evidence of a thrombin alteration was evident from the other coagulation studies completed.

Simultaneously, two different tests of adhesiveness of the platelets, which apply two different technical approaches to this phenomenon, indicated significant decreases of platelet adhesiveness. Therefore, it is conceivable that the anticoagulant effect, observed by Shimamoto et al. in earlier experiments, was due to altered platelet function. Indeed these authors have, in their recent publication, indicated that nialamide prevented the aggregation of platelets on the injured endothelial surface. The conventional anticoagulants, phenindione and warfarin, were significantly less effective than nialamide in the prevention of thrombosis in the rabbit even when the "prothrombin time was less than 6 per cent."

The present data indicate that nialamide produced a coagulation defect, probably at the platelet level, associated with deficient prothrombin consumption and decreased adhesiveness of the platelets.

The presently introduced technic for measuring the adhesiveness of the
platelets had fewer sources of error for the measurement of this phenomenon than previously described methods. This technic eliminated the influence of several extrinsic factors on the platelets.

The fact that nialamide diminished the adhesiveness of the platelets and simultaneously diminished its contribution to prothrombin activation suggested that the adhesiveness of the platelets represented a phenomenon which parallels the action of platelets, partially or totally, in contributing to the intrinsic clotting mechanisms.

SUMMARY

Nialamide at a single oral dose of about 3 mg./Kg. body weight produced decreased prothrombin activation by rendering the platelets less effective. It also decreased the adhesiveness of the platelets as measured by two different technics. A new technic for measuring the adhesiveness of the platelets has been described. It utilized a principle by which venous blood directly circulated through a column of fine glass beads, after which the platelets were reduced in number.

SUMMARIO IN INTERLINGUA

Nialamida, administrate in oral doses solitari de circa 3 mg per kg de peso corporee, resultava in un reducite activation de prothrombina como effecto de su render le plachettas minus efficace. Illo etiam reduceva le adhesivitate del plachettas, secundo mesurationes per duo differente technicas. Un nove technica pro mesurar le adhesivitate del plachettas es describite. Su principio es que sanguine venose circula directemente a transverso un columna de fin perlas de vitro, lo que resulta in un declino del numero de plachettas.

ACKNOWLEDGMENT

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